Galloway Lab Protocols

Galloway Lab

Sep 15, 2026

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CHAPTER

ONE

GENERAL LAB MAINTENANCE

1.1 Replacing microcentrifuge motor mounts

The microcentrifuges have rubber motor mounts which can dry up and crack over time. This causes the rotors to be off-balance and very loud when the centrifuge is turned on. Replacing these rubber motor mounts cuts down on noise and extends the lifetime of the instrument.

1.1.1 Required materials

Part	McMaster part number
Rubber vibration mounts	9376K21
Nuts	95462A029
Washers	90107A029
Lock washers	95584A207

1.1.2 Procedure

1. Buy more McMaster parts if needed. We should have extras of most in the cabinet with the toolbox.
2. Remove the rotor from the body of the microcentrifuge by unscrewing the nut in the center of the rotor. We have a specific 10mm wrench for this step.

Fig. 1: The top of the microcentrifuge with the rotor removed.

3. Unscrew the four side screws that attach the plastic shell and carefully lift it off, setting it to the right of the main body. Take special care not to disrupt the electronics.
4. Remove the old motor mount assemblies, replacing them with the new parts above. As seen in the picture, you should place washers on either side of the motor mount, and use a lock washer and a nut to secure it.
5. Put the outer shell, side screws, and rotor back into place. Take care not to press down hard on the rotor as this can cause the rubber mounts to split.

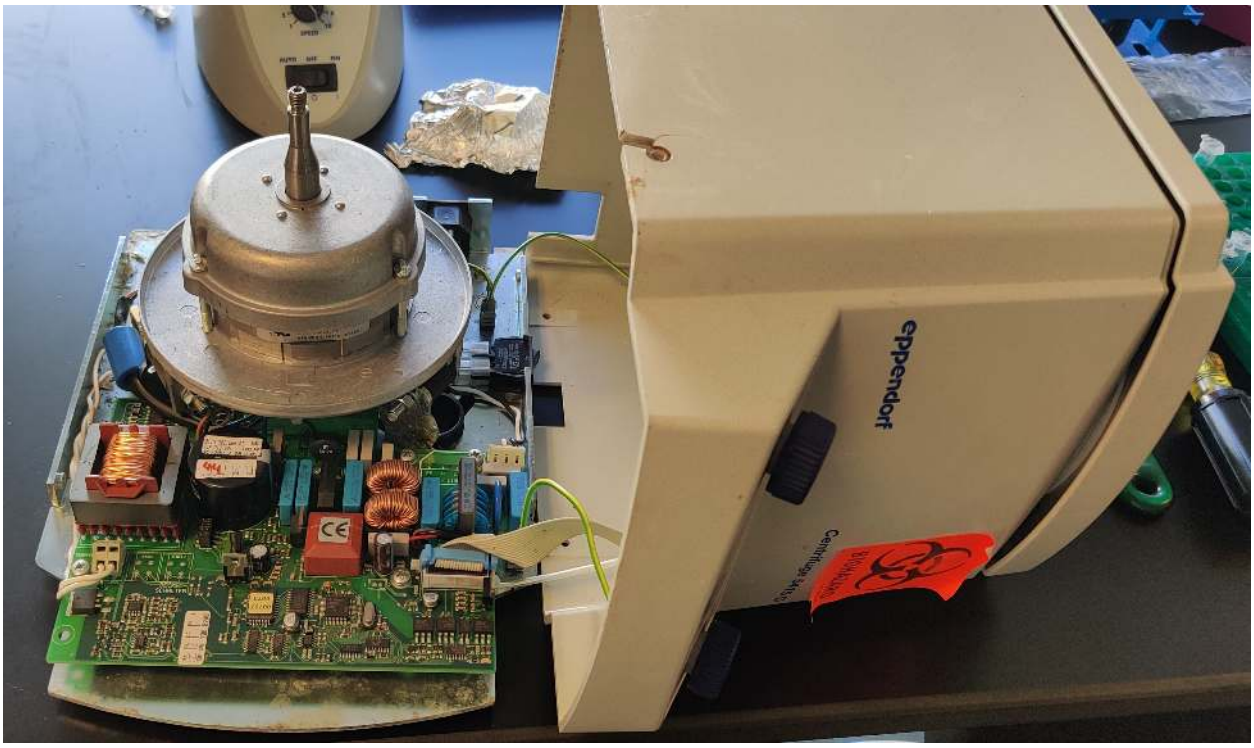


Fig. 2: The centrifuge with the shell removed. You should not disconnect the wires; just leave the shell on the right.

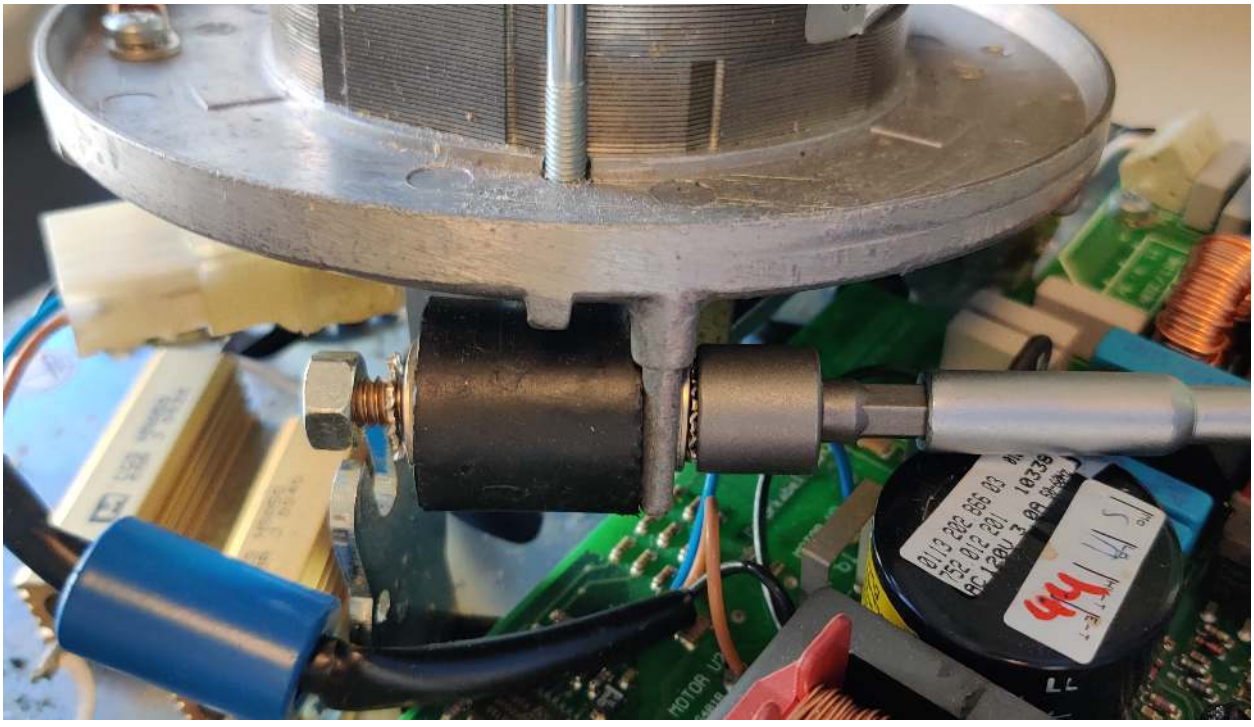


Fig. 3: A mount half-replaced. You need to attach the mount into the two slots for it. It is likely easier to attach it to the rotor first, then attach it to the base.

1.2 Cleaning and autoclaving guidelines

1.2.1 Washing glassware and tools

We dishwash all glassware using the dishwasher in the autoclave room (56-414), or wash item by hand using Alconox. Both are described below. The cleaning procedure for glassware, glass beads, and dissection tools are also described below.

Operating the dishwasher

1. Check that the dishwasher is free and fill out the log (date, time, name, phone number, office number, and PI initials) on the metal racks to the left of the autoclave.
2. Confirm that there are enough detergents in the large containers on the floor to the right of the dishwasher.
3. Prepare glassware as described below.
4. Load glassware into the dishwasher.

- If needed, the shelves/racks in the dishwasher can be swapped.
- Avoid overfilling the dishwasher, as this can lead to glassware hitting each other during the run and breaking.
- Place bottle lids in the metal mesh basket, and fit the mesh lids snugly over top to secure them in place. Otherwise, the lids will fall to the bottom during the cycle.

1. Select the "B2 Standard" cycle and press the button (play button icon) to start the cycle. The cycle takes 93 minutes to run.
2. When the cycle is complete, remove the glassware from the dishwasher. Note that it will be hot!
3. Store clean, dry glassware in lab as described below.

Washing glassware by hand

For any items that need to be washed by hand, we use Alconox as our soap.

1. As needed, dilute Alconox powder in water in the labeled squirt bottle by the sink (next to the Elga water dispenser). Alconox powder is stored under the sink, and there is no precise measurement for mixing it with water (a little bit of powder is sufficient).
2. Add Alconox and water to dirty glassware, and shake/swirl vigorously to clean. Use the brushes by the sink for tough spots.
3. Rinse thoroughly with tap water until no soap bubbles are left.
4. Perform a quick rinse with Elga water.
5. Place glassware on the drying rack above the sink to dry.
6. Once dry, remove promptly to free up space and to reduce the chance that it will be accidentally knocked off the drying rack.

Glass bottles and flasks

Note

If you empty a glass bottle or flask (media, PBS, gelatin, ELGA water, etc.), **you are responsible for bleaching and rinsing it.** If you cannot bleach and rinse the bottle immediately, keep the bottle on your personal lab bench.

1. If the glassware had cells in it (e.g., midiprep culture) first add bleach to a concentration of 10% and let sit for at least 15 min.
2. Rinse the glassware to remove the bleach.
3. Remove the tape label from the glassware. A razor blade can help to remove stubborn tape fragments—if these are left on when dishwashing, they become even harder to remove!
4. Glassware can now be placed in the area to be dishwashed, to the left of the Elga water dispenser. Alternatively, follow the steps above to wash by hand with Alconox.
5. Place clean, dry glassware from the dishwasher or drying rack on the shelf labeled "Clean glassware" outside of the TC room. To prevent dust accumulation, store beakers upside down and add foil to flask mouths.
6. For sterility, flasks that will be used to grow cultures are additionally autoclaved. Place autoclave tape on the foil on the flask mouth, then run the flasks in a typical dry autoclave cycle (see below). Running the dry autoclave cycle is typically a separate lab job from dishwashing.

Glass beads

Note

The dirty bead box should always be filled with 10% bleach.

1. If bleach levels are low, add more bleach to dirty bead box and allow to sit for at least 15 minutes. Dump out beads and bleach into mesh strainer found in cabinet to the left of the sink.
2. Soap with Alconox and then rinse with tap water.
3. Perform a quick rinse with Elga water.
4. Let beads fully dry in strainer overnight.
5. Use funnel to put beads into glass bottle for autoclaving on a dry cycle.

Dissection Tools

Note

Dissection tools should be washed as soon as dissection is finished. Dirty tools should not be left at the autoclave station.

1. After dissection, spray with ethanol and remove any visible residue.
2. Wash with Alconox and then rinse with tap water.
3. Perform a quick rinse with Elga water.
4. Let dry and wrap tools individually in tin foil.
5. Place tools back into the metal container and wrap the whole container in foil. Add autoclave tape.
6. Place the container in the autoclave area next to the Elga water dispenser, to be run in a dry autoclave cycle.

1.2.2 Autoclaving

Glassware and plastics are autoclaved as needed, usually twice a week as part of a lab job. Clean glassware on the appropriate storage shelf (i.e., wooden shelves next to the entrance to main TC) will be autoclaved as well as any empty tip or tube boxes in the autoclave area next to the Elga water dispenser. Prep materials as described below and place in an autoclave-safe bin (located on the rack to the right of the sink). The autoclave is located in room 56-414 and requires card access and completion of a training module[1].

Operating the autoclave

Note

When running the autoclave, it is okay to keep a cart in the room, pushed out of the way, since we have two carts. However, **do not leave heat-proof gloves in the autoclave room**, otherwise they might disappear.

1. Ensure there is not a program currently running. If someone else is currently running the autoclave, fill out your information in the autoclave log (date, name, phone number, office number, PI initials, and cycle time) on the metal shelves and write "next" to the left. Once the autoclave is free, cross out "next" and fill in the start time for your desired cycle.

   Note

   Only write "next" if no one else has done so already. This keeps the queue at one person. If someone has already written "next", come back later.

2. Open the door by turning the wheel fully counterclockwise, then turn the lever counterclockwise.
3. Place the bins on the racks inside the autoclave and close the door, first turning the lever and then the wheel fully clockwise.
4. Using the screen on the autoclave, ensure the jacket is turned on and select your desired cycle:
   - For dry cycles (glassware, plastics, etc.): Gravity >> 20 min / 20 min (starred cycle, runtime ~45 min)
   - For wet cycles (liquids such as PBS, LB, etc.): Liquid >> 20 min / 0 min (starred cycle, runtime ~45 min)
5. When the cycle is complete, **remove items promptly** to be courteous to other users. Open the door by first turning the wheel counterclockwise and then the lever. Be cautious of hot steam escaping from the open door.
6. Remove bins from the autoclave using heat-proof gloves.

[1] http://web.mit.edu/training/course.html?course=EHS00254w&sys=PS1

7. Close the autoclave door using the lever, but do not tighten the wheel when the autoclave is not in use.

Tip boxes

1. When a box of tips is emptied, *recycle* (page **??**) the empty plastic insert and throw away any autoclave tape, then place the empty box in the area next to the Elga water dispenser.

> **Note**
>
> Filter tip boxes do not need to be reused and the whole thing can be recycled when empty. *Recycle* (page **??**) any broken tip boxes.

2. Add inserts with new tips to empty boxes.
3. Add strip of autoclave tape where the lid meets the side of the box.
4. Autoclave on a dry cycle.

Tube containers

1. When a container of 1.7 or 0.6 mL tubes is emptied, throw away the autoclave tape and place the empty container in the area next to the Elga water dispenser.
2. Fill with new tubes.
3. Screw on top of container **loosely** to avoid warping while in the autoclave.
4. Add a strip of autoclave tape where the lid meets the side of the container.
5. Autoclave on a dry cycle.

Glass pipette containers

1. Make sure no glass shards remain in metal container (shake out over sharps container), throw away the autoclave tape, and place the empty container in the area next to the Elga water dispenser.
2. Carefully fill metal containers with glass pipettes with the thin end pointing down (i.e. larger handle side points outwards when you open it).
3. Add strip of autoclave tape where the lid meets the side of the container.
4. Autoclave on a dry cycle.

Toothpicks

1. When a container of toothpicks is emptied, throw away tin foil and autoclave tape and place the empty container in the area next to the Elga water dispenser.
2. Fill container with toothpicks.
3. Cover with foil and mark with autoclave tape.

4. Autoclave on a dry cycle.

Flasks

1. See instructions *above* (page **??**) for washing glass flasks. Clean, dry flasks should be stored covered with foil in the "Clean glassware" shelf next to TC.
2. Add strip of autoclave tape to the foil on the top of the flasks.
3. Autoclave on a dry cycle.

Dissection tools

1. Make sure tools are clean, dry, and individually wrapped in tin foil inside their metal container. See instructions *above* (page **??**).
2. Wrap the outside of the metal container with tin foil and mark with autoclave tape.
3. Autoclave on a dry cycle.

Glass bottles containing liquids or glass beads

1. Prepare solutions as described in the recipe protocol, or glass beads as described *above* (page **??**).
2. Loosely screw on bottle cap.

> **Warning**
>
> Do NOT screw caps on all the way! This could cause an explosion in the autoclave.

2. Use autoclave tape to further attach the cap to the bottle.
3. Cover cap and top threads with foil. This helps keep the edges of the top sterile while the cap is not fully screwed on, so make sure the foil is snug around the threads.
4. Mark the top of the cap with autoclave tape.
5. Autoclave bottles containing liquids on a liquid cycle, or glass beads on a dry cycle.

1.3 Cleaning BSCs

As part of quarterly lab jobs, the biosafety cabinets (BSCs) used for TC should be thoroughly cleaned. Do your best to scrub away any stains or residue on the BSC surfaces. This includes all 4 BSCs in the main TC and the quarantine hood in 66-219.

> **Important**
>
> There is a dedicated Swiffer to reach the back/far surfaces hanging on the wall in TC.

1. Remove any unnecessary items from the BSC, such as stray tubes or extra plastic tube racks.

2. Clean the walls and work surface: Use the dedicated Swiffer with paper towels (in place of where the Swiffer pad/towel would go) to clean the back and side walls and work surface of the BSC. Spray with Pre-empt and wipe, then spray with 70% ethanol and wipe to remove the Pre-empt residue.
3. Clean all items in the BSC: Spray all items in the BSC thoroughly with Pre-empt and wipe, then spray with 70% ethanol and wipe to remove the Pre-empt residue.
4. Clean the sash: Spray the inside and the outside of the sash with 70% ethanol and wipe with a paper towel.
5. Clean under the main work surface: Use the two knobs on either side of the main work surface to lift and clean underneath.
6. Once done cleaning, you should put your lab coat in hamper to be washed.

Tip

It is convenient to bring the carts into the room to have a place for all the items in the BSC. Be sure to give everything a Pre-empt and 70% ethanol spray before returning to the hood.

1.4 Cleaning microcentrifuges

1. Remove the rotor from the microcentrifuge by unscrewing the center bolt that holds the rotor in place. Use the magic wrench shown below that is labeled “For Cleaning Centrifuge”.
2. Wipe the inner surface of the microcentrifuge with 70% ethanol to remove any built up grime.
3. To clean the rotor, use a squeeze bottle to add soap to each of the samples holes and scrub using the small scrub brush located on the drying rack.
4. After cleaning the rotor with soap and water, dry the rotor and holes using compressed air. Tubing maybe attached to any of the “Air” nozzles and inserted into the holes of the rotor to blow out any water left in the rotor.
5. Using 70% ethanol and paper towels, clean the groove where the rotor lid sits during normal operation. Dried salts tend to build up here.
6. When the rotor is clean and dry, reattach the rotor to the centrifuge base. Tighten using the magic wrench.

1.5 Regular computer maintenance

One of the quarterly lab jobs is to do regular computer maintenance. This makes sure our lab computers stay up to date and aren’t accumulating junk.

1. Run Windows Update. Go to the Start menu / hit the Windows key and search for “Windows update” or “Check for updates”. Do not update to Windows 11 (yet), but install any other required/recommended updates. You do not have to install optional driver updates unless that piece of hardware is causing problems.
2. Reboot if necessary to install updates.

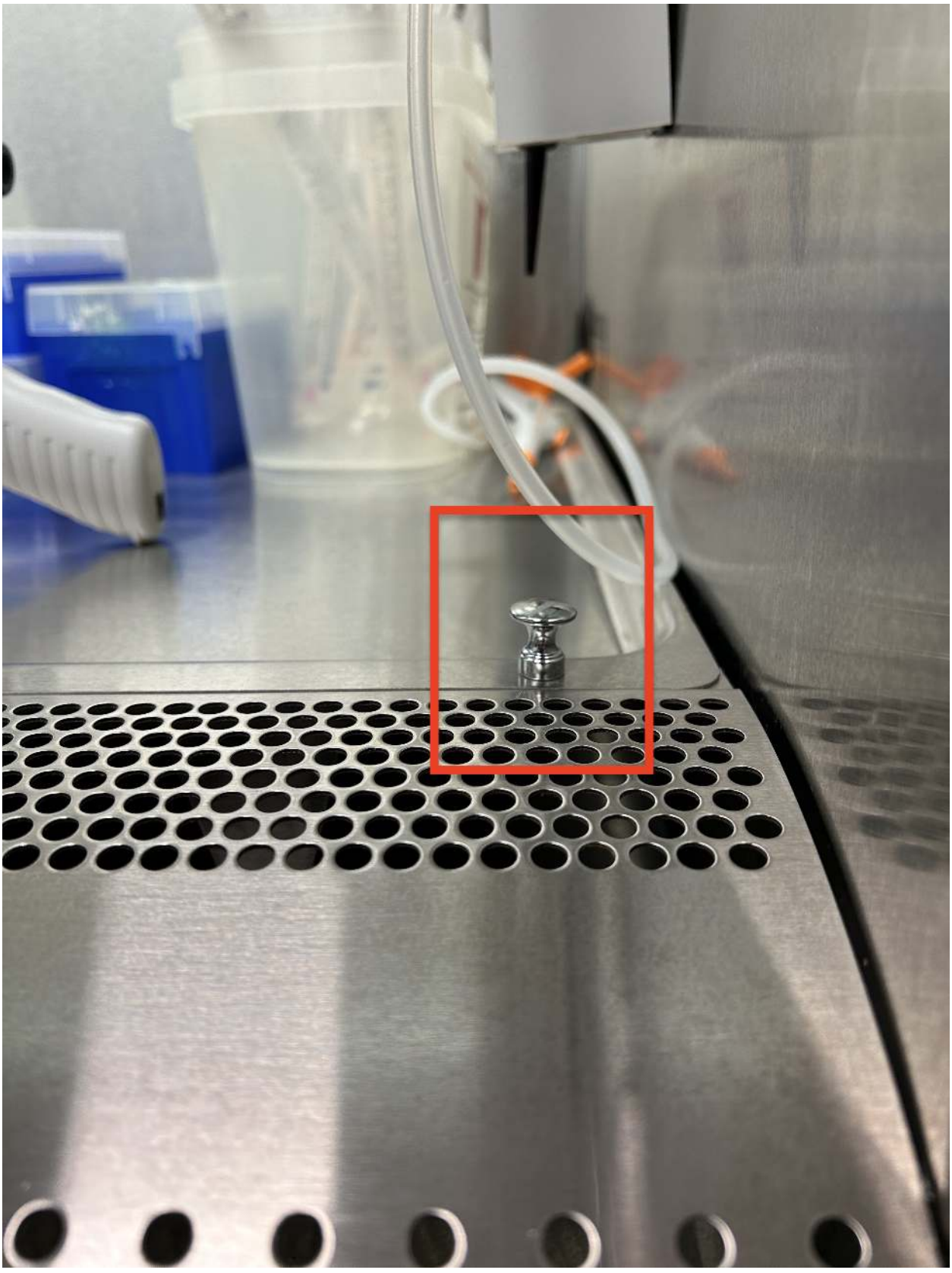


Fig. 4: One of two knobs (red box) that can be lifted to clean underneath the main work surface in the BSCs.


For cleaning centrifuge
BIOHAZARD
eppendorf
Centrifuge 5415 D
Time
Speed
1.0 min
16.1 rcf
x 1000

3. Check the disk storage available on each computer. You can use WinDirStat[2] to easily check how much storage is actually being locally used for the synced OneDrive folders.
    - Make sure that the boot drive (`C:\`) is not filling up. If it is, see what is causing this and correct it (e.g. do stuff like clear unneeded downloads)
    - See if there are unnecessary files locally synced, such as large timelapses. Right click and hit "Free up space" to remove them from the local cache.
4. Check for data files stored e.g. on the desktop, on the `C:\` drive, really anywhere besides the OneDrive. Move all such files you find into a folder and inform people about this. Delete these files after a week or so so we keep stuff tidy and all contained in the OneDrive folder.
5. Clean up the "quick access" menu on each computer (the top most part of the explorer window). If there are too many e.g. personal folders pinned here so that you can't see the main "keyence" / "attune" ones, unpin some of them.
6. Compare the Attune software version with that available online[3]. You have to fill out the form to get to the software page: I recommend using "this-is-a-dumb-requirement@mit.edu" as your email :) Do **not install** this update yourself; send a message to the lab and plan when to do this.

1.5.1 Installing Attune software updates

The Attune software updates takes a while, and can, in the worst case, temporarily leave you with a non-functioning Attune.

While that sounds scary, we do need to keep the software up to date, and we can always have a service call if we really break the software. **Do not ignore these software updates**, just plan them around non-critical experiments.

1. Before beginning, make sure that everyone has exported their FCS files into the OneDrive. Go into the software About page and write down the current software versions.
2. Close the Attune software, if it is open. Make sure the Attune is in the sleeping/idle mode.
3. Launch the "Attune Database Utility"; search in the Start Menu for it. Login with user account `admin`.
4. Go to the Backup tab and hit "Backup user data". Choose a folder outside the OneDrive to backup to.
5. When the backup is complete, close the database utility and unzip the software update installer.
6. Read the PDF inside the update installer package. **This is not optional**.
7. Run the installer and follow the prompts. This could involve doing many different things, which is why the instructions are important.
8. If the update fails, it is sometimes possible to downgrade by rerunning an earlier installer. You can use the Attune Database Utility to restore your userdata backup as well.

[2] https://windirstat.net/
[3] https://www.thermofisher.com/us/en/home/global/forms/attune-nxt-software-download-registration.html

9. If all else fails, ask for a service call to fix it.

1.6 Checking data backups

1.6.1 Onedrive backups

The OneDrive and timelapse OneDrive are the main place we store data. Each OneDrive has 10TB of storage. We can create more OneDrives, but eventually storing much greater than 20TB will run into logistical issues because of all of the OneDrive juggling.

Files in the OneDrive are chiefly backed up for us, through the normal interface. Deleted files stay in a special OneDrive recycle bin for around 90 days, after which they are deleted.

What happens if someone accidentally deletes data after it expires, or we need to get an older version of data? We now have a Network Attached Storage (NAS) with about 18TB of storage space. The Keyence computer has a sync client that duplicates both OneDrives onto this storage device. This means we have a secondary backup of everything in the OneDrive.

Once a year checks

Once a year, during the IAP infrastructure day(s), you should check and restore an older version of a file using the NAS, just for practice.

- Login to the NAS at https://ashurbanipal.mit.edu and ???
- Using the Synology sync client on the Keyence computer, test recovering a file by ???

1.6.2 NGS backups

When we do next generation sequencing, we can easily generate terabytes of data. Storing this in the OneDrive would quickly exceed the space available. To safely keep raw sequencing data, we duplicate this data onto pairs of physical hard drives, and use a backup helper to store *parity* data, which allows for recovery in the case of file corruption.

The pairs of hard drives are stored in fireproof/waterproof safe boxes. Ideally, we store one of the safe boxes somewhere in lab/in an office, and store another one offsite (with Katie).

Which NGS datasets are stored on which pair of hard drives is listed in a spreadsheet in the OneDrive[4].

Adding a new NGS dataset

1. Compress the dataset, either into a `.tgz` or a `.zip` file.
2. Check the hard drive spreadsheet, and determine if the dataset fits on one of the existing disk pairs (including an extra 3-5% for parity data).
3. If there is not enough room, order a new pair of hard drives. There are suggestions listed in the backup helper instructions[5]; as of 2023, it is recommended to get 6TB Western Digital Blue CMR (conventional magnetic recording) hard drives. This might change in the future, so read the instructions!
4. Locate and bring both fireproof safes to lab. One is likely with Katie or otherwise offsite from Building 66.
5. Copy the dataset onto both drives, into the *data* folder.
6. Follow the backup helper instructions[6].

Once a year checklist

The main instructions on how to use the backup helper are available at https://github.com/GallowayLabMIT/cold_backups

In short, once a year you should:

1. Bring both fireproof safes to lab. One is likely with Katie or otherwise offsite.
2. For each pair of drives, load each into the external hard drives enclosures and connect to the same computer.
3. Follow the backup helper instructions[7] to run the auto-verification.
4. Do the suggested spot-check manual check on one of the files both as practice and to make sure the automated helper is working.

[4] https://mitprod.sharepoint.com/:x:/s/GallowayLab/ERLZeBrX4OVIpMRjG9Q5foIBV0iK1xhWT1DEF2Qg1dgGCA?e=0OhHi8
[5] https://github.com/GallowayLabMIT/cold_backups
[6] https://github.com/GallowayLabMIT/cold_backups
[7] https://github.com/GallowayLabMIT/cold_backups

5. Check the desiccant packs. You can either weigh them to see how much water they have absorbed, or look at the color of the beads. You can regenerate the desiccant by microwaving the packs for the listed time (or until they lose water mass).

1.7 Incubator decontamination

Please refer to the manuals for detailed decontamination instructions. Below are additional tips and instructions not stated in the manuals. Decontamination should be performed at least twice per year, or after significant contamination occurs.

Note

Because the incubators heat up significantly during decontamination, an entire stack (top and bottom) should be decontaminated simultaneously.

1.7.1 Moana and Maui (Main TC)

Follow instructions on the incubator display.

1.7.2 Hei Hei and Pua (Main TC)

Estimated time

Decontamination takes approximately **two days**, with about **30 minutes** of hands-on time.

The Hei Hei/Pua incubator manual is located in the drawer to the left of the sink in the main TC room. Refer to Chapter 9 (page 53) for detailed instructions.

Day 1

1. Turn off the incubator (switch located below the door on the left) and turn off CO2 flow (silver levers on the wall).
2. Remove and empty the metal water tray. This tray may be autoclaved.
3. If any water remains in the bottom of the incubator, use the water pump hanging on the side of the incubator to empty.

   To operate the water pump:

   1. Place the water pump on the lowest shelf of the incubator and feed the inlet hose through a hole in the shelf to the water (see diagram below).
   2. Place the outlet tube into a bucket located below the lowest shelf.
   3. Pump about four times to start the flow of water.
   4. Let the water run into the bucket via gravity.
   5. Use a paper towel to wipe any remaining water from the floor of the incubator.
4. Remove and disassemble incubator components (3 shelves, 1 water tray per incubator)
5. Disinfect the incubator components by wiping down with 10% bleach followed by ethanol. Also wipe down the interior of the incubator.
   - If necessary, the blower wheel can be removed to be more thoroughly cleaned. Refer to the manual (page 56) for instructions.
6. Replace the metal water tray and fill with autoclaved DI water.
7. Follow the manual instructions (page 58) for starting the 90ºC decontamination cycle. This will take **approximately 25 hours** to complete.
   - Heating: 2 hours
   - Decontamination: 9 hours
   - Cool down: 11 hours
   - Postheating: 3 hours

Day 2

8. Once the decontamination routine is complete, wipe up any water on the bottom of the incubator and make sure the water tray is still filled.

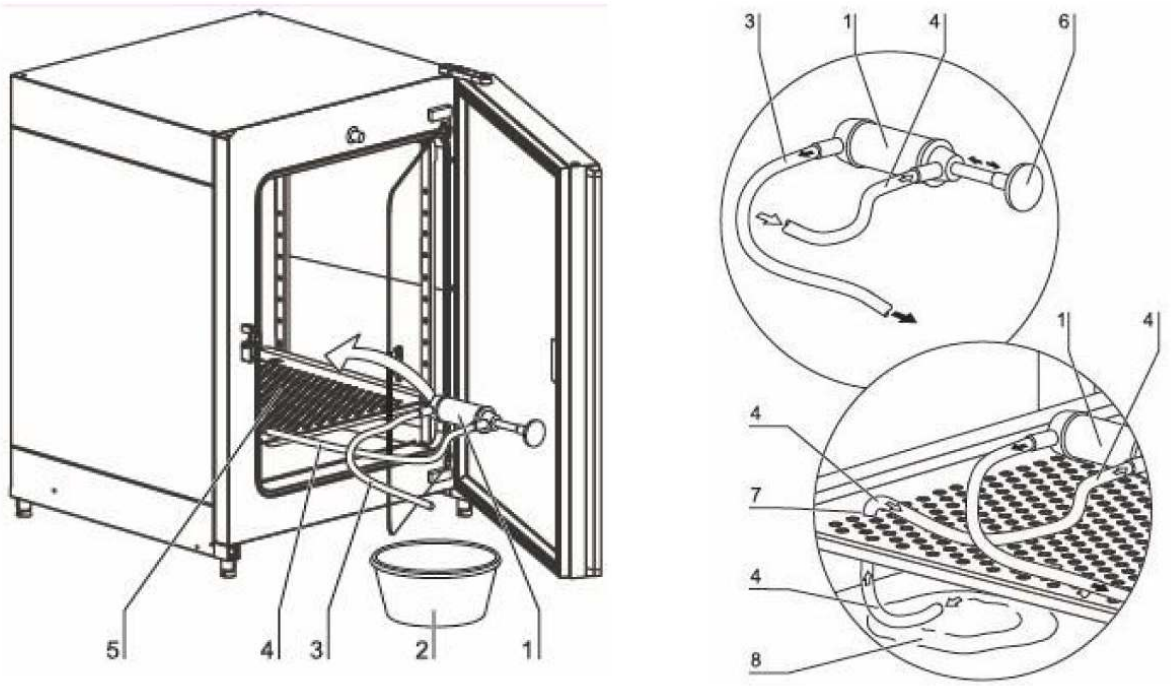


Fig. 5: Diagram of the water pump setup and parts: (1) pump, (2) bucket, (3) outlet hose, (4) inlet hose, (5) lowest incubator shelf, (6) pump hand grip, (7) incubator shelf hole, (8) water on incubator floor.

9. Reconnect CO2 flow.
10. Finish the decontamination cycle by holding down the 90ºC button for 5s (see manual instructions, page 59). The display should switch from 0h to the CO2 level.
11. Follow manual instructions (page 45) to begin the auto-start routine. This will take **approximately 5 hours**.
12. After the auto-start routine finishes, the incubators can be used.

1.7.3 Te Kā (Quarantine) and Te Fiti (66-219)

Estimated time

Decontamination takes approximately **two and a half days**, with about **two hours** of hands-on time.

Warning

Decontaminating these incubators requires the HEPA (1 per incubator) and inlet (2 per incubator) filters to be replaced. Ensure that you have replacements before beginning!

Materials

Filter information:

- HEPA filter: Thermo Scientific, 760175[8] (source from Ebay)
- Inlet filter: Thermo Scientific, 770001[9]

The incubator decontamination instructions are located in a plastic sleeve on the side of the incubators. See Te Kā/Te Fiti incubator manual for additional instructions.

Day 1

1. Turn off the incubators (power switch is located on the top left side of each).
2. Turn off carbon dioxide flow by screwing the knob counterclockwise until the pressure gauge reads zero. The carbon dioxide tanks are located on the wall to the left of the incubators.
3. Disconnect the carbon dioxide tubing from the back of the incubators. Be sure the tubing is not touching the incubator—the incubators get very hot during decon!
4. Remove and discard used filters in biowaste: 1 HEPA and 2 inlet filters.
5. Remove and disassemble all internal components from each incubator. Items below are listed in the order to remove:
    - 1 water tray
    - 3 shelves
    - Side walls: 2 wall pieces, 6 shelf support bars
    - Outer ceiling component: 1 large metal piece with rubber hose, 2 large wing nuts
    - Inner ceiling component: 1 white plastic air circulator, 1 metal piece, 1 hex nut, 4 small wing nuts

[8] https://www.fishersci.com/shop/products/hepa-main-filter-3110-series-310-series-w-hepa-option-steri-cycle-series-incubators-hepa15497022?crossRef=760175&searchHijack=true&searchTerm=760175&searchType=RAPID&matchedCatNo=760175

[9] https://www.fishersci.com/shop/products/thermo-scientific-midi-40-co-sub-2-sub-incubator-accessories/11687203

Fig. 6: Carbon dioxide knob (black, center) and pressure gauge (top, currently at 0 psi).

Fig. 7: Carbon dioxide tubing connected to the back of Maui (top incubator).

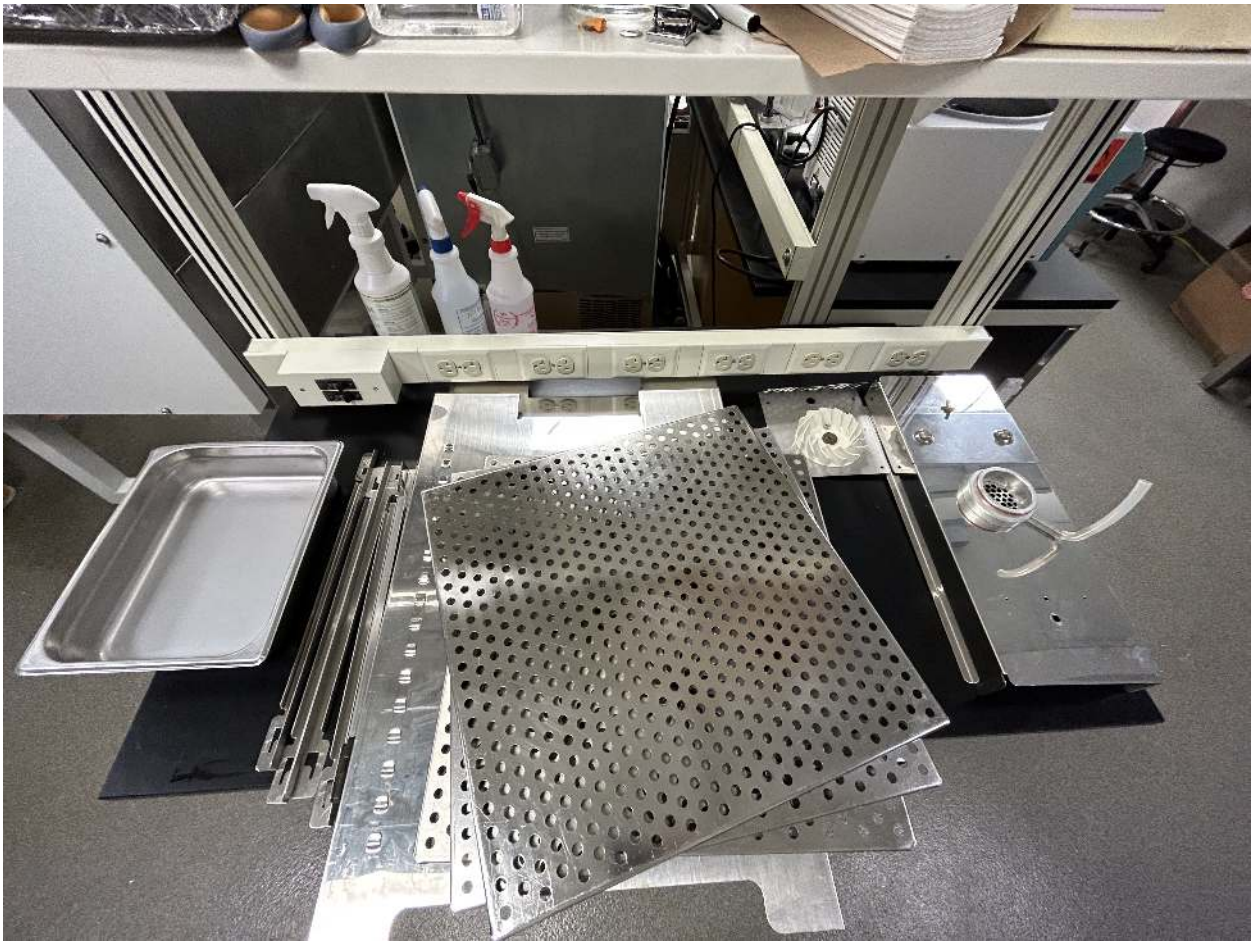


Fig. 8: Disassembled parts from one incubator.

Fig. 9: Interior of a disassembled incubator.

6. Disinfect the incubator components with a dilute bleach solution followed by ethanol. Also disinfect the inside of the incubator and glass door.
    - Tip: Fill one of the water trays with 10% bleach and dip paper towels into the solution to clean
    - Wear a lab coat for this cleaning step to prevent bleach from staining your clothes. Be sure to put the dirty lab coat in the laundry hamper once you finish!
    - It is recommended that 2 or more people help with this cleaning step (**approximately 1 hour**)
7. Replace all internal components (reverse order of the above list, this does NOT include filters).
8. Turn the incubators on and initiate the sterilization cycle by holding down the green button on the right for 3 seconds. The screen will flash "Remove HEPAs" and "Remove water", press Enter to continue.
9. Place the "Incubator decon in progress" sign on the incubators and allow the sterilization cycle to proceed. This will take **approximately 12 hours**.
    - Heat up: 2-4 hours
    - Sterilization: 2 hours
    - Cool down: 6-8 hours

Day 2

10. Once the cycle is complete, open the chamber and install new filters (1 HEPA, 2 inlet; see diagram below). Insert the temperature sensor into the metal HEPA collar behind the large metal ceiling piece.
11. Fill the water tray with autoclaved DI water but DO NOT RECONNECT CARBON DIOXIDE. Allow the incubators to equilibriate for **approximately 12 hours**. If necessary, a shorter equilibriation period may suffice.

Day 2 or 3

12. Recalibrate carbon dioxide levels to zero on the incubator display.
    1. Press "Mode" button
    2. Toggle left/right arrow buttons until the display reads "CO2 CAL"
    3. Press up/down arrow buttons to set the calibration value to 0.0
    4. Press "Mode" button to finish
13. Reconnect the carbon dioxide tubes to both incubators (see image at step #3). Before turning on the carbon dioxide flow, it is recommended to turn off the incubators (power switch is located on upper left of each). This aids in finding any leaks/incomplete seals.
14. Turn on carbon dioxide flow by screwing the knob clockwise until the pressure gauge reads 15 psi (see image at step #2). If any audible leaks are detected, determine the source of the leak, turn off the carbon dioxide flow, resolve the issue, then turn the flow back on.

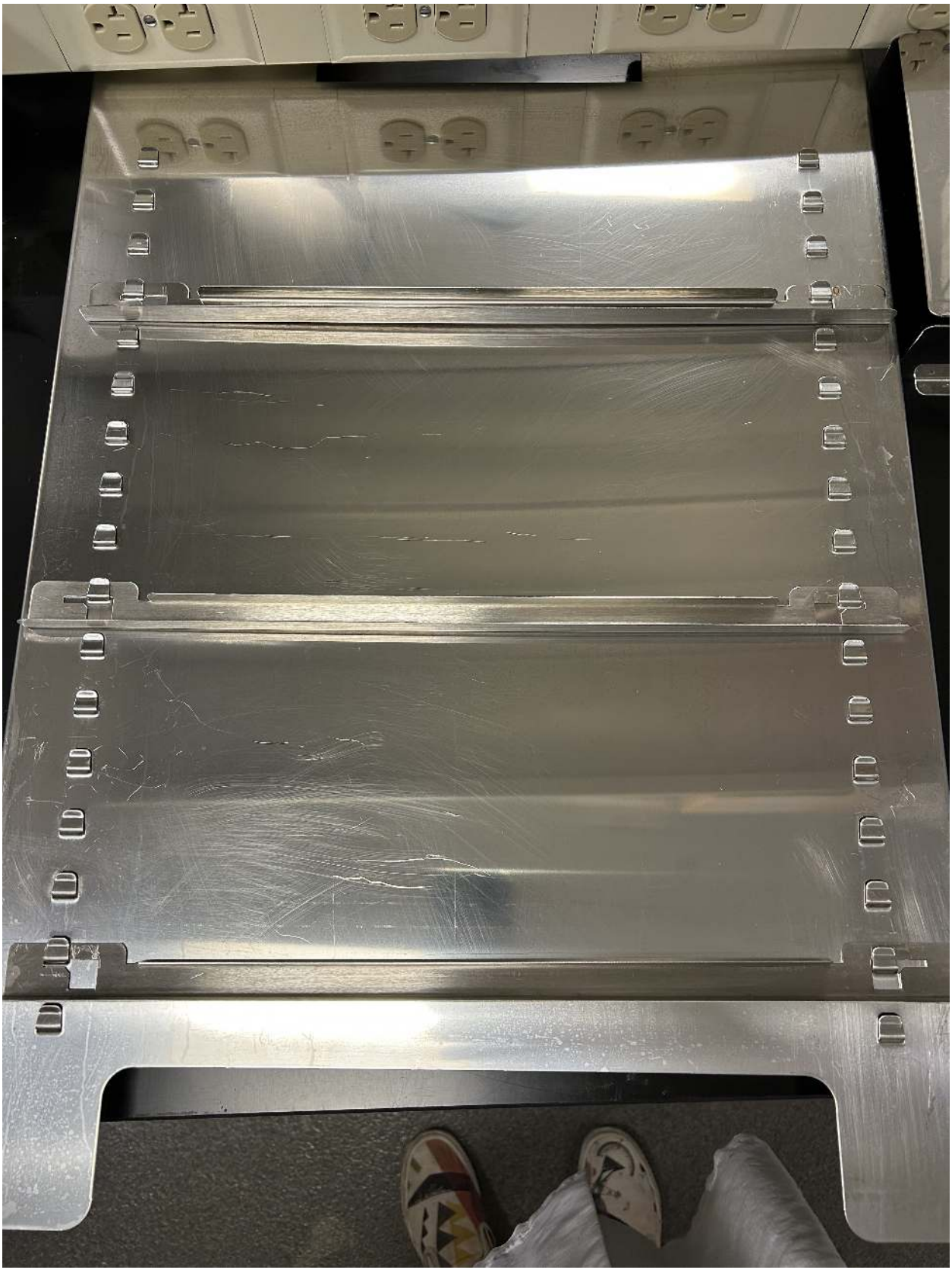


Fig. 10: Location of shelf holder bars.

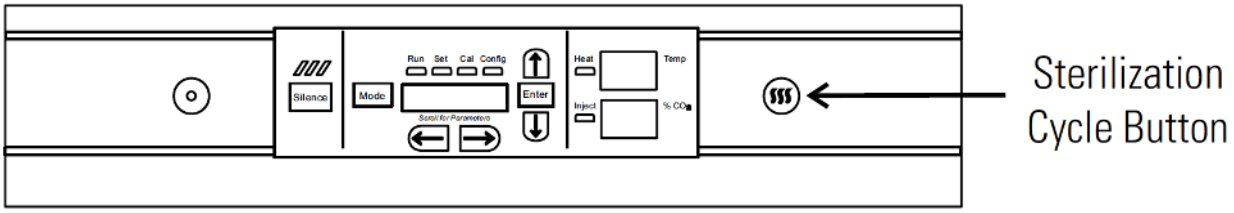


Fig. 11: Diagram of the incubator display with the sterilization cycle button indicated.

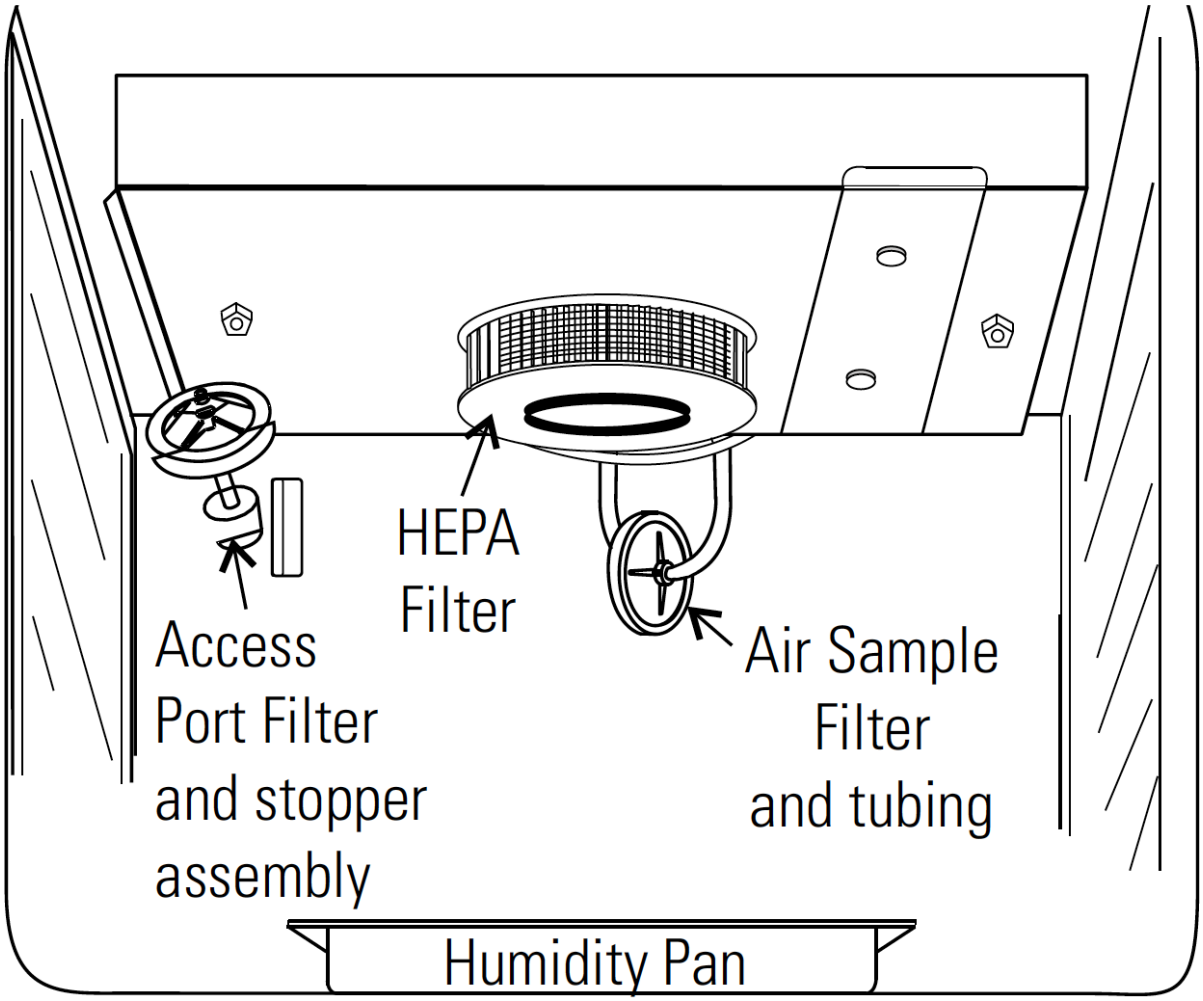


Fig. 12: Diagram of the incubator filters. The inlet filters are labeled “Access port filter” and “Air sample filter”.

15. Turn the incubators back on. Once carbon dioxide levels reach 5%, the incubators may be used.

1.8 Lab Safety Information

1.8.1 General Information

- Important phone numbers:
  - MIT Medical: 617-253-4481
  - MIT Urgent Care: 617-253-1311
  - MIT Emergency Number: 617-253-1212
  - MIT Facilities: 617-253-4948

Note

Emergency contact phone numbers are listed on the green card outside every lab room.

- Procedure if you are injured or exposed to biosafety/chemical hazards:
  1. Make sure area is safe to walk away from.
  2. Wash/clean the wound.
  3. Inform Katie and the EHS Rep (Chris) over Slack.
  4. Go to MIT Medical or the hospital for evaluation.

Note

Research-related medical costs are paid by MIT's occupational health fund.

- Injuries must be reported by the Department EHS coordinator (Brian Smith), the PI (Katie), and the EHS Rep (Chris).
- Evacuation routes:
  - The two closest evacuation routes are to the left towards Ames St or to the right towards Building 56.
  - In the case of a large chemical release in the building, the stairwell in 66-201 is under positive air pressure.

1.8.2 Room-Specific Information

66-257A (Shared space with the Coley Lab)

- Fire extinguisher and safety shower are located on the Coley Lab side of the room.
- Eye wash station is located next to the water baths.
- Satellite Accumulation Areas are located in the fumehood (formaldehyde and formamide only) and underneath the gel runners (gels only).
  - DNA gels containing ethidium bromide should be disposed of in the black container. A dilute rinse of the flask used to pour the gel can be put down the sink. DO NOT let pieces of gel go down the sink or sit in the sink strainer.

66-225 (Cloning area)

- Eye wash station is located to the right of the Elga water machine.
- Bleach storage is underneath the sink. The 10% bleach container should be used for the Attune and refilling bleach bottles next to media/cells aspirators. When it is empty, follow the recipe listed on the bottle and replace the "last filled" label.
- Satellite Accumulation Area is located under the Elga water machine:
  - Liquids such as miniprep waste (guanidium chloride), paraformaldehyde, acrylamide, and trizol must be collected in an SAA.
  - Decontaminated liquid biowaste, DNA/enzyme solutions, and salt buffers can go down the sink.
- Vacuum pumps closer to 66-219 are for miniprep waste (supernatant buffers AFTER cells have been lysed). Vacuum pumps closer to 66-257A are for media and have flasks containing bleach.

> **Important**
>
> Bleach should NEVER be added to miniprep waste since it will generate toxic fumes.

  - After aspirating into the media/cells aspirators, you should run 10% bleach through the line from the bottles in each bin.
  - If you swap between the multichannel and single-channel aspirator, flush the line with 10% bleach before switching.
- When decontaiminating liquid biowaste, you should add bleach to make a 10% bleach solution and let it sit for at least 20 minutes before pouring down the sink.

66-225B (Tissue culture area)

- Fire extinguisher and safety shower are located near the Keyence.
- Eye wash station is located by the sink.
- Sharps containers are next to each hood. Use these to dispose of glass aspirating pipettes (after flushing with bleach).

- Bleach is stored in the cabinet to the left of the sink.
- If you swap between the multichannel and single-channel aspirator, flush the line with 10% bleach before switching.
- When finished with a BSC, you are required to clean it with:
  - Pre-empt if you used virus or are the last user of the day. Prep-empt requires a 1 minute contact time to decontaminate the surface.
  - 70% ethanol always.
  - Rinse the aspirator line with 10% bleach from the bottle inside the BSC. If the bottle is empty, refill it with 50 mL concentrated bleach and 450 mL water.
- Liquid in the aspirator waster bottles should ALWAYS be clear. If it is red or pink, add more bleach from the cabinate under the sink.
- If the aspirator waste is filled to the indicated line, add more bleach for 20 minutes before pouring down the sink. Fill the empty bottle with a small amout of bleach.
- Gloves should be disposed of in large or small biowaste bins.

Important

BSL2+ Information

- Official indication that the room is BSL2+ is the black and orange sign on the door. If this is present, UROP and HIP-SAT students MAY NOT enter the room.
- BSL2+ samples can be stored in:
  - Incubators that are labeled with a sign, within a secondary containment tray.
  - Screw-top containers in a labeled plastic container in the fridge.
  - Screw-top containers in a labeled plaster container in the -80 freezer.
  - The bottom centrifuge when labeled.
- UROP and HIP-SAT students MAY NOT touch any equipment, cells, or samples that are BSL2+.
- When beginning BSL2+ work, you must flip the BSL2/BSL2+ sign, add the expected time to the lab Google calendar, send a message in the BSL2+ slack channel, and confirm that there are no non-BSL2+ trained people in the room.
- All human-infectible viruses that express known oncogenes are to be treated as BSL2+ including: Lenti p53DD/hRas, Retro p53DD/hRas (not made in PlateE cells), starting from 1 day after infection with a BSL2+ virus.
- Do not use glass aspirating pipettes when doing BSL2+ work.
- Biosafety centrifuge caps are required when spinning a BSL2+ virus or infected sample. Buckets should only be opened in the BSC and must be decontaminated after use.

For more information on BL2+ protocols, see the *Virus safety* (page **??**) page.

66-218 (Atrium/Hallway area)

- Fire extinguisher and safety shower are located near the shelving unit.

66-219 (Quarantine/Genomics area)

- Eye wash station is in the far-right corner.
- Bleach is stored under the sink.
- The "EHS corner" has red waste tags, waste containers, and sharps containers. A red waste tag should be added to a waste bottle when you first add waste to it.

1.9 OneDrive sync problems and replacing the logged-in user

1.9.1 OneDrive sync problem debugging

Warning

The OneDrive sync client is very sensitive and does not like being logged into from multiple user accounts! Keep track of who is logged in on each computer. If you want to switch, following the instructions at the bottom.

Unfortunately, after an authentication change that MIT made, we have to regularly log back into the computers to get them to sync, roughly once every other month.

Sometimes, the OneDrive client on the computers will either lose its sync connection or get stuck in a state where files are not syncing.

The first debugging step is always to run the OneDrive reset. A OneDrive reset does not change the logged-in user, it just clears the database that OneDrive uses to keep track of files. After a reset, OneDrive will rescan **all** of the files in the synced folder and figure out which it has to sync.

To reset:

1. Hit `Win-R` on the keyboard to open the run dialog. Enter `%localappdata%\Microsoft\OneDrive\onedrive.exe /reset`
2. You should see the OneDrive client close and disappear from the taskbar.
3. After a few seconds, launch OneDrive again from the start menu. The first start after a reset does some additional reset stuff, so the client will close itself.
4. After a few more seconds, launch OneDrive again. This time, it should stay up.

Note

A full resync takes a long time, upward of several hours. You can use the PowerToys toolbar icon and set it to "Keep computer awake -> Indefinitely" until it finishes.

It still doesn't work!

If syncing is still not happening, or it is forcing users to login, then the user cache probably needs to be fixed. Information on this is in the Microsoft documentation[10].

1. If possible, log out of the OneDrive client, unlinking it.
2. Run the OneDrive reset command from above (`%localappdata%\Microsoft\OneDrive\onedrive.exe /reset`), but do **not** start the client again.
3. Using Windows Explorer, navigate to `%localappdata%/Microsoft` (paste into the address bar). Delete the subfolders `IdentityCache` and `OneAuth`
4. Start the OneDrive client.
5. Log back in. When it asks where to put the OneDrive folder, pick:
   - for the Keyence computer (Edward): `D:\`
   - for the Attune computer (Jacob): `D:\Users\INSTR-ADMIN`

   After making your selection, the dialog should read "Your onedrive folder is `D:\OneDrive - Massachusetts Institute of Technology`"
6. Following the linking instructions from *the IAP sync instructions* (page **??**). This involves hitting "Sync" in the web client.
7. The client should recognize that there is already data in the selected folder. Accept this by hitting "use this folder".

[10] https://learn.microsoft.com/en-us/sharepoint/troubleshoot/sync/sign-into-onedrive-error-0x8004dec5

1.9.2 Unable to pin OneDrive folders to the quick access

If you are unable to re-pin folders like the Keyence or Attune folders, it may be due to a corrupt Quick Access folder. The solution is documented on the Microsoft forums[11]

Using a **administrator command prompt** (search for command prompt in the windows-key / start menu, then right click and hit "Run as administrator". This won't work in Powershell) run the following:

1. `del /F /Q %APPDATA%\Microsoft\Windows\Recent\*`
2. `del /F /Q %APPDATA%\Microsoft\Windows\Recent\AutomaticDestinations\*`
3. `del /F /Q %APPDATA%\Microsoft\Windows\Recent\CustomDestinations\*`
4. Reboot the computer

[11] https://answers.microsoft.com/en-us/windows/forum/all/cant-pin-images-and-documents-folder-to-quick/7c0380af-8d9b-4b85-ae0d-d7481dc67efe

1.9.3 User replacement

IST does not provide "service users", so a lab member has to be logged into the lab computers in order for OneDrive syncing to work. Occasionally, someone will graduate or swap roles, so we need to change the logged in user.

Important

Make sure that the OneDrive is fully synced, e.g. shows no pending files, before swapping users!

1. Select "Free up space" on the entire OneDrive, and on any folders still present. This both confirms that the files are uploaded and frees up space on the disk.
2. Wait for the OneDrive to finish syncing these changes.
3. Confirm with WinDirStat[12] that none of the files are actively present (e.g. not taking up space). WinDirStat should already be downloaded on the lab computers.
4. Logout the old user out of OneDrive, by clicking through the OneDrive settings, accessible via the blue cloud icon.
5. Close OneDrive, and exit out of any file explorer windows.
6. Using Powershell, rename the old OneDrive and Sharepoint folders, as stored in the data directory.

```
> cd D:
> mv "./Massachusetts Institute of Technology" "./Massachusetts Institute of␣
↪Technology - old"
> mv "./OneDrive - Massachusetts Institute of Technology" "./OneDrive -␣
↪Massachusetts Institute of Technology - old"
```

7. Login as the new user. It will ask you where to put the new OneDrive folder. **You have to select the D drive**, such that the OneDrive path is `D:\OneDrive - Massachusetts Institute of Technology`
8. Uncheck the backup syncing options (e.g. backup local documents / pictures).
9. Hit the Sync button in the web GUI for both the main OneDrive[13] and the timelapse OneDrive[14]. It should create a new `D:\Massachusetts Institute of Technology` folder that the `D:\data` symlink points at.
10. After confirming that the new user has the same synced view of the OneDrive, you can delete the `- old` folders you created in the above step.

[12] https://windirstat.net
[13] https://mitprod.sharepoint.com/sites/GallowayLab/Shared%20Documents/Forms/AllItems.aspx
[14] https://mitprod.sharepoint.com/sites/GallowayLab-Timelapse/Shared%20Documents/Forms/AllItems.aspx

1.10 Ordering

Important

Check with Katie which cost object to use if you are unsure. Too many charges can trigger an audit unnecessarily.

There are several ways to order/request new lab reagents. For all lab reagents, materials, etc., be sure to enter the information in Quartzy so that inventory can be tracked. (This excludes oligos and Addgene plasmids, everything else including Amazon orders should be tracked.) See the *receiving instructions* (page **??**) for proper tracking and storage of delivered items.

For a more comprehensive introduction to ordering in Coupa (buy-to-pay, or B2P), see this training series[15] from MIT Office of the Vice President for Finance. The finance office also has prepared a quick-guide document[16] that you can reference.

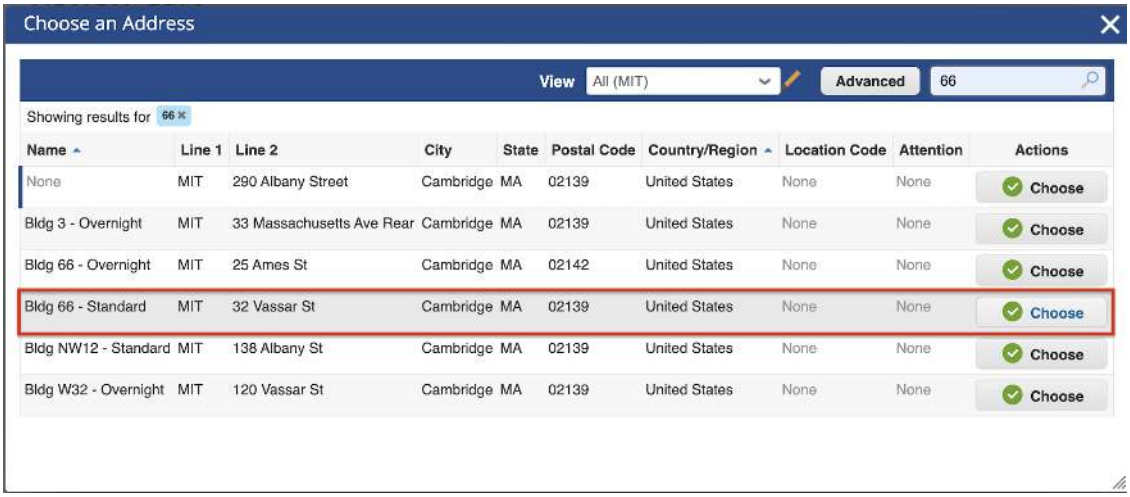
1.10.1 Direct ordering through Coupa

MIT's ordering system is accessible at https://mit.coupahost.com. After logging in, there is a large list of suppliers on the right that are available for "punchout", i.e. you can just add to cart and checkout inside Coupa.

On the Coupa checkout screen, fill out the following information:

Address

Under the 'Ship To' tab, click the magnifying glass. Search for Building 66 by typing '66' and select the standard delivery option. This is 32 Vassar St, the main receiving area for MIT deliveries.



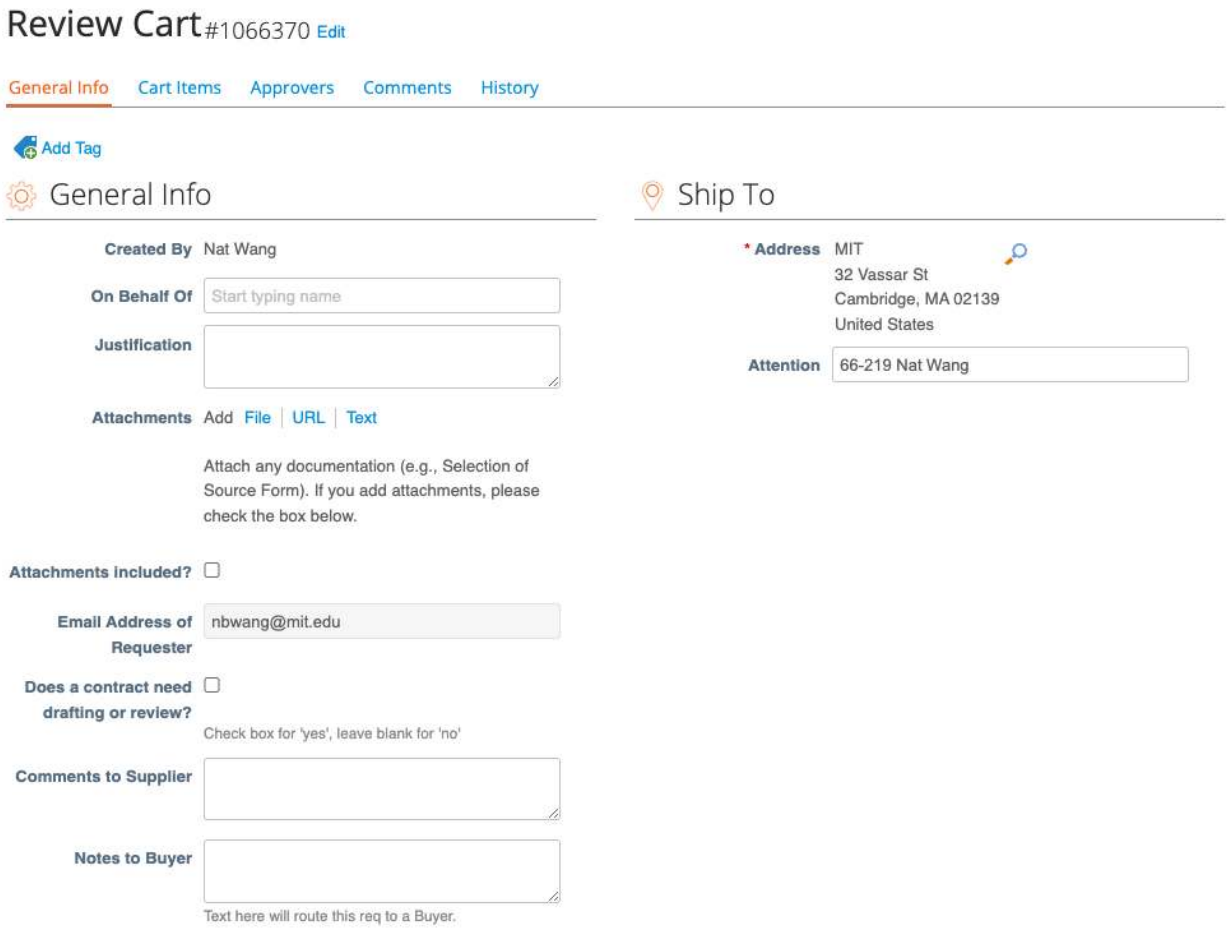
[15] https://vpf.mit.edu/buy-to-pay-b2p-training-series

[16] https://mitprod.sharepoint.com/:w:/s/GallowayLab/EaUDIQAoABlNgQVU6CKdbI0BTHDcVh3Q1q_cIBM6CRJa7w?e=OHAWtY

In the 'Attention' box, add lab address and your name, i.e., '66-219 – Your Name'.

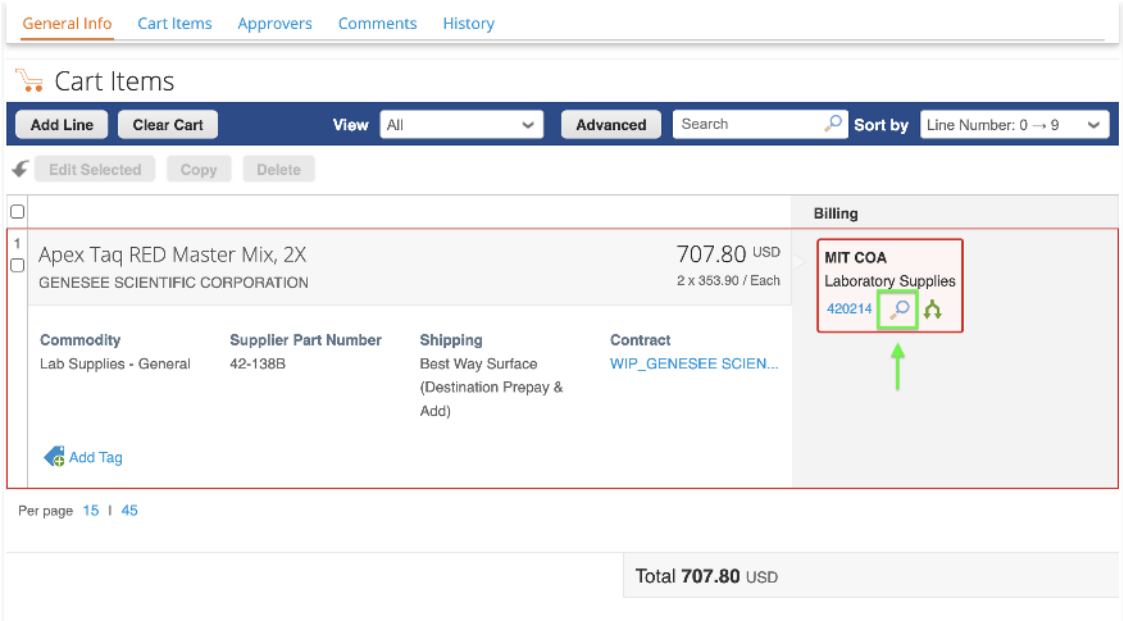
Important

Be sure to write the lab address first! Occasionally, Amazon deliveries will truncate this field.



Billing

For each item, choose the correct item type (e.g., General Lab Supplies) and click the magnifying glass to choose the PO to charge.



Tip

Almost all materials should be categorized as 'Laboratory Supplies', including office supplies (pens, notebooks, etc. — do NOT use the ‘Office Supplies’ category)

The PO can be found by searching 'Galloway' and selecting an option from the list (e.g., 'Start-Up Funds').

Important

Be sure to double-check with Katie if you don't know which account to use.

To apply the same cost object to multiple items:

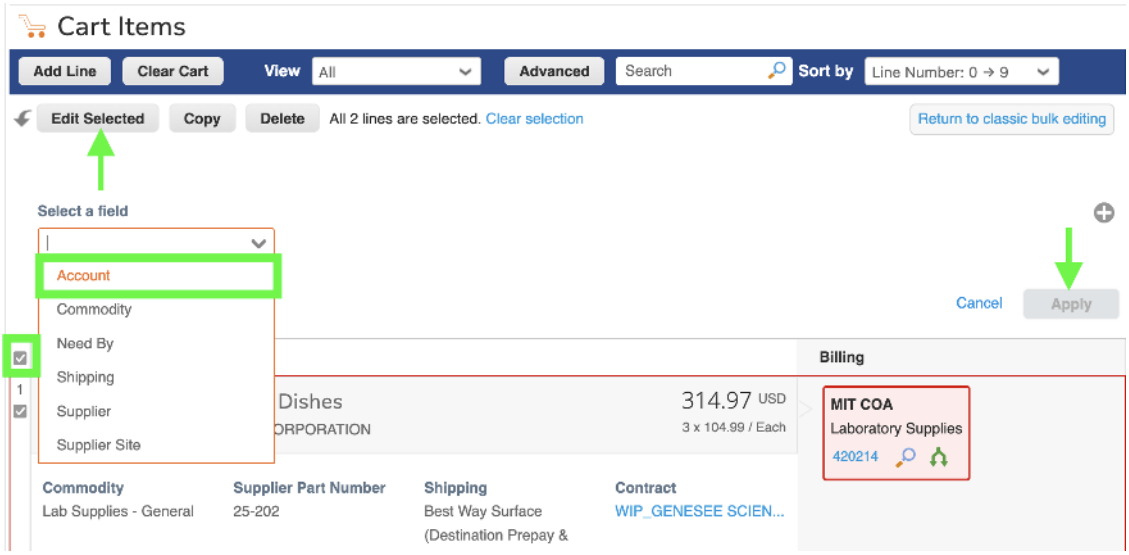
a. Select all using the checkbox in the upper left, or select relevant items individually

b. Click the 'Edit Selected' button

c. Choose 'Account' as the field from the dropdown menu

d. Choose the correct cost object, as described below

e. Click 'Apply' on the right to save the changes



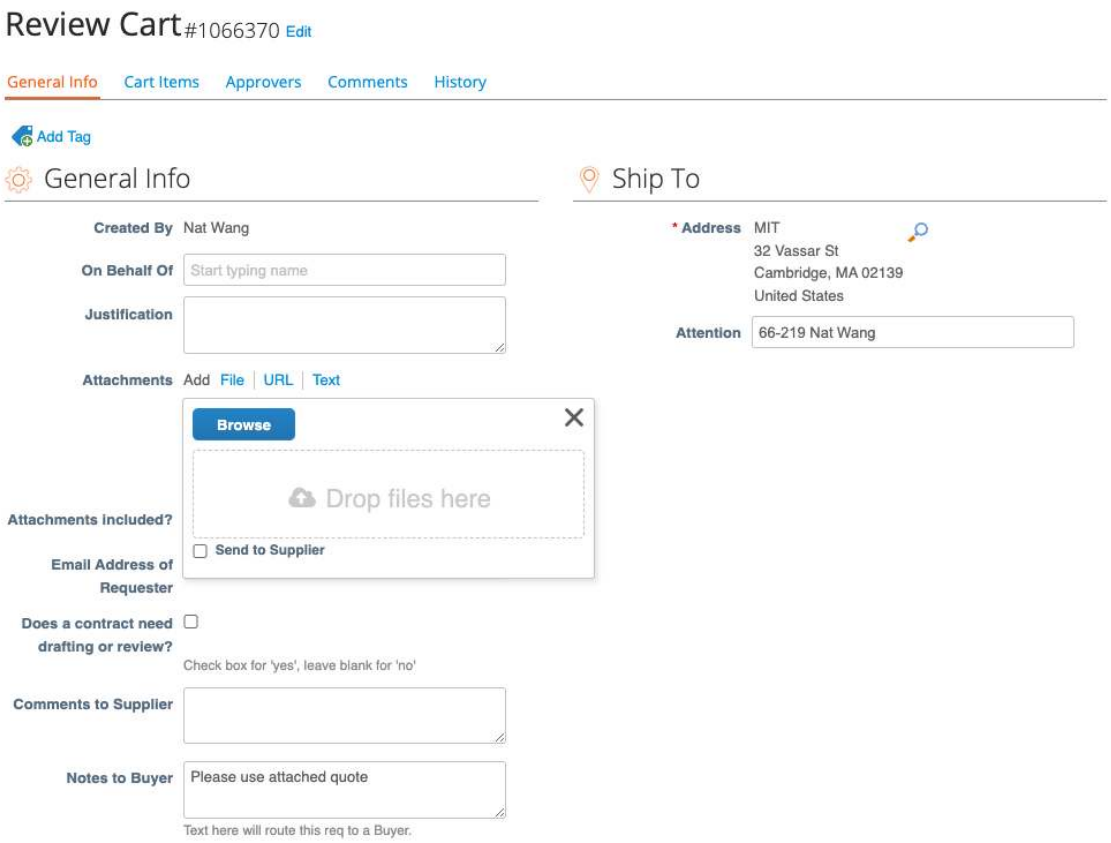
Approver

At the bottom of the page, double check that the Approver workflow is accurate (i.e., through ChemE) and submit the order.

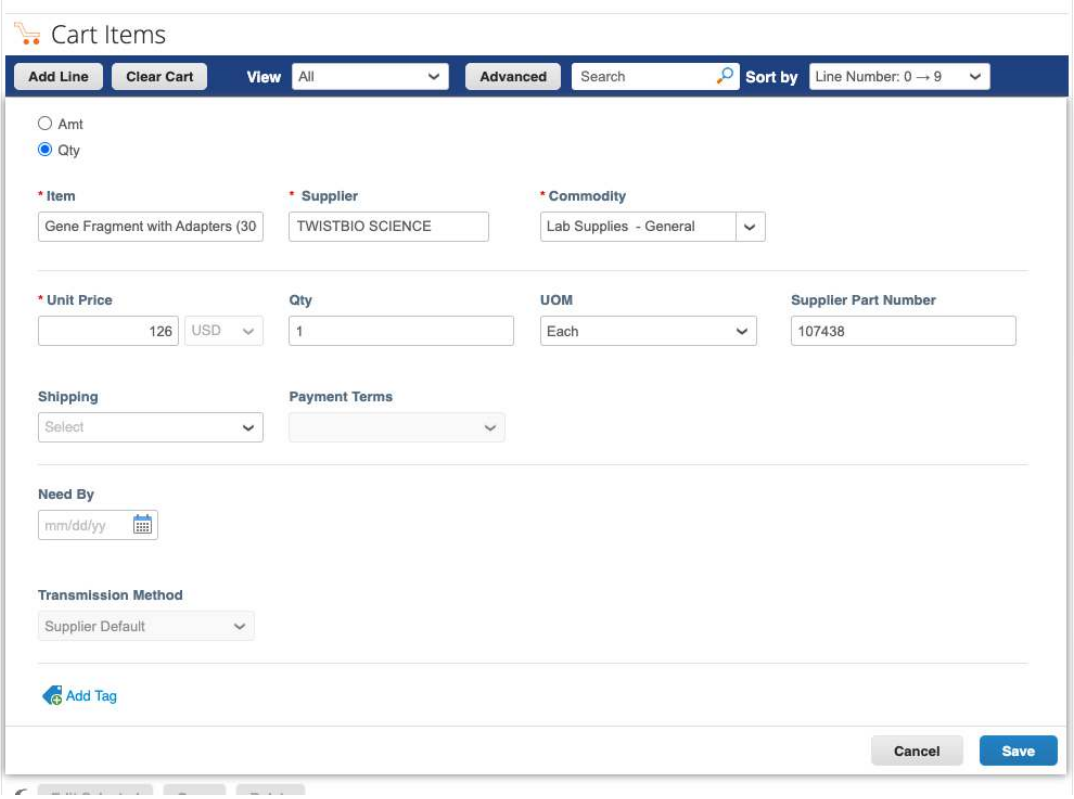
After placing the order through Coupa, be sure to add the items to Quartzy (see below). This helps organize the lab inventory and facilitates re-ordering.

1.10.2 Attaching a quote to a Coupa order

1. Navigate to the cart
2. Click `Add file` under Attachments
3. Drag the quote there
4. Add a buyer note to `Notes to buyer`: “Please use attached quote”



5. Manually add the item into the cart. Scroll down, click `Add line`, put in the info for your item and save


Cart Items
Add Line
Clear Cart
View
All
Advanced
Search
Sort by
Line Number: 0 → 9
Amt
Qty
* Item
Gene Fragment with Adapters (30
* Supplier
TWISTBIO SCIENCE
* Commodity
Lab Supplies - General
* Unit Price
126
USD
Qty
1
UOM
Each
Supplier Part Number
107438
Shipping
Select
Payment Terms
Need By
mm/dd/yy
Transmission Method
Supplier Default
Add Tag
Cancel
Save

1.10.3 Requests through Quartzy

There are three scenarios for adding requests to Quartzy: re-ordering an item in the inventory, making a new request for an item to be ordered, and adding an item already ordered through Coupa.

Tip

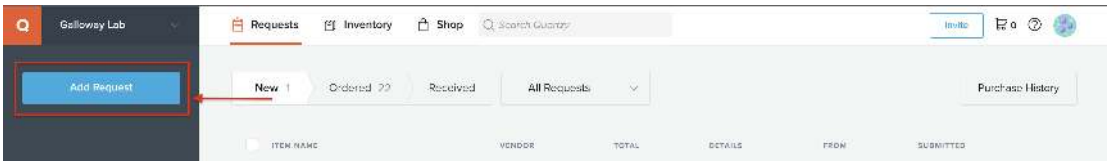
To check the current item price, search for the item from the relevant supplier in Coupa. Be sure to log in to see the discounted MIT prices!

Re-Ordering

To request an item already in the inventory, select the item and click on the 'Request' button. Double check that the catalog number, price, and quantity are correct, then click 'Request' to submit the request for Tseganesh to order.

New Requests

To request a new item, select the 'Requests' tab and click the 'Request' button in the top left corner of the page. Search for the item by Manufacturer and Catalog Number, and enter the correct price and quantity. There are two suppliers to choose from: the 'Quartzy Shop', in which Quartzy fulfills the order (can be cheaper but may experience delays), or the manufacturer directly. Then click 'Request' to submit the request for Tseganesh to order.

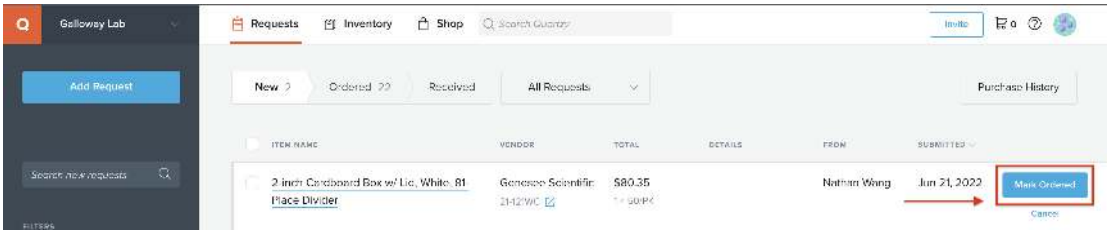


Important

> In most cases, we order materials directly from manufacturers/suppliers, NOT through the Quartzy Shop.

Adding Ordered Items

To add an item ordered directly through Coupa, follow the same process as for a new request. The item will appear in the 'New' section under the 'Requests' tab. Then, select the 'Mark Ordered' button to move the item to the 'Ordered' tab.



Then, add the PO and/or requisition numbers to the order to facilitate tracking.

1.10.4 Bulk Plastics Order

The following items are ordered in bulk from Genesee Scientific several times per year:

- Serological pipettes
- Cell culture plates, flasks, and dishes
- Syringe filters
- Barrier tips and reach tip reloads
- Reagent reservoirs
- Tubes (0.6mL 1.7mL, 15mL, 50mL, cyrovials, culture, PCR)
- Petri dishes
- Gloves

Information for the bulk order can be found in the `lab_jobs` folder in the Sharepoint[17]. To coordinate a bulk order:

1. Calculate the number of units needed for each item based on the previous bulk order, any extra items ordered on Quartzy, and expected usage (e.g., a busy upcoming summer).
2. Input this information into a new spreadsheet with the prices from the previous bulk order. Prices tend to increase annually, but this will be a good starting point.
3. Directly email this spreadsheet to the Genesee Scientific representative (Brad Sloan, as of January 2025) and request a quote; copy Tseganesh on this email so she can arrange the PO(s).
4. Once the order is placed, print the order spreadsheet and post it on the door to the atrium to track the plastics as they are delivered. Be sure to inform the lab so they can help track items.

1.11 Plasmid Database

1.11.1 Quartzy

The shared lab plasmid database is hosted on Quartzy[18]. Each item is assigned a five-digit `pKG` number, in order.

Tip

To make sure you add new plasmids with the correct next number, click the "Item Name" column header to sort by this field. (It should now have a downward arrow icon.) Alternatively, use the following URL: https://app.quartzy.com/groups/190392/inventory?sort=-name

To add a new plasmid to the database, click the the "Add Item" button in the top left corner of the Quartzy page. Then, click "Skip Lookup" in the top right of the pop-up that appears.

[17] https://mitprod.sharepoint.com/:f:/s/GallowayLab/EriJnL4G8UFBpwqUE48fWWUBn18cOK8C8pbRpWJOhlgWOQ?e=RiPRFm

[18] https://app.quartzy.com/groups/190392/inventory?sort=-name

Fill out fields as follows:

Field	Instructions
Vendor	Company that the plasmid was purchased from (e.g., Addgene)
Catalog #	Addgene number OR secondary plasmid name/ID (e.g., pGEEC100)
Item Name	`pKGNNNNN`, where `NNNNN` is the unique five-digit `pKG` number
Upload File	Upload the plasmid map
Type	Bacterial Stock
Date Stored	Date glycerol stock made
Plasmid	Short description of construct (e.g., pLentiX1-EF1a-mGL-bGH)
pKG#	`NNNNN`
Plasmid type	Select from options
Resistance markers	Select from options
Species	Bacterial cell type (e.g., NEB Stable)

Then, click the "Add Item" button in the lower left corner to add the item to the database.

Warning

Double check that all the fields are correct, especially "Item Name", "pKG#", and "Upload File"! These are the most common sources of entry errors, particularly when adding a many plasmids at the same time.

Tip

If the file upload fails, it is likely that the file is too large. The easiest way to fix this is to trim the history tree in SnapGene ('History' tab -> 'Edit History' -> 'Trim History Tree', or 'File' -> 'Batch trim file histories' to select several at once).

1.11.2 Plasmid website

In addition to hosting the database on Quartzy, we also export plasmid information to a separate website[19]. This way, we can group plasmids by alternate names, search plasmid names more easily, and add error/warning flags to improperly formatted entries.

The website displays plasmids with errors/warnings by `pKG` number, but it is often useful to group these entries by user so that the errors/warnings can be fixed. This list can be generated from the same project that builds the website: https://github.com/GallowayLabMIT/plasmids

1. Clone the git repo, then create and activate a virtual environment. See *Startup checklist when working with repositories* (page **??**) for instructions.
2. Create a file called `credentials.json` in the main directory of the repo. This should contain Quartzy login information in the following format:

```
{
    "username": "your-username",
    "password": "your-password"
}
```

3. In the terminal, run `python -m quartzy_parser` to print a list of `pKG` numbers with errors and warnings by user.
    - To print only errors or only warnings, add the flag `--only-errors` or `--only-warnings`, respectively.
    - To print only for a subset of users, add the flag `--users` followed by the list of users (full names).
    - The numbers printed are the values in the `pKG` field, not the "Item Name" field.

Note

This code takes a while (~5 min) to run, since it has to scrape thousands of items from Quartzy.

1.12 Receiving packages

Packages are usually delivered to the atrium (lab entrance near 66-219), or occasionally to the lab office. When a package arrives, do all of the following:

1. Find the packing slip or open the package to determine the contents of the item.

Important

It is important to immediately determine whether the item is temperature sensitive, so that it can be properly stored. Thus, receive all packages promptly!

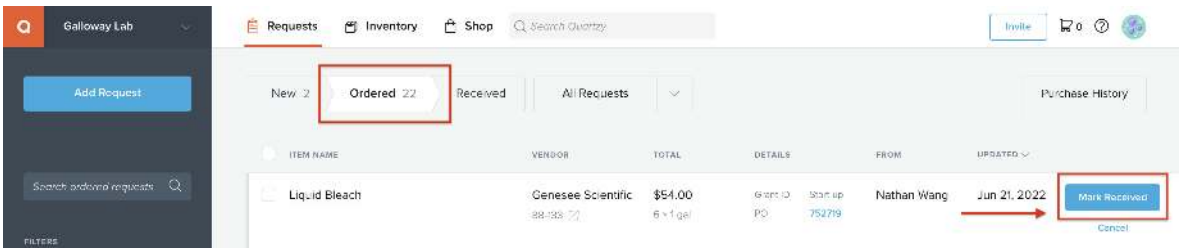
2. If you know where the item is stored, put it away properly.

[19] https://gallowaylabmit.github.io/plasmids/en/latest/

3. If you don't know where the item is stored, determine whether the item is temperature sensitive.
    - **If the package is temperature-sensitive, open the package immediately** and store it in an appropriate fridge/freezer:

Temperature	Type	Location
4°C	Cloning	Kristoff (deli fridge)
4°C	TC	Oaken
-20°C	Cloning	Anna
-20°C	TC	Olaf, top shelf
-80°C	Cloning	Elsa, bottom shelf
-80°C	TC	Nokk, top shelf

    - For items that are not temperature sensitive, leave the package on the recipient's lab bench.
4. If the item appears urgent (e.g., temperature-sensitive items) or is not a common lab item, message the recipient that you received the package and indicate where you put the item.
5. Place the packing slip in the box in the atrium near 66-219. This helps us diligently track received items for financial record-keeping and alerts us to inquire about missing packages.
6. Mark the item as received on Quartzy. To do so, navigate to the 'Ordered' section under the 'Requests' tab and click the 'Mark Received' button.

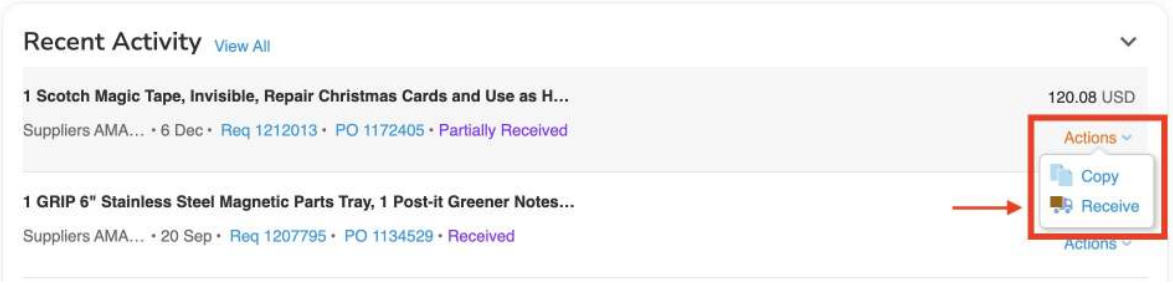


Note that you can add comments to an order or mark the order as 'Partially Recevied' if the packing slip doesn't match the Quartzy request.

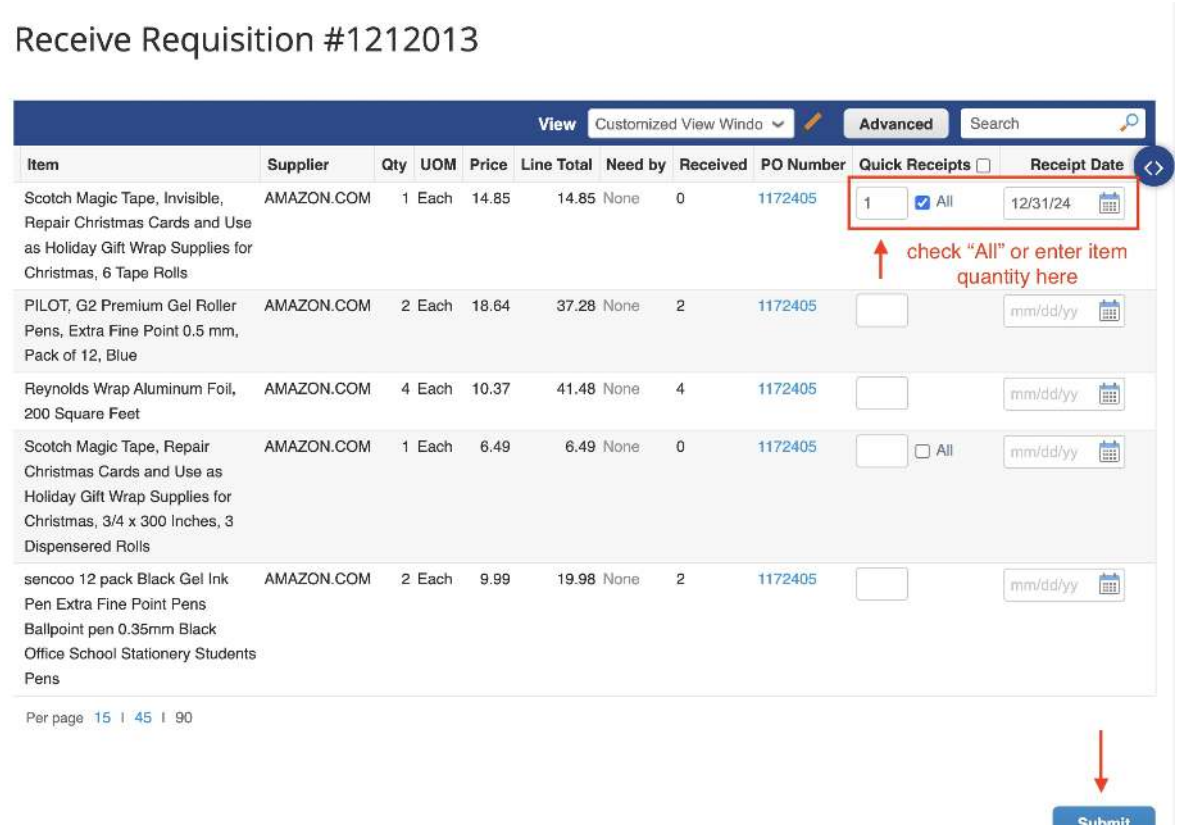
1.12.1 Receiving orders in Coupa

If you ordered items through Coupa (punch-out or manually), you need to personally mark those items as received in Coupa. When the package arrives, follow the steps above to receive the item physically and in Quartzy, then additionally check it off in Coupa.

1. Log in to Coupa. On the home screen, you should see your orders under 'Recent Activity'.
2. For the relevant order, under 'Actions' select 'Receive'.



3. For each item, enter the quantity received (or check 'All') and the receipt date, then click 'Submit'.



1.13 Recycling

MIT has several recycling programs for various lab plastics, packaging, and so on. The up to date Facilities information site[20] should be checked for details.

Currently, we recycle the following items in the following ways:

- **Cardboard**: break down the box and place it in/near a blue recycling bin, especially the one in the atrium.
- **Hard plastics #1, #2, #5**: place in a blue recycling bin.

[20] https://web.mit.edu/recycling

- **Pipette tip boxes and conical racks**: place in the specific pipette tip/rack collection box. We schedule pickups through this Quickbase link[21]. This box goes to a local startup who recycles these.
- **Styrofoam**: Leave clean coolers/boxes/lids made of Styrofoam **next to** a blue recycling bin. Bag Styrofoam into clear plastic bags for pickup.
- **Film plastic**: Collect film plastics (e.g., palette wrap, the plastic around filter tips, any other stretchy & clear film) *into the labeled bin in main TC, not into the standard blue recycling bins*. The blue recycling bins go to the single-stream recycling provider, and films can gum up those machines. We can move our film to the plastic bag recycling in the basement room (66-017).

> **Important**
>
> Film plastic must not contain paper of any kind, including stickers. Sticker labels should be cut/ripped out before placing the plastic in the bin.

We do NOT recycle the following:

- Anything bio- or chemically-contaminated.
- Gloves, even if they are clean.

1.14 Remote desktop for lab computers

It is sometimes convenient to be able to remote into lab computers, such as as when a microscope computer is running a timelapse.

At the same time, it is **not recommended** to use software such as TeamViewer to enable remote access. In addition to being a company with a bad security record, TeamViewer and similar software open you up to the entire internet.

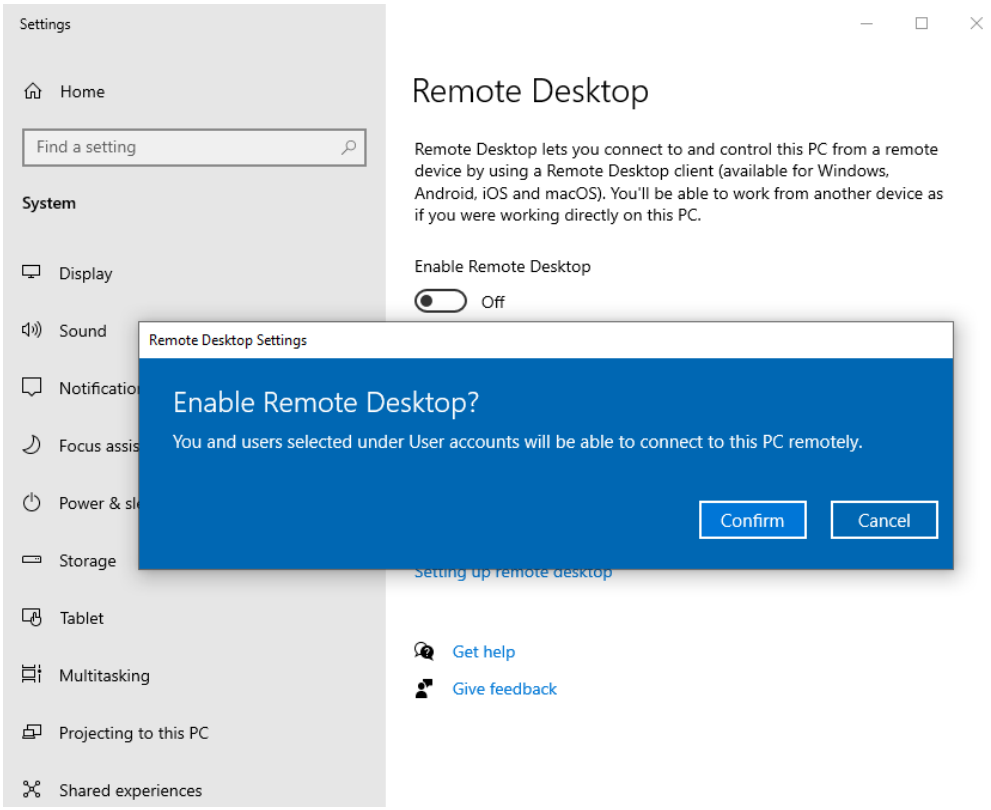
The better solution is to use the Remote Desktop Protocol (RDP) built into Windows, and use the MIT VPN if you are off-campus to keep access to the computers secure.

1.14.1 Enabling remote desktop

> **Important**
>
> Only keep the remote desktop setting active while you are expecting to use it. When you are done, turn it off.

1. Hit the Windows key or click the start menu search. Search for “Remote desktop settings”.
2. Enable remote desktop by clicking on the switch. It will pop up a modal to confirm this action.

[21] https://mit.quickbase.com/db/bq2rx8ncp?a=nwr



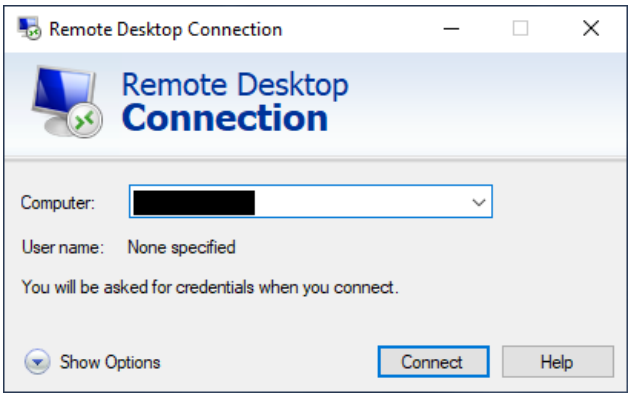
3. Verify that the default setting “Keep my PC awake for connections when it is plugged in” is set.
4. Setup your timelapse, etc. At this point, the computer is accessible if you are either on the MIT network or using the VPN (if off-campus), but from nowhere else on the internet.
5. Check the note card on the physical computer for server name / username / password information.
6. When done, go back to “Remote desktop settings” and disable the switch.

1.14.2 Remoting in to lab computers

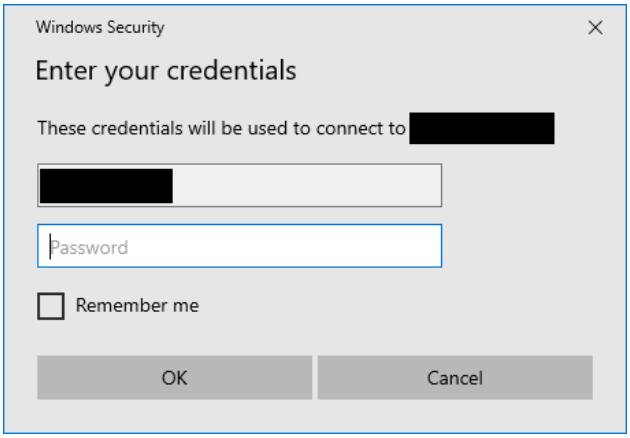
Instructions vary from operating system to operating system.

Windows

1. If you are not plugged in over Ethernet at MIT / using MIT Secure, connect to the MIT VPN.
2. Hit the Windows key or click the start menu search. Search for "Remote desktop connection".
3. Enter the full computer domain name (from the notecard on the computer) into the box.

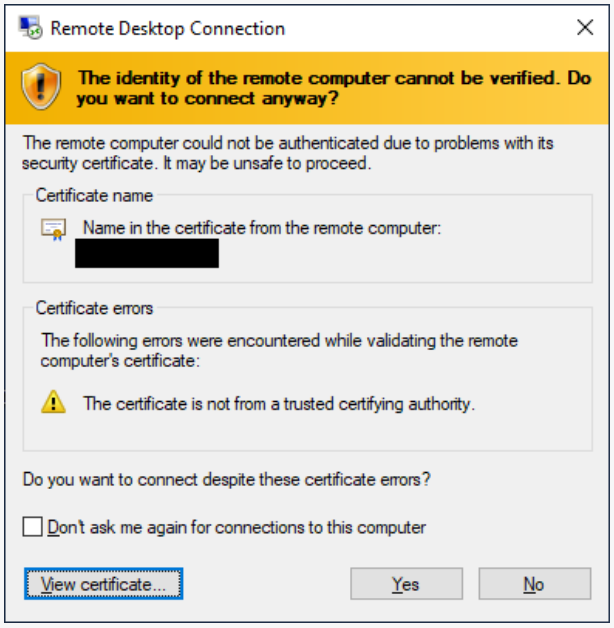


4. When prompted for credentials, use the username and password from the notecard.



5. If there is a certificate warning, you can accept it because we are connected through MIT's network.

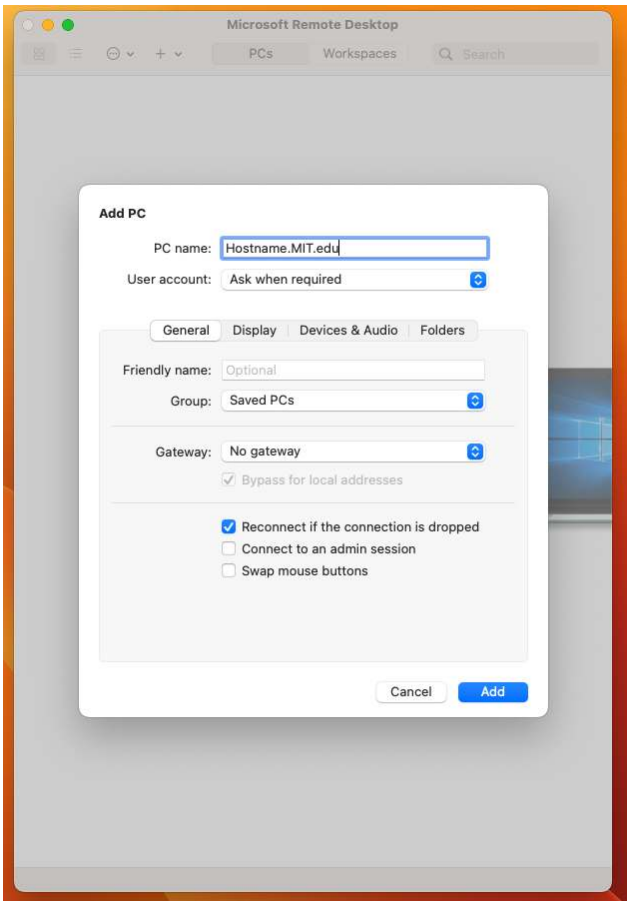
Note



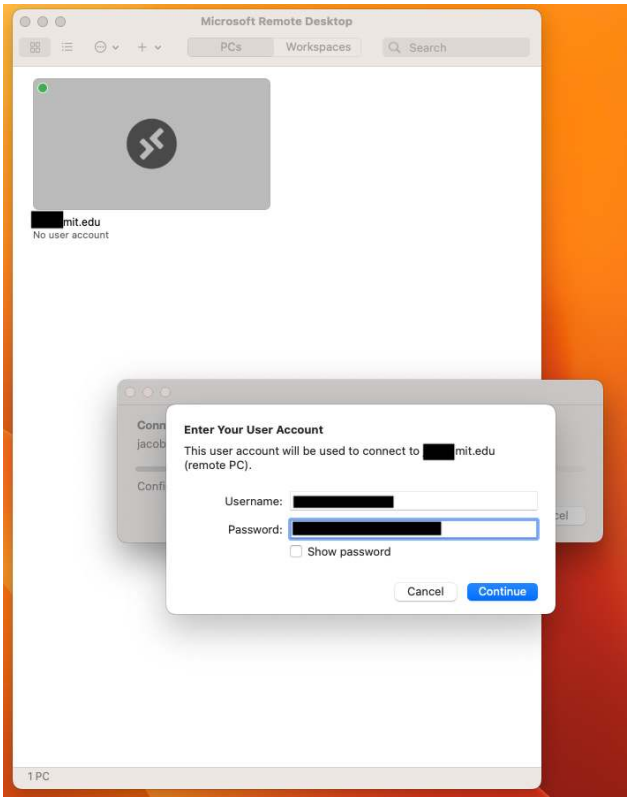
The certificate warning comes from the fact that we are using full domain names, but the computers aren't publicly accessible at that domain name, so can't get something like a Lets Encrypt certificate.

MacOS

1. Go to the App Store and download "Microsoft Remote Desktop" app.
2. If you are not plugged in over Ethernet at MIT / using MIT Secure, connect to the MIT VPN.
3. Hit the plus sign and "Add a PC", and enter the full host name / domain name from the notecard:



4. Double click the connection tile to connect. When prompted for credentials, use the username and password from the notecard.



5. If there is a certificate warning, you can accept it because we are connected through MIT's network.
6. Hit `command-q` to exit the remote desktop connection when done.

Linux

FreeRDP works great; look up from your distro instructions how to install the client this (`apt install xfreerdp`)

The command you want to run is

```
$ xfreerdp /u:"USERNAME" /v:HOSTNAME.mit.edu
```

1.15 Deli fridge starter replacement

When the deli fridge refuses to start, it is possibly due to the starter components. The starter components are responsible for getting the initial burst of energy needed to turn over the compressor motor. Luckily, both of these parts are easy to replace!

1.15.1 Background

The starter consists of two parts:

1. A current-sensitive starter relay: this video[22] discusses the details, but this is effectively a relay that turns on when it senses current above a certain threshold, entirely mechanically.
2. A starter capacitor: this is an extra energy store that the starter relay switches on, and provides enough energy to turn on the compressor.

We can reorder the parts for relatively cheap:

- A Danfoss/Kemet 117U5023 240V capacitor[23], $23.49 from Zoro. The capacitor fails by not having the correct capacitance anymore (e.g. through dielectric breakdown or other effects).
- An additional capacitor that is available to try is the Dayton E224674 Motor Start Capacitor, 216-259 MFD, Round[24], $24.39 from Zoro. This is a larger capacitor than the previous and could be used as an additional replacement. Lead wires are not attached to this capacitor and must be done so separately.
- Lead wires for the above capacitor, Dayton Capacitor Jumper Wire Set[25], $18.65 from Zoro.
- A Danfoss 117U7020 current-starting relay[26], $36.19 from Zoro. The relay fails when the contacts begin getting pitted / arcing and either the resistance is too high or it does not properly switch.

[22] https://www.youtube.com/watch?v=PRq1WPH1sRg
[23] https://www.zoro.com/danfoss-capac-240-mfd-125v-round-wbkt-117u5023/i/G4044761/
[24] https://www.zoro.com/dayton-motor-start-capacitor-216-259-mfd-round-2met8/i/G2244356/#specifications
[25] https://www.zoro.com/dayton-capacitor-jumper-wire-set-6-in-pk10-2mev9/i/G2684324/
[26] https://www.zoro.com/beverage-air-relay-danfoss-117u7020-for-312155d-comp-314-075d/i/G806895395/

1.15.2 Disassembly

1. Unplug the deli fridge from the wall. Wait about 5 minutes for capacitors to discharge.
2. Take the front grill off. It just pops off if you pull it horizontally out.
3. Using a **plastic-handled screwdriver, holding on using only the plastic** and after **ensuring the power is off**, fully discharge the capacitor by using the metal part of the screwdriver to briefly connect the two capacitor leads. There might be a spark or popping noise; this should be fine.

Fig. 13: Lightly touch a metal screwdriver between these contacts while only touching the plastic part of the screwdriver.

4. Loosen the capacitor from the plastic bracket. This plastic piece acts like a ski/snowboarding binding; you can lift up on the end to release it.
5. Find the two, two-input Wago lever nuts that connect to the relay. One should be connecting a pair of white wires, and one should be connecting a pair of black wires. Open the lever and remove the wires that lead to the relay.

Note

The starter relay and capacitor are wired into the rest of the system using something called Wago lever nuts[27].

A spring is compressed when you open the levers. You should be able to open the levers until they are perpendicular to the incoming wires.

4. Pull the relay off of the motor, taking the capacitor with it. There are three pins that go into the back of the relay, so you just pull out horizontally.

[27] https://www.youtube.com/watch?v=n8gLG6c-iKc

Fig. 14: The lever nuts are in the foreground, in their engaged position. You only need to lift the lever on the one wire we are removing for each lever nut.

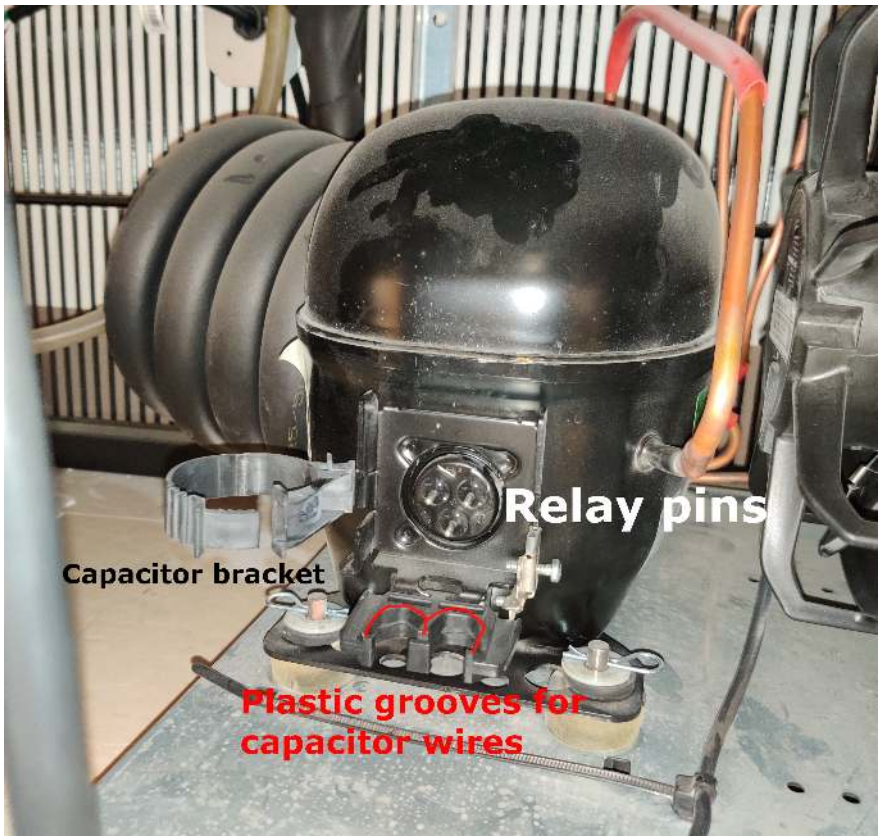


Fig. 15: The motor after removing the starter components.

1.15.3 Debugging

You don't need to disconnect any wires between the capacitor and relay to test it.

1. Check if the relay is bad or not. The relay switches via a metal piece that gets pulled up into the electromagnet. There is no spring, so testing this is as easy as inverting the relay. Attaching a multimeter in resistance mode, it should be an open circuit when the relay is right-side-up and a low resistance around 1 ohm when it is inverted.

Note

The following shows a successful test of a relay. In the up position, the contacts are not connected (OL means an open connection). In the upside down position, the resistance is low, under one ohm.

2. Optionally check if the capacitor is bad or not. This is more difficult to test unless you have a multimeter with a capacitance mode. The resistance over the capacitor leads should read around zero, but it will also likely read near zero if the capacitor is bad.

If the relay resistance is fine and switches when you invert it, then try swapping the capacitor.

1.15.4 Assembly

1. After deciding which part to replace (possibly both!) connect the replacement capacitor and relay by using the existing connectors. This should not require any wire stripping; just reuse the wires with the push-on connectors. Assemble it as in the picture. It is **important which terminals you connect the white and black wires to**, but it does not matter which orientation you attach the capacitor (it's an AC capacitor so no polarity).

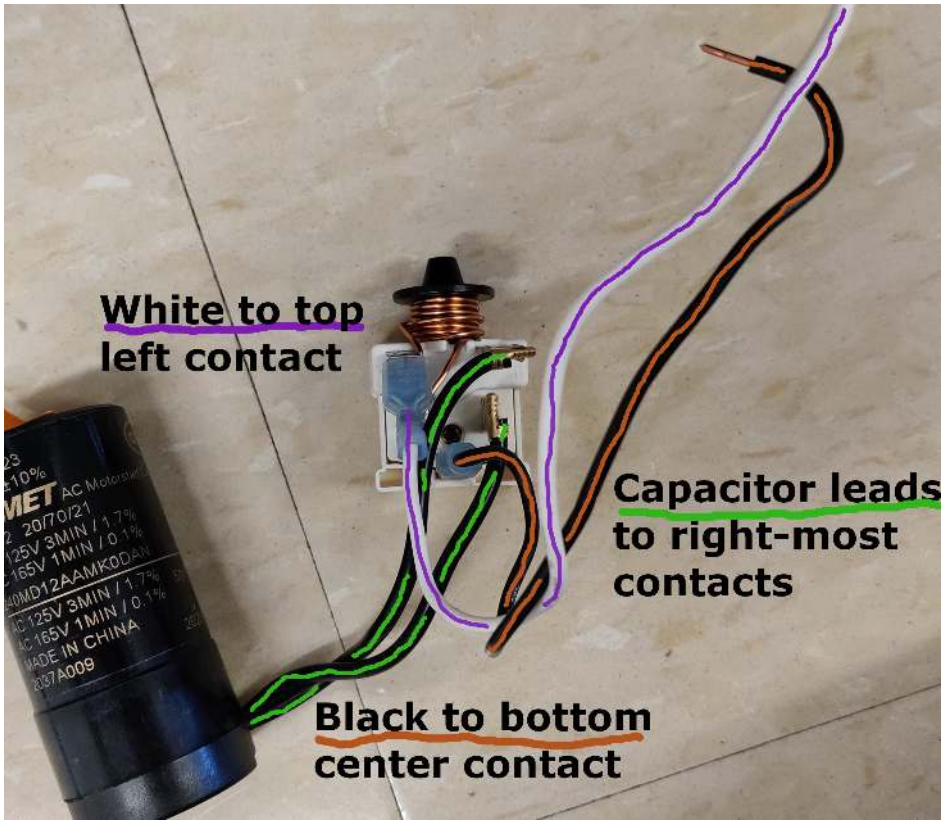


Fig. 16: The correct connections are shown.

2. After ensuring that the deli fridge is unplugged, take the capacitor/relay assembly and secure the capacitor by sliding it into the existing holder and latching it.
3. Align the three holes on the back of the relay and lightly push it onto the motor. Ensure that the copper/black plastic "hat" is pointing up. The wires leading to the capacitor route through the grooves in the plastic beneath.
4. Ensure that the green wire is plugged into the terminal to the lower right of the relay. This ensures that the metal of the deli fridge is safely grounded.
5. Reattach the stripped ends of the white and black wires to the Wago lever nuts, **matching white to white and black to black**. Make sure all levers are in the down position and the wires are securely attached.

Fig. 17: Properly reassembled starter components

1.15.5 Replacements:

- 2023.11.10 - Capacitor
- 2024.06.24 - Capacitor
- 2025.02.20 - Capacitor
- 2025.09.06 - Capacitor

1.16 Replacing shake incubator drive belts

Note

If the shake incubator reports that it is actively shaking but isn't, its drive belt may need to be replaced. Top shake incubator drive belt replaced by CJ and MC 7/17/2025.

1.16.1 Required McMaster parts

Part	McMaster part number
Ultra-Flexible Banded V-Belt 28-5/16" Outer Circumference	9003K47[28]
Stainless Steel Hex Drive Flat Head Screw	92210A622[29]

[28] https://www.mcmaster.com/catalog/131/1348/9003K47
[29] https://www.mcmaster.com/catalog/131/3487/92210A622

1.16.2 Procedure

1. Buy more McMaster parts if needed. We should have extras of most in the cabinet with the toolbox.
2. Acquire tools from toolbox:
    - ?? sized hex key
    - 14mm deep socket
    - socket extender
    - ratchet
3. Remove top cover.
4. Use hex key to undo countersunk screws.
5. Use ratchet to remove all of the nuts and remove counter weight.
6. Undo electrical connector and lift out motor assembly.

Warning

The motor assembly is very heavy. Ideally, two people should remove it.

7. Remove drive belt from underneath motor assembly and replace with new drive belt. May need to loosen screws holding motor in place in order to get the drive belt around it.
8. Place motor assembly back in incubator, ensuring power supply is threaded underneath motor assembly and through the hole near the front, beside the power connection. Plug in power.

9. Secure the motor assembly with the washer + lock washer + nut 14mm combination shown below.

10. Place counter weight back in and turn on to confirm the incubator now shakes.

Warning

Do not turn incubator on without counterweight in place; it will unbalanced and possibly cause damage.

11. Turn off the incubator and replace the top cover.

1.17 Shipping

As of June 30, 2026, MIT has a new policy for shipping materials on behalf of MIT. This includes sending reagents like plasmids or cells to collaborators.

We are now required to do the following:

Fig. 18: Top cover

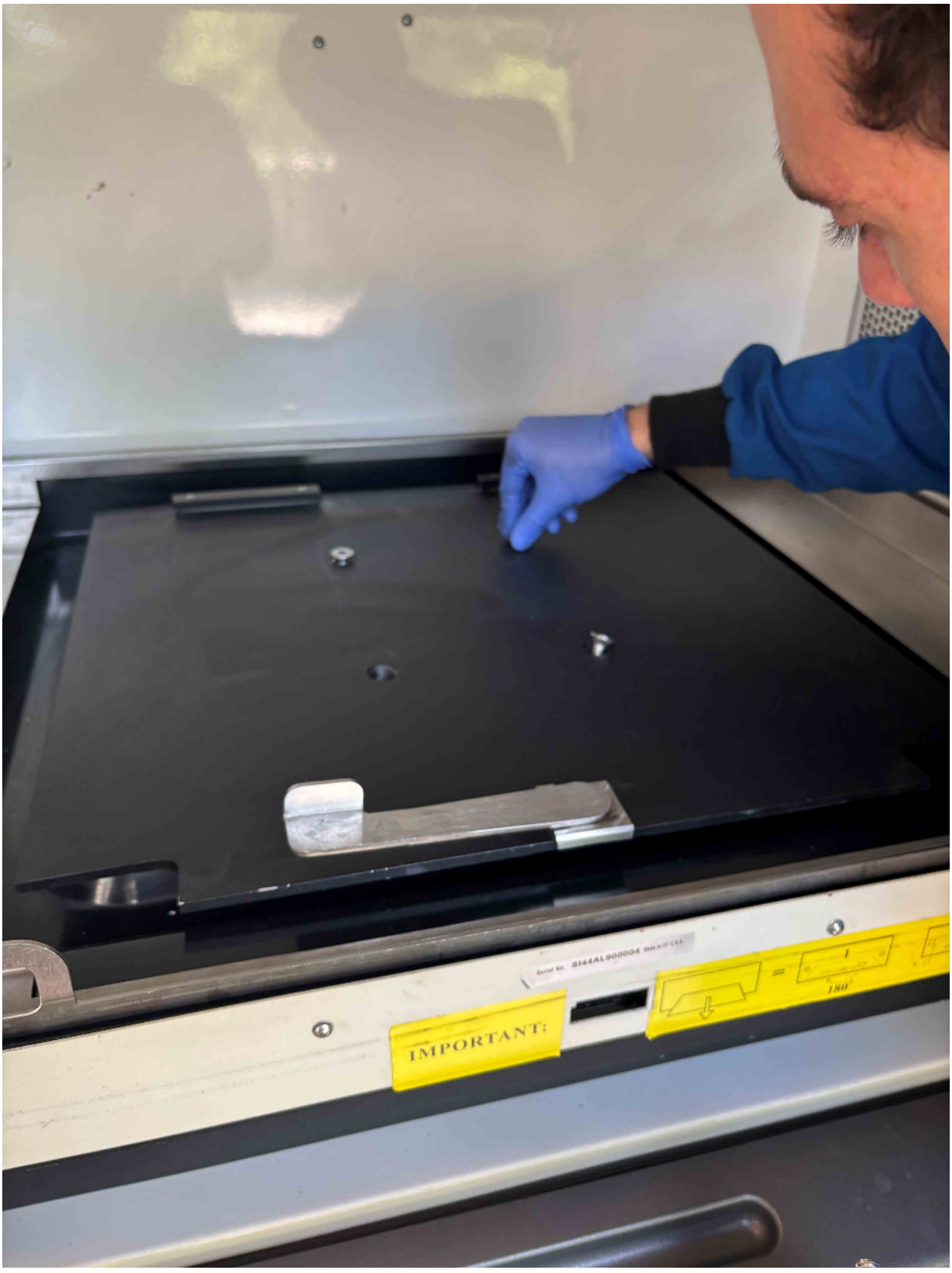


Fig. 19: Counter weight. Screws pictured correspond to McMaster part number 92210A622.

Fig. 20: Motor assembly

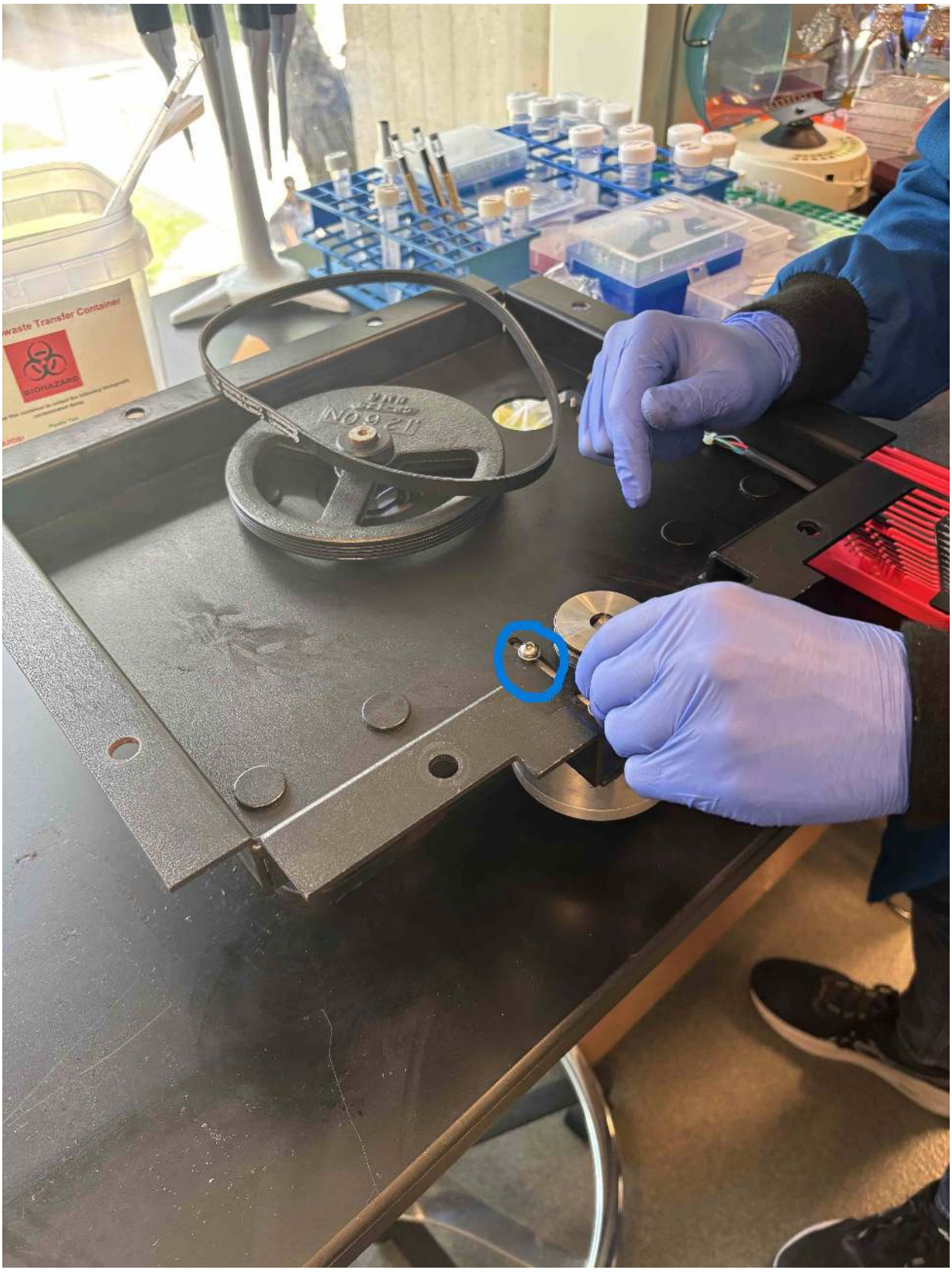


Fig. 21: May need to loosen screws circled in blue and move motor a little closer to rotator in order to get the belt around.

Fig. 22: Place new drive belt around rotator and motor; be sure to adjust screws again to ensure the belt is taught.

Fig. 23: Note the 3-3-2-pronged orientation that must match when being plugged in.

1. Complete the Shipping Training[30] on Atlas.
2. Use eShipGlobal[31] for all shipments involving **research materials** (e.g., biological samples, but *not* documents or items like mugs) and **all international** shipments.

See the full guidelines here[32].

[30] https://us.list-manage.com/x50viiGiYi6?e=3aa52f1b11&c2id=e3a0eacb0edf1dd8510d3cfd3e0194a2
[31] https://us.list-manage.com/k6mf9RaMqB1?e=3aa52f1b11&c2id=e3a0eacb0edf1dd8510d3cfd3e0194a2
[32] https://procurement.mit.edu/mit-shipping-policy#guide

CHAPTER

TWO

INSTRUMENT USE AND CORE FACILITY ACCESS

2.1 Attune operation

2.1.1 Attune startup and shutdown

Quick startup checklist

1. Make sure fluid bottles are full except for waste bottles. If necessary, empty waste bottle and refill with 10% bleach (100 mL for Attune, 200 mL for Cytkick). Be sure to wipe off bottles with any exterior liquid.
2. Fluid lines are all plugged in correctly (especially Cytkick autosampler waste!! should hear double click)
3. Log in and run start up procedure
    a. If the Attune has not been run for > 3 days, run another startup procedure to further flush the lines (don't shutdown, just immediately hit startup again).
    b. If the Attune has not been run for > 1 week, run Debubble with the debubble solution.
4. Run performance test
    a. Make sure optical filter config is correct (no red-stripes, order is correct)
    b. Verify bead lot number
    c. Put 3 drops of performance beads in 2 mL of focusing fluids and mix (should be ~100 ev/s at first)
    d. If Delta PMT is high (>10-20), check Attune maintenance guide pg. 41
5. Run SIP sanitize to wash beads out

Quick shutdown checklist

1. Place a clean 96 well plate into the autosampler (or reuse an old plate with no salt crusts - remove with water/toothpick if there is).
2. Empty the waste containers and refill with 10% bleach (100 mL for Attune, 200 mL for Cytkick).
3. Refill the shutdown and wash solution bottles if needed.

4. Fill a clean tube with **3 mL of 10% bleach** and load it into the tube lifter.
5. Run the following depending on how quickly the next user will be using it:
    a. (**DEFAULT**) If next user will be using it > 2 hr, run Shutdown - standard (40 min)
    b. If next user will be using it < 2 hr, run Deep Clean - quick (20 min)
    c. If last user of the day: i. if there is a user scheduled for the next morning, start a SIP sanitize with Hellmanex. 3. Once the script is running, please pay attention to the volume in the tube. The volume will go down and then up 3X as the fluid is being aspirated into the flowcell. Once you see the volume in the tube decrease the third time, power off the Attune from the back (both main machine and autosampler). ii. If there is no user scheduled for the next morning, run SIP sanitize with Hellmanex, then run Shutdown - thorough (60 min)
6. Log out of the software but do **not** close the software or log out of the INSTR-ADMIN Windows user or shutdown will fail.

Initial inspection and startup

Estimated time
30 minutes

1. Visually inspect the Attune.
    a. Check the main fluidics syringe for the presence of a salt ring.

Fig. 1: A normal syringe without salt ring. The salt ring would be visible at the bottom of the syringe travel, e.g. near the printed label.

    b. Check the internal and external fluid bottles for any leaks or salt residue. Clean up any salt residue with ethanol and a Kimwipe.
    c. Check that other components are in good order (focusing fluid filters, SIP, etc).

Warning

Always grip fluid bottle and data connections by the connector, e.g. as close to the connection as possible. This prevents stress on both the fluid and data lines!

2. Check that all internal fluidics bottles except waste are reasonably full.

    a. If the waste bottle needs to be emptied, empty it into the sink, and refill with **100 mL of 10% bleach**. See *Attune operation* (page **??**) for definition of 10% bleach.

    b. The focusing fluid bottle can be refilled by **carefully** moving the focusing fluid bottle forward just enough to be able to take the cap off, without disconnecting the fluid or data lines.

Fig. 2: The focusing fluid bottle can be moved slightly out for refilling without stressing the fluid or data line.

    c. If disconnecting fluid bottles, the connectors have disconnect "buttons" that must be pressed before disconnecting the bottle. After pushing the disconnect button, carefully remove the line, gripping at the **connector**, not the fluid line itself to prevent putting stress on the system.

    d. If a fluid line does not reconnect with a click sound, do not force the connection. Check to make sure the instrument-side connector has not been tripped. If it has, press down on the top connector button to reset.

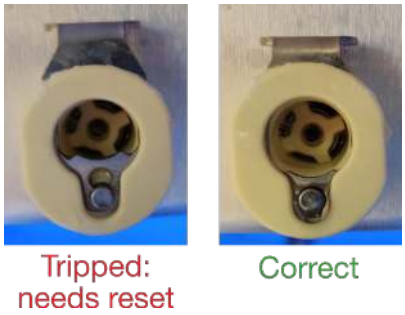


Fig. 3: Visual appearance of tripped and normal connectors.

3. Check that the autosampler focusing fluid bottle is full and the waste bottle is empty.

   a. The focusing fluid bottle has a single click lock.
   b. The waste fluid bottle requires a double “click” for locking purposes.
   c. After emptying the waste bottle, fill with **200 mL of 10% bleach**.
4. If the instrument is off, turn on the Cytkick first, wait ten seconds, then turn on the Attune.
5. Remove all plates from the Cytkick.
6. Login to the **INSTR-ADMIN** user, start the software, then login to your specific software user.
7. Run the startup procedure.
   a. If the Attune has not been run for > 3 days, run another startup procedure to further flush the lines.
   b. If the Attune has not been run for > 1 week, run Debubble with the debubble solution.

Performance test

Estimated time

10 minutes, run before the first flow run of the day.

1. Open the top of the Attune, to check the optical filter setup. Replace any red-stripe filters with their stock filters.
2. Click the performance test button in software.
3. Verify the bead lot # of the performance beads.
4. Add 3 full drops of performance beads to a new, clean tube. Dilute with 2 mL of focusing fluid.
5. Run the performance test. In the first part of the performance test, the events per second should be near 100 ev/sec.
6. After performance test, wash the beads out of the sample loop by running **SIP sanitize**.

Delta PMT	Status
> 10	Run Deep Clean
> 20	Indicates an issue
> 50	Performance will fail

Important

The bead lot number changes roughly every four years and requires a new set of parameter files to be downloaded.

Bead lot changes also requires a new baseline reference to be set.

Instructions for the install/baseline reference and bead lot data files can both be downloaded from ThermoFisher[33].

Shutdown

Estimated time

30 minutes total, ~2 minutes hands-on time.

1. Place a clean 96 well plate into the autosampler. If reusing a shutdown plate, make sure there are no salt crusts in wells; if so, they can be removed with a toothpick and water.
2. Empty the waste containers and refill with 10% bleach (100 mL for Attune, 200 mL for Cytkick).
3. Refill the shutdown and wash solution bottles if needed.
4. Fill a clean tube with **3 mL of 10% bleach** and load it into the tube lifter.
5. Run the following depending on how quickly the next user will be using it:
    a. If next user will be using it < 2 hr, run Deep Clean - quick (10 min)
    b. (**DEFAULT**) If next user will be using it > 2 hr, run Shutdown - standard (40 min)
    c. If last user of the day, run SIP sanitize with Hellmanex, then run Shutdown - thorough (60 min)
6. Once the shutdown is started, you can log out of the software (but do *not* close the software or log out of the INSTR-ADMIN Windows user!).

Note

Hellmanex III is the generic version of the very expensive Attune cleaning solution. The Fisher Scientific[34] version is diluted at a 1:2 ratio.

[33] https://www.thermofisher.com/order/catalog/product/4449754
[34] https://www.fishersci.com/shop/products/fisherbrand-hellmanex-iii-liquid-cleaning-concentrate/14385864

2.1.2 Attune operation

Note

"10% bleach" refers to a final concentration of 0.525% sodium hypochlorite (5250ppm chlorine). Use ELGA water instead of DI water to dilute the bleach.

This was originally a 10x dilution of 5.25% sodium hypochlorite bleach, but in lab we typically stock higher concentration bleach.

Concentration	Dilution	Vol bleach (100mL)	Vol ELGA water (100mL)
5.25%	1:9	10 mL	90 mL
6%	1:10.4	8.8 mL	91.2 mL
8.25%	1:14.7	6.4 mL	93.6 mL

Procedure descriptions

Through the **instrument tab**, several different user-runnable procedures are listed. From left to right in the instrument tab, these are:

- **Rinse**: Rinses the sample loop and connecting sample lines with focusing fluid.
- **Sanitize SIP/Sanitize Autosampler SIP**: Washes and sanitizes the Sample Injection Port (SIP) on the Attune or on the autosampler. Use this especially after running sticky samples, including the performance beads.
- **Deep clean**: Cyclicly runs Wash solution and 10% bleach through the flow cell and sample lines. This is the wash steps done in the shutdown step, without the final flush with shutdown fluid.
- **Startup**: Flushes the shutdown solution from all fluidic lines, replacing it with focusing fluid.
- **Shutdown**: Uses bleach to backflush the flow cell and all sample lines, rinses all lines with water, then runs Wash solution and 10% bleach cyclicly through all lines. *Quick* does 5 cycles/10 minutes, *standard* does 15 cycles/40 minutes, *thorough* does 25 cycles/60 minutes.
- **Debubble**: Clears bubbles in the fluidics lines.
- **Unclog**: Backflushes the sample probe and flow cell.
- **Decontaimate system**: Runs bleach through all backend and frontend fluidic lines. The focusing fluid filters must be replaced after decontamination.

Weekly system cleaning

The proprietary "flow cell cleaning solution" is actually a Hellmanex solution[35]. Hellmanex is an alkaline, viscuous detergent for cleaning glass. It can be corrosive to metals; make sure to clean up spills!

1. Dilute 1 mL of Hellmanex with 2 mL of DI water (3 mL total).

[35] https://www.fishersci.com/shop/products/fisherbrand-hellmanex-iii-liquid-cleaning-concentrate/14385864

2. Run Deep Clean - Standard (40 min) with 1:2 diluted Hellmanex (instead of standard 10% bleach)
3. Run Deep Clean - Quick (10 min) with 10% bleach to ensure that any Hellmanex is flushed out of the system.

Note

10% bleach or diluted Hellmanex can also be used with the debubble protocol instead of debubble solution to clean the flow cell (however, don't expect it to debubble! We just use the debubble procedure because it washes and backflows the flow cell). If you use Hellmanex, however, run a debubble with 10% bleach after to flush it out.

Every ~3-6 month system decontamination

Estimated time

2.5 hours

This is not a "fire and forget" protocol; you will have to be actively doing things for nearly two hours!

Warning

The focusing fluid filters must be replaced after decontamination, so ensure we have replacements before beginning!

Note

The Attune maintenance manual (page 21) is no longer up-to-date with what the Attune decon protocol is!

When in doubt, always follow the on-screen prompts. There is only one divergence from the on-screen prompts that makes replacing the focusing fluid filters easier.

Note

Over the course of the decontamination, all of the fluid is poured out. You may save some of the fluids if they are not contaminated, but it is good to fully remove all liquids somewhat regularly to prevent reservoir contamination.

It is helpful to run the bottles closer to empty before performing the decontamination to waste less fluid.

1. Sanitize
    a. Rinse out the focusing fluid bottles (internal and autosampler) and the shutdown bottle with deionized water. Fill the focusing fluid bottles with 500 mL of fresh 10% bleach and fill the shutdown bottle with 100 mL of fresh 10% bleach.
    b. Make sure the wash bottle is filled with at least 100 mL of wash solution.
    c. Ensure all fluid connections are connected securely.
    d. Hit **Next** in the software.
    e. Load a clean, empty tube onto the tube lifter and load a clean 96 well plate into the autosampler.
    f. Hit **Next** in the software to start phase 1.
2. Rinse
    a. Lower the tube lifter, and remove the plate from the autosampler.
    b. Hit **Next** in the software.
    c. Remove all liquids from all fluid bottles. If you have time, rinse bottles that have not yet been bleached with 10% bleach.
    d. Rinse all with deionized water.
    e. Replace the normal liquids in the fluid bottles.
    f. Hit **Next** in the software.
    g. Replace the focusing fluid filters, as detailed in *User-replaceable parts* (page **??**).

> **Note**
>
> Replacing the focusing fluid filters before reconnecting the bottles, as suggested here, makes your life much easier.

    h. Clean the Attune with ethanol (wipe up any spills inside the cabinet, clean the metal parts, etc).
    i. Replace all bottles and reconnect all fluid connections.
    j. Hit **Next** in the software to start phase 3.
3. Run 3 startup procedures, 2 debubble procedures, and 2 rinse procedures while observing for leaks from the newly replaced filters.

Focusing fluid refill

Empty focusing fluid bottles should be kept and placed on top shelf over the attune. No other bottles should be kept (e.g., throw away shutdown bottles when empty).

Important

Once you get to the last 1X focusing fluid bottle, follow this procedure so we aren't refilling with contaminated solutions.

1. Rinse sides of bottles with 10% bleach squeeze bottles. Let sit for 15 min.
2. Rinse out bleach.
3. Rinse bottles with Alconox.
4. Rinse out the Alconox with tap *until no more soap*. MAKE SURE NO SOAP REMAINS AND GETS INTO ATTUNE.
5. Rinse bottles with DI water. Let dry overnight.

Important

Wash large 1X focusing fluid reservoir spigot with Alconox and DI water before using to prevent refilling with contaminated focusing fluid.

6. Refill bottles with 1X fcocusing fluid from the large reservoir using the *clean* spigot.

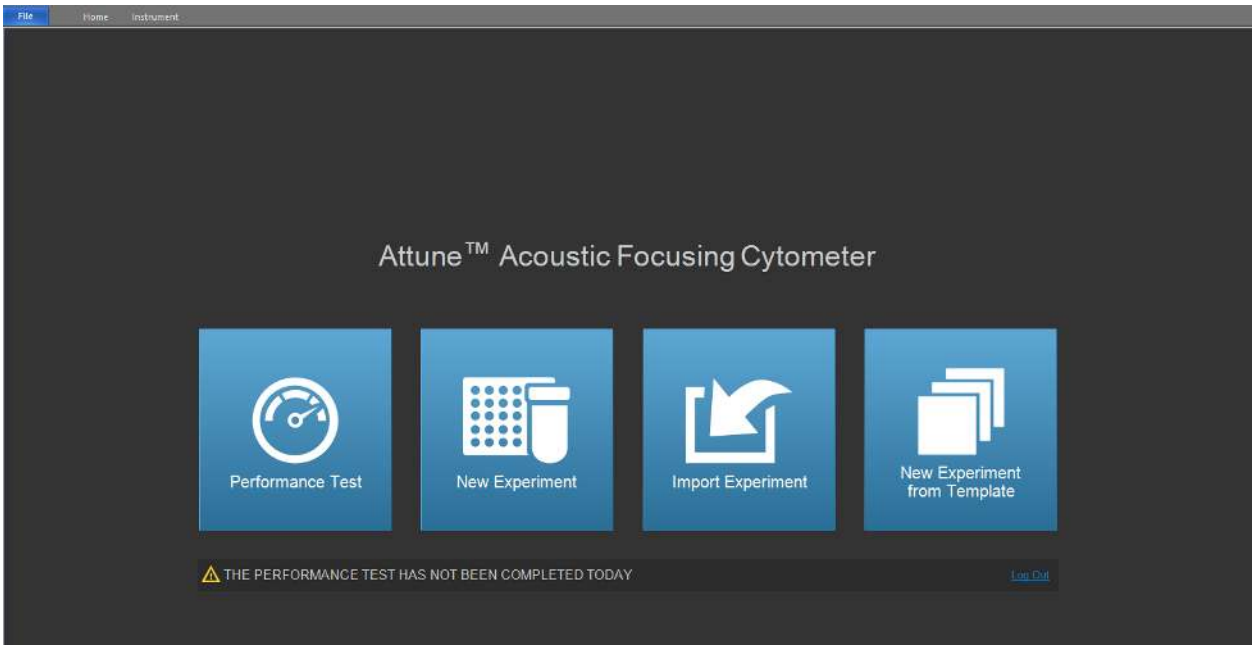
Full Attune guides

You can download the Attune software manual or the Attune maintenance manual.

2.1.3 Attune software

Common processes

When first logging into the Attune software, you will see the screen below with options to run a performance test, create a new experiment, import an experiment, or create an experiment from a template. Below these options, the software will display whether or not a performance test has been completed already today.



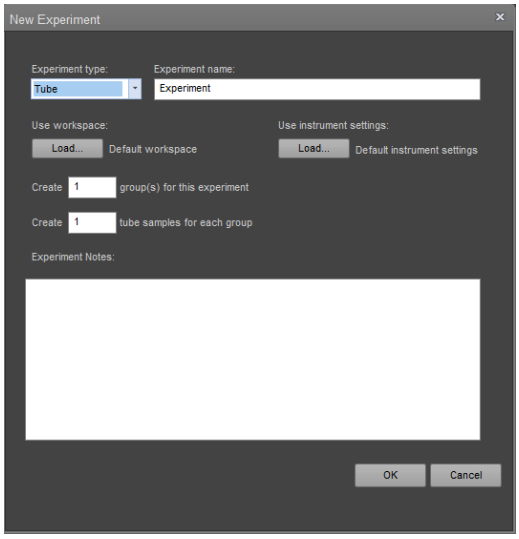
Clicking on the instrument tab at the top of the window will reveal the options to run various processes as detailed in *Attune operation* (page **??**).

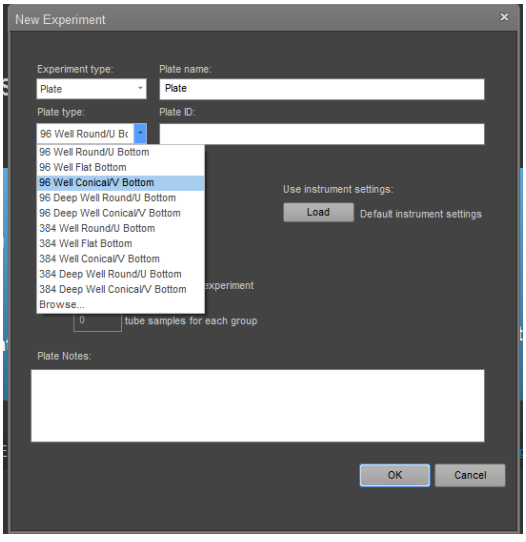
Once the startup process is completed, the other processes will be available to run. To access variations of the processes, such as "Deep Clean - quick" and "Shutdown - thorough" (*Attune startup and shutdown* (page **??**)), click the dropdown arrow next to the default process name.

Creating an experiment

Once you have followed the startup steps and run the performance test (*Attune startup and shutdown* (page **??**)), you can create a new experiment by clicking the second option on the homepage. Here, you have the option to create a tube experiment or a plate experiment. For a tube experiment (below), you only need to specify an experiment name.

For a plate experiment (below), you must specify a plate name/ID as well as the plate type. Our lab currently uses 96 Well Conical/V Bottom plates, so be sure to update this option to ensure the autosampler functions correctly.


New Experiment
Experiment type:
Tube
Experiment name:
Experiment
Use workspace:
Load...
Default workspace
Use instrument settings:
Load...
Default instrument settings
Create 1 group(s) for this experiment
Create 1 tube samples for each group
Experiment Notes:
OK
Cancel

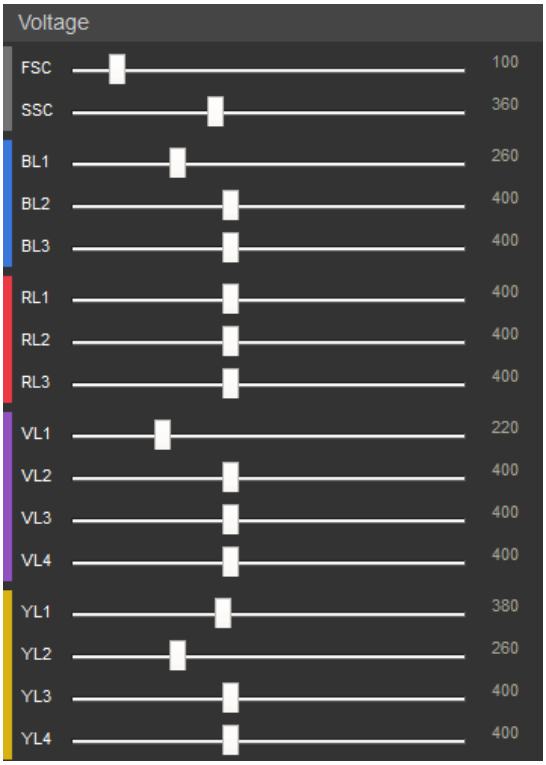

New Experiment
Experiment type:
Plate
Plate name:
Plate
Plate type:
96 Well Round/U Bc
Plate ID:
96 Well Round/U Bottom
96 Well Flat Bottom
96 Well Conical/V Bottom
96 Deep Well Round/U Bottom
96 Deep Well Conical/V Bottom
384 Well Round/U Bottom
384 Well Flat Bottom
384 Well Conical/V Bottom
384 Deep Well Round/U Bottom
384 Deep Well Conical/V Bottom
Browse...
Use instrument settings:
Load
Default instrument settings
tube samples for each group
Plate Notes:
OK
Cancel

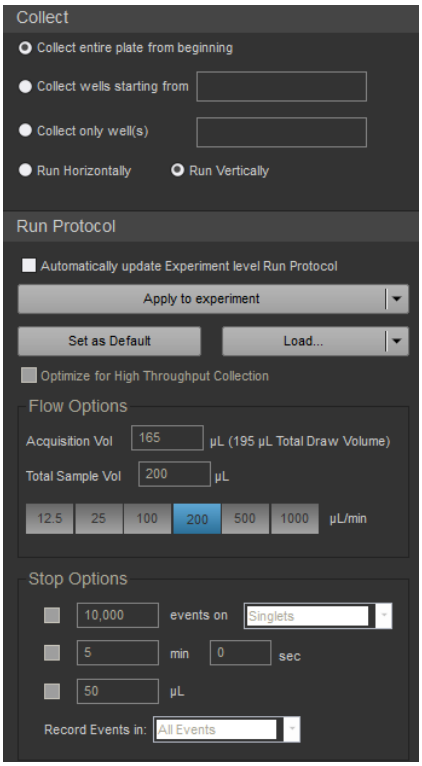
In a plate experiment, you can highlight your desired wells in the Heat Map View, right click, and add the wells to a new sample. This also gives you the option of designating a manual well, which can be used to test laser settings before running your experiment.

Instrument settings can be accessed using the tabs on the lower left corner of the screen. In the laser settings tab, you can select which channels to collect using the left checkboxes and add labels to the channels corresponding to your fluorophores.

Below these settings, you can change the voltages of each laser using the slider or type a number in on the right. Be sure to apply these settings to the experiment after finding the optimal levels.

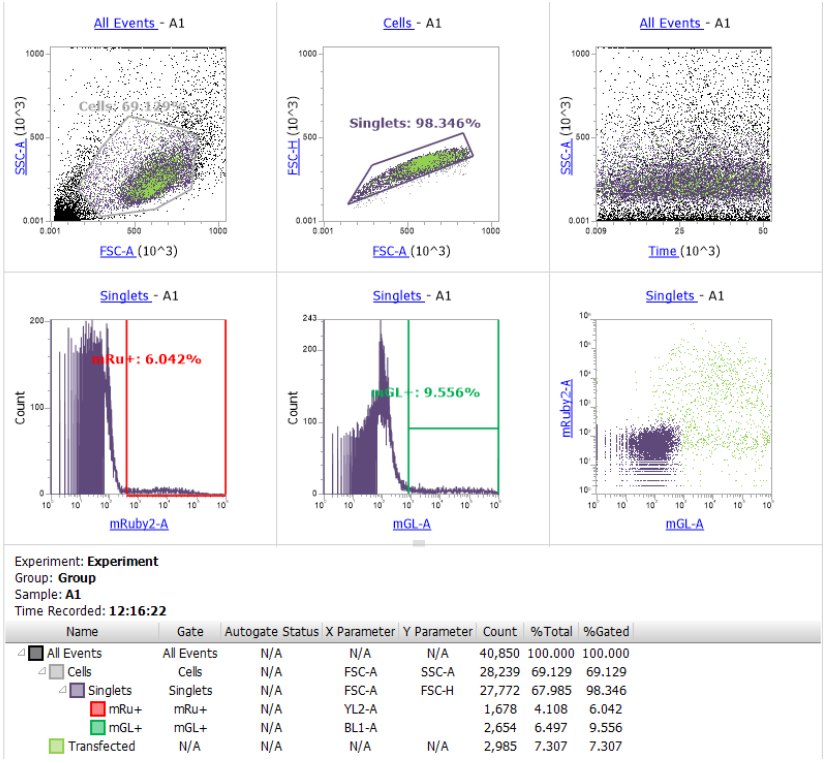
Finally, the collection settings tab allows you to specify the flow conditions for your experiment. For a plate experiment, you can choose which of the defined sample wells to run and whether to run horizontally or vertically. In flow options, you can edit the acquisition volume and sample flow rate. Finally, in stop options, you can have the data collection stop after a certain number of events, time, or volume.


Voltage
FSC
100
SSC
360
BL1
260
BL2
400
BL3
400
RL1
400
RL2
400
RL3
400
VL1
220
VL2
400
VL3
400
VL4
400
YL1
380
YL2
260
YL3
400
YL4
400


Collect
Collect entire plate from beginning
Collect wells starting from
Collect only well(s)
Run Horizontally
Run Vertically
Run Protocol
Automatically update Experiment level Run Protocol
Apply to experiment
Set as Default
Load...
Optimize for High Throughput Collection
Flow Options
Acquisition Vol
165
µL (195 µL Total Draw Volume)
Total Sample Vol
200
µL
12.5
25
100
200
500
1000
µL/min
Stop Options
10,000
events on
Singlets
5
min
0
sec
50
µL
Record Events in:
All Events

Customizing the workspace

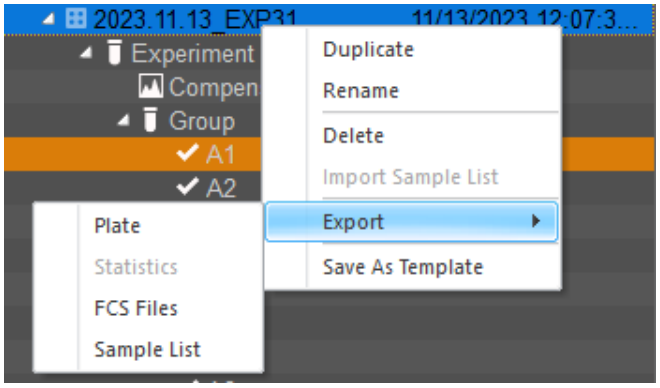
As your experiment runs, your data will be displayed in the experiment workspace. The workspace tab can be used to customize what information is shown here. For example, you can create joint plots and histograms for gating as well as show statistics. The data displayed in each plot can be modified by clicking the words highlighted in blue. These allow you to change the plot axes and which gated population to plot.



Name	Gate	Autogate Status	X Parameter	Y Parameter	Count	%Total	%Gated
All Events	All Events	N/A	N/A	N/A	40,850	100.000	100.000
Cells	Cells	N/A	FSC-A	SSC-A	28,239	69.129	69.129
Singlets	Singlets	N/A	FSC-A	FSC-H	27,772	67.985	98.346
mRu+	mRu+	N/A	YL2-A		1,678	4.108	6.042
mGL+	mGL+	N/A	BL1-A		2,654	6.497	9.556
Transfected	N/A	N/A	N/A	N/A	2,985	7.307	7.307

Exporting data

To export data from your experiment, right click on the name of your experiment in the right-most panel and select the FCS files to export.



2.1.4 User-replaceable parts

Main syringe

See page 16 in the Attune maintenance guide for full details.

As detailed in *Attune startup and shutdown* (page **??**), before every run the syringe should be examined for a build-up of salt. Salt buildup occurs when micro-clogs occur in the sample loop. Due to the back-pressure applied to the syringe, small drops of liquid can be forced around the syringe. These drops get pushed to the bottom of the syringe travel, where they dry and form a salt ring.

When a salt ring forms, the syringe should be replaced.

1. Shut down the Attune.
2. Unscrew the bottom thumb-screw, to release the ball of the syringe.
3. Carefully remove the ball from the piston, then rotate the syringe counter-clockwise to unscrew it.
4. Insert the ball of the new syringe into the bottom mechanism.
5. Align the syringe with the syringe port, rotate clockwise until the syringe bottoms out on the valve.

Warning

Do not apply excessive force; if the syringe does not easily screw in with light finger-only force, you may be cross-threading the syringe!

6. After bottoming out (e.g. when you first feel resistance), screw **only an additional quarter of a turn!**
7. Tighten the thumbscrew to secure the syringe ball.
8. After replacement, run 3 startup procedures, 2 debubble procedures, and 2 rinse procedures before use!

Sample Injection Probe (SIP)

See page 18 in the Attune maintenance guide for full details.

If too much force is applied, the tube SIP may be bent. If the bend is minor, it can be carefully bent back. If the bend is severe, order a new SIP.

1. Unscrew the old SIP tube.
2. Move the plastic fitting to the new SIP tube.
3. Hand tighten the new SIP until the fitting audibly clicks.
4. Run 3 startup procedures, 2 debubble procedures, and 2 rinse procedures before use.

Focusing fluid filters

See page 15 in the Attune maintenance guide for full details.

Note

The focusing fluid filters must be replaced after a system decontamination.

1. Remove all internal fluid bottles from the Attune.
2. Place Kimwipes or paper towels beneath the focusing fluid filters to catch any drips.
3. Undo the velcro securing the focusing fluid filters to the Attune.
4. Gently pull the filter slightly out to an angle from the machine.
5. Unscrew the top tubing by rotating it counter-clockwise.
6. Holding the filter between your fingers, unscrew the bottom fitting by rotating it counter-clockwise.
7. Unscrew both the black section of the top female-to-male luerlock adapter and the bottom male-to-female luerlock adapter.

Warning

The new filters do not contain the luerlock adapters! If you throw them away/lose them, you will have to obtain replacements!

8. Orient the new filter with the arrow pointing downward. Screw the luerlock adapters in.
9. Screw in the bottom part of the filter into the amber threaded adapter at the bottom.
10. Using a syringe, flush 10 mL of focusing fluid into the top of the filter. Do not apply any backpressure to the system!
11. Carefully screw in the top luerlock adapter into the top of the tubing until a click is felt.
12. Run 3 startup procedures, 2 debubble procedures, and 2 rinse procedures while observing for leaks.

2.1.5 Attune troubleshooting

The following troubleshooting tips and explanations are a result of empirical observations and discussions with our support engineers. Additional troubleshooting is available in the Attune maintenance manual.

Delay before events start running

If you notice that there is a delay before events appear, but the events appear normal (e.g. there isn't a clog), check that the thumbscrew and screw at the top of the main sample syringe are fully tight. You can add about a quarter-turn if they are loose.

Explanation: The main sample syringe is responsible for pushing liquid, including your sample, through the fluidics system. If the two screws that attach the servo motor to the syringe are loose, then when the servo motor initially starts moving, that motion instead goes towards the slop in the system and the main syringe doesn't start moving until after a delay.

This delay actually affects any procedure that starts from the fully-down syringe position, but is most noticeable when capturing events. The delay is not harmful in any way.

Fluid leak from the autosampler SIP onto a running plate

First, take the plate out and clean up the spilled liquid. Carefully, **while the Attune is idle or off**, you can open the autosampler door and clean up any spilled liquid with a paper towel.

Then, ensure that the two waste bottle connections are fully connected. Make sure you re-seat the autosampler waste bottle connection unti you hear **two clicks**.

Explanation: The autosampler fluidics are relatively simple. The autosampler syringe directly pulls focusing fluid from the autosampler focusing fluid bottle to use as sheath fluid. After aspirating a sample, the sample syringe is actually *pushing* fluid through the system to move your sample into position, with the excess fluid "in front" of your sample redirected into the waste bottle.

If the autosampler waste bottle is not fully connected, you instead end up with the syringe trying to push against a closed connection, which leads to high backpressure. The fluid goes through the easiest escape route, which is back out of the SIP onto your plate :(

Wells skipping and the autosampler fails to draw focusing fluid

If you directly observe that fluid is not successfully being taken up by the autosampler syringe, or you observe that no events happen or wells get entirely skipped, the autosampler is likely not taking up focusing fluid correctly.

Check that the autosampler focusing fluid bottle connection is fully seated. Also check that the autosampler waste bottle connection is fully seated (two clicks on insertion).

Explanation: The syringe has to be able to hold suction. A leak on the focusing fluid bottle side will prevent the syringe from getting fluid in (unless you elevate the focusing fluid bottle). A not fully connected waste line will cause pressure to build up in the system and prevent it from working.

Power cycling the Attune

If the machine is behaving oddly in a way not described above, or if the software prompts you to do so, power cycle the machine. To turn the Attune off, switch both the autosampler and the main machine off, and let them sit for 30 seconds. Then, to turn the machine on, **first turn on the autosampler** (switch is labeled on top with a 1) then turn on the main machine (switch is labeled with a 2).

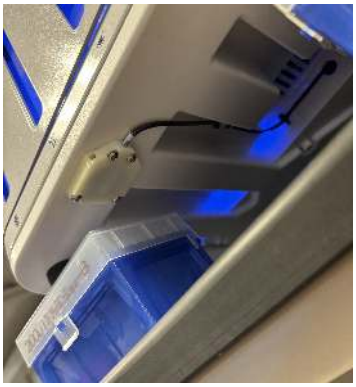
Explanation: The autosampler should be turned on first so that the main machine is able to sense that it is connected and initiate the correct settings.

Spill detected by bottles

If a spill occurs when refilling focusing fluid or emptying waste, the attune will detect this and report an error message.

First, wipe up any visible spills. If the error has still not cleared, it is likely that the spill sensor still has liquid. The sensor is directly underneath the focusing fluid bottle on the exterior of the attune (see below).

1. Elevate the attune with pipette tip boxes.
2. Using a 5/64 long arm wrench, unscrew the sensor.
3. Dab the top of the sensor with a Kimwipe to remove any liquid.
4. Rescrew the sensor and lower the attune back down.



2.2 Nikon operation

2.2.1 Microscope Components

2.2.2 Startup/Shutdown

Note

The microscope controller and epifluorescence light source can remain on all the time, although it may be useful to power cycle them for troubleshooting. If they are on, skip their corresponding steps below.

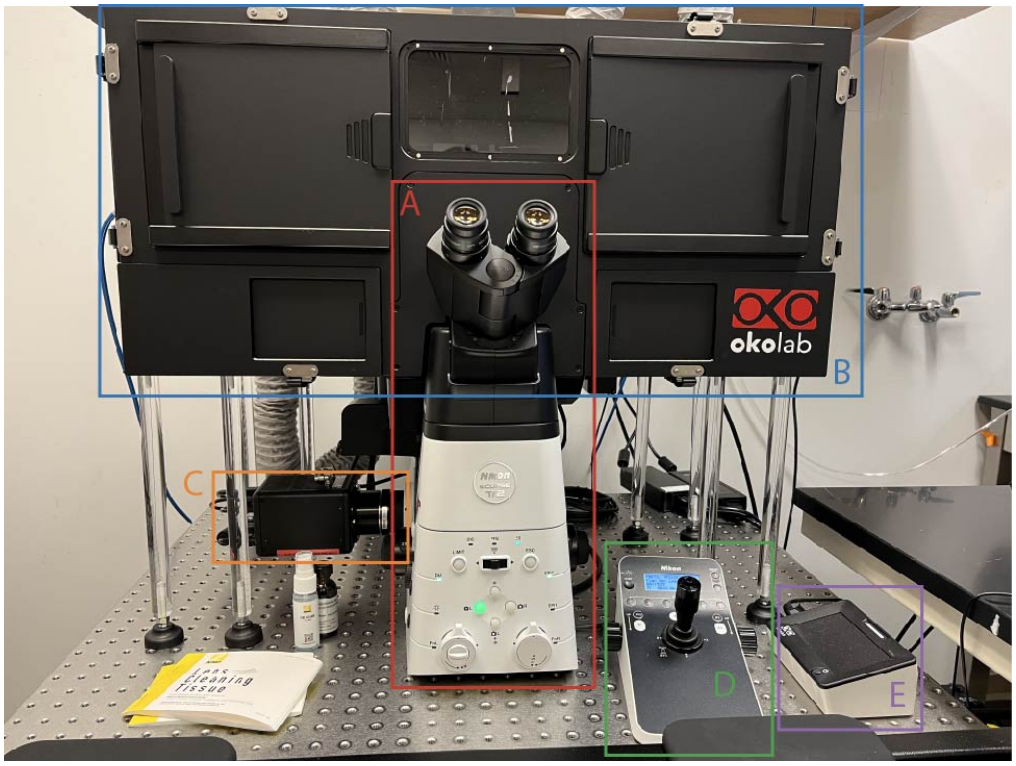


Fig. 4: A (red): Main body of the microscope, B (blue) Okolab incubation chamber, C (orange): Digital camera, D (green): Joystick/Display, E (purple): Okolab controller display

Turning the power on

1. Turn on the auxiliary devices: digital camera (C) and epifluorescence light source (K)
2. If the controller is not already on, turn on the controller (J).
3. Turn on the main body of the microscope (A). The switch is located at the back corner of the right side.
4. **During initialization, the screen on the display (D) should illuminate and display the status.**
    1. If initialization fails (screen fails to illuminate, microscope starts beeping, etc.), try power cycling the components above as well as the computer.
    2. If that does not work, turn off all the components and reseat the connections on the back of the microscope controller (J).
5. Open the NIS-Elements software (center of desktop) in acquisition mode. **DO NOT check the box to select automatically on startup.**

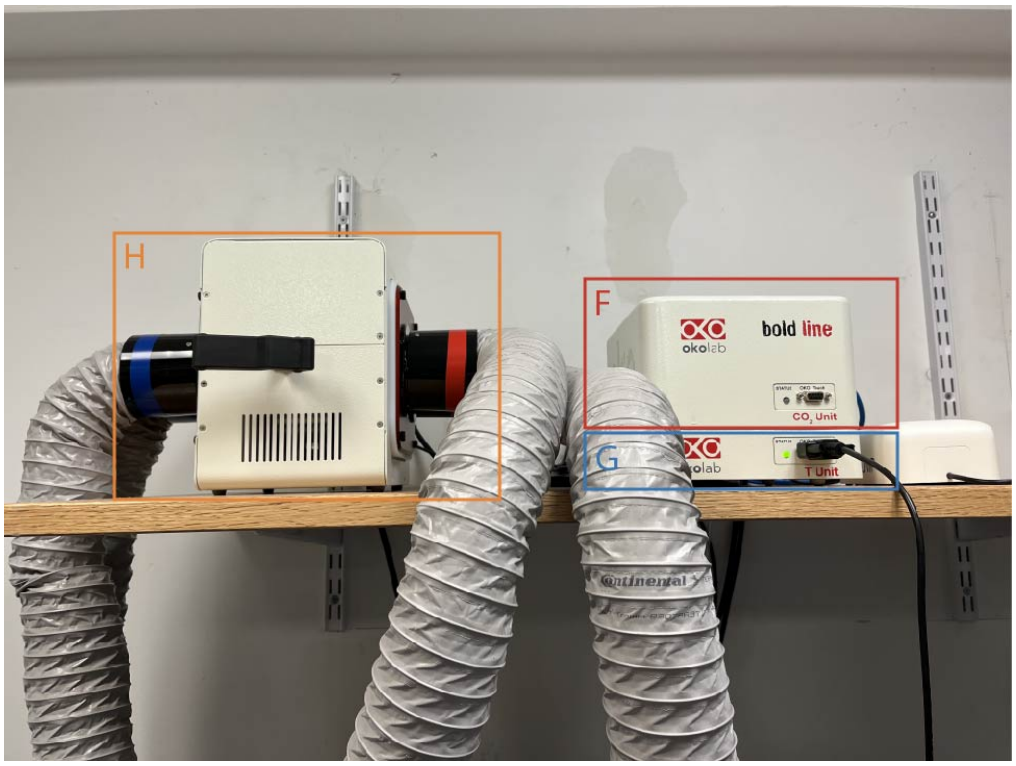


Fig. 5: F (red): Incubator CO2 unit, G (blue): Incubator temperature unit, H (orange): Incubator heating box

Turning the power off (last user of the day)

1. Close the NIS-Elements software, making sure to save all of your data.
2. Turn off the the main body of the microscope (A). Check the top, front, and sides of the incubator enclosure to make sure all of the doors are closed.
3. Turn off the digital camera (C).
4. Optional for troubleshooting: Once the lights on the front of the microscope main body turn off, you may turn off the microscope controller (J).

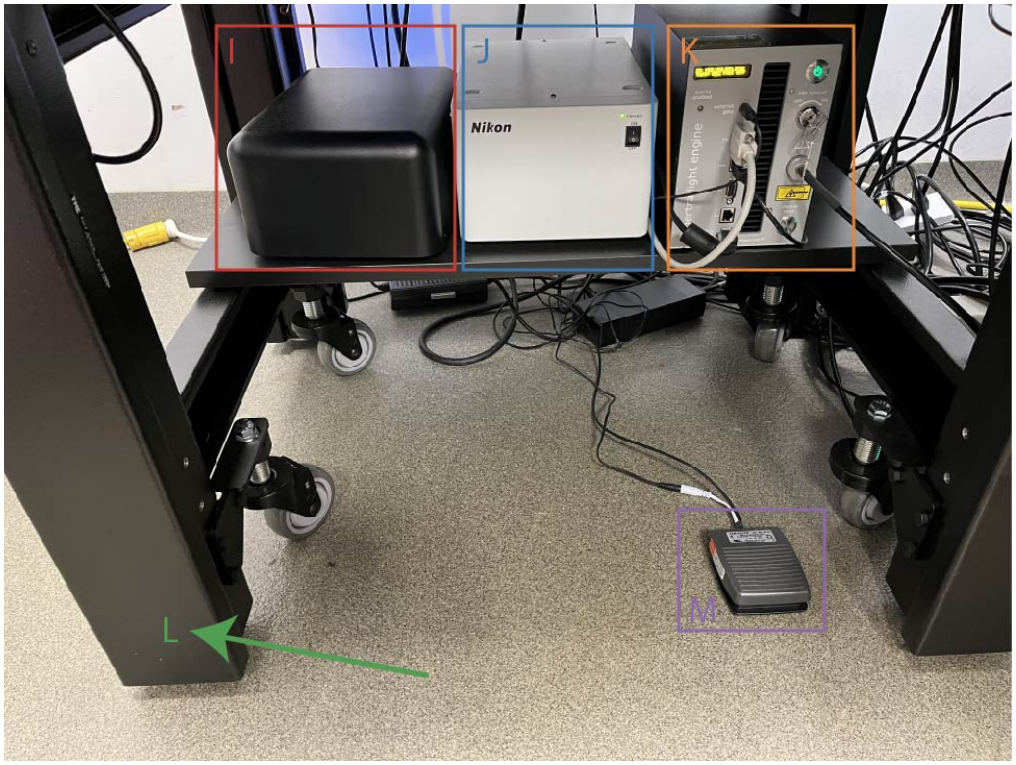
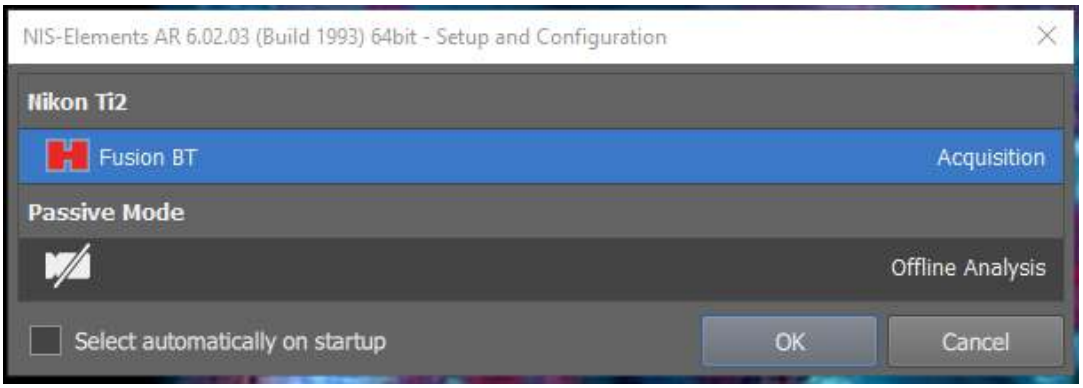


Fig. 6: I (red): Digital camera controller, J (blue): Microscope controller, K (orange): Epifluorescence light source, L (green): Air table (compressor not pictured), M (purple): Incubation chamber light switch



2.2.3 Simple single image acquisition

Important

Glass-bottom plates/dishes or glass slides must be used for imaging on the Nikon. Plastic plates/dishes may be okay for use with the low power objectives (4x, 10x), but the images will be low quality.

Sample setup

1. Remove the magnetic cover from the front window of the enclosure. Slide open the front left and right doors to facilitate bringing samples inside the enclosure. Slide open the top covers around the microscope to allow the microscope pillar to tilt back. You may use the foot pedal to turn on the light inside the enclosure, but be sure to turn this off when imaging.

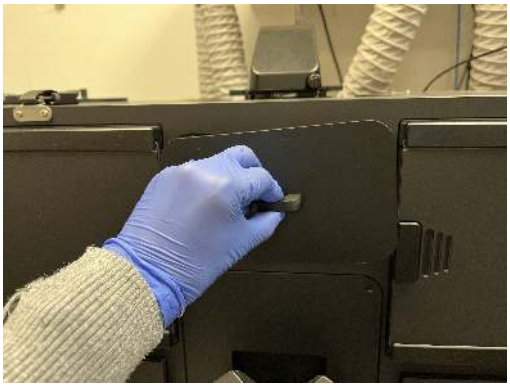


Fig. 7: Removing front magnetic window cover

Fig. 8: Sliding open side enclosure door

2. With the microscope pillar down, there is not enough room to safely place your samples on the stage. Carefully, tilt the microscope pillar back to allow for more room.
3. Choose the appropriate plate holder for your sample (TI2-S-HW for well plates and TI2-S-HU for slides and other plates). The Okolabs plate holders should only be used for timelapse imaging. If you must change the plate holder, move the objectives to the lowest z-level

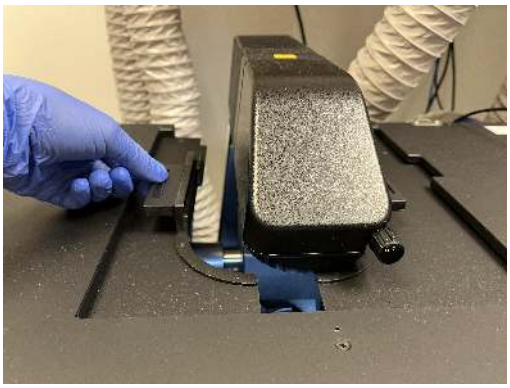


Fig. 9: Sliding open top doors

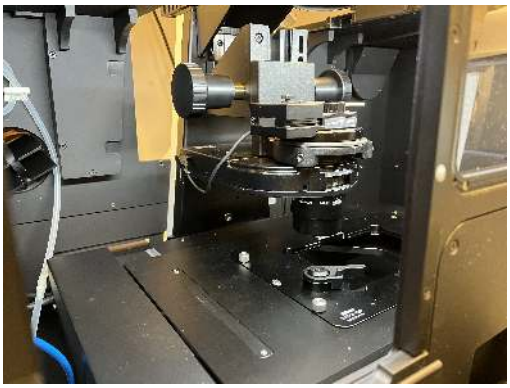


Fig. 10: Microscope pillar in “down” position relative to stage

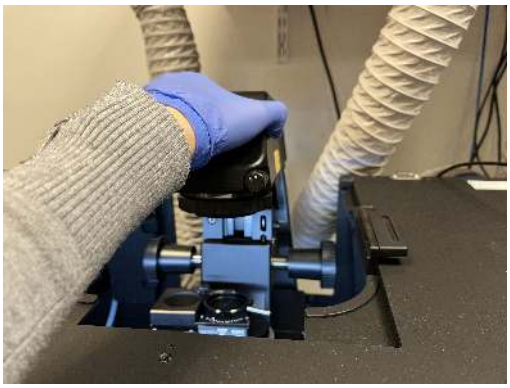


Fig. 11: Tilting microscope pillar up by grasping at the top

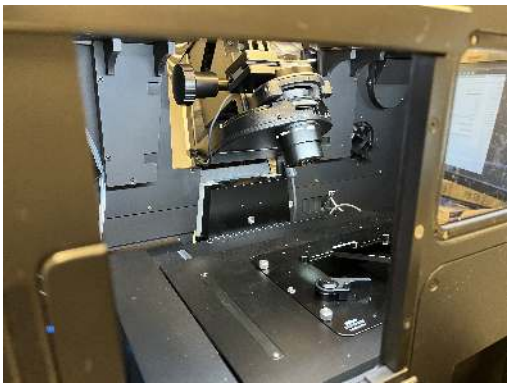


Fig. 12: Microscope pillar in “up” position relative to stage

by hitting the “ESC” button on the front of the microscope. This prevents the objectives from being damaged. Carefully tighten the six screws (three by hand and three with the labeled Allen wrench). Press the “ESC” button again to return the objectives to their previous position.

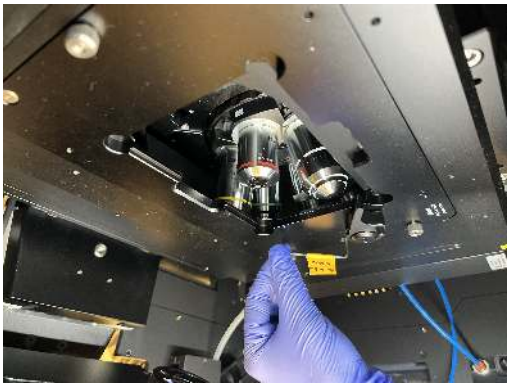


Fig. 13: Using labeled Allen wrench to tighten plate holder

4. If you are using any of the oil immersion objectives (60x, 100x), place a drop of immersion oil on the lens.
5. Carefully place your sample in the holder, making sure to avoid contact with the objectives or the microscope pillar. Tilt the microscope pillar back down into place and close the doors on the front and top of the enclosure. Optionally, place the magnetic cover back on the front window to block out all light.

Basic microscope controls

1. Find the well plate navigator on the left. Ensure that the correct plate holder is listed on the bottom. If not, click on the gear icon so select the correct one.
2. Hitting the “Detect” button should automatically detect what kind of well plate you have an update the map. This function has been spotty, but Emma has found it works reliably with an empty plate. When in doubt, the plate icon at the bottom left of the screen can be used to manually switch between well plate maps.

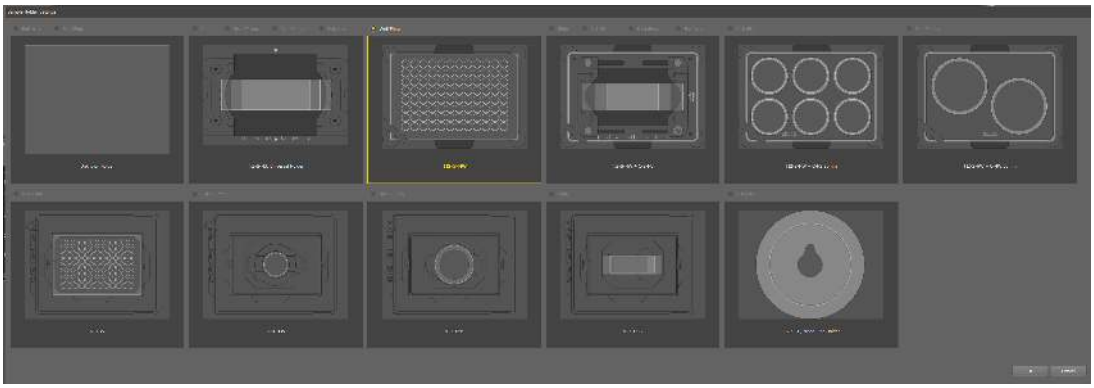


Fig. 14: Options for plate holder

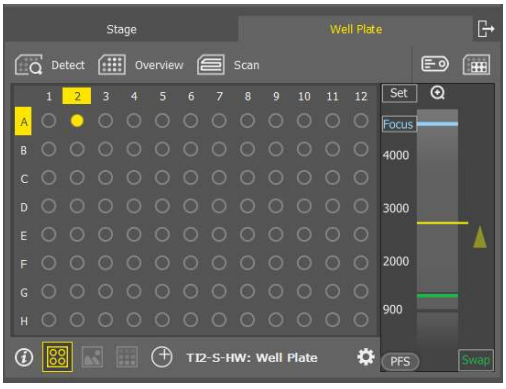


Fig. 15: Well plate map and well plate detection

3. The panel on the right has the controls for optical configuration (which channels are active), exposure times, light intensities, and objectives. To capture a simple image with Brightfield and fluorescence choose “Standard + BF” from the dropdown menu.

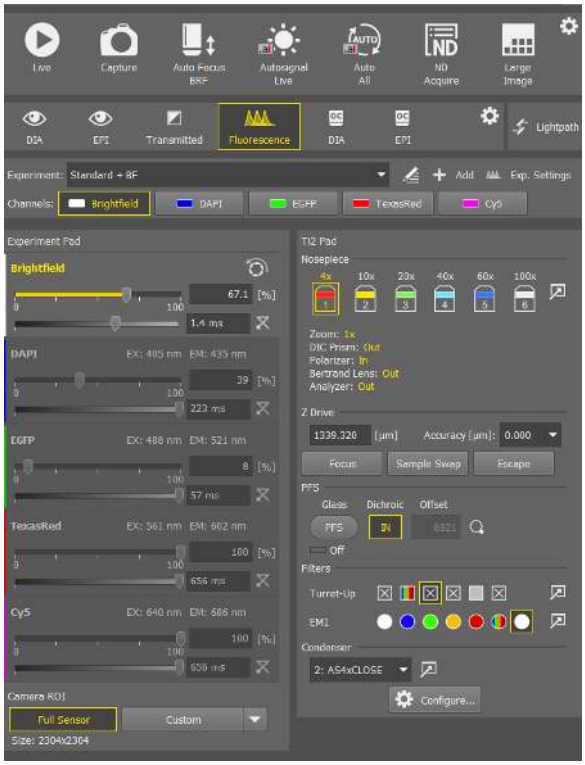


Fig. 16: Optical configuration menu

4. Choose your desired objective by clicking on the icon.

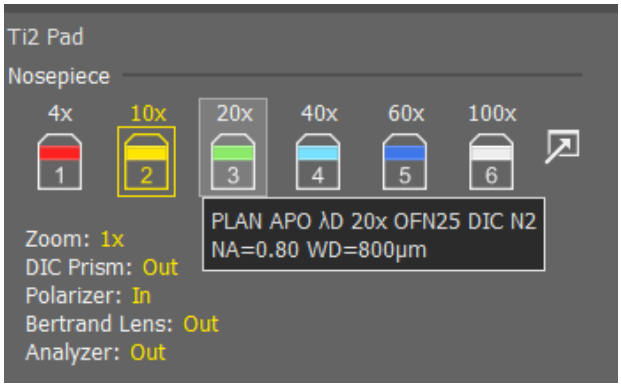


Fig. 17: Objective plate_holder_selection

5. Choose which channels you want on by right clicking on the channel names.

6. Navigate to your desired well either by using the joystick or by double clicking on the plate map. The “XY^” button directly above the joystick determines how coarse or fine the XY movements are. Similarly the “Z^” buttons on the right and left sides determine how coarse or fine the Z movements are.

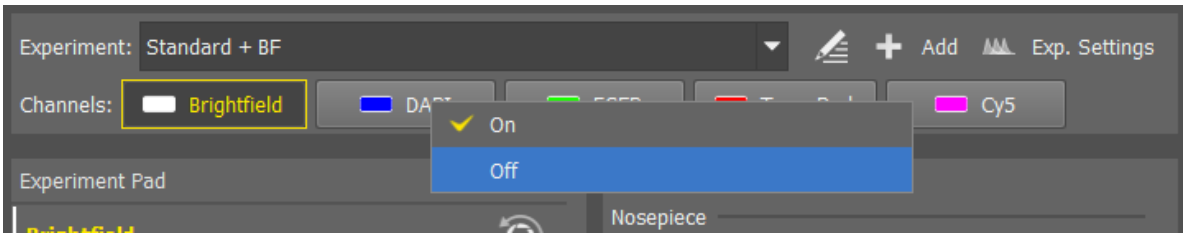


Fig. 18: Fluorescent channel selection

Fig. 19: Joystick controls to move stage

7. Select the Brightfield channel, hit "Live" at the top, and use the focus wheels to find your cells. Be careful not to hit the objective on your plate. Starting at lower objectives and gradually moving to higher magnification can help. Click "Freeze" to pause imaging.

8. Hit "Auto All" to autofocus and autosignal all channels. This works the best with your highest expressing condition. Importantly, this sets the parafocality offsets for each channel, which ensure all your channels are in focus. Alternatively, you can select a single channel and individually perform "Auto Focus" or "Autosignal". You can also manually set lightsource power and exposure times for each channel.

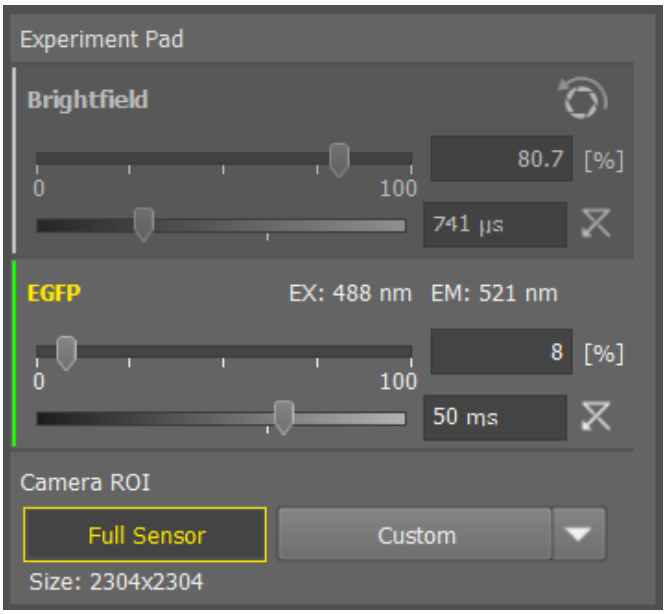


Fig. 20: Optical configuration settings for a sample requiring Brightfield and green fluorescence channels

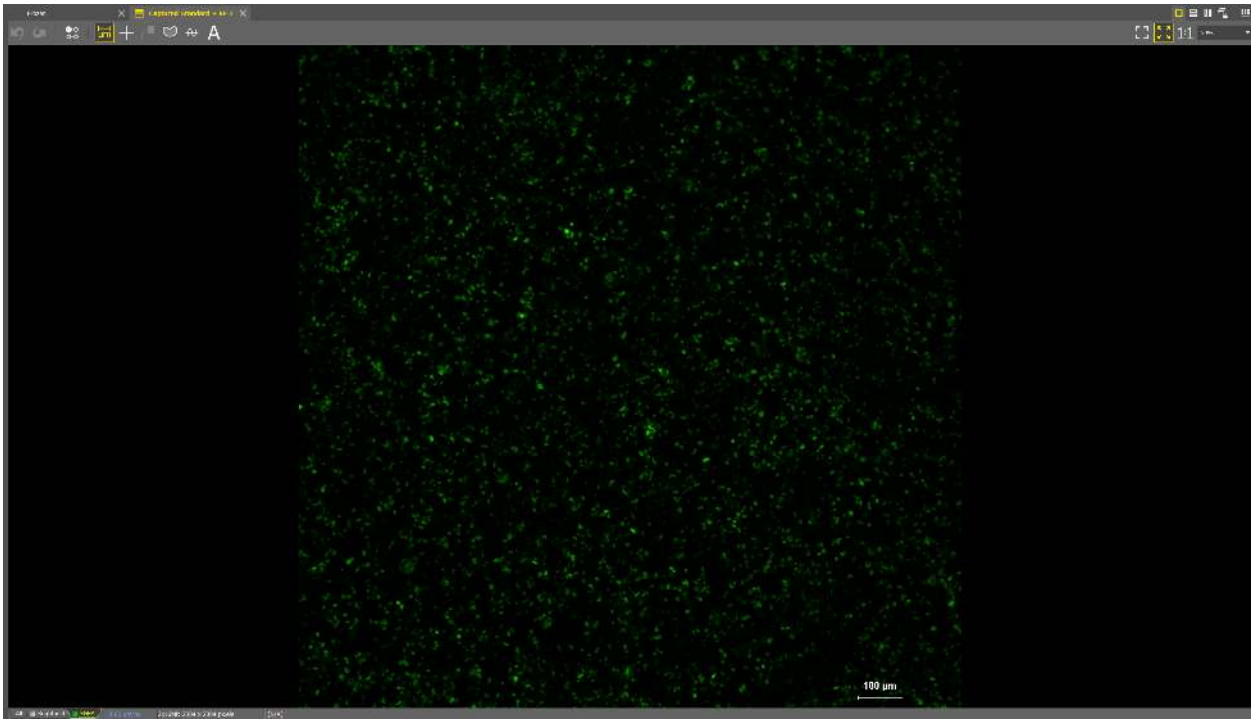
Simple image acquisition and exporting

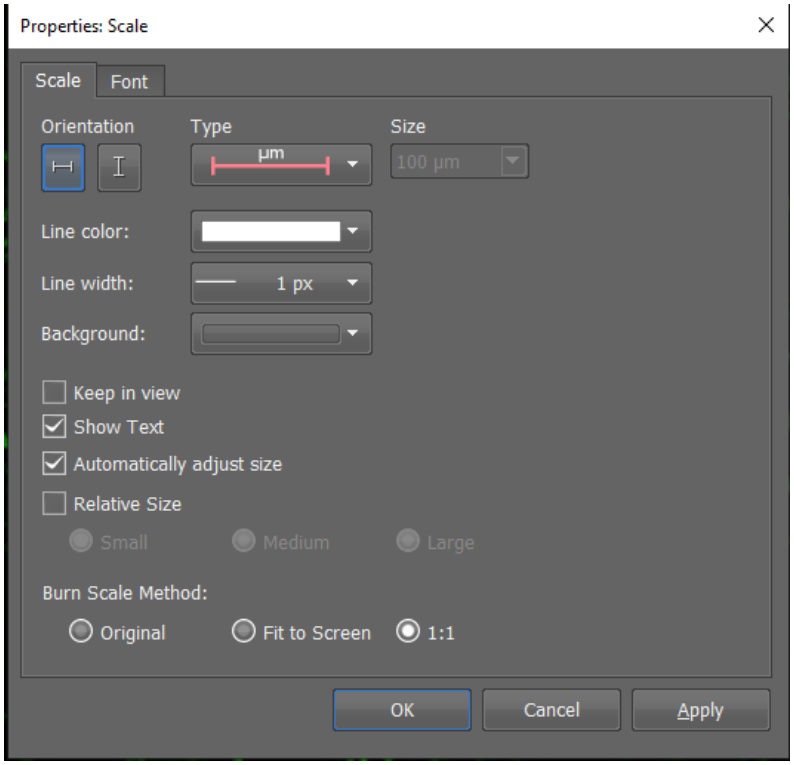
1. Once you are satisfied with the focus and image settings, hit "Capture." Your image will appear as a new tab in the center workspace. AT THIS POINT THE IMAGE IS NOT SAVED ANYWHERE YET!

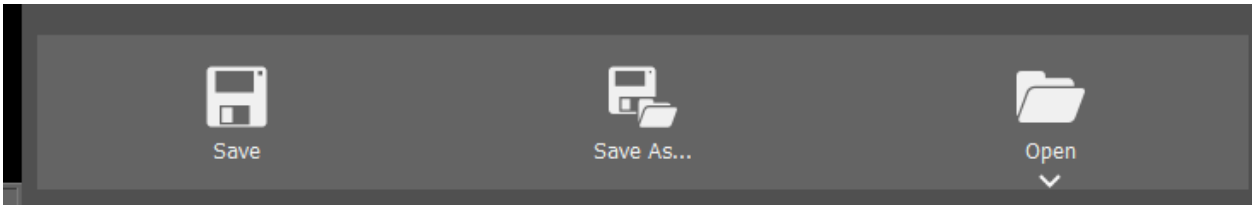
2. You can add a scale bar by clicking on the scale bar icon under the tabs. Right click on the scale bar itself to edit the settings.

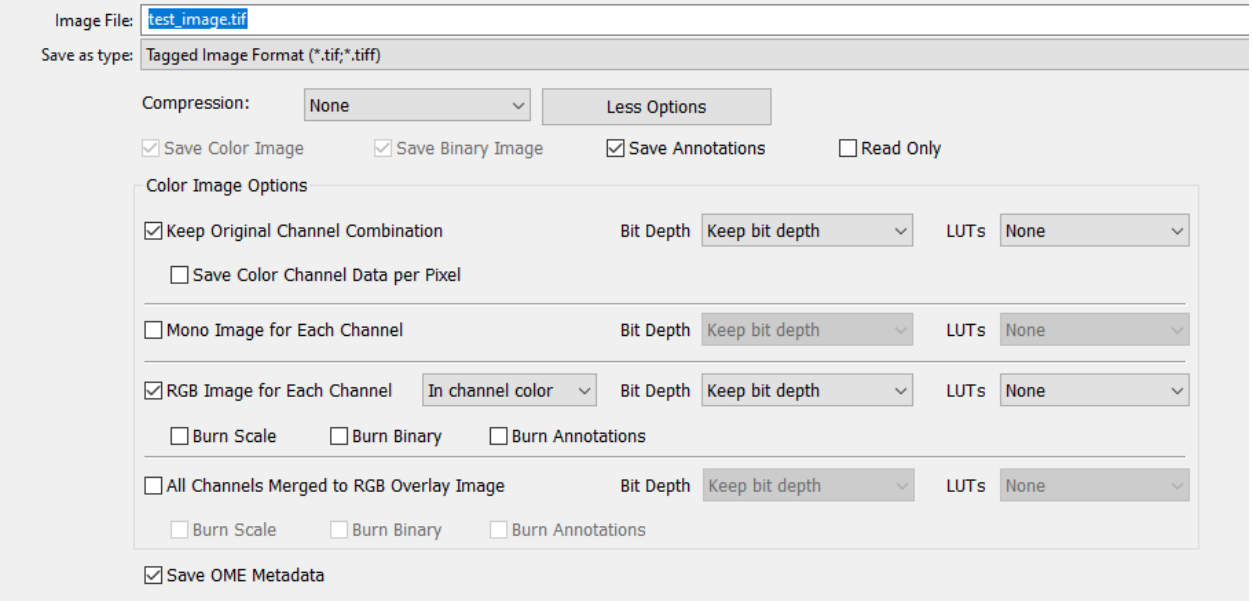
3. Select "Save As. . . " in the bottom right corner of the screen, and save the image as a .nd2 file. This is format the Nikon software uses for any subsequent analysis and editing.

4. You may also save as a .tif file. This will create a multipage .tif containing the individual channel images. You can additionally save .tif files of individual channels by selecting "RGB Image for Each Channel" and an overlay by "All Channels Merged to RGB Overlay Image."


100 µm


Properties: Scale
Scale
Font
Orientation
Type
Size
µm
100 µm
Line color:
Line width:
1 px
Background:
Keep in view
Show Text
Automatically adjust size
Relative Size
Small
Medium
Large
Burn Scale Method:
Original
Fit to Screen
1:1
OK
Cancel
Apply


Save
Save As...
Open



Cleaning up

1. Once you have completed your imaging, open the enclosure doors and tilt the microscope pillar back.
2. Carefully remove your sample.
3. If you used an oil objective, gently clean it with the provided cleaner and wipes. Do not use Kim wipes to clean.
4. Return the microscope pillar to its normal spot and close all doors on the enclosure.
5. If you are the last user of the day, refer to the instructions for powering the microscope off.

2.2.4 Live Cell Tracking

Important

Take images in as few fields of view as possible, with as few conditions as possible. Live tracking generates large quantities of data.

1. At least 1 hour before imaging, turn on CO2 tank. Turn top knob to be completely open, and open valve so that it is inline with the tubing. Left gauge should read ~15-20 psi, right should read over 600 psi.
2. Also at this time, turn on the Okolab controller display (E) so that it can start getting up to temp.
3. Follow directions for *startup* (page **??**).
4. Ensure water tank is full to max line. Refill with sterile water and a serological.
5. Replace stage with incubator stage (completely enclosed) if necessary.
6. Turn on stage sensing (often the empty plates by the computer are better for sensing).
7. Use the experiment suitable to your fluorescent needs (under the Fluorescence tab on the right panel).
8. Find PFS offset (under PFS tab in rightmost panel).
9. Click on Job Wizard (in the bottom left corner).
10. If using an experiment from a previous run, double click to continue editing. If you click run and edited anything, you must save it under a new name.
11. Turn on autofocus on brightfield (or whatever channel is constant between all conditions).
12. Autofocus on one point.
13. Turn on use autofocus when PFS fails.
14. Do a test run (even if using a previous template, you must click on each module before it will let you proceed).
15. Let the plate sit for ~20 min before starting image collection.
16. To do media changes, hit the pause button in the corner (hit resume when plate is back in place).
17. When live track is completed, follow directions for *shutdown* (page **??**).

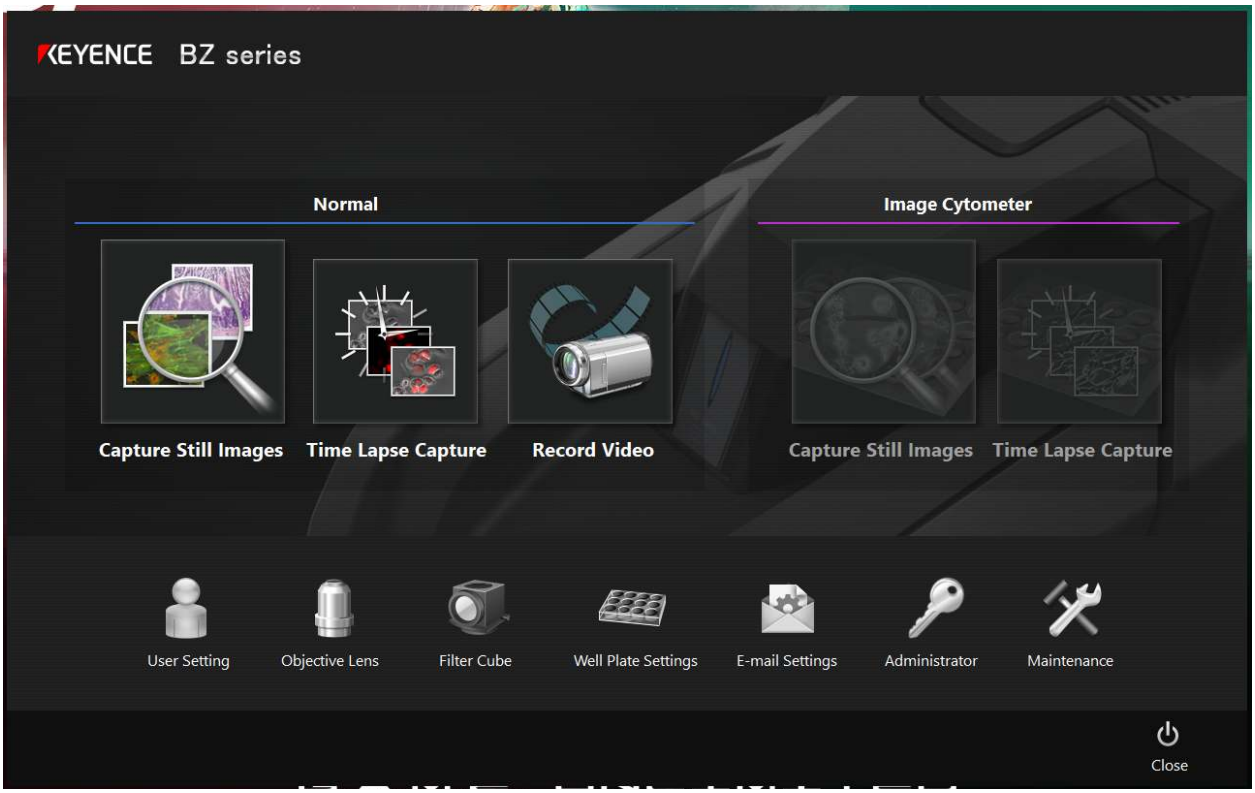
Note

MC has noticed the glass bottom plates get external gunk more easily than the plastic. If you're noticing this in your BF, clean off all surfaces with a kim wipe and EtOH.

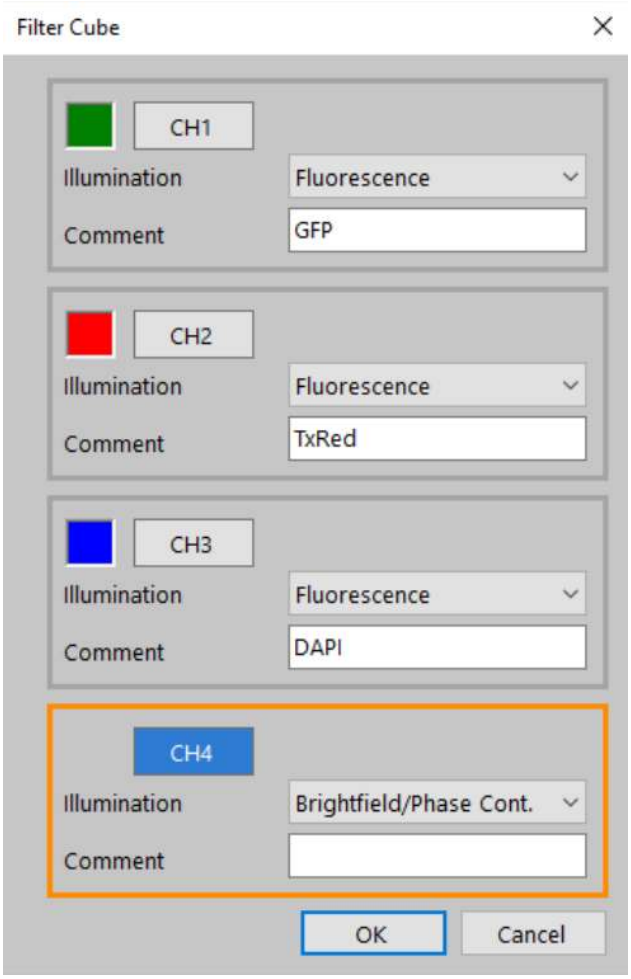
2.3 Keyence beginner's user guide

2.3.1 Basic Operation

1. Turn on the Keyence using the power button.
2. Open the BZ-X800 program from the desktop shortcut. You will see the following menu. Note: you must turn on the Keyence before you are able to navigate the software.

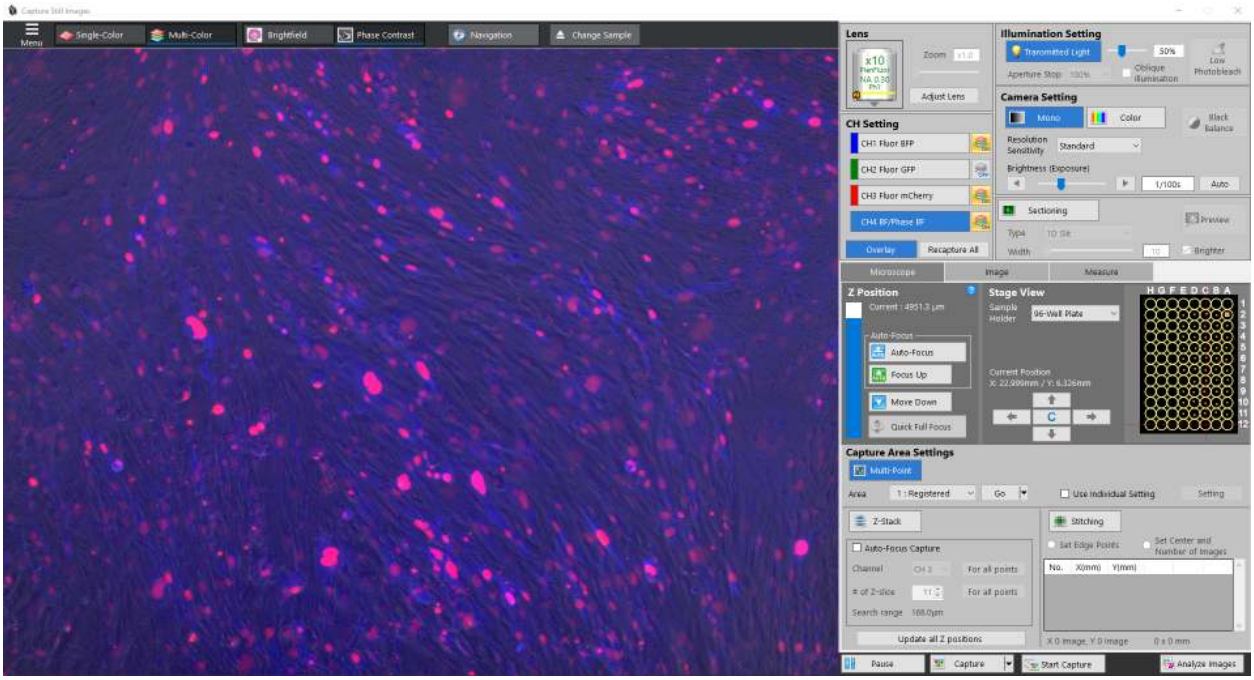


3. The **Filter Cube** button can be used to change which 4 filters are used when capturing images.



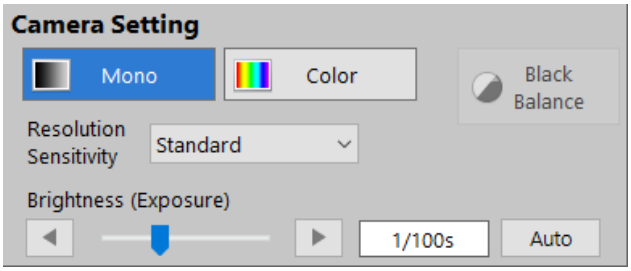
4. If you are using a plate, the **Well Plate Settings** button can be used to select the number of wells on your plate. This can also be changed within the capture window.

5. The most frequently used capture setting is **Capture Still Images**, and selecting this option leads to the window below:

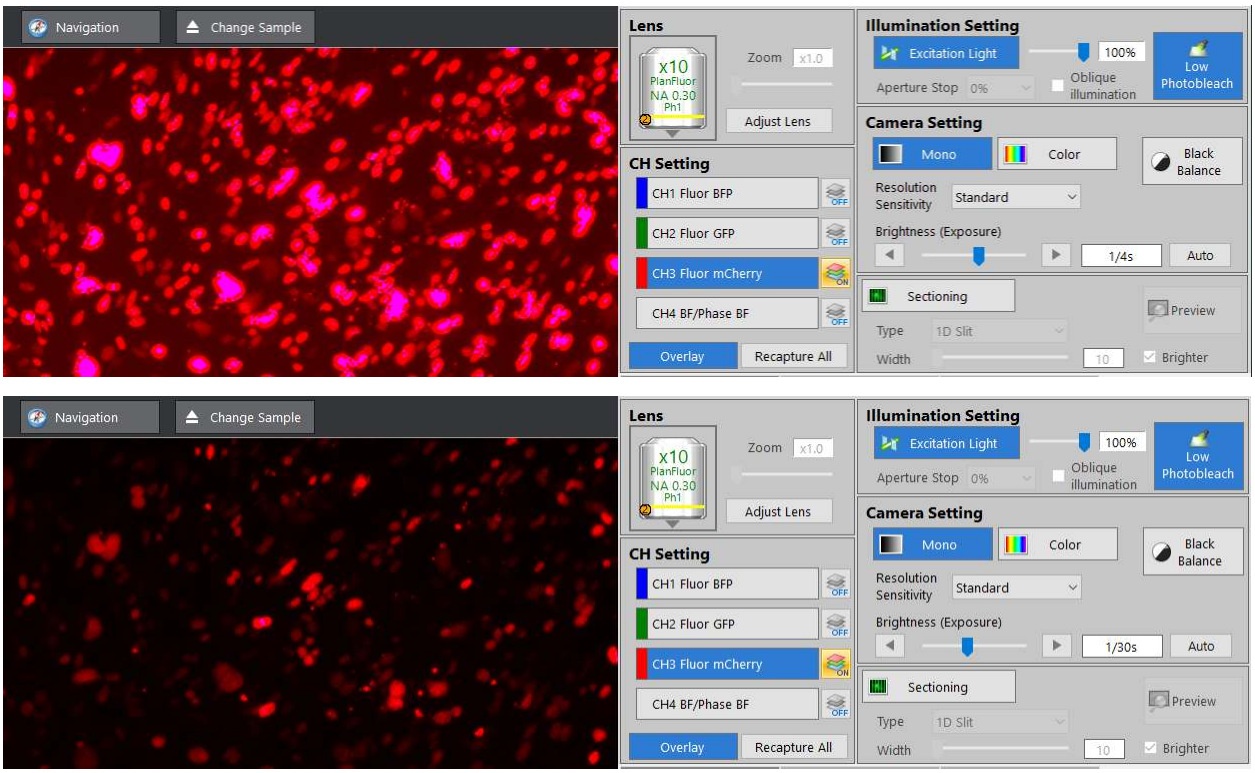


6. **Focusing Images**: To manually focus the microscope image, use the mouse scroll for fine adjustments. For moderate adjustment use ctrl + mouse wheel. For large adjustments use ctrl + shift + mouse wheel. There is also an option to auto-focus under the Z Position menu, but this method can take longer.

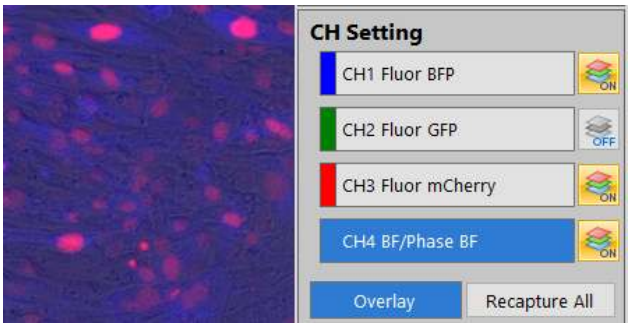
7. **Exposure Time / Brightness**: To change the brightness of the selected channel, change the exposure time under the Camera Setting menu. In general, it is best to stay under an exposure time of 1s for fluorescent channels because any longer typically starts to show autofluorescence.



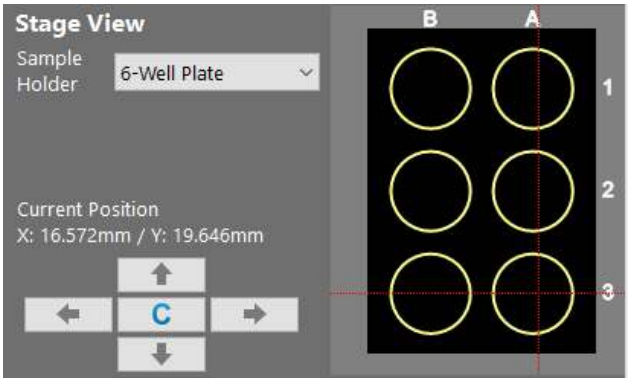
8. **Overexposure**: When the exposure time is too high, the images can be overexposed. When this happens, the image has purple spots in the areas that are overexposed. It is best to decrease exposure time until no more purple is seen.



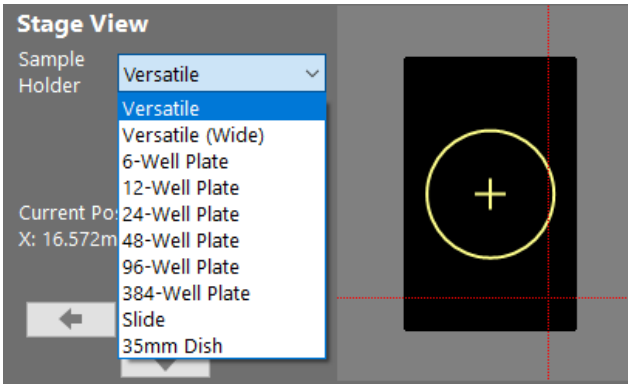
9. **Channel Settings**: Select which color channel to view by clicking on the buttons labeled CH1, CH2, CH3, or CH4. Only the selected channel will captured when changing X/Y/Z position or exposure times. Multiple channels can be viewed at once by clicking the ON/OFF button next to each channel. However, you must select each channel in turn to update the each channel's image to the current X/Y/Z position.



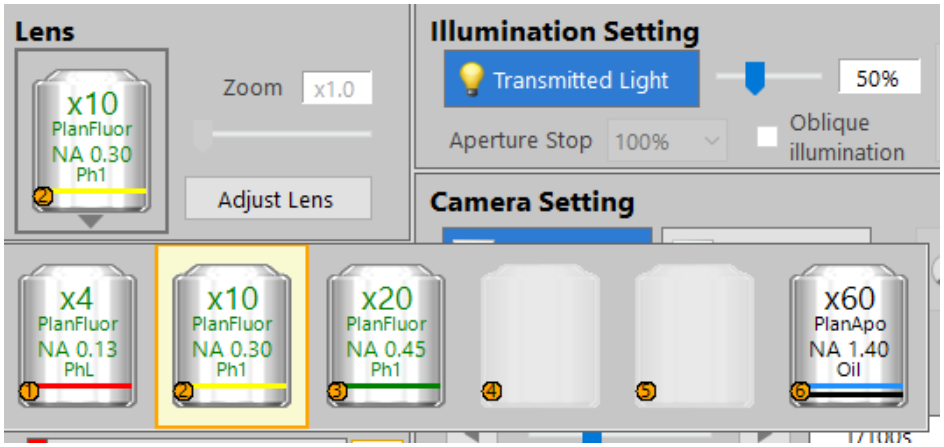
10. **Navigation**: There are several options to navigate a plate. (1) Click and drag on the microscope image. (2) Use the arrows in the Stage View menu. (3) Click to the desired location in the plate layout in the Stage View menu. The red perpendicular lines indicate the current position.



11. The stage view menu can also be changed here to match the plate layout.



12. **Magnification**: To change the magnification, click on the objective lens button in the Lens menu on the left, and the objective lens options will appear. Select the desired magnification.



Note

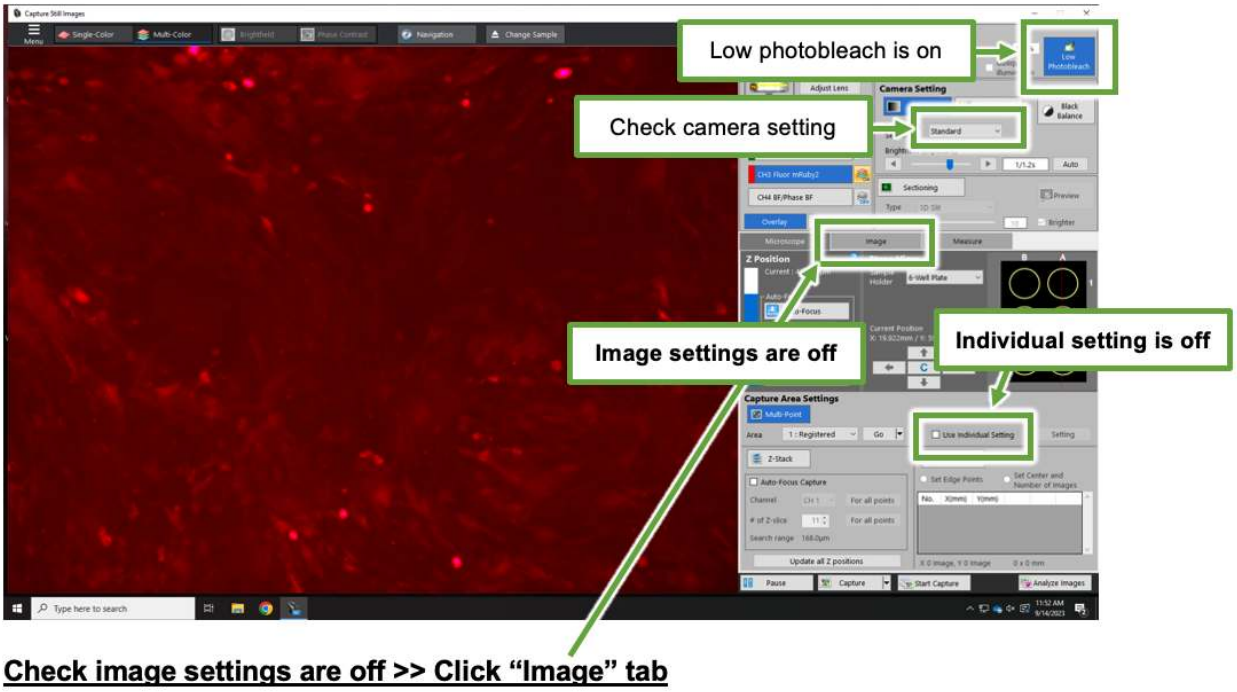
The 60X lens requires adding oil to the lens. See *60X Magnification* (page **??**)

2.3.2 Default image settings

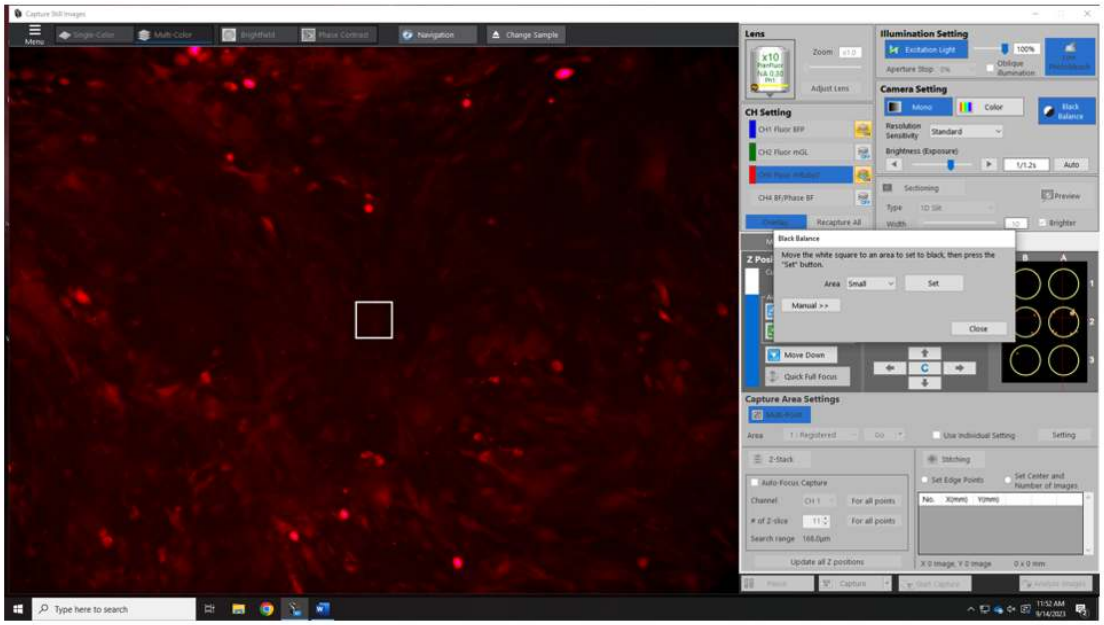
Important

Check to make sure the image settings are off. It is best practice to take un-edited images and then make trackable adjustments with softwares like Fiji.

Here are some common things to check for.



As an example, this is what the same cells look like with black balance on. While it helps clarify which cells are mRuby2+, it can also be misleading.


Menu
Single-Color
Multi-Color
Brightfield
Phase Contrast
Navigation
Change Sample
Lens
Zoom
Adjust Lens
Illumination Setting
Excitation Light
Aperture Stop
Oblique Illumination
Camera Setting
Mono
Color
Black Balance
Resolution Sensitivity
Standard
Brightness (Exposure)
Auto
CH Setting
CH1 Fluor BFP
CH2 Fluor mGL
CH3 Raw Artifact
CH4 BF/Phase BF
Sectioning
Preview
Recapture All
Brighter
Black Balance
Move the white square to an area to set to black, then press the "Set" button.
Area
Small
Set
Manual >>
Close
Move Down
Quick Full Focus
Capture Area Settings
Use Individual Setting
Setting
Z-Stack
Stitching
Auto-Focus Capture
Set Edge Points
Set Center and Number of Images
Channel
For all points
# of Z-slice
Search range
Update all Z positions
Type here to search

2.3.3 60X Magnification

The 60X lens works best with glass coverslips/plates.

Important

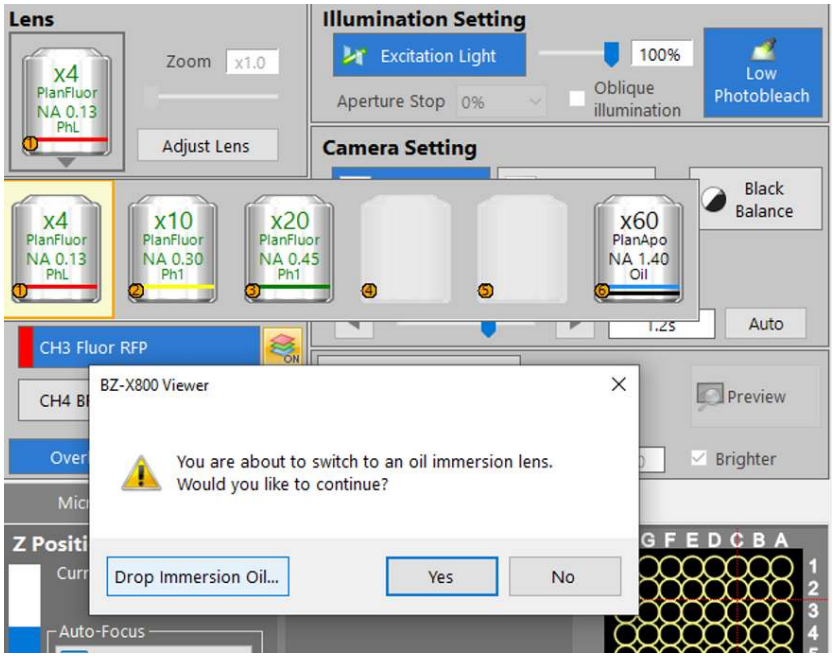
The 60X lens is very sensitive. Exercise extreme caution when using and only clean with the special *Ross Optical Lens Tissue* (doesn't leave behind lint).

Tips for cleaning

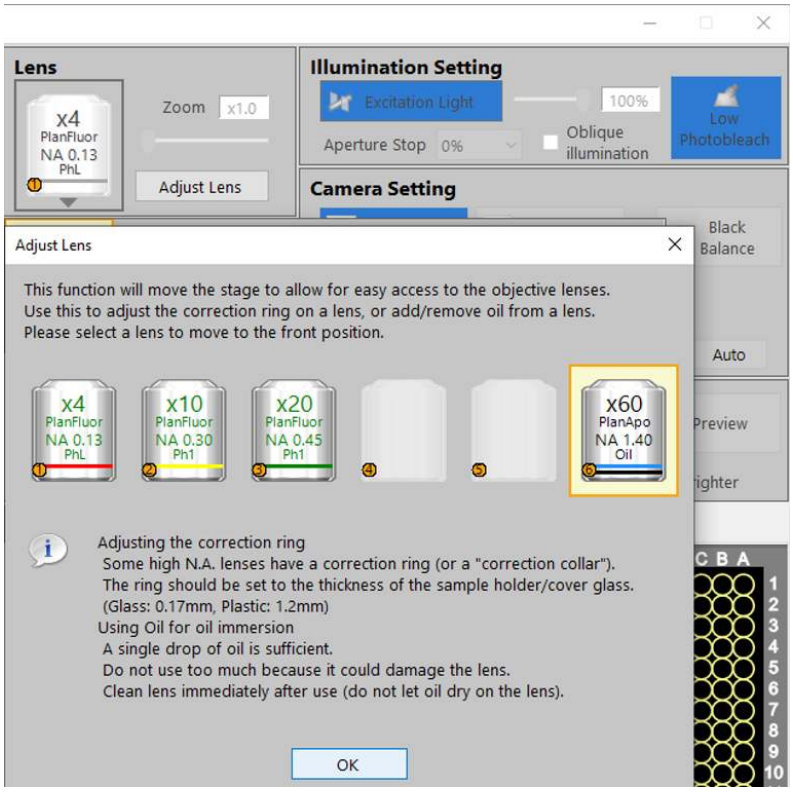
1. Dust is the most common contaminant and can usually be removed using pressurized gas. Use a dust-free blower or a soft optical cleaning brush to remove large dirt particles before attempting to clean the optic with lens tissue, as these larger particles trapped under the tissue will scratch the surface.
2. Take a single piece of tissue and fold it in half until it is about 1 inch wide. **Moisten the tissue with ethanol**. The tissue should be moist but not dripping wet.
3. Place the moist portion of the tissue on the optic. With gentle pressure from your index finger, drag across the surface with a circular motion, starting from the center of the lens and moving outward. Discard this piece of tissue. Reusing the same tissue can cause recontamination of the cleaned surface.
4. Repeat steps 2 and 3 until the surface of the optic is clean.

How to use the 60X lens

1. Click the "x60 lens" icon to change the lens
2. Click `Drop Immersion Oil...`. The 60X lens will move forward for you to be able drop oil on it.



2. Follow the instructions that are presented. Use the Nikon immersion oil and carefully place no more than one drop of oil. Wipe off excess with a Ross optical tissue so oil doesn't spill when the plate is added.



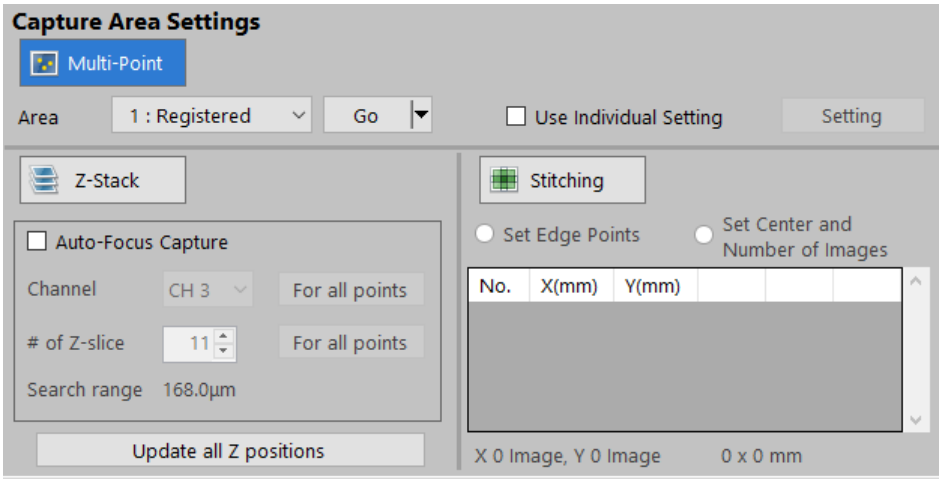
3. Click Ok and then proceed to change the lens.

Note

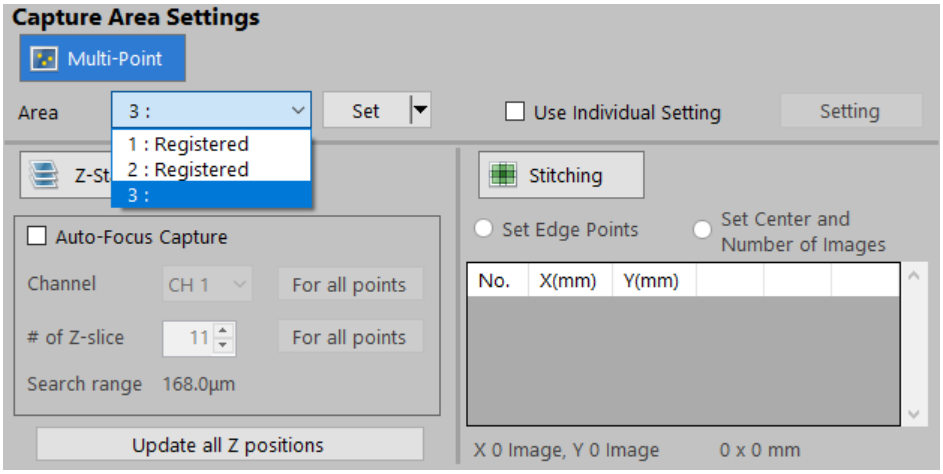
If doing lots of imaging, you may have to add more oil as it will dry out from the heat.

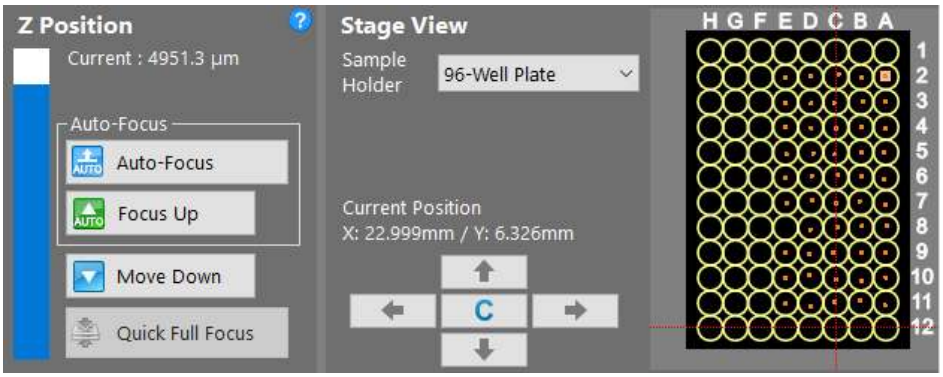
2.3.4 Multi-point capture

1. To capture multiple X/Y/Z points on your plate, instead of capturing points individually, select the **Multi-Point** button under the Capture Area Settings menu.



2. Navigate to a position on the plate and focus the image. To set a capture point, click **Set**. This point will then be Registered as a capture point and assigned a number. Orange dots will appear on the Stage View for each set capture point.





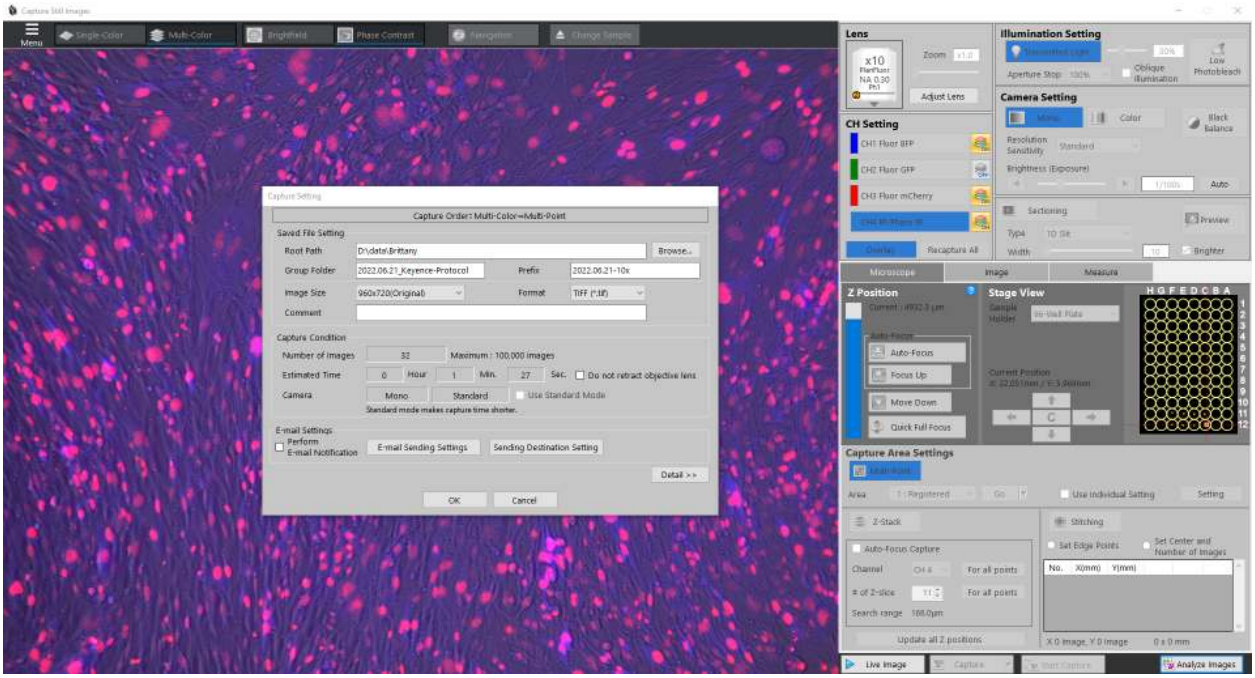
3. Once all the capture points are set, turn ON all the color channels to capture and ensure the exposure settings for each channel are correct. Then click **Start Capture** at the bottom of the

screen. A dialogue box will appear to select where you want the images to be saved (Root Path), name the folder the images will be grouped into (Group Folder), and whatever prefix you want to give the images such as the date or magnification. Click OK to capture images.

Note

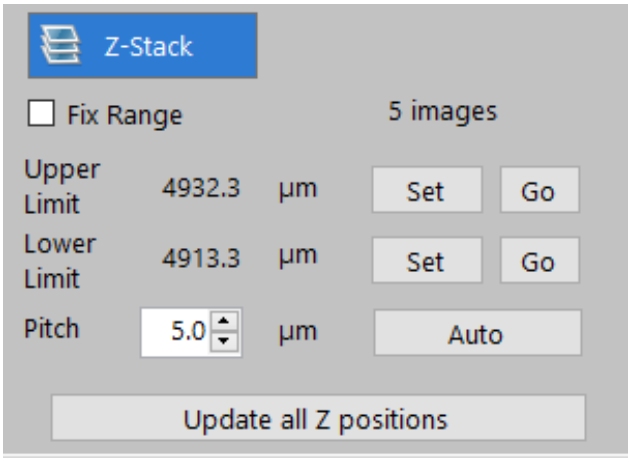
Usually, you *do not* want the **Use Individual Setting** box to be selected when using Multi-Point Capture. This ensures that whichever channels are turned ON and the exposure times for those channels are the same for all the images captured. The same exposure settings between images is required for the direct comparison of brightness between conditions.

If the **Individual Capture Setting** *is* selected, the Multi-Point Capture will record which channels are turned on and their exposure settings *when the point is originally set*. This can be useful if you want different exposure settings for some conditions or different wells require different color channels to be captured. However, *it is important to remember this each time you set a capture point*.

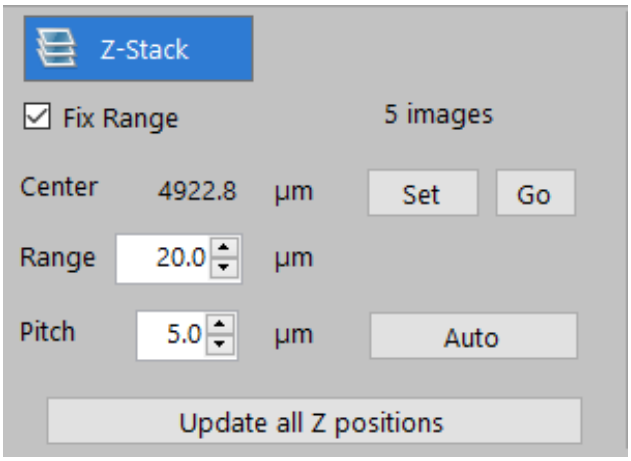


2.3.5 Z-stack

1. To capture multiple focal planes, click the **Z-stack** button under Capture Area Settings.
2. The default Z-stack method is to set the Upper and Lower Z coordinates. First, focus the image to the highest Z position to capture and click **Set** next to **Upper Limit**. Next, focus the image to the lowest Z position to capture and click **Set** next to **Lower Limit**. Finally, select the desired height between images (Pitch). If also using Multi-Point Capture, you may want to set these values for each capture point.



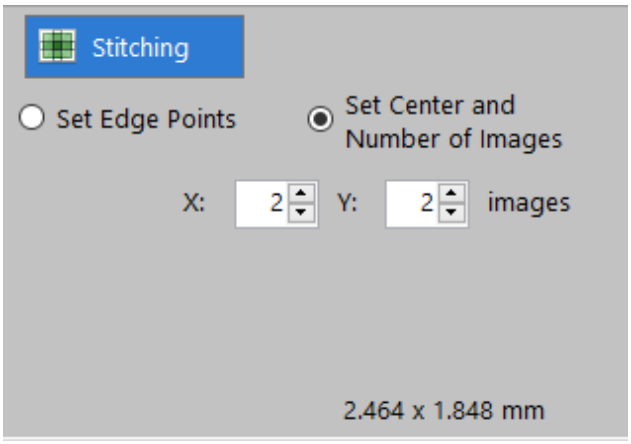
3. Alternatively, select the box next to **Fix Range**. Then set the Z position to be the center of the Z-stack by focusing the image and clicking **Set**. Enter a range to capture. For example, a range of 20um will capture 10um above and below the center, for a total range of 20um. Additionally set the desired height between images (Pitch).



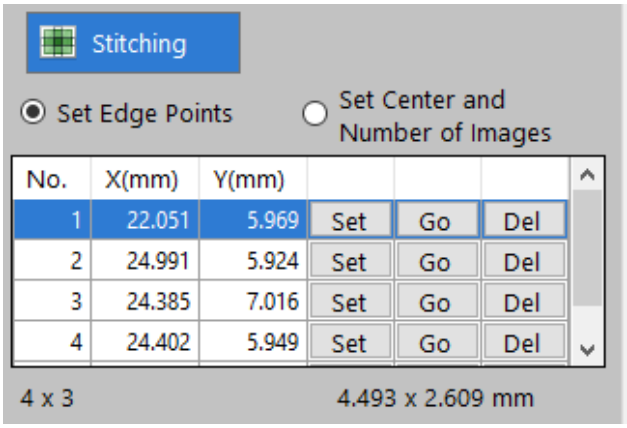
2.3.6 Stitching

1. To capture a larger total area at a given magnification, click the **Stitching** button under Capture Area Settings. This will capture a series of slightly overlapping images that cover the desired area. There is the option to **Set Center and Number of Images** or to **Set Edge Points**.

2. **Set Center and Number of Images**: When you set your capture point, this will be the center of the stitching region. Next, choose the size of the grid to capture. For example a 2x3 grid will capture an area 2 images wide and 3 images tall, 6 images in total.



3. **Set Edge Points**: Click set at the locations at the edge of the field you wish to capture. It will automatically determine the number of images to capture.



Note

The images will be saved separately. If the images need to be stitched together, this can be done using the BZ-X800 Analyzer software or using a custom script.

2.4 Keyence Time Lapse Imaging

2.4.1 Setting up the incubation chamber

The Keyence comes with a special chamber / plate holder and stage top incubator controller[36] for time lapses that maintains temperature, humidity, and CO2 levels for the cells.

1. Place the stage adaptor in the Keyence and use a screwdriver to tighten the screws.
2. Place the chamber on top of the stage adaptor. It just sits in place.
3. Secure the cords/tubes to the plate using the clasps.
4. Remove the chamber lid and add Elga water to the "moat" until it is full. You can do this using the syringe located by the Keyence. Be careful not to spill water into the Keyence!
5. Turn on the stage top incubator controller that is attached to the incubation chamber.
6. Turn on the flow of CO2 by opening the valves on the tank. There are 3 different values you may need to open: the valve directly on top of the cylinder, the large valve on pressure regulator, and the small blue valve on the underside of the pressure regulator.
7. Check there is enough CO2 tank pressure (right pressure gauge) for your time lapse (should be >600 psi). The left pressure gauge should be between 14.5-21.5 psi; to change this pressure you can turn the large blue valve on the CO2 tank. Ensure the CO2 is flowing by looking at the process value (PV) on the controller display.

[36] https://spectraservices.com/mm5/graphics/00000001/MA-STX-EN.pdf

2.4.2 Using the Keyence Software

1. Open the Keyence software and select “Time Lapse Capture”.
2. Set capture points, z-stacking, and stitching as normal.
3. Click “Start Capture”.
4. Choose where you want to save the images. You should save them onto the Galloway Lab - Timelapse OneDrive that is specifically for time lapses.
5. Choose how frequently you want to capture images. They software will show the limit for how frequently you are able to capture images. This limit depends on the total number of images you want to capture at each time point.
6. Choose how many times you want to capture images. The software will display the total capture time in the window based on imaging frequency and number of time points.
7. There is also a limit to the total number of images you can take in a single time lapse (100,000 images). You might also be limited by how much storage space is available on the Keyence computer. You can free up space on the computer if you need more storage space up to ~1.8TB.
8. Once all the capture conditions are set, you can begin the time lapse.

2.4.3 Tips and Tricks

- Mix light cells (fluorescent) with a population of dark cells (no fluorescence). This is helpful for tracking since it reduces crowding of the light cells. The ratio of light to dark you want will depend on your experiment.
- You can also try to seed cells more sparsely than usual to prevent crowding of cells which helps with tracking.
- Imaging as frequently as possible is best for tracking so the cells move less between time points.
- Free up space on the Keyence computer the morning before you start the time lapse. This can be done by right clicking on OneDrive folders and selecting "Free up space". It can take several hours for OneDrive to remove files from the computer.
- Let the plate sit in the Keyence for a while (1-2 hours) before starting the time lapse. This will help keep the cells in focus during the time lapse. If you start imaging immediately, the cells will likely go out of focus within the first few hours.
- Use a z-stack to ensure you keep the cells in focus throughout the time lapse. Brittany has previously used a z-stack with 60um range with a 12um pitch.

2.5 Koch Flow Core FACS Training

2.5.1 Sorting Machines

There are currently two types of FACS machines available at the Koch Flow Core.

1. Sony MA-900 (recommended)
    - Easy to use.
    - Doesn't require extra training on an analyzer.
    - However, the green + red lasers and the blue + far-red lasers are collinear (not spatially separated), meaning if you want to use green with red or blue with far-red at the same time, you will want to learn how to use the compensation wizard.
2. Aria
    - You need to be trained on an analyzer first, then you can be trained on the Aria.
    - However, lasers are spatially separated like on the Attune.

Training

You will need to do several meetings and trainings before you can use the flow core independently, expect it to take a few weeks to get fully trained. The main steps are:

1. Submit the biohazard form on iLabs
2. 1-hour consultation meeting with Glenn
3. 1-hour startup and QC training

4. 2-hour flow sort training (you need to bring your own samples)
5. 1-hour flow sort with supervision (this can be part of an experiment)

Training Details

Note

Much of this training information is also on iLabs or you will hear about it during your consultation meeting with Glenn.

1. Make sure you have an iLabs account and PO for payment: https://mit.ilabsolutions.com/account/login
2. In iLabs, under Core Facilities, you can find the Flow Cytometry Core.
3. Go to Schedule equipment tab.
4. Fill out the linked biohazard form.

- You only need to do this once unless your biosafety requirements change.

5. Schedule a consultation meeting with Glenn.

- 1 hour long
- Typically M-F from 11am-12pm
- Under Staff Calendars for Training and Assisted Work heading, book yourself on Glenn's calendar.
- You will need to know what fluorophores and cell types you plan to use during your training.
- He will go over a lot of the information about flow sorting and the flow core.

6. After the consultation meeting, you will be given access to the instrument calendar to book your training with the staff.
7. Next you will need to sign up for a time for Startup & QC training.

- This is 9-10am W-F on days when someone is signed up to use that FACS machine (which is most days).

8. Next you will sign up for a 2-hour time block to actually be trained how to sort.

- The staff are usually available for training from 10:30am-12:30pm and 1-3pm each day.
- Since training takes extra time, it is not recommend to use this time to sort cells for an important experiment since you might not have enough time to sort everything.
- You will need the following samples:
  - Unstained - same cell type as samples without any fluorophores. Needed for autofluorescence.

  - Single color - one for each fluorophore in your experiment. Needed for spectral overlap (compensation).
  - FMO - all fluorophores minus one. Important for dim populations and gating.

9. Finally, you will sign up for a 1-hour assisted sorting. After this, you can sort on your own anytime.

- If you need to do a longer sort for an experiment, you could sign up for the 1-hour assisted use, then separately sign up for more time immediately after to finish your sort.

Sony MA-900

The Sony MA900 cell sorter has a SOP[37] that you can follow for startup and shutdown.

Startup

You only need to start up the Sony if you want to use it over the weekend, on holidays, or outside of normal hours. During the week, core staff will do the start up in the morning. The Sony is generally very easy to startup once you've seen how to; you mostly follow automated prompts and switch out the sorting chip. However, the auto-alignment and equivilant of the Attune performance test takes around 45 minutes.

1. Empty the waste container. Put it in the sink and add 1 bleach tablet. Wait at least 20 minutes before emptying. They have labels to the right of the sink if you want to label with the time.
2. Refill sheath fluid. Boxes can be found in the closet and on a rolling cart. Ethanol the tip of the tube before putting in the sheath fluid reservoir.

- Sony 3 rubber O ring often falls off, so be cautious.

3. Turn on the air (blue valve) if it is not already open, then wait 1-2 minutes.
4. Open the BSC and turn on Sony with power button.
5. Log into computer (login info is on the monitor) and open the Sony software.
6. Select User 1.
7. Scan chip QR code.

- Each chip is good for 24 hours, so write time and date on package after opening it.
- You don't need to change out the chip if it has been less than 24 hrs since it was opened (e.g. someone used it at 5pm on a Saturday and you are using it at 9am on a Sunday)

8. Push to open the top of the Sony. Take out the old chip and put new one in. It's like an SD card, so push gently on top of chip and eventually the instrument will pull it in.
9. Follow prompts on screen.

- May need to change out tube holder from 15 mL to 5 mL.
- Beads are run in 5 mL tubes.

[37] https://docs.google.com/document/d/1toqMY_qnDy0_YDkcEr2ktDJWcteKe0Pj42_scukqT5s/edit

10. Beads are located in the mini fridge near the sink in a brown cardboard box tray.
    - They make new tubes every week.
    - New beads can be found in the white boxes on the right side of the fridge. Make sure the bead lot matches if you get a new dropper bottle.
    - Sony 2 and 3 needs about ~10 drops.
    - Sony 1 needs ~15-20 drops.
11. Shake the dropper bottle well and vortex tubes before use.
12. On software during QC, make sure the droplets look good (e.g. no vibrations or fuzzy looking drops).
    - If droplets look weird, you can try a chip exchange and just put the same chip back in. If you do this, you need to skip autocalibration and then calibrate later after the chip exchange.
13. Sony 2 and 3 takes ~30 minutes to start up, and the Sony 1 takes ~45 minutes to start up
    - The calibration step takes ~20 minutes.
    - You don't need to include startup time when signing up to sort on weekends/holidays (e.g. don't pay for startup time).

Sample Prep and Sorting

1. Get ice. Keep your samples on ice as much as possible after dissociation.
2. Dissociate cells using appropriate method. Centrifuge the cells to pellet them.
3. Aspirate the supernatant and resuspend cells in ~5-10 million cells / mL of media.

> **Tip**
>
> Start with resuspending in 0.5-1 mL of media per 6-well. For reprogramming, try 1 mL for proliferative conditions and 0.5 mL for less proliferative conditions.

4. Gather useful items to bring to the sort:
    - Your samples
    - 5 mL round bottom tubes with strainer caps (at least 1 per sample, plus some extra)
    - Collection tubes of appropriate size
    - Extra media if you need to further dilute cells prior to sorting
    - FBS or 5% BSA for collection tubes
    - P1000 pipette with filter tips
    - Gloves
    - Lab markers

5. Head over to the Koch flow core.
6. On the computer, open a workspace for sorting.
    - If you don't have a template yet, you can select **File** > **New** and then Blank or Existing template. If you have sorted before and saved a template, you can import that instead.
    - You can uncheck any unused fluorophores (e.g. FL1 through FL4) if needed. If you need to use the compensation wizard, then you *must* uncheck the unused fluorophores.
    - The 488-nm laser *must* stay on, but other unused lasers can be unchecked. It may be easiest to just keep all lasers on.
    - Click Create Experiment.
7. Set gates by running some of your controls and samples. If needed, start the compensation wizard and run your single color controls.
    - Select begin experiment and start collecting first filtered tube.
    - You can make a new plot from the button in the upper left or by double clicking the gate.
    - As needed, adjust the Gain Settings (voltages) under **Detector & Threshold Settings**.
    - Add sort gates and rename them as needed.
    - The compensation wizard will give you prompts for getting it set up.
8. Place labeled collection tubes (with BSA or FBS) in the tube holder in the collection chamber and close the door.
    - You may need to change the tube holder to fit the tube size you wish to sort into.
    - Vortex your collection tube to allow BSA or FBS to coat the sides of the tube. This is supposed to help with recovery.
9. Under **Sort Control**, click **Load Collection**.

Tip

Remember to click **Load Collection** *before* starting to run your sample. If you start running your sample first, you have to stop or pause it, click **Load Collection**, then restart running the sample. You can only begin to sort when the collection tube has been loaded.

10. Set the desired settings under Sort Control.
    - **Method** should match the collection tube size.
    - Set the sort **Mode** to your desired option (e.g. Normal, Purity, or Yield). There is a tradeoff between the two (e.g. better purity leads to lower yield and vice versa).
    - Under **Sort Settings**, select the location and gate for each collection tube, and optionally add a stop count. A stop count of 0 leads to non-stop sorting.
    - Also set your desired recording conditions (e.g. stop recording after 100k events). It is recommended to have Auto Record checked.

11. Filter your sample and load tube. Make sure the filter cap is off!
12. Click start and verify your gates.
13. Once the flow rate is stable, click **Sort & Record Start**.
14. You can change the **Sample Pressure** to adjust the flowrate and events/second.
    - Do not go over 5k events/second.
    - AMB recommended to run at $\leq$2-2.5k/sec for MEFs because he had issues at higher event rates.
    - Sample pressure of 4 is standard.
    - Increasing sample pressure can decrease sort efficiency. Aim for a sort efficiency of 80% or greater.

Warning

Watch out for the event rate suddenly dropping quickly. This can mean the tube is close to running dry which can damage the machine.

Always keep an eye on sample volume!

1. If you did not set a stop condition for the sort, once you have sorted the desired number of cells, click Stop.
2. If you have more samples to sort, click **Next Tube** to create a new sample. You can rename each sample to reflect your condition name.
3. After you have sorted all your samples:
    a. If there is a user signed up after you, run **Bleach Cleaning** ($\sim$6 min) and then **DI Rinse** ($\sim$6 min) and follow the software's prompts for each step. You don't have to stay while the DI Rinse if there is another user after you.
    b. If you are the last user of the day, from the **Cytometer** tab, select **Hardware and Software Shutdown** to start the cleaning wizard. Follow the prompts to complete the Bleach Cleaning and DI Rinse. Then select Shutdown to turn off the instrument.
4. Export your FCS files to the computer. There should be a data folder on the desktop with a folder for each month of the year, and you can add a folder with your name for the month.
    - Right-click on the experiment (at the top of all the tube names) and select **Export as FCS Files**.
    - A PDF of the experiment layout can be saved by clicking **Custom Print** (in **Worksheet Tools** ribbon).
    - To export the entire experiment, go to **File** > **Database**, then click on the experiment you want to export, then on the arrows to move it to the export window on the right. This will save the data, plots, and gates into one file.
5. Optionally, save your workspace as a template for future experiments.

6. Login and then upload your data and/or template to the Galloway Lab Onedrive under instruments/data/koch_flow_core.

Note

- Each droplet is 1 nL, and you have 2 droplets per sorted cell. (e.g. 100k sorted cells = ~200 μL)
- Sorting at 5k cells / sec is a safe rate, but you will lose more cells the more you put through.
- You want your BSA or FBS to be at least 20-30% of the final volume after sorting. For example, if you want to sort 300k events, the sorted volume will be ~600 μL, and therefore you want to put ~200-300 μL of BSA or FBS into your collection tube.
- Don't use a larger collection tube than needed. You can lose cells on the sides of your tube.
- Leave 1-2 cm at the top of the collection tube to reduce cell loss.
- The Sony can keep your sample and collection tubes cold, just remember to turn on that feature.
- Strain cells *immediately* prior to sorting. This prevents clogs. If you get a clog, you can lose all your sorting time.
- Use polypropylene tubes; this material is less "sticky" compared to polystyrene and can reduce cell loss.
- DO NOT run the sample tube dry! This will damage the machine!
- If fluorophores are dim, a live/dead stain is recommended.
- It takes ~30 seconds from clicking start for cells to begin appearing in plots.

Tip

How long should you sign up for flow sorting?

Example: I have 6 samples to sort, and I want ~100k sorted cells.

1. Assuming 40% yield (could expect between 30-50%), I want to sort at least 250k events in order to get 100k live cells after my sort.
2. From my gating, I expect 50% of events to be Live Cells, 90% of those to be Single Cells, and 60% of those to be positive for my fluorophore.
3. I plan to run at ~2000 events / second. Based on the percentages above I expect to sort ~540 events / second.
4. Therefore, to collect 250k sorted events, it will take about 8 minutes to run each sample.
5. For 6 samples, it may take about 48 minutes of just sorting time.
6. Add on extra time for setting up the workspace, setting voltages and gates, swapping

samples, the final Bleach Cleaning and DI Rinse steps, and exporting your data.

Shutdown

Follow the shutdown SOP, with the exception that on step 7 ("Turn off the air compressor and blue switch on the air-line"), do **not** turn off the blue switch on the air-line, and turn off the air compressor by turning off the power strip that is below you to the left, when sitting at the computer. **Do not** turn off the air compressor by turning off the switch on the back of it.

2.6 Running Genomic pre-analysis using SnakePipes in Supercloud

We have snakePipes module set-up in the Galloway Supercloud account. To read more: https://snakepipes.readthedocs.io/en/stable/. This protocol aims to guide the user how to create an alignment script using the SnakePipes module and instructions on running it in Supercloud.

1. Log in to your SuperCloud from Terminal.
2. Navigate to `~/galloway_shared/data/NGS_DATA/raw_reads` and confirm the path to the FASTQ files that you intend to analyze.
3. Go back to your local directory and create a bash script using

```
vim alignment.sh
```

4. Type the following lines into the bash script, and don't forget to modify the directory paths, organism genome , Read_1 and Read_1 extensions , accordingly.

```
#!/bin/bash
# SBATCH --output=snakePipes-%j.txt
# Loading the required module
source /etc/profile
module load anaconda/2023a
# Activate local conda module
eval "$(conda shell.bash hook)"
conda activate /home/gridsan/groups/galloway/conda_envs/snakepipes
## SnakePipes for DNA mapping command ##
DNA-mapping -i ~/galloway_shared/data/path/to/FASTQ_files -o ~/galloway_
↪shared/data/path/to/output_files -v --ext ".fastq" --reads '_1_
↪sequence' '_2_sequence' mm10_gencodeM19
```

5. Save and exit from script by pressing `:wq` . You can always open and edit the script using `vim alignment.sh`
6. To run the script in Supercloud, you need to submit to SLURM via LLsub (See Supercloud user guidelines) using the following command:

```
LLsub -i alignment.sh
```

7. Once submitted, you can check the status of the run via `LLstat`. To verify the success/failure of the run, you can check the log `file-alignment.sh.logXXXXX` for more details. Usually the log file contains the progress of the alignment run and/or the details if the alignment failed.

2.7 Animal Facility

1. Go to https://atlas.mit.edu and go to the learning center, through the tab on the left.
2. In the upper right, select **My Profile**, then **Join a Group** and select **68N: Mouse**.
3. Complete all the required online trainings/forms that appear for group **68N: Mouse** under the **My Training Needs** tab.
    - General Biosafety for Researchers
    - DCM Facilities Pre-Orientation
    - DCM Cage Cards
    - CITI Working with Mice in Research
    - CITI Working with the IACUC
    - Occupational Health Questionnaire
    - Any others
4. Use the Atlas Learning Center to sign up to attend live training sessions for group **68N: Mouse**. You can register for these sessions by clicking the "Book Session" button. If your schedule absolutely prevents you from attending normally scheduled classes, or if no sessions are listed, email the instructor directly.
    - Rodent Refresher (available once a month, *must be completed once each calendar year*)
    - Animal Handling Orientation: MICE
    - Facility Orientation: 68N
5. Once all the **required** trainings are complete, the current mouse colony manager will need to submit a personnel amendment to the current CAC protocol in CAC-CONNECT. All trainings must be completed within 6 months of the amendment being submitted. If trainings are more than 6 months old, you will be required to complete them again.
    - For expired CITI trainings, they need to be manually reset by DCM in Atlas. Email dcmtrainings@mit.edu to do so.
6. To gain access to the facility, you will need to email dcmaccess@mit.edu. In the email include the facility in which you will be working (68N) and your MIT Kerberos ID.
7. Once granted access to the 68N facility, you can be trained on lab-specific CAC protocols.

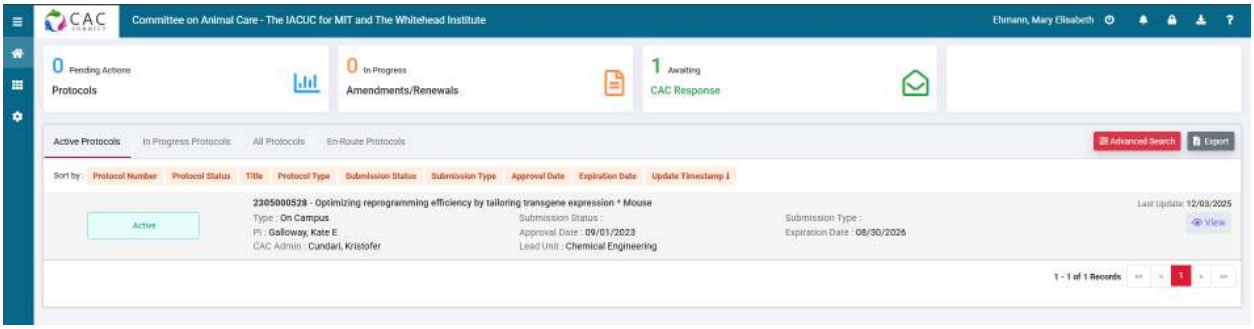
2.7.1 Mouse Colony Manager Lab Job

A lab job is to manage our mouse colony. Responsibilities include:

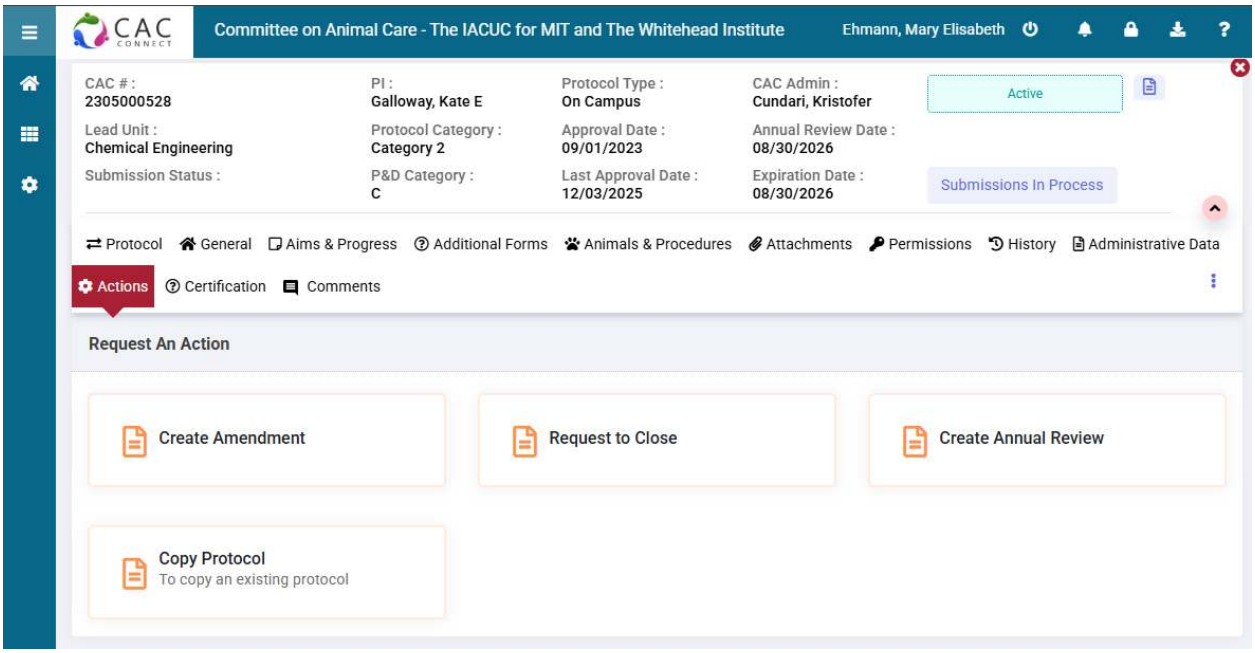
- Completing annual CAC renewal (or 3 year renewal)
- Submitting amendments for new mouse strains or protocols
- Ordering new mouse strains
- Setting up timed matings with our vet tech Yinghui Ning (yhning@mit.edu)
- Attending monthly DCM meetings (usually the first Tuesday of each month)

How to submit a CAC amendment

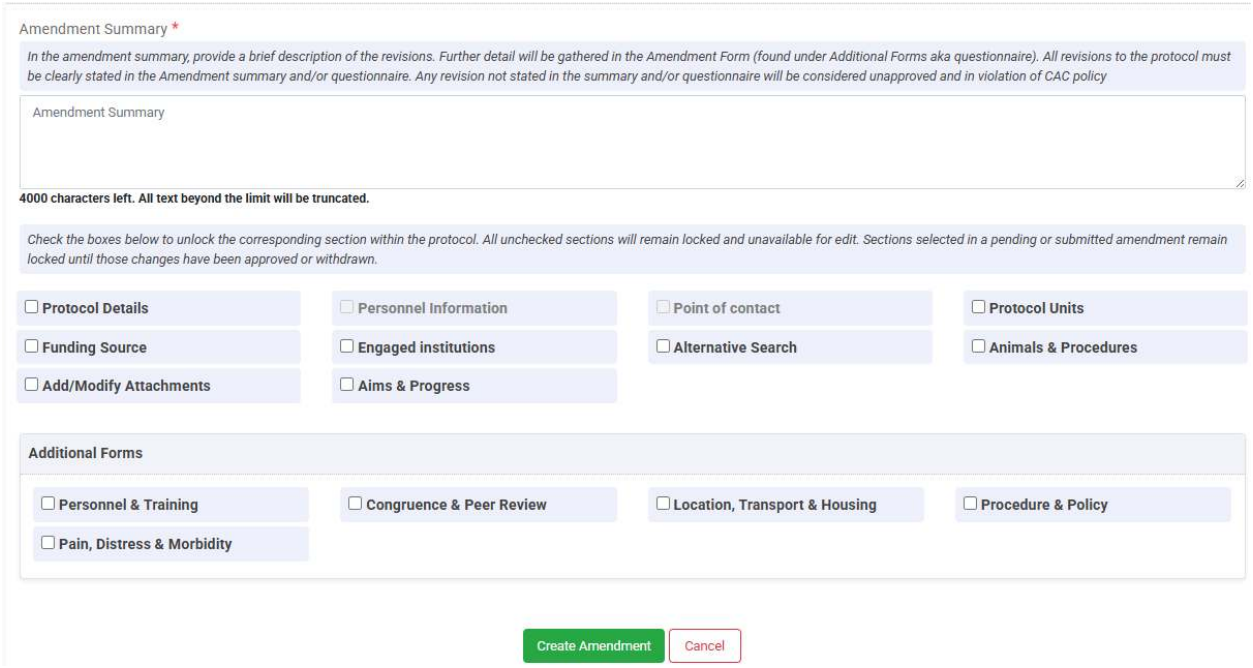
There are multiple types of CAC amendments that you can submit. The most common are personnel and new mouse strain amendments. Below is the CAC Connect dashboard. Here you can access the active protocol and any amendments that are in progress or submitted to the CAC.



To create an amendment, click “view” on the active protocol. Then produce to Actions >> Create Amendment



After opening an amendment, you will need to first complete the **Amendment Summary** to specify the purpose and type of the amendment. Below is an image what the summary looks like.



After filling out this summary, you will need to fill out the follow sections.

For Personnel or Administrative Amendments:

- Additional Forms: answer all questions marked under an incomplete icon
- General: change personnel information or points of contact
- Certification: completed by the PI before the amendment can be submitted

For Animal or Protocol Amendments:

- Additional Forms: answer all questions marked under an incomplete icon. Include animal justification here if adding a new strain.
- Animals & Procedures:
  - Procedures: If requesting to make a new strain (or purchasing a cryopreserved embryo), include a "Transgenic/KO Animal Production" form
  - Animal Groups: Add new strain to respective mouse groups (usually groups B and C)
  - Animal Justification: A written justification for new strain and number of animals expected in each group per protocol year
  - Certification: completed by the PI before the amendment can be submitted

After completing all required sections, you can submit the amendment by clicking Actions >> Submit Amendment

For more general information, the CAC provides a small online course on how to submit certain amendments[38].

Ordering a new mouse strain

To order new mouse strains, an amendment needs to be added to the CAC protocol and approved. Once approval has been granted you can proceed with ordering. If ordering from an approved DCM vendor (i.e. Jackson Labs, Charles River, Taconic), email animalrequest@mit.edu. For atypical vendors (i.e. MMRRC), these are covered by animal-import-export@mit.edu. They will send you a form that requires you to fill out some standard information:

- PI name: Kate E. Galloway
- Department: Chemical Engineering
- Contact person & email (current mouse manager)
- Building/Room: 66-219
- Protocol Title: Optimizing reprogramming efficiency by tailoring transgene expression
- CAC Protocol Number: 2305000528
- Campus housing location: 68N

The form will also require information about the strain, vendor, expected pain category, age, and sex of the mice. When ordering both male and female mice, they require separate ordering forms.

Note

When ordering throught MMRRC, a COU agreement will need to be filled out. This form will be sent in an email to either DCM or animal-import-export. They will forward this email to you. The form (even if they say to fill it out) needs to be sent to Susan Watts in the TLO office (wattss@mit.edu) for them to sign.

Recovering a cryopreserved embryo

Certain mouse strains are only available for purchase as a cryopreserved embryos. While companies do offer the service to rederive the strain in house, it is usually expensive. DCM offers assisted reproductive services where they can implant cryopreserved embryos. You must fill out a form[39] to request the services. The cost is currently around $300.

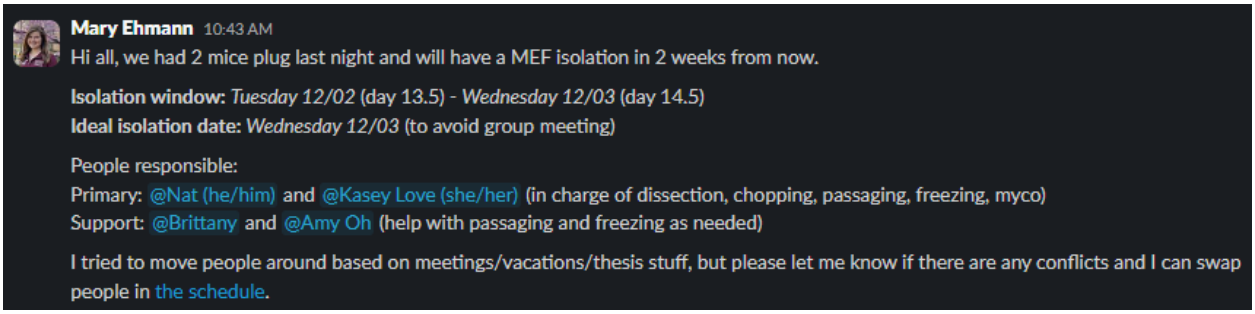
Timed matings for embryo isolation

In the lab we use mouse embryonic fibroblasts (MEFs) to assess our direct conversion protocols. The MEFs that we use are isolated from embryos at day E13.5 to E14.5. Therefore, we need to set up timed matings to ensure we isolate embryos on these specific days during gestation. How to set up timed matings:

[38] https://cac.mit.edu/cac-connect/cac-connect-guidance
[39] https://comp-med.mit.edu/form/webform-707

- Contact our vet tech (Yinghui Ning, yhning@mit.edu) to schedule timed matings. Reach out the week before you want to the matings set up. Provide how many mice you would like to plug (become pregnant) and the pairings you would like. (ex. Hb9+ male w/ WT female)
- Yinghui will send an email regarding how many mice plugged. The day the mice were separated and a plug is found is day E0.5
- Send a message in the mef_isolation channel with the isolation days (E13.5 or E14.5) and people responsible
- The day before the isolation, check to see if the mice are pregnant. Send updates in the mef_isolation channel.
- If mice do not appear pregnant, leave the cage and email Yinghui to let her know.

Below is an example of a message in the mef_isolation channel. The schedule can be found here[40]



2.8 Cluster Computing

MIT houses several computing clusters that are available for the lab to use. As of 2025, we use the Engaging cluster, though this may change in the future.

The most common use case in our lab is RNA-seq analysis because reading and aligning millions of transcripts is computationally intensive, as you can imagine. The general workflow (in detail below) is using a Snakefile to execute a list of commands for trimming, aligning, and/or analyzing transcripts. These commands may point to RNA-seq-related packages or to user-defined python scripts that run analysis. At a high level, you upload your raw reads and your project repo housing your Snakefile, run snakemake on the cluster, the cluster will compute, and then you will extract the data you need (usually gene counts and/or differentially expressed genes) to make plots locally.

2.8.1 First-time setup

0. Create an account

Following the instructions on the MIT ORCD docs page[41], log in to the Engaging cluster through the web portal using your Kerberos ID and password (instructions here[42]). This will automatically trigger a new account to be created.

[40] https://mitprod.sharepoint.com/:x:/s/GallowayLab/Eaa1DDMFM65IsCM5oHc59DUBvyd7G7xggmkQJ7QRqa_RPg?e=eNfced

[41] https://orcd-docs.mit.edu/orcd-systems/#how-to-get-an-account-on-engaging

[42] https://orcd-docs.mit.edu/accessing-orcd/ondemand-login/

Note

There may be a delay of a day after creating your account before you can start any jobs. However, you should still be able to log in.

Confirm you can log in to Engaging via your terminal or PowerShell using `ssh`. Replace `[your-kerberos]` with your Kerberos ID:

```
$ ssh [your-kerberos]@orcd-login.mit.edu
```

This will prompt you for your Kerberos password and Duo authentication.

1. Add an `ssh` shortcut

Once you've confirmed that you can log in, create an `ssh` shortcut to the cluster.

On your computer (not in the cluster), add the following to your config file using `nano ~/.ssh/config`:

```
Host engaging
    HostName orcd-login.mit.edu
    User [your-kerberos]
    ForwardAgent yes
```

While you're at it, add a shortcut to the BioMicro Center cluster. This is where they'll temporarily store your sequencing data.

```
Host bmc
    HostName bmc-150.mit.edu
    User galloway_ill
```

Important

You can't use `nano` on Windows. Instead, navigate to the folder directly in the File Explorer and edit your config file with a text editor:

1. In PowerShell, run `cd ~/.ssh`
2. Get the directory path by `pwd`
3. Copy this path into "File Explorer". This might look like `C:\Users\ChemeGrad2025\.ssh`
4. Once you've located the hidden .ssh directory, edit the config file with "Notepad" (or "VS-Code", etc.) and add in the above.

See MIT ORCD docs SSH key setup[43] for help.

Now, check to confirm that the shortcut runs:

[43] https://orcd-docs.mit.edu/accessing-orcd/ssh-setup/

```
$ ssh engaging
```

This should generate the same prompt for your Kerberos password and Duo authentication as above, just via a simpler command.

2. Set up an SSH key with forwarding

Based on MIT ORCD docs "SSH key setup"[44] and GitHub docs "Using SSH agent forwarding"[45].

At a high level: SSH agent forwarding can be used to make deploying to a server simple. It allows you to use your local SSH keys instead of leaving keys (without passphrases!) sitting on remote servers, like the Engaging cluster.

You can set up `ssh-agent` for your local computer which runs in the background and keeps your SSH key loaded into memory so you don't need to enter a passphrase every time you need to use the key. Then, you can give remote servers, like the Engaging cluster, access to your local `ssh-agent` as if they were running on the server. This is sort of like asking a friend to enter their password so that you can use their computer.

The end result basically means you get use `git clone` and other things without having to re-enter passphrases every time while on the Engaging cluster.

We'll start with GitHub docs "Using SSH agent forwarding"[46]. Check to see if your own SSH key is set up and working by entering `ssh -T git@github.com` in the terminal. If successful it will look like:

```
$ ssh -T git@github.com
# Attempt to SSH in to github
> Hi USERNAME! You've successfully authenticated, but GitHub does not provide␣
↪shell access.
```

If not, next make sure your local computer has an SSH public key for GitHub.

1. Check for an existing SSH key on your local computer: GitHub docs "Checking for existing SSH"[47]
2. If no key exists, then generate and add a new SSH key: GitHub docs "Generating a new SSH key and adding it to the ssh-agent"[48]
3. Now add the SSH key from your local computer to your Github account: GitHub docs "Adding a new SSH key to your GitHub account"[49]
4. Confirm that the SSH key works by entering `ssh -T git@github.com` in the terminal. You should see the message above.

[44] https://orcd-docs.mit.edu/accessing-orcd/ssh-setup/#__tabbed_1_2
[45] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/using-ssh-agent-forwarding
[46] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/using-ssh-agent-forwarding
[47] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/checking-for-existing-ssh-keys
[48] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/generating-a-new-ssh-key-and-adding-it-to-the-ssh-agen
[49] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/adding-a-new-ssh-key-to-your-github-account

Note

Your public key is likely `id_ed25519.pub` but may alternatively be `id_rsa.pub` or `id_ecdsa.pub`.

Now GitHub has your public key but you still need to let `ssh-agent` get access to your private key. This way, when a remote server with `ForwardAgent true` needs to sign something with your private key, the request gets funneled back to your `ssh-agent` which returns the signed request so the private key never leaves your local computer. By copying the public key onto remote systems—such as copy-pasting onto Github like we just did or using `ssh-copy-id`—your public key gets pre-loaded onto remote systems but you can still control access to your private keys for each individual remote server.

To make your key available to `ssh-agent`:

1. Check that your key is visible to `ssh-agent` by running the following command on your local computer: `ssh-add -L`
2. If the command says that no identity is available, you'll need to add your key with the following command: `ssh-add .` This will add any "default" keys. You can also add a specific key. For NBW this looks like `ssh-add ~/.ssh/id_rsa` which is different than the public key, `~/.ssh/id_rsa.pub`!
3. On macOS, `ssh-agent` will "forget" this key, once it gets restarted during reboots. But you can import your SSH keys into Keychain using this command: `ssh-add --apple-use-keychain YOUR-KEY`

Great! You should be done now! The secret was in something we added before:

```
Host engaging
    HostName orcd-login.mit.edu
    User [your-kerberos]
    ForwardAgent yes
```

The `ForwardAgent yes` tells your `ssh-agent` to let the Engaging cluster use your local keys. This is known as "SSH agent forwarding".

Check to make sure it's set up correctly:

1. Log in to the Engaging cluster using `ssh engaging`, enter your Kerberos password, and authenticate with Duo.
2. On the Engaging cluster, test to see if the SSH key is set up and working with Github by entering `ssh -T git@github.com` in the terminal. It should show the same successful response as above.

If it's not working, check GitHub docs "Using SSH agent forwarding: Troubleshooting SSH agent forward"[50] for tips.

[50] https://docs.github.com/en/authentication/connecting-to-github-with-ssh/using-ssh-agent-forwarding#troubleshooting-ssh-agent-forwarding

3. Link the shared data directory

We have a 20-TB shared data directory on the Engaging cluster. It is located at `/orcd/data/katiegal/002`, which is an annoying path to type. Instead, it is convenient to put a link in your home directory, which is the place where you start when you SSH in.

You only have to create this symbolic link (symlink) once. To make the symlink, run:

```
$ ln -s /orcd/data/katiegal/002 ~/katiegal_shared
```

The relevant directories here are:

- `data/raw_reads`: where we put all our raw data
- `projects`: where we clone git repos for analysis pipelines, etc.
- `hpc_infra`: infrastructure scripts and other useful items.

```
katiegal_shared/
├── data/
│   └── raw_reads/
├── hpc_infra/
└── projects/
```

Note

Before December 2025, we used to have another directory at `/orcd/pool/003/katiegal_shared/`. If something is missing in the main shared folder, it is likely here. You should symlink each set of data into the new directory, e.g.:

```
$ ln -s /orcd/pool/003/katiegal_shared/data/raw_reads/250425Gal/ ~/katiegal_
↪shared/data/raw_reads/250425Gal
```

2.8.2 Per-project setup

TODO

Needs description of other files in the suggested repo layout. Pipeline templates are in progress.

After getting your `ssh-agent` set up as described above, you should clone your project repo into `~/katiegal_shared/projects/`. This will let you edit your script files locally or on the server, and track changes. You will want to make a new directory to house all of your Engaging cluster files. You can either copy a `cluster` folder from someone else's pipeline (CJ is working on an incoming template repo) or make a new one.

To clone your repo:

1. Log in to the Engaging cluster via `ssh engaging`
2. Navigate to the projects directory by `cd katiegal_shared/projects`
3. Clone your project repo by using the `ssh` URL, which you can get from GitHub. This might look like:

```
$ git clone git@github.com:GallowayLabMIT/your_repo.git
```

Next, make a new cluster directory in your `your_repo` (if not using an existing template) and symlink the raw reads:

```
$ mkdir ~/katiegal_shared/projects/your_repo/cluster
$ mkdir ~/katiegal_shared/projects/your_repo/cluster/data
$ ln -s ~/katiegal_shared/data/raw_reads/ ~/katiegal_shared/projects/your_repo/
↪cluster/data/raw_reads
```

Ultimately, your cluster file structure should look something like this:

```
katiegal_shared/
├── data/
├── hpc_infra/
└── projects/
    └── your_repo/
        ├── ...             # everything else in your repo, like Python data␣
↪analysis, figures, etc.
        └── cluster/
            ├── config      # TODO DESCRIPTION - Metadata for configuring
            ├── data/       # Data you don't want tracked, like genomes
            │   └── raw_reads
            ├── envs/       # TODO DESCRIPTION
            ├── inputs/     # Inputs that should be tracked, like transgenes or␣
↪metadata
            ├── profiles/   # TODO DESCRIPTION
            ├── scripts/    # Scripts for analysis
```

(continues on next page)

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```
            ├── .gitignore  # TODO DESCRIPTION
            └── Snakefile   # Runs pipeline
```

2.9 Keck Microscopy Facility

The W.M. Keck Microscopy Facility[51] at the Whitehead Institute has confocal, lightsheet, and widefield microscopes that are available for use. Obtaining access to the facility and training on the equipment takes several weeks to schedule, so plan ahead.

1. Fill out a new user form[52]. The lab's Whitehead Cores Customer Number is `6289`.
2. Email Tseganesh to authorize you on an associated PO for the lab's Whitehead account.
3. After you submit the new user form, you'll meet with one of the core specialists for an initial consultation. At this meeting, be prepared to share information on cell type, fluorophores, desired microscopes, live/fixed sample, etc.
4. The core specialist will also send you a **list of trainings and paperwork** to complete. Follow the steps they outline. This may include:
    - Paperwork to obtain card access
    - Picking up a physical Whitehead ID card
    - Building safety trainings
5. Once you have access to the facility, you can make an appointment for a first training session with a specialist (using your own test samples—don't bring any precious experiments, though!).
6. The next time you book a session, it must be when one of the core staff is present in case you have questions. Check with the core staff about their hours and availability.
7. After that, you can book whenever you want using the online calendar system (the "Kecklendar"[53]).

2.10 Myco Testing

Myco testing is run by the Preclinical Modeling Core Facility in the Koch Institute most Tuesdays at 11am. If testing will not be run for a given week, an announcement will be posted on the iLab page where requests are submitted. Samples and requests through iLab need to be submitted before

[51] https://wi.mit.edu/core-facilities/microscopy/equipment-software
[52] https://nematode.wi.mit.edu/kecklendar/intake?newuser=true
[53] https://wi.mit.edu/core-facilities/microscopy/kecklendar

10am on Tuesday.

2.10.1 Getting Access

1. Create an iLab account with your MIT email and select to join the Galloway Lab.
2. If needed, request internal access to the Koch by emailing the Preclinical Modeling staff (Aurora and Noranne) at escore@mit.edu and explain why you need access. They will need to sponsor anyone outside of the KI for those who need access.

2.10.2 Submitting Testing Request in iLab

1. Requests for myco testing can be submitted through the iLab website. The request form can be found under Core Facilities >> ES Cell and Transgenics Facility >> Request Services >> MYCOPLASMA TESTING >> Request Service.
2. Complete the table with matrix vial barcode numbers, cell line names, email, PI, and TC Room # (66-225b). Adding the passage number is optional. If submitting a non-conditioned media sample, indicated “media only” in the passage number box. Once completed, save the table.
3. Enter the quantity of samples to be submitted. This should match the number of completed rows in the table. The cost is $71 per sample as of December 2025.
4. Under payment information, select the desired payment number.
5. Submit the request to the core and drop off samples before 10am on Tuesday.

2.10.3 Submitting Samples for Testing

1. Matrix vials can be found outside of 76-195.
2. Collect exactly 500 uL of media from cells and store in matrix vials. The media should be incubated on cells for at least 3-4 days (or 5-7 days if possible, no antibiotics is ideal) prior to testing, and cells should be >80% confluent prior to sampling. Usually this means the media has turned orange or even yellow. That is not a problem.

> **Note**
>
> Recommendation from the KI Core:
>
> Ensure that your cells have been growing in the same media (no "feeding" or refreshing the media) for at least 72 hours, though for mycoplasma testing, longer is always better. We recommend seeding the cells to one well of a 6-well plate or one 6cm dish at very low density (5-10% coverage) on Thursday or Friday and let them sit, untouched until Monday afternoon or Tuesday morning.

3. If you are submitting media-only samples (eg. no cells, just media, FBS, etc), indicate that clearly on the request form. There are parallel processes for handling those types of samples.
4. Samples must be dropped off in the 96-well box in the white styrofoam box outside of 76-195 by Tuesday morning between 7am and 10am. Samples can be stored at 4°C overnight or -20°C if longer (up to 2 weeks). DO NOT leave samples overnight at room temperature in the drop-off box.

2.10.4 Results

- Results will be emailed to you by the end of the day on Tuesday.
- The results from the MycoAlert PLUS Assay include a Read A and a Read B. The ratio of these reads determines the results. B/A < 1.0 is considered negative, between 1.0-1.2 should be quarantined, monitored and re-tested, >1.2 is considered positive.
- The KI Core Facilities use the Lonza MycoAlertTM PLUS Assay, a selective biochemical test that exploits the activity of mycoplasma enzymes which are found in all six of the main mycoplasma cell culture contaminants and the vast majority of 180 mycoplasma species, but are not present in eukaryotic cells.

2.11 Qubit

The Qubit Fluorometer (Invitrogen 1.0 model[54]) quantifies nucleic acid concentration with greater precision than the Nanodrop. The instrument is located in the BioMicro Center (BMC)[55] and requires a kit (reagent + tubes) for diluting samples.

The protocol below describes the workflow for the dsDNA HS Assay Kit (Invitrogen Q32851[56]).

2.11.1 Materials

- **Your DNA sample**, with concentration ranging from 5 pg/μL to 120 ng/μL
- **Qubit dsDNA HS Reagent**: light-sensitive orange solution, stored in a black bag in the drawer next to the genomics hood
- **Qubit dsDNA HS Buffer**: stored in bottle at room temperature in the genomics hood
- **Qubit dsDNA HS Standards 1 and 2**: tubes stored in Sven 4°C door in Qubit box
- **Qubit Assay Tubes** (Invitrogen Q32856[57]): special 0.5-mL tubes compatible with the instrument, stored in the cabinet next to the genomics hood

[54] https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/qubit-technical-resources/previous-qubit-models.html
[55] https://biomicro.mit.edu/
[56] https://www.fishersci.com/shop/products/qubit-dsdna-hs-br-assay-kits/Q32851
[57] https://www.fishersci.com/shop/products/invitrogen-qubit-assay-tubes/Q32856

2.11.2 Prepare dilutions

1. **Prepare the Qubit working solution**: Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer. Prepare enough for 200 μL per sample plus 400 μL for the standards, e.g., for 8 samples, dilute 10 μL of reagent in 1.990 mL buffer (this includes enough extra to account for pipetting loss).
2. **Prepare the standards**: Dilute 10 μL each of Qubit dsDNA HS Standards 1 and 2 in 190 μL of working solution in the Qubit Assay Tubes, one tube per standard.

> **Note**
>
> When labeling the tubes, write on the cap, NOT on the side.

3. **Prepare your samples**: Dilute each of your DNA samples in working solution in the Qubit Assay Tubes. Choose an appropriate dilution factor based on your expected concentration. The Qubit dsDNA HS Assay is sensitive in the range 0.5-600 pg/μL (concentration of the diluted sample).
    - For samples 10-100 **pg**/μL, dilute 1:20 (i.e., 10 μL sample plus 190 μL working solution)
    - For samples 10-100 **ng**/μL, dilute 1:200 (i.e., 1 μL sample plus 199 μL working solution)
    - For samples with expected concentrations in between, choose any dilution between 1:200 and 1:20, inclusive
4. Vortex the diluted standards and samples. Incubate the samples at room temperature for 2 minutes. (This is as long as it takes to walk to the instrument in the BMC.)

2.11.3 Measure samples

1. Bring your samples to the BMC. The Qubit Fluorometer (Invitrogen 1.0 model[58]) is in the back room on one of the lab benches.
2. Select the "Quant-IT dsDNA, HS" assay, then follow the prompts on the instrument to insert each standard and then your diluted samples, one at a time. Simply place the tube in the chamber (under the black lid, no need to push), close the lid, and press "Go" to measure each tube.

Fig. 21: Left: Choose the "dsDNA, HS" assay. Right: The instrument first prompts you to insert and measure Standard 1.

3. Record the measured concentration for each diluted sample. It will display in ng/mL, which is equivalent to pg/μL. Convert these values to the actual concentration of your samples based on the dilution factor.
4. When finished, insert the blank tube back into the chamber. You can dispose of the diluted samples and standards.

[58] https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/qubit-technical-resources/previous-qubit-models.html

2.12 Data Transfer of Sequenced files from BioMicro Center to Supercloud

FASTQ files for Samples submitted for sequencing at the BioMicro center are stored in the BMC drive Luria. We usually transfer these files to our Supercloud account for processing and analysis. To transfer sequenced files from the BioMicro center to the Galloway Lab Supercloud account, do the following steps.

1. Log in to your SuperCloud from Terminal.
2. Navigate to `~/galloway_shared/data/NGS_DATA/raw_reads`
3. Create a folder for your sequenced files in the `raw_reads` directory. This is where you'll copy the sequenced files.
4. Load and configure Rclone using the following commands:

```
module load ~/galloway_shared/modules/rclone_module
rclone config
```

5. Make sure that `BMC` is listed in the current remotes
6. To begin copying files from BMC, navigate first to newly created folder and use the following command:

```
rclone copy -progress BMC:~/path/to/sequenced_folder/ .
```

7. You can do a test run of the copying process by adding in `--dry-run` .

```
rclone copy --progress --dry-run BMC:~/data/230717Gal/ .
```

8. Confirm that the data transfer is successful by checking the accuracy of copied files.

2.13 MIT SuperCloud Access

1. Go to http://supercloud.mit.edu/requesting-account and complete account request form. On this form, answer "Do you plan to use any data that is not publicly available?" as "Yes". Indicate that any such data will be generated by you/our lab and will be made publicly available upon publication. Additionally, indicate you should be added to the "galloway" group.
2. After completing the form, email supercloud@mit.edu and copy Katie for PI confirmation of the account.

Example

"Hello, I am writing to request confirmation of a supercloud account. I will be using this account to run Julia simulations using combined ODE and stochastic modeling for my PhD thesis work. My PI, Dr. Galloway, is copied here. Please let me know if you need any other information. Thank you, [Your Name]"

3. Once approved, you will receive an email with instructions to set up the account.

Tip

For more information about how to use the SuperCloud, complete the Practical High Performance Computing Course: https://learn.llx.edly.io/course/practical-hpc/

CHAPTER

THREE

PROTOCOLS

3.1 Biochemical and analytical protocols

3.1.1 Genomics and sequencing methods

Genomics methods give us a way to interrogate any DNA you can place adaptors on, at very high scale and resolution. Short-read sequencing is increasingly a commodity; it costs roughly $1000 for 500 million reads, and even less per-read for billions of reads.

Genomics methods are very "mix and match", as they all follow the overall trend of:

1. **Generating fragments.** These steps take in all of the chromatin (or intact nuclei, or cells) and somehow fragment the DNA. Alternatively, when doing an RNA method, the transcripts are already fragments.
2. **Post-fragment methods.** Fragments of interest (transcripts, chromatin, etc) can be selected and enriched.
3. **Adding PCR adaptors.** To the fragments, adaptors are added, enabling downstream PCR steps. Tagmentation methods combine fragment generation and PCR adaptor addition.
4. **Library generation.** Using the adaptors, just enough PCR is performed to reach submission minimums without over-amplifying the library (which biases towards smaller fragments). These library PCRs also tend to add per-condition barcodes.
5. **Post-library methods.** Specific library members can be selected and enriched.
6. **Library pooling, QC, and submission.** Multiple libraries with different barcodes can be combined into the same sequencing run.

We can break down our in-lab protocols in the following way:

Protocol	Fragment generation	Post-fragment	Adaptors	Post-library
ATAC-seq	Tn5 transposition		Added by Tn5	
ChIP	Sonication	Antibody pulldown	Ligation	
CUT&RUN	Targeted MNase digestion		Ligation	
CUT&Tag (page **??**)	Targeted Tn5 transposition		Added by Tn5	
GapRUN (page **??**)	GapR-targeted MNase digestion		Ligation	
MicroC	MNase digestion	Proximity cross-linking	Ligation	
RCMC (page **??**)	MNase digestion	Proximity cross-linking	Ligation	Region capture
RNA-seq	RNA isolation, cDNA synthesis		Ligation	

Single-cell genomics methods take one of two paths:

1. **Droplet-based methods** like 10X encapsulate individual cells in droplets that have all of the required components for the assay.
2. **Combinatorial/Split-pool methods** do successive rounds where cells are split across multiple PCR plates, then re-pooled and split again. These rely on combinatorial indexing to statistically add single-cell barcodes.

The following protocols we have done in lab fall into these categories:

Method	Single-cell technique	Effort
sci-ATAC-seq (page **??**)	Combinatorial	Very high
sc-RNA-seq	Droplet	Low (core submission)

CUT&Tag

Important

This protocol is modified from the `Active Motif protocol`. Our modifications typically make the process more efficient or are gentler on the cells. Additionally, the final PCR step diverges, as we perform a *test PCR* (page **??**) first.

We follow the same numbering as the linked protocol for easy comparison, i.e., step 1 in this protocol maps to step 1 of the actual protocol. You may want to print out both. Steps labeled with letters (e.g., step a) are steps we've added to streamline the protocol in our hands.

We buy the following kits from Active Motif:

- CUT&Tag-IT Express[59] (Active Motif 53175/53177): the main kit. The kit contains components stored at room temperature (cupboard under the genomics bench), at 4ºC (drawer in Sven), and at –20ºC (Active Motif boxes in Sven). Be sure to unpack and store components properly when they arrive.
- CUT&Tag Spike-in Control[60] (Active Motif 53168/53173): spike-in nuclei and corresponding primary antibody. Pick the spike-in control kit that **matches the secondary antibody** you will be using for the marks of interest. Active Motif sells anti-rabbit and anti-mouse controls. The control primary antibody is stored at –20ºC (with other Active Motif antibodies in Sven), and the nuclei are stored at –80ºC (currently in a bag in the red bin in Queen Iduna).

Background

CUT&Tag uses Tn5 conjugated to protein-A/protein-G to tagment DNA at sites determined by primary/secondary antibody binding. The advantage of this is a higher signal-to-noise ratio relative to CUT&RUN, which uses MNase conjugated to protein-A. CUT&Tag libraries are directly tagmented with PCR-compatible adapters, which allows you to skip the library ligation step.

However, Tn5 has a related accessibility bias (like the bias measured in ATAC-seq!). CUT&Tag buffers do the tagmentation in a higher-salt buffer than ATAC-seq, which increases electrostatic screening and thus disrupts nucleosome and chromatin interactions, lessening the accessibility signal.

This protocol can be reasonably split over three days.

- **Day 1**: cell counting, bead binding, primary antibody binding overnight. For 32 samples, this takes ~3 hours after cell dissociation and counting.
- **Day 2**: secondary antibody binding, tagmentation, DNA cleanup. For 32 samples, this takes ~9 hours.
- **Day 3+**: test PCR, PCR amplification, library quantification. These steps can easily be split across several days if needed.

Experimental setup

Active Motif does *not* recommend using an IgG control antibody condition. Instead, use a common histone mark (H3K27me3, H3K27ac) as your control. If your experiment already calls for one of these histone marks, then you do not need an explicit separate control.

Select primary antibodies that have the same species, either rabbit or mouse. Then, order a spike-in nuclei kit from Active Motif with the matching species.

Design your experiment such that you have in excess of **500k cells per antibody** for each cell condition of interest at the day of collection. For example, if you have four antibodies, culture cells such that you collect more than 2M cells. The protocol below refers to your different cell treatments as **conditions** and each combination of condition and antibody as a **sample** or **reaction**. For example, with eight cell conditions and four antibodies each, you'd have 32 reactions (aka samples).

[59] https://www.activemotif.com/catalog/1395/cut-tag-it-express
[60] https://www.activemotif.com/catalog/1377/cut-tag-spike-in

Preparation (N days before)

- Order the CUT&Tag-IT Express and CUT&Tag Spike-in Control kits linked above. These typically ship within a few days of ordering. Store the reagents at the proper temperature:
  - **Room temperature** (open bottles inside genomics hood, extras in cabinet): Tn5 Release Solution, DNA Purification Binding Buffer, DNA Purification Wash Buffer, DNA Purification Elution Buffer
  - **4ºC** (Sven drawer): 1X Binding Buffer, Dig-Wash Buffer, Dig-300 Buffer, Antibody Buffer, Tagmentation Buffer, ConA beads (tube), silica beads (tube), SPRI beads
  - **–20ºC** (Sven Active Motif box): Protease Inhibitor Cocktail (PIC), 5% Digitonin, secondary antibodies (alpaca anti-rabbit, rabbit anti-mouse), pA-Tn5 Transposomes, Glycogen, Proteinase K, i5 and i7 index primers
  - **–20ºC** (Sven CUT&Tag antibodies sleeve): primary antibodies, Spike-in control antibody
  - **–80ºC** (Queen Iduna red bin): Spike-in nuclei
- Check the Concanavalin A (ConA) beads for aggregation. To do so, pipet thoroughly to resuspend the beads, then dilute 1 μL of beads in 9 μL of 0.1x TE (or water). Transfer the 10 μL to the hemocytometer (there's one by the microscope in 66-219) and look under the microscope. You should not see large clumps of beads; if you do, then the beads are NOT good to use. If you got the beads from Active Motif (e.g., in the kit linked above), contact customer support: they will ship you new beads overnight for free. Otherwise, order more from a vendor of your choice (e.g., Cell Signaling Technology 93569S[61]).

Warning

Do **NOT** store Concanavalin A (ConA) beads below 4ºC. These beads are extremely sensitive to freezing.

Some protocols tell you not to vortex ConA beads, to avoid shearing the ConA from the beads, so we avoid vortexing the beads in the initial steps.

Day 1

Estimated time

~3 hours plus time to collect and count cells; usually **~4 hours total**

Prepare buffers

[61] https://www.cellsignal.com/products/cut-run-kits-reagents/concanavalin-a-magnetic-beads-and-activation-buffer/93569

Note

These steps are not numbered in the original protocol. We use letters for steps that do not appear in the original protocol.

Note

For all the solutions listed, make extra to account for pipetting loss across multiple samples. A good rule of thumb is to make **10% extra** for all the recipes listed. For the Transposomes Master Mix, make a bit less than this, since the kit does not provide much extra of these reagents.

a. Defrost Protease Inhibitor Cocktail (PIC) on ice and 5% Digitonin at room temperature.

b. Prepare Complete Antibody Buffer and Dig-Wash + PIC. Make extra of each of these!

Complete Antibody Buffer (51 μL / reaction)

Component	Volume
Antibody Buffer	50 μL
Protease Inhibitor Cocktail	0.5 μL
5% Digitonin	0.5 μL

Dig-Wash + PIC (101 μL / reaction)

Component	Volume
Dig-Wash Buffer	100 μL
Protease Inhibitor Cocktail	1 μL

Activate ConA beads

Note

The original protocol suggests you do this before preparing the cells, but does not order the protocol in this way.

6. Resuspend the ConA beads via trituration and aliquot 10 μL per sample in low-binding 1.7-mL tubes. Batch up to 80 μL (for 8 samples) per tube.
7. Place tubes on the magnet. Once clear, remove the supernatant.
8. Remove tubes from the magnet and resuspend in 100 μL of activation / binding buffer (Active Motif 1X Binding Buffer) per sample. This should be 10x the original bead volume. Incubate at room temperature for 10-15 minutes.

9. Place tubes on the magnet and discard the supernatant once clear.

c. If using the Cell Signaling Technology ConA beads, repeat the 100 μL wash step, as CST provides enough reagents for two washes.

10. Remove tubes from the magnet and resuspend in 10 μL of activation / binding buffer per sample. Aliquot each 10-μL sample of beads into fresh PCR tubes (one tube per sample).

Prepare cells

1. Collect your target cells **without using trypsin** or other enzymatic methods. For iPSCs, this means using Gentle Cell Disassociation Reagent. Sort cells if required. Then, resuspend cells in media that allows for good pelleting (for iPSCs: DMEM/F12 + 1% FBS) and count live cells using Trypan Blue.

d. After counting, take spike-in nuclei out of the –80°C freezer and defrost on ice. Combine enough nuclei for all samples together into a single tube for better reproducibility.

> **Note**
>
> We want the spike-in reads to be 5-10% of the overall sequencing reads. Use a spike-in nuclei : cell ratio of 1:25 for common marks, increasing to 1:10 for rare marks. For H3K27me3, H3K27ac, H3K4me3, we used 20,000 spike-in nuclei for 500,000 cells.
>
> Active Motif provides vials of 160 μL at 500 nuclei/μL, so 20,000 nuclei is 40 μL (4 reactions per vial).

e. Calculate the volume of cells needed to obtain 500k cells per CUT&Tag reaction. If you are running multiple reactions (antibodies) per cell condition, you can make a cell "master mix" (e.g., calculate volume for 2M cells if you have 4 antibodies). It is helpful to use a spreadsheet for these calculations.

> **Note**
>
> If you do not have 500k cells per reaction, you can use fewer cells. However, you need to adjust the amount of spike-in nuclei for each reaction! It is very important that the cell : spike-in-nuclei ratio is constant.
>
> For example, if most of your conditions have 500k cells, but one only has 400k cells, then you should use the entire 400k while only adding in 4/5th of the amount of spike-in nuclei.

2. Aliquot 500k cells per reaction into low-binding 1.7-mL tubes.

f. Add spike-in nuclei to the cell aliquots.

4. Centrifuge cells for 3 minutes at 600xg at 4°C and discard the supernatant.

Warning

Since these are still live cells, collect the supernatant as BL2 waste and decontaminate with bleach. It is simplest to do this in the quarantine BSC.

All other waste from this protocol (unless otherwise noted) can be collected in a 50-mL conical in the genomics hood for sink disposal.

5. Resuspend cells in 100 μL Dig-Wash + PIC per sample.

11. Add 100 μL of cells per sample to the Con A beads in PCR tubes prepared earlier. Resuspend via trituration and incubate on a Nutator (e.g., this[62]) for 10 minutes at room temperature.

Bind primary antibody

g. Prepare Complete Antibody Buffer plus the chosen antibodies on ice. Prepare slight excess (e.g., 17x for 16 samples), and make one master mix per antibody:

Complete Antibody Buffer plus Antibodies (52 μL / condition)

Component	Volume
Complete Antibody Buffer	50 μL
Target antibody	1 μL (or manufacturer recommendation)
Spike-in control antibody	1 μL

12. Remove tubes from the Nutator and briefly spin down. Place the tubes on the magnet and discard the supernatant.

13. Resuspend the beads/cells in 52 μL of cold Complete Antibody Buffer plus Antibodies.

15. Place samples on a Nutator overnight at 4°C.

Day 2

Estimated time

- For 32 samples and one person: ~5 hours through tagmentation, ~4 hours for DNA extraction; **total ~9 hours**
- For 5 and 2 samples: 7 hours and 5.5 hours total, respectively

Prepare buffers

[62] https://www.vwr.com/us/en/product/4787436/vwr-nutating-mixer

Note

The proposed volumes in the Active Motif documentation are greatly in excess! As described above, a good rule of thumb is to make **10% extra** for all the recipes listed. For the Transposomes Master Mix, make a bit less, since the kit doesn't provide much extra of this reagent.

h. Defrost Protease Inhibitor Cocktail (PIC) on ice and 5% Digitonin at room temperature.

i. Prepare Complete Tagmentation Buffer, Complete Dig-300 Buffer, and Complete Dig-Wash buffer per condition. Make some excess.

Note

Some volumes are small; if you are only doing 1 sample, scale up just for the purpose of pipetting no less than 0.5 μL.

Complete Tagmentation Buffer (40 μL / reaction)

Component	Volume
Tagmentation Buffer	40 μL
Protease Inhibitor Cocktail	0.4 μL
5% Digitonin	0.08 μL

Complete Dig-300 Buffer (500 μL / reaction)

Component	Volume
Dig-300 Buffer	500 μL
Protease Inhibitor Cocktail	5 μL
5% Digitonin	1 μL

Complete Dig-Wash Buffer (700 μL / reaction)

Component	Volume
Dig-wash Buffer	700 μL
Protease Inhibitor Cocktail	7 μL
5% Digitonin	7 μL

Bind secondary antibody

16. Remove tubes from the Nutator and briefly spin to collect solution at the bottom. Place on the magnet and remove the supernatant.

17. Wash the beads by resuspending in 200 μL of Complete Dig-Wash Buffer. Transfer the solution to fresh PCR tubes and place the tubes back on the magnet.
18. Prepare a secondary antibody master mix:

    Secondary Master Mix (100 μL / reaction)

Component	Volume
Complete Dig-Wash Buffer	100 μL
Secondary antibody	1 μL

    Remove the supernatant from the beads. Resuspend each sample in 100 μL of Secondary Master Mix.
19. Place the tubes on a Nutator at room temperature for 15 minutes.
20. Pipet gently to resuspend beads, then place back on the Nutator for another 15 minutes.
21. Remove tubes from the Nutator and briefly spin to collect solution at the bottom.
22. Place the tubes on the magnet, then discard the supernatant.
23. Resuspend in 200 μL of Complete Dig-Wash Buffer, then place back on the magnet.
24. Repeat the wash step by removing the supernatant, adding Complete Dig-Wash buffer, mixing, and placing back on the magnet.

Bind CUT&Tag-IT Assembled pA-Tn5 Transposomes

25. Dilute CUT&Tag-IT Assembled pA-Tn5 Transposomes in Complete Dig-300 Buffer. Make very slight excess.

    Transposomes Master Mix (101 μL / reaction)

Component	Volume
Complete Dig-300 Buffer	100 μL
Assembled pA-Tn5 Transposomes	1 μL

26. Discard the supernatant.
27. Add 100 μL of Transposomes Master Mix and pipet to resuspend the beads.
28. Place tubes on a Nutator at room temperature for 15 minutes.
29. Pipet gently to resuspend beads, then place back on the Nutator for another 15 minutes.
30. Remove tubes from the Nutator and spin briefly to collect solution at the bottom.
31. Place the tubes on the magnet, then discard the supernatant.
32. Add 200 μL of Complete Dig-300 Buffer and gently pipet to resuspend the beads.

33. Repeat the wash step by placing on the magnet, discarding the supernatant, and resuspending in Complete Dig-300 Buffer.

Tagment

34. Place the tubes on the magnet and discard the supernatant.
35. Add 40 μL of Complete Tagmentation Buffer. Gently pipet to resuspend the beads.
36. Incubate at 37°C in a thermocycler for one hour.

> **Note**
>
> This is the `C&T/tagment` protocol.

j. Thaw Glycogen at room temperature and Proteinase K on ice.

k. Prepare Complete Tn5 Release solution on ice. Vortex the Tn5 Release Solution for at least 30 seconds.

Complete Tn5 Release Solution (40 μL / reaction)

Component	Volume
Tn5 Release Solution	40 μL
Glycogen	0.8 μL
Proteinase K	0.8 μL

Extact DNA

37. Place the tubes on the magnet and discard the supernatant.
38. Vortex the Complete Tn5 Release Solution, then add 40 μL of Complete Tn5 Release Solution per sample.
39. Mix well via pipetting.

> **Note**
>
> It is typical for the beads to form a large clump during the incubation. Do your best to mix here and in the next few steps; it will be difficult to fully homogenize the solution. This requires **at least 1 minute** of pipetting per sample at this step.

40. Incubate the reaction for 1 hour at 55°C in a thermocycler, with the heated lid set to 65°C.

> **Note**
>
> This is the `C&T/tag_stop` thermocycler protocol.

l. If the extraction buffers are new, follow the instructions on the bottles: add 9 mL IPA to the DNA Purification Binding Buffer and 40 mL ethanol to the DNA Purification Wash Buffer.

41. Add 40 μL of DEPC-treated water to each sample and mix well via pipetting. Place back at 55°C for 5 minutes.

> **Note**
>
> This is the `C&T/release` thermocycler protocol.

44. While the samples are incubating, prepare the silica beads. Begin by vortexing the silica beads for at least 30 seconds.

45. Pipet 25 μL of silica beads per sample into fresh PCR tubes. Place tubes on a magnet.

m. Discard the supernatant from the silica beads, then take the beads off the magnet. Add 35 μL of DNA Purification Binding Buffer (yellow solution).

n. When the samples are done incubating, pipet well until the bead/cell clump is broken up. Add 100 μL of DNA Purification Binding Buffer (60% IPA) to each sample to help with homogenization. Pipet with the volume set below 180 μL to avoid air bubbles. Place the samples on the magnet and ensure the beads pellet well.

48. Remove ~180 μL total supernatant from the samples while still on the magnet, adding it to the silica bead tubes. Pipet well to mix and incubate at room temperature for 5 minutes.

> **Warning**
>
> It is important that as few magnetic beads as possible are transferred! You don't need to transfer all 180 μL of supernatant if it comes with some beads.

49. Place the tubes on the magnet and discard the supernatant.

50. Resuspend in 200 μL of DNA Purification Wash Buffer.

51. Place tubes back on the magnet and discard the supernatant.

52. Repeat the wash step by adding wash buffer, pipetting to resuspend, placing back on the magnet, and discarding the supernatant.

53. Leave the tubes on the magnet with the caps open, and use a 10-μL pipet to remove any remaining wash buffer. Air dry until the beads transition from shiny to matte, not more than 5 minutes.

54. Remove the tubes from the magnet and resuspend in 24 μL of DNA Purification Elution Buffer. Incubate for 1 minute.

55. Place tubes on the magnet and transfer 23 μL of supernatant to fresh PCR tubes.

> **Note**
>
> At this point, pre-amplification libraries can be stored at –20°C. Alternatively, further steps can be performed on the same day if desired.

Day 3+

> **Estimated time**
>
> **Total: ~8 hours**
>
> - Test PCR ~2.5 hours
> - "Real" PCR and cleanup ~2 hours
> - Library QC ~2.5 hours
> - Library pooling <1 hour

PCR amplify

o. Follow the *Test PCR protocol* (page **??**) with the following parameters:

- Use 3 μL of the 23 μL elution for the test PCR; 20 μL will be used in the "real" PCR
- Use the `tagmentation_fwd` and `tagmentation_rev` primers
- Perform the test PCR with cycle counts 16, 19, 22
- For the "real" PCR, target 100 ng of the final amplified libraries; this should require ~12 cycles

56. Set up the "real" PCR with indexed primers. Choose a unique set of indexed primers for each library. For instance, use a different i7 primer for each cell condition and a different i5 primer for each antibody. Use the primers that come with the CUT&Tag kit (Sven –20°C kit box). For the master mix, use the NEBNext reagent (NEB E7645[63], Sven –20°C "Library prep reagents" box) instead of the one that comes with the kit.

Component	Volume
2x NEBNext Ultra II Q5 Master Mix	25 μL
i5 indexed primer (Nextera compatible)	1 μL
i7 indexed primer (Nextera compatible)	1 μL
Water	3 μL
Pre-amplification library	20 μL

[63] https://www.neb.com/en-us/products/e7645-nebnext-ultra-ii-dna-library-prep-kit-for-illumina

57. Using the optimal cycle counts from the test PCR, run the "real" PCR with the following program (`lib_prep/pcr`):

Temp (°C)	Time (MM:SS)	Description
72	5:00	Polymerase activation
98	0:30	Initial denaturation
–	–	*Optimal number of cycles of:*
98	0:10	Denaturation
65	1:15	Extension
–	–	*[end cycle]*
4	Hold	Final hold

    It is possible that the cycle counts will differ across samples. If that is the case, use the `lib_prep/test_pcr` thermocycler protocol, removing samples at the hold steps as needed.

p. Vortex SPRI beads well, at least 30 seconds. Prepare fresh 80% ethanol for the bead wash steps, 400 μL per sample.

58. Perform a double-sided SPRI bead clean-up. Add 25 μL SPRI (0.5x) to each sample, pipet to mix, and incubate at room temperature for 5 minutes.

59. Place tubes on the magnet and move the supernatant to new PCR tubes. Discard the beads.

60. Add 35 μL of SPRI beads to the sample (0.7x sample volume, for a 1.2x final ratio), mix well via pipetting, and incubate at room temperature for 5 minutes.

61. Place tubes on the magnet and discard the supernatant. Wash the beads twice with 200 μL of 80% ethanol, without disturbing the beads or removing the beads from the magnet (i.e., do not resuspend).

62. Allow the beads to dry until they transition from shiny to matte in appearance, not longer than 5 minutes. Add 22 μL of DNA Purification Elution buffer, pipet to mix, and incubate at room temperature for 1 minute.

63. Place the tubes back on the magnet, and transfer the 22 μL to a fresh low-binding 1.7-mL tube, one tube per library. Since these are the final libraries, label the tubes well: include the date, genomics technique (e.g., "C&T lib"), and sample identification on a sticker on the cap. Store at –20°C.

Perform in-house QC

q. Quantify the resulting libraries via *qPCR with the NEBNext Library Quant Kit* (page **??**).

r. Run a sample of each library on the Fragment Analyzer in the BMC (protocol TODO).

Pool libraries for sequencing

s. TODO

GapRUN

This protocol is based on the work of [**?**] and [**?**].

Measure MNase activity

A key variable in any genomics assay with MNase is the ratio of MNase to cells required to properly digest the DNA. Before using the nanobody-MNase protein (fusion of an anti-GFP nanobody to MNase, produced in-house according to *this protocol* (page **??**)) in the GapRUN assay, benchmark its activity against commercial MNase. This only needs to be performed once per batch of protein, but should be performed for each cell type (iPS11, Leiden iPSCs, induced neurons, etc.). It is not necessary to perform a titration per genetic edit.

You will need two 5-million (5M) cell aliquots to perform the titration, one aliquot each for the nanobody-MNase and the commercial MNase. The cells can be fixed and snap-frozen any time prior to the titration.

Preparing fixed, snap-frozen cell aliquots

1. Grow up at least 10M cells using normal culturing conditions. On the day of harvest, proceed accordingly.
2. Prepare sufficent 100x BSA (200 μg / mL) to aid to help cells pellet and limit cell losses. Cool a centrifuge to 4°C and place sufficient PBS on ice.
3. Collect cells following normal passaging conditions. Collect into and wash in the same 50 mL tube. After collecting and spinning down the cells, perform a PBS wash before resuspending and counting cells. Spike in 100x BSA prior to spinning down the cells in the PBS wash.
4. Count the cells. We want as high cell counts as possible; it is good to crosslink and fix as many cells as possible instead of throwing away cells at this step.

> **Warning**
>
> Collect all of the following wash steps as formaldehyde waste until step the cells are re-suspended prior to aliquoting.

5. In a fumehood, add 1 mL of fresh 16% formaldehyde per 15 mL of cells (15M cells), in a dropwise but relatively rapid manner. Use the 10mL and 1mL ampules either opened fresh or opened and stored in the last ~48 hours.
6. Incubate for 10 minutes at room temperature on the plate shaker.
7. Add pH 7.5 Tris dropwise to a final concentration of 0.375M. This is 3.45 mL of 2M Tris per 15M cells.

8. Incubate for 5 minutes at room temperature. Spike in 100x BSA prior to spinning at 400 xg at 4°C for 5 minutes.
9. Remove the supernatant, wash with cold PBS at a concentration of 1M cells / mL. Spike in 100x BSA prior to spinning at 400 xg at 4°C for 5 minutes.
10. Resuspend the cell pellet to 20M cells / mL, in cold PBS with spiked-in 100x BSA. Make the desired aliquots of these cells. For the MNase titration, you will need at least one aliquot of 5M cells. If processing the rest of the cells, in the same reaction, you can freeze down a "large" aliquot.

> **Note**
>
> For smaller aliquots, make sure you use **low-binding** 1.7 mL tubes instead of normal 1.7 mL tubes.

11. Spin down aliquots, aspirate the supernatant and snap-freeze the cell pellets in liquid nitrogen. Store at -80°C. For consistency, snap-freeze and store the main samples even if you are proceeding on the same day. There is no benefit to having "fresh" un-snap-frozen cells.

> **Note**
>
> To snap-freeze, using the LN2 PPE, fill the small dewar with LN2. Then, pour some LN2 into a styrofoam container. Place the tubes to be snap-frozen in the foam holders we use for water baths, then float them in the LN2.
>
> Use tongs / some similar tool and the LN2 gloves to remove the samples.

MNase titration

> **Estimated time**
>
> 2 hours to pause point (reverse crosslinking)

> **Note**
>
> At this point, the cell pellets are fixed. All waste from the titration can be collected and sink-disposed.
>
> Use low-binding 1.7 mL tubes for all steps requiring this tube size.

1. Prepare MB1 following the instructions in the *RCMC protocol* (page **??**).
2. Prepare fresh Complete MB1 and place on ice. For the standard 5M cell titration, you need 1.5 mL of this buffer per 5M cells, but this can be scaled up or down.

   Complete MB1 (2 mL):

Component	Volume
MB1	1940 μL
10% NP-40 alternative	40 μL
100x PIC (in MB1)	20 μL

3. Thaw two 5M cell pellet on ice and resuspend in 500 μL of Complete MB1 (1M cells per 100 μL).
4. Incubate for 20 minutes on ice. While waiting, unfreeze a fresh MNase aliquot on ice to compare to.

> **Note**
>
> You can technically reuse MNase aliquots 2-3 times, but MNase is cheap compared to redoing an experiment because the aliquot you used went bad.
>
> You will need roughly 0.5 μL of MNase per million cells, so unfreeze accordingly.

5. Centrifuge at 1750 xg for 5 minutes at 4°C.
6. Remove the supernatant, leaving ~10-20 μL in the tube.
7. Wash the nuclei pellet with 500 μL of Complete MB1. Centrifuge at 1750 xg for 5 minutes at 4°C.
8. Removing as much supernatant as possible, resuspend the cell pellet in 500 μL of Complete MB1 (1M cells per 100 μL).
9. Split the cells into 5 low-binding 1.7 mL tubes, with 100 μL of cells each, keeping them on ice.
10. Decide on your titration series. A common series is 2U, 4U, 7U, 12U, and 20U for the commercial enzyme and 5U, 10U, 15U, 20U, 40U (assuming 20U/μL) for our in-house enzyme.
11. Add MNase to each 1M cell aliquot. Briefly vortex the tubes to ensure uniform MNase distribution, then incubate at 20 min at 37°C with shaking at 1000 rpm.
12. Transfer the digested nuclei to ice and add 0.8 μL of 500 mM EGTA. to reach a final concentration of 4 mM EGTA.
13. Briefly vortex the tube, then incubate for 10 minutes at 65°C with no shaking (but using the Thermomixer).
14. While waiting on the inactivation, prepare 0.9 mL of Reverse Crosslinking Solution **at room temperature and outside of the genomics hood** because of the RNase A. The SDS will precipitate out if you put it on ice.

    Reverse Crosslinking Solution (1.8 mL)

Component	Volume
1x TE	1440 μL
10% SDS	180 μL
5M NaCl	72 μL
20 mg/mL Proteinase K	90 μL
10 mg/mL RNaseA	18 μL

15. Centrifuge at 1750 xg for 5 minutes at 4°C.
16. Discard the supernatant and resuspend each pellet in 150 μL of Reverse Crosslinking Solution.
17. Reverse cross-links for a minimum of 2 hours to overnight at 65°C with 1000 rpm shaking. This step does not noticeably improve after 2 hours, you just have the option to stop.
18. Clean up the resulting DNA fragments using the genomics-only Monarch DNA cleanup kit. Use a 5:1 ratio of binding buffer : sample. Elute in 25 μL.
19. Quantify the DNA via Nanodrop. Load 1-5 μg of sample per thin-comb well using Orange Loading Dye onto a 1.5% agorase gel. Run at 100V for 30 minutes, then image.
20. Compare the digestion bands to estimate the activity on the nanobody-MNase, in the same units as the commercial MNase. Expect that the in-house nanobody-MNase has at least 5x worse activity.

GapRUN

Stock buffer preparation

1. Prepare and check that there is sufficient amounts of the following *shared genomics buffers* (page **??**):

- 1M HEPES, pH 7.5
- 5M NaCl
- 2.5M CaCl2
- 0.5M EDTA, pH 8.0

2. Prepare 500 uL of **500 mM spermidine**, aliquoted and stored at -20C

Component	Concentration	g / L	Amount / 500 uL
Spermidine trihydrochloride	500 mM	254.6	63.7 mg
Elga water	to 500 uL		

3. Prepare 500 uL of **500 mM EGTA stock solution**

Component	Concentration	g / L	Amount / 10 mL
EGTA	500 mM	389.36	0.0973 g
Elga water	to 0.5 mL		

Two days before Day 1 (Day -1)

Infect cells (or otherwise turn on expression) of the GapR-EGFP fusion protein two days before collection.

Day 0 (N days before)

Check the Concanavalin A (ConA) beads for aggregation. To do so, after pipetting to resuspend the beads, dilute 1 uL of beads with 9 uL of 0.1x TE (or water). You should not see large clumps of beads with the hemocytometer. This is your last chance to order more ConA beads!

Day 1

Estimated time

3 hours

1. Prepare the following buffers fresh:
    - 5 mL of **Wash Buffer** (enough for 12 samples, e.g. ~400 uL per sample, excess already included):

Component	Concentration	Amount / 5 mL
1M HEPES-NaOH	20 mM	100 uL
5M NaCl	150 mM	150 uL
500 mM spermidine	0.5 mM	5 uL
Elga water	to 5 mL	4.74 mL

2. Ensure there is at least 50 uL of 100x PIC dissolved in HEPES-NaCl (Wash Buffer without spermidine). If there is not enough, dissolve 1 Pierce EDTA-free Protease Inhibitor Cocktail tablet in 500 uL of Wash Buffer and store at 4C.
3. Defrost digitonin (CST #16359) and pipette to mix. Freshly transfer 20 uL to a PCR tube. Heat the tube to 95°C for 5 minutes, then place on ice.

> **Note**
>
> This thermocycler protocol is `GapRUN/digprep`

4. Make the following two buffers immediately before the protocol calls for it. The following recipes are given per 100 uL.

   - **Wash Buffer + PIC**

Component	Amount / 100 uL
Wash Buffer	100 uL
100x PIC	1 uL

   - **Complete Wash Buffer (Wash + PIC + digitonin)**

Component	Amount / 100 uL
Wash Buffer	100 uL
100x PIC	1 uL
CST digitonin	2.5 uL

> **Note**
>
> Cell Signaling Technologies digitonin is batch normalized to an activity level, not a concentration. This is why we specify CST's digitonin and give the amount as a volume ratio.

5. Non-enzymatically detach cells and count them in FBS-containing media suing Trypan Blue. For iPSCs, this means using Gentle Cell Disassociation Reagent and counting in DMEM/F12 + 1% FBS.
6. While spinning down the cells as described, activate the Concanavalin A beads. You can activate the ConA beads in larger batches (e.g. activate 8 reactions worth in 1.7 mL tubes).
   a. Resuspend ConA beads via trituration, and aliquot 10 uL per sample.
   b. Place the beads on the magnet and, once clear, remove the supernatant.
   c. Add 100 uL of activation / binding buffer per 10 uL sample. Let sit at room temperature for 10-15 minutes.
   d. Place the beads on the magnet, remove the supernatant.
   e. If using the Cell Signaling Technology beads, repeat this 100 uL wash step, because CST provides enough reagents for two washes.
   f. Resuspend beads in 10 uL of activation/binding buffer per sample.

7. Wash aliquots of 200-500k cells twice with 100 uL of Wash Buffer + PIC. Resuspend the washed cell pellets in 100 uL of Wash Buffer + PIC.

> **Warning**
>
> Collect these two washes as biowaste, since the cells are not fixed. Bleach the waste and sink-dispose.

8. Combine the 100 uL of cells with 10 uL of activated ConA beads. After mixing well via trituration, place the tubes on a Nutator at room temperature for 10 minutes.

> **Note**
>
> You can optionally take some of the supernatant and look with a hemocytometer to confirm that the concentration of cells has been reduced by ConA bead binding.

9. Magnetically separate the cells bound to ConA beads, and resuspend each in 50 uL of Complete Wash Buffer (Wash + PIC + **digitonin**). Place the cells on ice.
10. On ice, spike in one microliter of the LaG16 MNase per 100k cells, mix well (optionally, with a multichannel), and nutate overnight at 4C.

Day 2

> **Estimated time**
>
> 4.5 hours

1. Prepare fresh Complete Wash Buffer (500 uL per sample, excess included), using the recipe from the previous day.
2. Magnetically separate and wash the beads with 200 uL of Complete Wash Buffer (Wash + PIC + digitonin), transferring the washed beads to a new PCR tube. When not pipetting, keep cells on ice

> **Note**
>
> Moving the beads reduces the amount of background.

3. Repeat the wash step.
4. Resuspend beads in 50 uL of Complete Wash Buffer, mixing via gentle pipetting, and place on ice for at least two minutes.
5. Dilute 2.5M CaCl2 to 150 mM in DEPC-treated water. Dilute 1 μL of 2.5M CaCl2 with 15.6 μL water.

6. Spike in 1 uL of 150 mM CaCl2. Place samples on the Nutator at 4C for 2 hours.
7. Prepare Stop Buffer, adding the RNase A and glycogen shortly *(and outside the genomics hood!)* before the 2 hours are up. You need 50 uL per sample (excess **not** included)

   Stop Buffer

Component	Concentration	Amount / sample (50 uL)
5M NaCl	340 mM	1.7 uL
500 mM EDTA	20 mM	2.0 uL
500 mM EGTA	10 mM	1.0 uL
CST digitonin		1.25 uL
Elga water	to 500 uL	43.4 uL
20 mg/mL glycogen	50 ug/mL	0.125 uL
20 mg/mL RNase A	100 ug/mL	0.25 uL

7. Remove the samples from the Nutator, keeping them on ice. Add in 50 uL of Stop Buffer to each sample **outside of the genomics hood**, and mix with gentle pipetting.
8. Incubate samples at 37C for 10 minutes in a thermocycler, with the lid set to 65C.

> **Note**
>
> This thermocycler protocol is `GapRUN/stop`

9. Place samples on the magnetic rack **outside the genomics hood**, and transfer the cleared supernatant to fresh low-binding 1.7 mL tubes.
10. Use the Monarch DNA cleanup kit (using the small, non-miniprep columns), following the instructions that **retains small fragments**.
    a. Add 200 μL of binding buffer to each 100 μL sample.
    b. Add 600 μL of IPA and mix well. Do not try to combine this with the previous step, SDS will crash out!
    c. Perform two washes plus a dry spin.
    d. Elute in 50 μL of 1x TE, for compatibility with the library prep kit.
11. Libraries can be stored at -20C until ready to perform library preparation (Day 3).

Day 3

Estimated time

2.5 hours

1. Follow the instructions for NEBNext Ultra II DNA Library Prep Kit, without performing size selection (e.g. follow steps 3B instead of 3A). Elute in 18 μL instead of 17 μL and stop before Step 4 (PCR enrichment).
2. Follow the *Test PCR protocol* (page **??**) with the following parameters:
    - Use 3 μL of the 18 μL elution for test PCR purposes. 15 μL will be used in the real PCR, so $\log_2(15/0.7) = 4.42$ delta cycles.
    - Use the `ligation_fwd` and `ligation_rev` primers.
    - Target at least 500 ng. Perform 16/19/22 test cycles.
    - The final cycle count should be around 12 cycles.
5. Perform indexed "full" PCRs, following the volumes and SPRI cleanup instructions given in the Ultra II DNA Library Prep Kit instructions from above. Elute in 33 uL of 0.1x TE. Eluted libraries can be stored at -20C.
6. Quantify the libraries and pool for sequencing.

References

Region Capture Micro-C

RCMC is a complicated protocol! Don't let this discourage you; there are plenty of intermediate QC steps you can do along the way to ensure that you are proceeding correctly through the process.

The big-picture protocol is:

- Cross-link and fix cells, which preserves the chromatin contacts.
- Run a test MNase digestion to select an optimal MNase concentration. This must be done fresh every time!
- Run the real MNase digestion. Then, cross-ligate fragments together and do DNA manipulations to biotinylate fragments.
- Extract and purify religated fragments. Perform library prep and PCR on the fragments.
- Region-capture the resulting library. PCR the region-captured library again for sequencing.

Protocol numbers will be sequentially numbered throughout the entire procedure, for easy referencing.

The overall days are split up:

Day -n: Buffer preparation

Shared genomics buffers

- Make sure there is sufficient amounts of the following *shared genomics buffers* (page **??**):
  - 5M NaCl
  - 1M Tris-HCl, pH 7.5
  - 0.5M EDTA, pH 8.0
  - 10% SDS
  - 1M MgCl2
  - 2.5M CaCl2

RCMC-specific buffers

10 mM Tris, 10 mM NaCl (1 mL)

Component	Volume
1 M Tris-HCl pH 7.5	10 μL
5 M NaCl	2 μL
DEPC-treated water	988 μL

2xBW (50 mL)

Component	Volume
Water (Elga or DEPC)	29.4 mL
5 M NaCl	20 mL
1 M Tris-HCl pH 7.5	500 μL
0.5M EDTA pH 8.0	100 μL
0.2 μm sterile filter	

1xTBW (50 mL)

Component	Volume
Water (Elga or DEPC)	25 mL
2xBW	25 mL
100% Tween-20	50 μL
0.2 μm sterile filter	

MB1 (50 mL)

Component	Concentration	Volume
5 M NaCl	50 mM	0.5 mL
1 M Tris-HCl pH 7.5	10 mM	0.5 mL
1 M MgCl2	5 mM	0.25 mL
2.5 M CaCl2	1 mM	20 μL
DEPC-treated water		48.7 mL

MB2 (50 mL)

Component	Concentration	Volume
5 M NaCl	50 mM	0.5 mL
1 M Tris-HCl pH 7.5	10 mM	0.5 mL
1 M MgCl2	10 mM	0.5 mL
DEPC-treated water		48.5 mL

MB3 (50 mL)

Component	Concentration	Volume
1 M Tris-HCl pH 7.5	50 mM	2.5 mL
1 M MgCl2	10 mM	0.5 mL
DEPC-treated water		47 mL

20 mg/mL BSA (prepare fresh each day)

Component	Amount
BSA	20 mg
DEPC-treated water	1 mL

MNase resuspension and storage

We want to avoid temperature cycles of the MNase! To prevent temperature cycles, we make aliquots of 20U / μL MNase.

1. Based on the documented units of lyophilized MNase (on the packing slip), prepare and resuspend the MNase to 40U / μL in the following buffer:

   2xMNase storage buffer (10 mL)

Component	Concentration	Volume
1M Tris-HCl, pH 7.5	20 mM	200 μL
5 M NaCl	100 mM	200 μL
500 mM EDTA, pH 8.0	2 mM	40 μL
DEPC-treated water		9.56 mL

2. Add an equal volume of 100% glycerol to reach 20U / μL. Mix well and aliquot, store in a **reliable** -80°C freezer.

Protease inhibitor cocktail

- Dissolve one Protease Inhibitor Cocktail tablet in 500 μL of MB1. The protease inhibitor cocktail will not fully dissolve, but aliquot the resulting slurry. This is your 100x PIC solution.

Day 1: MNase titration

Buffer preparation

Cell harvest and fixation

Note

It is convenient to have someone helping you on Day 1.

In particular, one person can harvest and wash the cells. Then, while one person is counting, the other person can prepare the crosslinking buffers.

1. Remove enough DSG from the refrigerator and allow it to come to room temperature before starting. Each 50 mg of DSG is enough for 50 mL of solution (50M cells).
2. Prepare sufficent 100x BSA (200 μg / mL) to aid to help cells pellet and limit cell losses.
3. Collect cells following normal passaging conditions. Collect into and wash in the same 50 mL tube. After collecting and spinning down the cells, perform a PBS wash before resuspending and counting cells. Spike in 100x BSA prior to spinning down the cells in the PBS wash.
4. Count the cells. We want as high cell counts as possible; it is good to crosslink and fix as many cells as possible instead of throwing away cells at this step.

5. Resuspend each vial of DSG in 500 μL of DMSO to make 100x DSG Stock Solution.
6. Dilute enough DSG Stock Solution in PBS for fixation of all cells: per 10M cells, 100 μL Stock Solution diluted to 10 mL.
7. In a fumehood, resuspend each cell pellet to 1 million cells per mL. Use a P-1000 to do the initial resuspension, then use a serological pipette to add the rest of the fixation solution.
8. Incubate at room temperature on a plate shaker for 35 minutes.
9. While waiting, cool a centrifuge to 4°C and place sufficient PBS on ice.

> **Note**
>
> The following steps need to be done in a timely manner. It is helpful to have someone helping you if you have more than a 2-3 conditions. Precompute volumes using a spreadsheet; all volumes can be calculated from the cell-count per condition.

> **Warning**
>
> Collect all of the following wash steps as formaldehyde waste until step the cells are resuspended prior to aliquoting.

10. In a fumehood, add 1 mL of fresh 16% formaldehyde per 15 mL of cells (15M cells), in a dropwise but relatively rapid manner. Use the 10mL and 1mL ampules either opened fresh or opened and stored in the last ~48 hours.
11. Incubate for 10 minutes at room temperature on the plate shaker.
12. Add pH 7.5 Tris dropwise to a final concentration of 0.375M. This is 3.45 mL of 2M Tris per 15M cells.
13. Incubate for 5 minutes at room temperature. Spike in 100x BSA prior to spinning at 400 xg at 4°C for 5 minutes.
14. Remove the supernatant, wash with cold PBS at a concentration of 1M cells / mL. Spike in 100x BSA prior to spinning at 400 xg at 4°C for 5 minutes.
15. Resuspend the cell pellet to 20M cells / mL, in cold PBS with spiked-in 100x BSA. Make the desired aliquots of these cells. For the MNase titration, you will need at least one aliquot of 5M cells. If processing the rest of the cells, in the same reaction, you can freeze down a "large" aliquot.

Note

For smaller aliquots, make sure you use **low-binding** 1.7 mL tubes instead of normal 1.7 mL tubes.

16. Spin down aliquots, aspirate the supernatant and snap-freeze the cell pellets in liquid nitrogen. Store at -80°C. For consistency, snap-freeze and store the main samples even if you are proceeding on the same day. There is no benefit to having "fresh" un-snap-frozen cells.

Note

To snap-freeze, using the LN2 PPE, fill the small dewar with LN2. Then, pour some LN2 into a styrofoam container. Place the tubes to be snap-frozen in the foam holders we use for water baths, then float them in the LN2.

Use tongs / some similar tool and the LN2 gloves to remove the samples.

MNase Titration

Estimated time

2 hours to pause point (reverse crosslinking)

A key variable is the ratio of MNase to cells required to properly digest the DNA. It is very important that this titration is performed every single time when running RCMC! This controls for batch-to-batch differences in fixation, snap freezing, and MNase activity.

For each cell type (iPS11, Leiden iPSCs, 293Ts, reprogrammed neurons), you should perform the titration. It is not necessary to have a titration per genetic edit.

You will need a single 5M cell aliquot to perform the titration on.

Note

At this point, the cell pellets are fixed. All waste from the titration can be collected and sink-disposed.

Use low-binding 1.7 mL tubes for all steps requiring this tube size.

17. Prepare fresh Complete MB1 and place on ice. For the standard 5M cell titration, you need 1.5 mL of this buffer, but this can be scaled up or down.

    Complete MB1 (2 mL):

Component	Volume
MB1	1940 μL
10% NP-40 alternative	40 μL
100x PIC (in MB1)	20 μL

18. Thaw a 5M cell pellet on ice and resuspend in 500 μL of Complete MB1 (1M cells per 100 μL).
19. Incubate for 20 minutes on ice. While waiting, unfreeze a fresh MNase aliquot on ice.

    Note

    You can technically reuse MNase aliquots 2-3 times, but MNase is cheap compared to redoing an experiment because the aliquot you used went bad.

    If performing the MNase titration and the "real" digestion on the same day, you can pool multiple unfrozen aliquot(s) on ice and use them for the real digestion. You will need roughly 0.5 μL of MNase per million cells, so unfreeze accordingly.

20. Centrifuge at 1750 xg for 5 minutes at 4°C.
21. Remove the supernatant, leaving ~10-20 μL in the tube.
22. Wash the nuclei pellet with 500 μL of Complete MB1. Centrifuge at 1750 xg for 5 minutes at 4°C.
23. Removing as much supernatant as possible, resuspend the cell pellet in 500 μL of Complete MB1 (1M cells per 100 μL).
24. Split the cells into 5 low-binding 1.7 mL tubes, with 100 μL of cells each, keeping them on ice.
25. Decide on your titration series. A common series is 2U, 4U, 7U, 12U, and 20U. For the small volumes, prepare a 1:10 dilution of the 20U / μL stock solution in 10 mM Tris-HCl, pH 7.5.

Important

Use the **exact same** digestion procedure here and in the "real" digestion! This means as close to 20 minutes as possible, working carefully but rapidly.

26. Add MNase to each 1M cell aliquot. Briefly vortex the tubes to ensure uniform MNase distribution, then incubate at 20 min at 37°C with shaking at 1000 rpm.
27. Transfer the digested nuclei to ice and add 0.8 μL of 500 mM EGTA. to reach a final concentration of 4 mM EGTA.
28. Briefly vortex the tube, then incubate for 10 minutes at 65°C with no shaking (but using the Thermomixer).
29. While waiting on the inactivation, prepare 0.9 mL of Reverse Crosslinking Solution **at room temperature and outside of the genomics hood** because of the RNase A. The SDS will precipitate out if you put it on ice.

    Reverse Crosslinking Solution (0.9 mL):

Component	Volume
1x TE	720 μL
10% SDS	90 μL
5M NaCl	36 μL
20 mg/mL Proteinase K	45 μL
10 mg/mL RNaseA	9 μL

30. Centrifuge at 1750 xg for 5 minutes at 4°C.
31. Discard the supernatant and resuspend each pellet in 150 μL of Reverse Crosslinking Solution.
32. Reverse cross-links for a minimum of 2 hours to overnight at 65°C with 1000 rpm shaking. This step does not noticeably improve after 2 hours, you just have the option to stop.
33. Clean up the resulting DNA fragments using the genomics-only Monarch DNA cleanup kit. Use a 5:1 ratio of binding buffer : sample. Elute in 25 μL.
34. Quantify the DNA via Nanodrop. Load 1-5 μg of sample per thin-comb well using Orange Loading Dye onto a 1.5% agorase gel. Run at 100V for 30 minutes, then image.
35. Select the MNase to cell ratio that is optimally digested: 80-90% monomer, 10-20% dimer, faint trimer band, no tetramer.

Day 1 continued: post-titration digestion and end repair

Buffer preparation

36. Prepare sufficient 20 mg/mL BSA if needed. You don't need super large amounts: 1 mL should be sufficient.

37. Prepare enough Complete MB1, MB2, and MB3 on ice for all of your samples. You will need 3 mL of Complete MB1 per 10M cells:

    Complete MB1 (10/50 mL):

Component	Volume per 10 mL	Volume per 50 mL
MB1	9.7 mL	48.5 mL
10% NP-40 alternative	200 μL	1000 μL
100x PIC (in MB1)	100 μL	500 μL

    You will also need 2 mL of Complete MB2 per sample (not per cell count):

    Complete MB2 (10 mL):

Component	Volume
MB2	10 mL
20 mg/mL BSA	50 μL

    and 1 mL of Complete MB3 per sample (not per cell count):

    Complete MB3 (5 mL):

Component	Volume
MB3	5 mL
20 mg/mL BSA	25 μL

    Then, prepare fresh 100 mM DTT. Prepare 1 mL of this buffer; do not go smaller because our balances have limited precision.

    100 mM DTT (1 mL):

Component	Amount
DTT	0.01543 g
1 M HEPES pH 7.5	25 μL
DEPC-treated water	975 μL

    Warning

    DTT stock should be collected as a separate waste stream.

38. On ice, start defrosting NEBuffer 2.1 and ATP for the end labeling and Biotin-dATP, Biotin-dCTP, dTTP, dGTP, and T4 DNA ligase buffer for the end labeling step.

MNase digestion

39. Thaw the cell pellets on ice and resuspend in Complete MB1 at a concentration of 1M cells per 100 μL.
40. Incubate for 20 minutes on ice. If you did not already unfreeze fresh MNase aliquots, unfreeze enough fresh aliquots for all of your cells given the titration ratio.
41. Centrifuge at 1750 xg for 5 minutes at 4°C.
42. Remove the supernatant, leaving ~10-20 μL in the tube.
43. Wash the nuclei pellet with complete MB1, at a concentration of 1M cells per 100 μL. Centrifuge at 1750 xg for 5 minutes at 4°C.
44. Removing as much supernatant as possible, resuspend the cell pellet in Complete MB1 (1M cells per 100 μL).
45. Using the optimal ratio of MNase to cells, add MNase to each tube. Briefly vortex the tubes to ensure uniform MNase distribution, then incubate at 20 min at 37°C with shaking at 1000 rpm.
46. Transfer the digested nuclei to ice and add 500 mM EGTA to a final concentration of 4 mM (0.8 μL per 100 μL / 1M cells).
47. Briefly vortex the tube, then incubate for 10 minutes at 65°C with no shaking (but using the Thermomixer).
48. Centrifuge at 1750 xg for 5 minutes at 4°C. Wash the nuclei pellet twice in 1 mL of cold Complete MB2.

Fragment end repair

49. Prepare a master mix for the end-chewing step on ice. You can scale this linearly up with cell count; if you have fewer than 2.5M cells, do not go below 45 μL of master mix per condition. Strictly add the components in the order listed.

    End Chewing Master Mix (90 μL / 5M cells)

Component	Volume
DEPC-treated water	50 μL
10X NEBuffer 2.1	10 μL
10 mM ATP	20 μL
100 mM DTT	5 μL
10U / μL T4 PNK	5 μL

> **Note**
>
> It is easiest to perform the math for the master mix by multiplying these numbers by (N+1), for N the number of samples, instead of adding 5 or 10%.

50. After removing the Complete MB2 supernatant from each sample, resuspend the cells in 90 μL of End Chewing Master Mix per 5M cells.
51. Incubate for 15 minutes at 37°C, with 1000 rpm shaking.
52. Add 10 μL of 5U/μL Klenow Fragment to each sample. Incubate again for 15 minutes at 37°C, with 1000 rpm shaking.

End labeling

53. While waiting on the incubation, prepare a master mix for end labeling on ice. As with the end chewing, do not scale below 45 μL per condition; if you have fewer cells, it is fine if they are more dilute.

    End Labeling Master Mix (50 μL / 5M cells)

Component	Volume
DEPC-treated water	22.75 μL
1 mM Biotin-dATP	10 μL
1 mM Biotin-dCTP	10 μL
10 mM dTTP	1 μL
10 mM dGTP	1 μL
10X T4 DNA ligase buffer	5 μL
20 mg/mL BSA	0.25 μL

54. Add 50 μL of End Labeling Master Mix to each sample, pipetting to mix.
55. Incubate for 45 minutes at 25°C with interval mixing (1000 rpm for 1 minutes, 3 minutes still, repeat).
56. Add 500 mM EDTA to a final concentration of 30 mM (9 μL per 150 μL / 5M cells).
57. Briefly vortex, then incubate at 65°C for 20 minutes, without shaking.

Warning

Be careful when removing the supernatant in the following two steps; the pellet will be very loose!

58. Centrifuge at 1750 xg for 5 minutes at 4°C and carefully remove the supernatant.
59. Wash once with 1 mL of cold Complete MB3. Centrifuge at 1750 xg for 5 minutes at 4°C.

Proximity ligation

60. Prepare enough Proximity Ligation Master Mix on ice:

    Proximity Ligation Master Mix (500 μL / 5M cells)

Component	Volume
DEPC-treated water	422.5 μL
10X T4 DNA ligase buffer	50 μL
20 mg/mL BSA	2.5 μL
400U/μL T4 DNA ligase	25 μL

61. Carefully remove the supernatant and resuspend each sample in 500 μL of Proximity Ligation Master Mix per 5M cells.
62. Incubate for at least 2.5 hours (but normally overnight) at room temperature on a nutator.

Day 2: Reverse crosslinking

Estimated time

1 hour in-lab

Now that we have properly biotin-labeled proximity-ligated fragments, the reverse crosslinking step reverses the fixation process. The solution should go from cloudy to clear as this process happens, and samples should be fully clear by the following day.

63. Defrost NEBuffer 1 on ice.
64. Centrifuge the samples at 3000 xg for 5 minutes at 4°C.
65. Prepare enough Biotin Removal Master Mix on ice. As before, do not scale down volumes by more than half if you have less than 2.5M cells.

    Biotin Removal Master Mix (200 μL / 5M cells)

Component	Volume
DEPC-treated water	170 μL
10X NEBuffer 1	20 μL
100 U / μL Exonuclease III	10 μL

66. Resupend the nuclei pellet in 200 μL of Biotin Removal Master Mix per 5M cells.
67. Incubate at 37°C for 15 minutes with interval mixing (1000 rpm for 1 minute, 3 minute still, repeat).
68. Prepare Reverse Crosslinking Master Mix at room temperature **outside of the genomics hood**. Do not scale volumes down by more than half.

    Reverse Crosslinking Master Mix (39 μL / 5M cells)

Component	Volume
5M NaCl	10.4 μL
20 mg/mL Proteinase K	26 μL
10 mg/mL RNase A	2.6 μL

69. **Outside of the genomics hood**, add 39 μL of Reverse Crosslinking Master Mix per 5M cells to the 200 μL / 5M samples. Mix via pipetting.
70. **Outside of the genomics hood**, add 26 μL of 10% SDS per 5M cells. This must be spiked in! Otherwise you would inactivate the Proteinase K. The final total volume is 265 μL / 5M cells.
71. Incubate at 65°C overnight in the thermomixer, without shaking.

Day 3: Fragment cleanup and library prep

Buffer preparation

72. Ensure there is enough 2xBW and 1xTBW. These have very long shelf lives and were previously prepared. You need 3 mL of 1xTBW per sample and 150 μL 2xBW per sample.
73. Take out the T1 streptavidin beads and let them equilibrate to room temperature.
74. Defrost End Prep Reaction Buffer, Ligation Master Mix, and Ligation Enhancer from the NEBNext Ultra II DNA Library Prep Kit on ice.

Bead cleanup

75. Clean up each sample following the NEB Monarch Clean & Concentrator kit. Use miniprep (25 μg capacity) columns instead of 5 μg columns. Use 6x binding buffer and elute twice with 40 μL of warm elution buffer (total of 80 μL).

> **Note**
>
> Use the Thermomixer to heat up the elution buffer.

76. Estimate the concentration of the library using the Nanodrop.
77. Perform a double-sided SPRISelect cleanup to select for dinucleosomes. Use a 0.7x right side and 0.85x left side selection and elute in 32 μL 0.1xTE.
    a. Vortex the room-temperature SPRI beads until well mixed, at least 30 seconds.
    b. To each 80 μL sample, add 0.7x of the initial volume (56 μL) of SPRI beads. Mix will by pipetting and incubate at room temperature for 1 minute.
    c. Place the tubes on the magnet and separate the beads. Move the **supernatant** to a new tube.
    d. Add 0.15x of the initial volume (12 μL) of SPRI beads to the supernatant. Mix well by pipetting and incubate at room temperature for 1 minute.

e. Place the tubes on the magnet and separate the beads. **Remove** the supernatant.

f. With the tubes still on the magnet, add 200 μL of **freshly prepared** 80% ethanol, incubate for 30 seconds, then remove the ethanol. Repeat this wash step

g. Use a P10 pipette to remove residual ethanol, while the tubes are still on the magnet.

h. Wait for the beads to dry, not longer than 5 minutes. The beads should go from "shiny" to "matte".

i. Elute by adding 32 μL 0.1xTE. Take the tubes off the magnet and mix well. After a 1 minute incubation, place back on the magnet until the beads are pelleted.

j. Move the 32 μL samples to new PCR tubes.

78. Quantify the concentration of your samples with Qubit. We are expecting at least 10 ng total.

    a. Dilute the Qubit light-sensitive reagent 1:200 in Qubit dilution buffer to make working buffer. For 8 samples + 2 standards, this is 10 μL reagent + 1.990 dilution buffer.

    b. In Qubit tubes, dilute 10 μL of Standard 1 and Standard 2 with 190 μL of working buffer.

    c. In Qubit tubes, dilute 2 μL of each sample with 198 μL of working buffer.

    d. Vortex all tubes to mix well.

    e. Measure at the BMC.

Note

If needed, you can store the eluted 30 μL samples at -20°C or -80°C and pause the protocol.

79. Vortex the T1 streptavidin beads well. For N samples, transfer 30 * N μL of beads to a low-binding 1.7 mL tube.

80. Pipetting up and down to mix well before taking each sub-sample, transfer 30 μL beads to separate low-binding 1.7 mL tubes.

Note

You will be using the same 1.7 mL tubes until you elution step after adapter ligation. Don't transfer samples to PCR tubes.

81. Add 1 mL of 1xTBW to each tube to wash the beads. Incubate for 1 minute, then place on magnet until beads separate.

82. Remove the supernatant while the tubes are still on the magnet.

83. Remove the 1.7 mL tubes from the magnet and resuspend in 150 uL of 2xBW.

84. Dilute the remaining 30 μL DNA samples to 150 μL by adding 120 μL of DEPC-treated water.

85. Add the samples to the tubes with beads. Mix well at room temperature with 300 rpm shaking for 30 minutes.

Note

If needed, you can leave the bead + sample solution mix overnight and start the steps the following day. With the enhanced speed of the SPRI-based cleanup, we merged Day 3 and Day 4 of the original protocol.

86. Add 950 μL of 1xTBW to each sample. Mix by pipetting and then shake at room temperature and 1200 RPM for 5 minutes.
87. Place on the magnet to settle the beads, remove the supernatant, and repeat the previous step.
88. After the second wash, remove the supernatant and quickly add 950 μL of 10 mM Tris-HCl pH 7.5 while the tubes are still on the magnet. Leave the Tris-HCl on the beads until right before the following steps.

End repair and A-tailing

89. Prepare enough End Repair Master Mix. This (and following steps) are no longer scaled per cell count; use the same volume for all samples.

    End Repair Master Mix (60 μL / sample)

Component	Volume
DEPC-treated water	50 μL
End Prep Reaction Buffer	7 μL
End Prep Enzyme Mix	3 μL

90. Remove the 10 mM Tris and carefully resuspend each sample in 60 μL of End Repair Master Mix, avoiding bubble formation.
91. Incubate for 30 minutes at 20°C with interval mixing (1000 rpm for 1 minute, 3 minutes still, repeat).
92. Incubate for 30 minutes at 65°C without mixing.
93. Transfer samples to ice.

Adapter ligation

94. Prepare enough diluted Illumina adapter and Reaction Master Mix.

    You need 1 μL of diluted adapter per condition. Use the following dilutions based on the estimated DNA concentration as measured by the Qubit. Dilute in 10 mM Tris-HCl pH 7.5 + 10 mM NaCl.

    Adapter dilution

DNA amount	Dilution
1 μg - 100 ng	None
100 ng - 5 ng	Dilute 1:10
< 5 ng	Dilute 1:25

Reaction Master Mix, (31 μL / sample):

Component	Volume
Ligation Master Mix	30 μL
Ligation enhancer	1 μL

95. Add 1 μL of diluted adapter into each tube, separately. Add in 31 μL of Reaction Master Mix, mixing with pipetting while avoiding bubble formation.
96. Incubate for 30 minutes at 20°C with interval mixing (1000 rpm for 1 minute, 3 minutes still, repeat).
97. Add in 3 μL of USER enzyme to each tube. Incubate for 15 minutes at 37°C with interval mixing (1000 rpm for 1 minute, 3 minute still, repeat).
98. Add 950 μL of 1x TBW to each tube, mix well, and incubate for 3 minutes at room temperature at 1200 rpm.
99. Briefly spin the tube, transfer to the magnet, and remove the supernatant while still on the magnet.
100. Repeat the wash step by adding 950 μL of 1x TBW, mixing well, incubating, spinning, and placing on the magnet.
101. While still on the magnet, rinse the beads wtih 950 μL of 10 mM Tris-HCl, pH 7.5. Incubate on the magnet for 1 minute, then remove supernatant while still on the magnet.
102. Resuspend the beads in 23 μL of 0.1x TE; the resulting bead slurry is used in downstream steps because the pulled-down fragments are relatively irreversibly bound to the beads. This does not interfere with the PCR.

> **Warning**
>
> Do not freeze the bead slurry! Store at 4°C or on ice.

Test PCR

103. Following the *Test PCR protocol* (page **??**), using the following variables:
    - Use 3 μL of the 23 μL bead slurry for test PCR purposes. 19 μL will be used in the real PCR, so $\log_2(19/0.7) = 4.76$ delta cycles.
    - Use the `ligation_fwd` and `ligation_rev` primers.

- Target at least 500 ng, erroring on the higher side. Perform 12/14/16 test cycles.
- The final cycle count should be between 6-9 cycles.

Real PCR

104. Prepare enough Master Master mix for each condition, 76 μL per condition.

Master Master Mix (76 μL / condition)

Component	Volume
DEPC-treated water	26 μL
2x NEBNext Ultra II Q5 Master Mix	50 μL

105. Assign dual-indexes to each condition. We buy the pre-indexed plates from NEB, so this means assigning a well of a 96-well to each condition (e.g. A4 is condition 1, B4 is condition 2, . . .).

106. Prepare reaction mixes per condition by combining the following:

Component	Volume
Master Master mix	76 μL
Premixed dual-index primers	5 μL
Bead slurry (add last)	19 μL

107. Split reaction mixes across two PCR tubes, e.g. so each tube has 50 μL total volume. Run the following PCR program:

Temp (°C)	Time (MM:SS)	Description
72	5:00	Polymerase activation
98	0:30	Initial denaturation
–	–	Optimal number of cycles
98	0:10	Denaturation
65	1:15	Extension
–	–	cycle end
4	Hold	Final hold

Note

If some of the conditions have a different optimal cycle count, you can use the same 42°C hold technique as the test PCR; e.g. do 7 cycles, have a 42°C hold where you remove the tubes that only need 7 cycles, resume for another cycle or two, and so on.

108. Pool the split reactions back together.

109. To each 100 μL sample, add 90 μL of Ampure beads. Mix well via pipetting.
110. Incubate at room temperature for 15 minutes.
111. Place tubes on the magnetic rack. Remove the supernatant, and wash twice with 200 μL of **freshly prepared** 80% EtOH without disturbing the beads (e.g. keep the tubes on the magnet).
112. Remove residual EtOH wash using a P10.
113. Air-dry the beads until the shiny-to-matte transition happens, not longer than 5 minutes.
114. Add 25 μL of 0.1x TE to elute. Mix well via pipetting and incubate off-magnet for 2 minutes at room temperature.
115. Place the tubes back on the magnet and transfer the supernatant to nicely labled, new low-binding 1.7 mL tubes. Store at -20°C. This is a good pause step.

Pre-amplification library quantification

116. Following the instructions for the NEB Next Library Quant Kit, quantify the concentration of each library.
117. Perform a 1:200 dilution Qubit quantification of each library.
    a. Dilute the Qubit light-sensitive reagent 1:200 in Qubit dilution buffer to make working buffer. For 8 samples + 2 standards, this is 10 μL reagent + 1.990 dilution buffer.
    b. In Qubit tubes, dilute 10 μL of Standard 1 and Standard 2 with 190 μL of working buffer.
    c. In Qubit tubes, dilute 1 of each sample with 199 μL of working buffer.
    d. Vortex all tubes to mix well.
    e. Measure at the BMC.
118. Based on these measurements, calculate volumes required to pool 500 ng of each condition together, up to a maximum of 4 μg per capture reaction. Trust the library quant kit result over the Qubit quantification, though both should be close. The Qubit measures total DNA, whereas the library quant kit measures only DNA containing amplifiable Illumina primers.

Day 4: Region capture

Estimated time

The first capture day has very short hands-on time. It ends with a 16 hour incubation step that should be precisely followed! Start the incubation step at the end of the day so that you will have sufficient time to prepare buffers and such the following day.

Warning

If you google the Twist Target Hybridization Protocol, there are multiple versions of the protocol! Make sure your protocol version matches the reagents you have.

We currently use the v2 protocol.

119. Combine 500 ng of each condition together into a single pool in a fresh PCR tube. Load another PCR tube with an equivilant amount of water.
120. Thaw required ragents on ice (Hybridization Mix, Hybridization Enhancer, Universal Blockers, Blocker Solution).
121. Use a lyophilizer to dry the indexed pool, in no-heat mode.
    a. Fill the condenser with liquid nitrogen.
    b. Place the PCR tubes **(with caps open!)** within 1.7 mL tubes, then place these inside the lyo centrifuge.
    c. Make sure the lid seals, then turn on the centrifuge, keeping the heater **off**.
    d. Turn on the vacuum pump and open the valve to the centrifuge. Wait at the centrifuge until the vacuum monitor reaches mill-Torr.
    e. Lyophilize the pooled library for at least 90 minutes.
    f. There should be a thin film of DNA visible after drying is complete, with no visible liquid.
121. Near the end of the lyophilization, set a water bath to 65°C and set a thermocycler to 95°C (lid temp 105°C).
122. Heat the Hybridization Mix in the water bath for 10 minutes. Cool at room temperature for 5 minutes.
123. In a clean PCR tube, prepare Probe Solution. Mix well.

    Probe Solution

Component	Volume
Hybridization mix	20 μL
Twist Custom Panel	4 μL
Water	4 μL

124. Resuspend the library pool with the following reagents. Flick and pipette to mix.

Component	Volume
Dried library pool	n/a
Blocker solution	5 μL
Universal blockers	7 μL

125. Heat the Probe Solution to 95°C for two minutes in the thermocycler (with the lid closed to prevent condensation), then remove tube and place on ice for 5 minutes.
126. While the Probe Solution is cooling, heat the library pool to 95°C on the thermocycler (with the lid closed to prevent condensation) for 5 minutes, followed by 5 minutes at room temperature.
127. Vortex and spin down the probe solution, then transfer the whole volume into the resuspended library pool. Mix well.
128. Pulse-spin the tube.
129. Add 30 μL of Hybridization Enhancer to the top of the reaction. This enhancer floats on the top like oil.
130. Pulse-spin the tube.
131. Incubate the hybridization reaction at 70°C for 16 hours in a thermocycler, with the lid at 85°C.

Note

Shoot for 16 hours. You can stop the hyrbidization between 15 and 17 hours, so you have some, but limited room.

Day 5: Region capture part 2

132. Follow the Twist instructions with the following changes:
    a. Instead of guessing on a number of amplification cycles, perform a test PCR. Twist has you elute in 45 μL, so if you perform 30 μL test reactions with 3 μL template, subtract off $\log_2(45/3) = 3.9$ cycles from the gel quantification. Use the NEBNext Ultra II mix for the test PCR.
    b. You can use either the provided Equinox library amplification mix, or the NEBNext Ultra II mix for the final PCR.
    c. Quantify the resulting 30 μL libraries (eluted in 10 mM Tris-HCl, pH 8) both with the NEB Library Quant kit and by submitting a sample to the FragmentAnalyzer in the BMC.

sci-ATAC-seq

This protocol is from the STAR Protocols paper, [**?**], with added commentary and suggestions.

You should print out and follow along to the STAR protocols paper while reading this protocol.

Background

At a high level, sci-ATAC-seq is a **split-pool** technique where four successive barcodes are added to each fragment in the library. This allows us to do single-cell analysis without requiring single-cell droplets. The two rounds of barcoding are:

- Barcoded transposition, to add condition barcodes. Cells are split at 1k cells / well onto a 96 well plate, where each well of the 96 well plate has a unique pair of transposition barcodes assembled with Tn5. After transposition, all transposed cells are pooled together.
- The cells are then split onto plates, where PCR is performed with another set of unique barcodes per well. These PCR rounds add additional barcodes. The cells are sparsely split over the plates such that it is very likely (e.g. via the Poisson distribution) that cells have unique barcodes at this point.

Preparation (N days before)

The following steps can be done at any time. The transposition mix and PCR primer plates quantities suggested here make enough mix for multiple experiments. However, check how much is left because it takes some time for large primer orders.

Primer order

Note

An Excel sheet that has rows that you can directly copy-paste into Azenta to order primers is here.

1. Order HPLC-purified transposition primers (sciAD1/sciAD2), in at least 25 nmol scale.
2. Order (optionally HPLC-purified, not required) PCR primers (sciP1/sciP2) in at least 100 nmol scale.
3. Order the mosaic end primer, MEComp, in at least 500 nmol scale. The sequence is `C*T*G*T*C*T*C*T*T*T*A*T*A*C*A`, and you should order this with HPLC purification, a `5'-Phosphate` 5' modification, and a `3'ddC` 3' modification.

Buffer preparation

Estimated time

Multiple hours; put on a good podcast and try to enjoy the pH'ing and measuring.

- Make sure there is sufficient amounts of the following *shared genomics buffers* (page **??**):
  - 1M Tris-HCl, pH 8.0
  - 1M Tris-HCl, pH 7.5
  - 0.5M EDTA, pH 8.0
  - 1M HEPES, pH 7.5
  - 1M MgCl2

All buffers except for the DTT can be stored at room temperature for a long time and can also be sink-disposed. You may want to remake if it's been longer than a year. The DTT is likely good for at least a month.

4. Prepare 1 mL of **5 mM EDTA**

Component	Concentration	Amount / 1 mL
500 mM EDTA	5 mM	10 μL
Elga water		990 μL

5. Prepare 10 mL of **1xTE**

Component	Concentration	Amount / 10 mL
10x TE	1xTE	1 mL
Elga water	to 10 mL	9 mL

6. Prepare 1 mL of fresh **100 mM DTT stock solution**:

Component	Amount
DTT	0.01543 g
1 M HEPES pH 7.5	25 μL
DEPC-treated water	975 μL

> **Note**
>
> DTT, as a reducing agent, will slowly degrade / lose its reduction potential. You need to remake this fresh every time you use it.

> **Warning**
>
> DTT stock should be collected as a separate waste stream.

7. Prepare the acetate salt buffers. These salts are *very* hygroscopic! You may have to reorder, and we should possibly store them with silica gel.

   Prepare 10 mL of **0.2M Tris-acetate stock solution**:

Component	Concentration	g / L	Amount / 10 mL
Tris-acetate	0.2M	36.24	0.3624 g
Elga water	to 10 mL		

Prepare 10 mL of **0.5M potassium-acetate stock solution**:

Component	Concentration	g / L	Amount / 10 mL
K-acetate	0.5M	490.7	4.9 g
Elga water	to 10 mL		Add in two rounds

Prepare 10 mL of **1.0M magnesium-acetate stock solution**:

Component	Concentration	g / L	Amount / 10 mL
Mg-acetate	1.0M	214.46	2.144 g
Elga water	to 10 mL		Add in two rounds

8. Prepare 5 mL of **10% IGEPAL CA-630**:

Component	Concentration	Amount / 5 mL
IGEPAL CA-630	10% (v/v)	500 μL
Elga water	to 5 mL	

Prepare 5 mL of **10% Tween20**:

Component	Concentration	Amount / 5 mL
Tween 20	10% (v/v)	500 μL
Elga water	to 5 mL	

9. Prepare 10 mL of **reverse crosslinking buffer (RCB)**

Component	Concentration	Amount / 10 mL
1 M Tris, pH 8.0	100 mM	1 mL
5 M NaCl	100 mM	0.2 mL
10% SDS	0.4%	0.4 mL
Elga water		8.58 mL

10. Prepare 1 mL of **2.5M glycine stock solution**:

Component	Concentration	g / L	Amount / 1 mL
Glycine	2.5M	187.67	0.1876 g
Elga water	to 1 mL		

11. Check that there is enough 7.5% BSA solution; much less than a mL is needed.

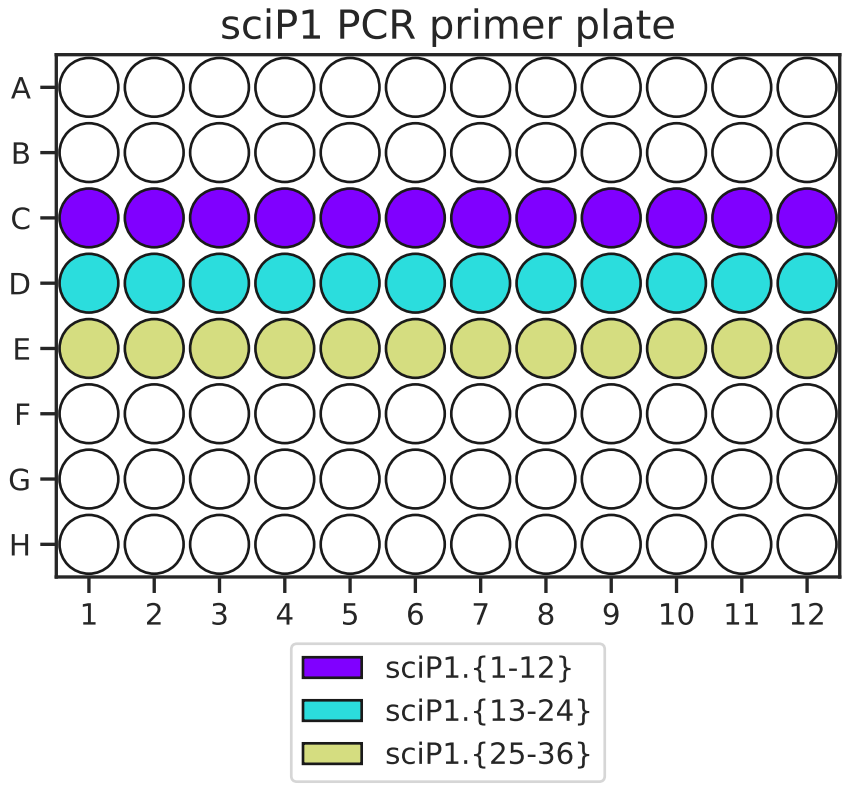
Component	Concentration	Amount / 1 mL
BSA	7.5% (w/v)	0.075 g
Elga water	to 1 mL	

Primer resuspension

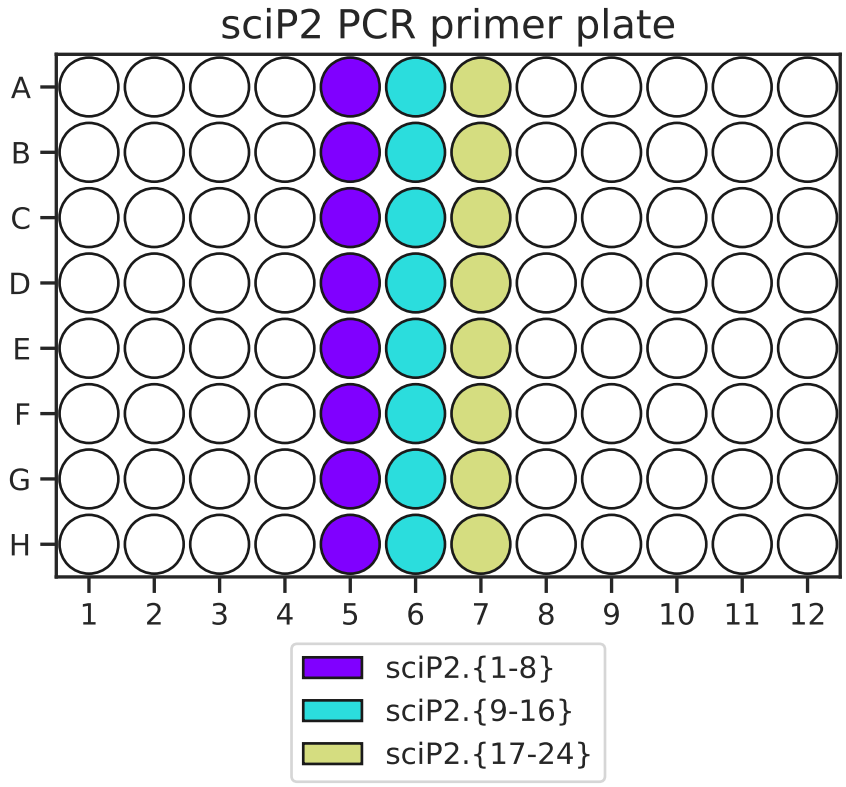
Estimated time

90 minutes

1. Resuspend all primers (sciAD1/sciAD2/sciP1/sciP2/MEComp) in 1xTE to a final concentration of 100 μM.
2. Pipette 50 uL of each PCR primer onto two plates. The recommended plate layouts are:



8. Make a transposition primer master mix:


sciP2 PCR primer plate
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
sciP2.{1-8}
sciP2.{9-16}
sciP2.{17-24}

Component	Volume per mix	Volume / 20 mixes (+ 5% excess)
100 μM MEComp	13 μL	273 μL
1M Tris, pH 8.0	0.26 μL	5.46 μL
5M NaCl	0.26 μL	5.46 μL

9. In a PCR plate / PCR tubes, combine 13.5 μL of the master mix with 13 uL of each 100 μM sciAD1.X or sciAD2.X oligos.
10. Heat the mixes in a thermocycler to 85C for 2 minutes, then cool down to 20C at -1C/minute, or as slowly as possible. When set to a 1% ramp rate, our thermocyclers cool at -3C/minute, which is sufficient.
11. While the thermocycler is cooling, aliquot ~600 uL of 100% glycerol and put on ice.
12. Pipette 25 uL of cold 100% glycerol into a PCR plate. Add 25 uL of each cooled mixture to create 20 transposition primer mixes.

Note

100% glycerol is very viscous. I recommend using a 20 uL pipette (it can go up to 25 uL without hitting the end stop) and pipetting very slowly.

The recommended transposition plate layout is:

Day 1: cell collection and fixation

Note

If you have multiple people helping, that person can perform the Tn5 assembly step detailed in the next session while one person is counting/fixing.

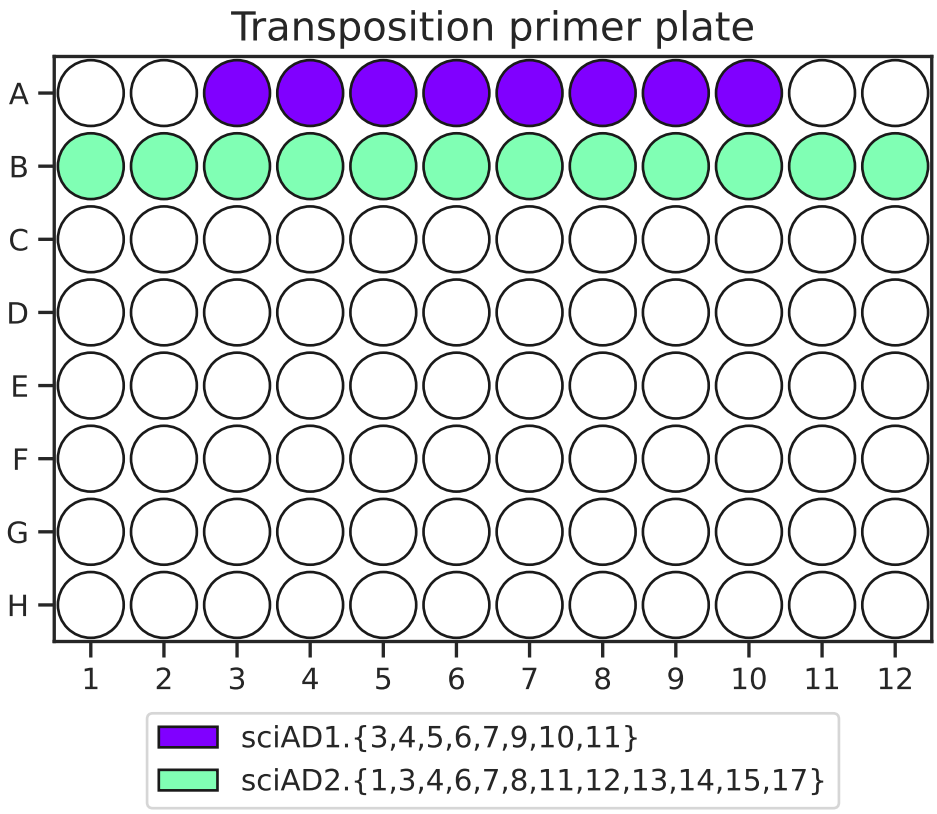
If you are doing this yourself, you can assemble the Tn5 either before of after fixation.

- Before fixation: this is slightly faster since you don't have a 30 minute wait step after fixation, but could be annoying if you are e.g. prepping for sorting.
- After fixation: conceptually simpler, and you can more accurately lay out your transposition plate based on sorting results, but is 30 minute slower.

Estimated time

2 hours (including cell counting time)

1. Pre-sort, prepare 7.5% BSA-coated tubes or sort with FBS-containing media. Cells should never be placed into non-coated tubes in non-FBS-containing liquid.


Transposition primer plate
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
sciAD1.{3,4,5,6,7,9,10,11}
sciAD2.{1,3,4,6,7,8,11,12,13,14,15,17}

Tip

To BSA coat tubes, place a small amount of 7.5% BSA into a tube, vortex the tube, then aspirate out the BSA.

2. Determine how many cells in each condition you would like to collect. This protocol allows you to transpose up to 96k cells, split among up to 96 conditions.
    a. If you have enough cells to spare, count at least 100k cells to make later fixation steps easier.
    b. If you do not have 100k cells per condition, count with some amount of excess. For example, if you have 10 conditions, you will need around 10k cells in the transposition. You then may want to sort at least 30k cells.
3. Sort and count cells, placing them into 7.5% BSA coated tubes. **Record the number of cells per tube**.
4. Pellet the cells at 300 g for 3 minutes. Use a bucket rotor if possible to get a tight pellet in the bottom.

Tip

Reducing the centrifuge deceleration speed will also maximize pellet stability.

5. Aspirate off the supernatant, and resuspend the pellets in 100 uL cold PBS.
6. Count the cells using a 1:1 dilution in Trypan Blue. Dilute the cells to 100k cells / 100 uL if needed. If you have fewer than 100k cells, leave it resuspended in the cold PBS (e.g. do not concentrate).
7. Pellet the cells at 300 g for 3 minutes. Do not aspirate the PBS.
8. Prepare small quantities of 1.6% formaldehyde dilution, at least 6.7 μL per sample. From our 32% stock, this is a twenty-fold dilution. You can alternatively prepare 2.7 μL of 4% formaldehyde per sample.
9. Freshly prepare a master mix to stop fixation:

Component	Amount per sample
2.5M glycine	5.6 μL
1M Tris, pH 8.0	5.0 μL
7.5% BSA	1.3 μL

11. Fix cells by adding 6.7 μL of 1.6% formaldehyde or 2.7 μL of 4.0% formaldehyde to each sample, simultaneously diluting the formaldehyde and resuspending the pellet via pipetting.
12. Incubate the samples at room temperature for 5 minutes. Get ice if you don't already have some.
13. Add 11.9 μL of the stop-fixation master mix to each sample.

14. Incubate on ice for 10 minutes.
15. Gently add 0.5 mL of cold PBS; try to not disturb cells that have settled to the bottom. Spin at 500 g for 3 minutes.
16. Carefully aspirate media and again gently add 0.5 mL of cold PBS, trying not to disturb the pellet.

Note

You can pause at this point, with the fixed pellet sitting in PBS. If time allows on day 1, you should proceed to transposition.

Day 1: transposition

Transposase assembly

Estimated time

1 hour

Note

Work in the genomics hood when preparing these buffers and performing these steps.

1. Prepare or defrost dilution buffer; this buffer can be stored at -20C for several months.

Component	Concentration	Amount / 100 μL
100% glycerol	50%	50 μL
1M Tris, pH 7.5	50 mM	5 μL
5M NaCL	100 mM	2 μL
5 mM EDTA	0.1 mM	2 μL
100 mM DTT	1 mM	1 μL
10% IGEPAL CA-630	0.1%	1 μL
DEPC water		39 μL

2. Based on the number of transposition reactions (which are the number of 1k cell aliquots, e.g. 50k cells requires 50 reactions), determine the desired plate layout and total number of Tn5 assembled mixes you need.

   There are several dilution steps, so we will name them:

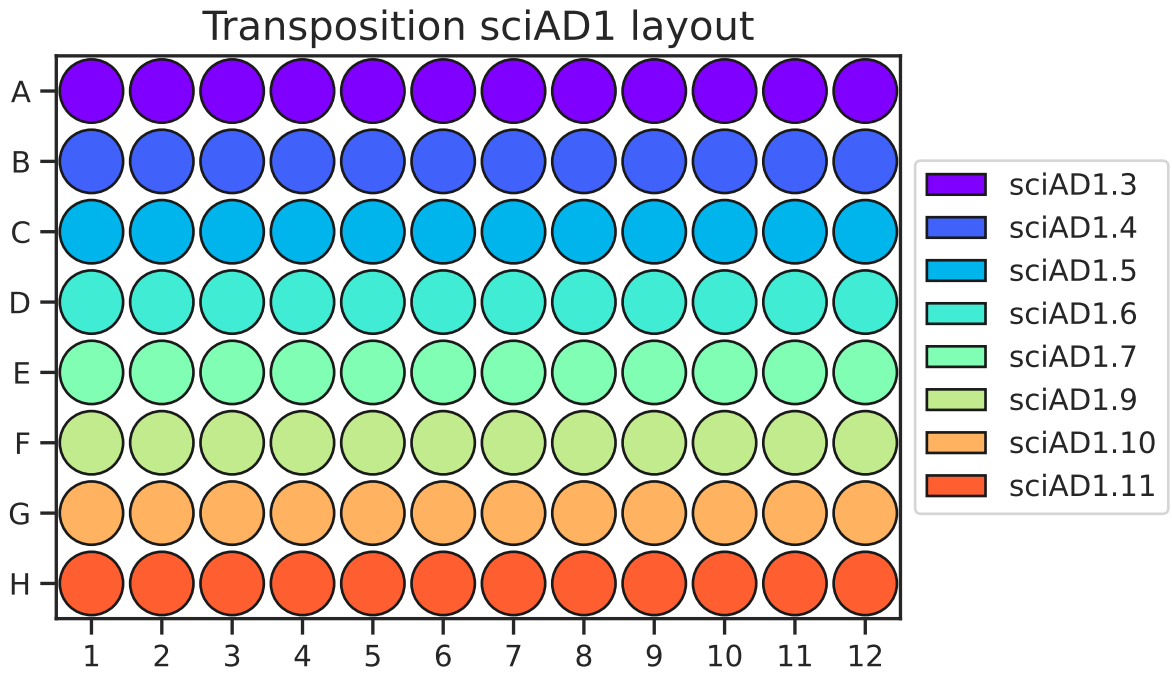
   - D1 (unloaded): The initial, unloaded Tn5 diluted in dilution buffer.
   - D2 (loaded): Dilution D1 is mixed with assembled adapters, in a two-fold dilution.

- D3 (final): Dilution D2 is further diluted in transposition buffer, then 1 μL of each mix is added to each well.

For every final well, we will be adding 1 μL of the sciAD1.X D3 dilution and 1 μL of the sciAD2.X D3 dilution. This means that we need:

- 0.5 μL per assembly mix / 1.0 μL total of D2 dilutions
- 0.25 μL per assembly mix / 0.5 μL total of D1 dilutions per well.

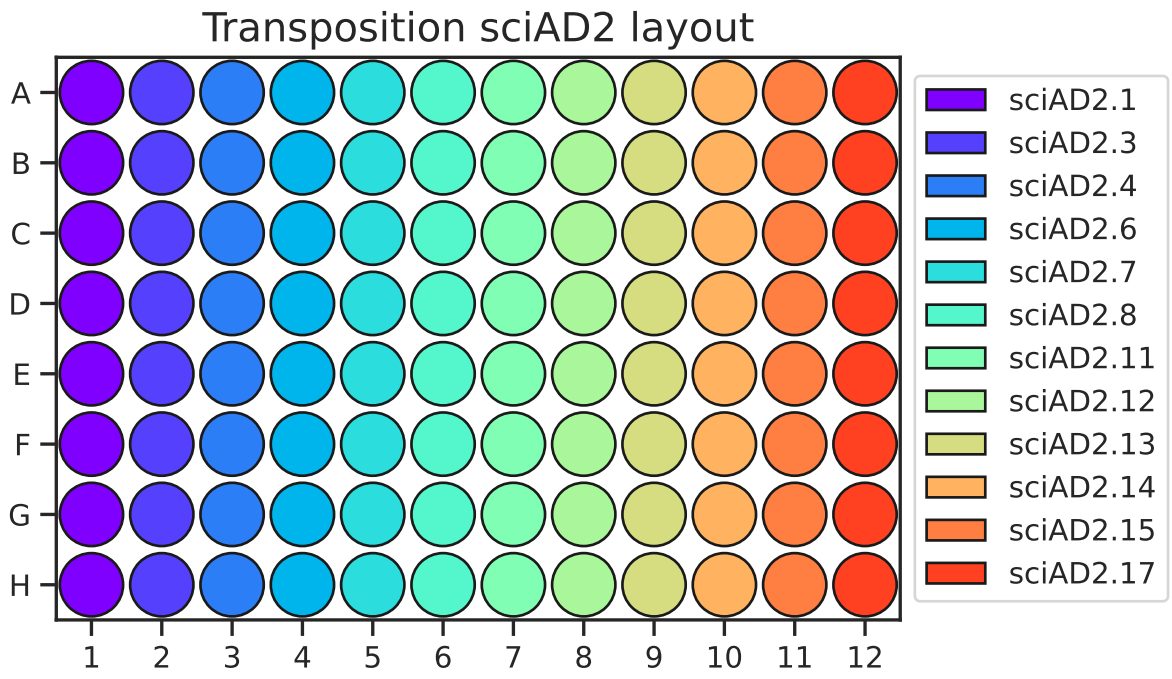
When planning your plate layout, the standard layout uses one sciAD1.X per row and one sciAD2.X per column. The intersection of these two gives the combinatoric barcoding per-well.



Note

For example, if you have 12 transposition reactions to do (12k cells split across conditions), the most efficient plate layout is a 4 x 3 rectangle. This means that we will use 3 of the sciAD1.X primers and 4 of the sciAD2.X primers.

In total, we need to make 7 Tn5 mixes, and each mix will be used in 3 or 4 wells. This means that we need at least 1.0 μL of D1 dilutions per mix. Accounting for excess, we


Transposition sciAD2 layout
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
sciAD2.1
sciAD2.3
sciAD2.4
sciAD2.6
sciAD2.7
sciAD2.8
sciAD2.11
sciAD2.12
sciAD2.13
sciAD2.14
sciAD2.15
sciAD2.17

could make 10.5 uL of D1 mix total (1.5 μL of D1 per mix, diluted to 3 μL D2, diluted to 6 μL D3).

Note

If you are doing an entire plate, then you can use $Z = 3.5$ μL for the sci.AD1 primers and $Z = 3.0$ μL for sci.AD2.

3. Prepare the unloaded D1 dilution of Tn5 by diluting Tn5 with dilution buffer. Using the Diagenode 2 mg/mL Tn5, the dilution ratio can be at least 1:10 (e.g. 9.0 μL Tn5 diluted to 90 μL). This has been verified to work at 1:10.
    - For a full 96-well plate, this is in the 70 μL range, depending on how much excess you need in the next step.
4. For each Tn5 mix required (20 mixes for a whole plate), prepare D2 dilutions by separately combing Z μL of D1 mix with Z μL of pre-annealed adapters. The minimum **without excess** for N reactions is $Z = \frac{N}{4}\mu$L; you should include excess.
    - For a full 96-well plate, we need at least 8 / 12 reactions worth of mix. This is at least 6 μL; the original protocol recommends Z = 4 μL so that we have 8 μL (2 μL of leftover mix). You can reduce this amount of excess.
    - For our example above, we can use Z=1.5 μL to have excess.
5. Incubate the D2 mixes at room temperature for 30 minutes.
6. While waiting on this, check to see if more 100x protease inhibitor cocktail needs to be made. Dissolve one tablet (one per 50 mL normally) in 500 uL of Elga water, and make small aliquots (25 μL) and store at 4C. The tablets will not fully dissolve; mix well before aliquoting.
7. Transfer the assembled D2 mixes to ice until right before use.

Transposition

Estimated time

2.5 hours

At this point, you should have a tight cell pellet. If you are resuming the protocol after a delay, spin down the cells at 500 g for 3 minutes before proceeding.

For greater accuracy, you can resuspend and re-count cells. Alternatively, you can use the cell counts you recorded pre-fixation.

1. Prepare fresh transposition buffer and nuclei isolation buffers.

    Transposition buffer, scale down as needed (1.44 mL per plate):

Component	Concentration	Amount / 1.44 mL
0.2 M Tris-acetate	41.25 mM	297 μL
5 M K-acetate	82.5 mM	23.76 μL
1 M Mg-acetate	12.5 mM	18 μL
DMF	20%	288 μl
10% IGEPAL CA-630	0.125%	18 μL
100x Protease inhibitor	0.5%	7.2 μL
DEPC water		788 μL

Nuclei isolation buffer:

Component	Concentration	Amount / 10 mL
1 M Tris, pH 7.5	10 mM	100 μL
5 M NaCl	10 mM	20 μL
1 M MgCl2	3 mM	30 μL
10% IGEPAL CA-630	0.1%	100 μL
Elga/DEPC water		9.75 mL

2. Carefully remove the PBS from the cell pellets, and resuspend the pellets in PBS to a final concentration of 1K cells / μL.
3. In a fresh PCR plate, fill 7 μL of transposition buffer into each reaction well.
4. Add 1 μL of fixed cells to each reaction well, bringing the volume to 8 μL. **Record which wells map to each condition**.
5. Dilute each assembled D2 Tn5 mix with an equal volume of transposition buffer to create the final D3 Tn5 mixes. E.g. if you made 4 μL D2 mixes, add 4 μL of transposition buffer.
6. Add 1 μL of the sciAD1.X mixes (row by row) and add 1 μL of the sciAD2.X mixes (column by column) to each well, matching your plate layout.

Important

This is the first round of combinatoric barcoding! You **must know** which conditions are in each well and which sciAD1/2 you added to each well; these barcodes are demultiplexed to conditions in the NGS data processing step!

7. Shake at 300 rpm for 30 minutes at 37C using a ThermoMixer.
8. Quench the reaction by adding 1 μL of 0.5M EDTA to each well, mixing well.
9. Shake at 300 rpm for 15 minutes at 37C using a ThermoMixer.
10. Pool all reactions into a BSA-coated Eppendorf tube. Add 38.4 μL 1M MgCl2 to quench the EDTA and mix well via pipetting.
11. Pellet cells by spinning at 500 g for 2 minutes.

12. Remove the supernatant and wash the cells with 1 mL nuclei isolation buffer.
13. Centrifuge at 500 g for 2 minutes.
14. Resuspend the cells in 0.5 mL of nuclei isolation buffer, and filter using a 40 μm cell strainer. You can use the 40 μm strainers that fit on top of 50 mL tubes.
15. Count the cells and dilute them to 13.3 cells / μL, diluting using nuclei isolation buffer.
16. Determine how many PCR plates you will need. There are enough barcoded PCR primers to split onto 9 PCR plates. Each PCR plate will amplify around 19k cells.

Reverse cross-linking

1. Calculate the amount of RCB you need (400 μL per PCR plate). Then, add 2 uL of 20 mg/mL proteinase K per 1 mL of RCB.
2. Determine which PCR primers sets you will use for each plate. There are three "sets" of sciP1 and sciP2 primers, which multiplexes onto up to 9 plates. For instance, if you have three PCR plates, you could choose to use sciP1.1-12 for all three plates, but then use sciP2.1-8 for plate 1, sciP2.9-16 for plate 2, and sciP2.17-24 for the third.
3. Prepare Master Mix 1.X and Master Mix 2.X. The following mixes are given per PCR plate. If you prepare these in a secondary PCR plate / PCR strips, you can use a multichannel in the next step.

   Master mix 1.X (one mix per primer):

Component	Amount / PCR plate
Transposed cells (13.3 / μL)	15 μL
10 μM sciP1.X	5 μL

   Master mix 2.X (one mix per primer):

Component	Amount / PCR plate
50 μL RCB + Proteinase K	50 μL
10 μM sciP2.X	10 μL

4. Distribute 2 μL of Master Mix 1 across the PCR plates. Each column should use the same Master Mix 1.X. **Record which wells got which master mix**.
5. Distribute 3 μL of Master Mix 2 across the PCR plates. Each row should use the same Master Mix 2.X. **Record which wells got which master mix**.
6. Incubate in a thermocycler at 55C for 1 to 16 hours (e.g. overnight).

Day 2: library amplification

Minimal amplification

Estimated time

45 minutes

1. Spin down the PCR plate briefly at 300 g for 10 seconds.
2. Add 5 μL of 10% Tween20 to each well, mixing well via pipetting.
3. Prepare PCR master mix by combining 1.25 mL of NEBNext PCR mix and 250 μL of Elga water per plate.
4. Add 15 μL of the PCR mix to each well, pipetting to mix.
5. Spin down the PCR plate briefly at 300 g for 10 seconds.
6. Run an initial amplification. This takes about 24 minutes per plate. You can run multiple batches of these, e.g. you don't need 9 thermocyclers to do so. Leave completed plates at 4C.

Step	Temperature (C)	Time	Cycles
Initial extension	72	5 min	
Initial denaturation	98	5 min	
Denaturation	98	10 s	5
Annealing	70	30 s	5
Extension	72	1 min	5
Hold	4		

7. Remove the plate and **keep it on ice or otherwise at 4C** until after the final amplification.

Quantification

Estimated time

2 hours

1. Randomly choose O(5) wells per PCR plate to use for qPCR, using **any** of the sciP1/P2 primers (this has been done with sciP1.01 and sciP2.01). This works because the primers share a 3' end.
2. Prepare qPCR master mix. Using the 2X master mix from the BioMicroCenter, the recipe for a full 96-well plate (with 5% excess) is:

Component	Amount	Amount with excess
2x SYBR master mix	500 μL	525 μL
100 μM stock sciP1.01	1.0 μL	1.05 μL
100 μM stock sciP2.01	1.0 μL	1.05 μL
DEPC/Elga water	498 μL	523 μL

3. Sample 1 μL from 3-5 wells of each PCR plate and put it in the qPCR plate, doing at least duplicate technical replicates. Include some blank water wells, where you just place 1 μL of water in each well.

4. Add 9 μL of master mix to every well, bringing the reaction volume to 10 μL.

5. Perform qPCR with the following programs, ensuring that you included some blank water wells, following the LightCycler instructions here.

   Programs:

Program	Cycles	Analysis mode
Pre-incubation	1	None
Amplification	35	Quantification
Melting curve	1	Melting curve
Cooling	1	None

   Details:

Program	Temp	Acquisition mode	Hold	Ramp rate	Acquisitions
Pre-incubation	95	None	00:05:00	4.4	
Amplification	95	None	00:00:30	4.4	
Amplification	60	None	00:00:45	2.2	
Amplification	72	Single	00:00:30	4.4	
Melting curve	95	None	00:00:05	4.4	
Melting curve	65	None	00:01:00	2.2	
Melting curve	97	Continuous			5
Cooling	40	None	00:00:10	1.5	

6. Export the raw fluorescence curves for analysis.

7. Calculate the average $\frac{C_t}{3}$ value (e.g. the cycle number to reach 1/3rd the plateau value), ensuring that the water wells do not show comparable amplification. **Keep the qPCR plates to run wells on a gel**.

8. Perform N additional cycles of PCR, where $N = \frac{C_t}{3} - 5$. This minimal amplification ensures that we generate a large enough library without introducing a bunch of PCR duplicates. The extra number of cycles should generally be less than 10. This PCR takes about 25 minutes.

Step	Temperature (C)	Time	Cycles
Initial denaturation	98	30 s	
Denaturation	98	10 s	N
Annealing	70	30 s	N
Extension	72	1 min	N
Hold	4		

9. While additional cycles are running, run some of the completed qPCR wells on a gel. You should see a smear of transposition products, with the peak around 800bp-1kb.

Note

A smaller peak indicates overtransposition and means that you can increase the cell loading and/or increase the Tn5 dilution. Over-transposition isn't necessarily bad, it just possibly makes the data harder to align.

10. Pool samples from each plate into separate 15 mL conical tubes.
11. Purify the DNA from each plate using the DNA Clean and Concentration kit (1 column per plate). Use between 3- and 5-fold binding buffer. This will be a lot of binding buffer; it's fine. Load the column multiple times as needed.
12. Elute in 18 μL of 1xTE.

Day 3: library quantification

Estimated time

2.5 hours

1. Following the instructions for the NEB Next Library Quant Kit, quantify the concentration of each library.
2. Perform a 1:200 dilution Qubit quantification of each library.
    a. Dilute the Qubit light-sensitive reagent 1:200 in Qubit dilution buffer to make working buffer. For 8 samples + 2 standards, this is 10 μL reagent + 1.990 dilution buffer.
    b. In Qubit tubes, dilute 10 μL of Standard 1 and Standard 2 with 190 μL of working buffer.
    c. In Qubit tubes, dilute 1 of each sample with 199 μL of working buffer.
    d. Vortex all tubes to mix well.
    e. Measure at the BMC.

References

Non-pA and pA transcript sequencing

This protocol allows you to do a targeted pulldown of both polyadenylated and non-polyadenylated transcripts, targeted to a set of genes or a region of interest. The resulting transcripts can be reverse transcribed into cDNA or directly used for long-read sequencing.

How do we do both this enrichment while maintaining the non-polyadenylated transcripts? The overall process is:

1. Extract RNA from cells, following normal RNA isolation instructions.
2. Non-specifically ligating on a known DNA adapter to the 3' end of all RNA. This includes things like rRNA, miRNA, etc.
3. Using a biotinylated probe library, bind capture probes to the adapter-ligated library.
4. Select for the library by mixing with streptavidin magnetic beads.
5. Release the resulting transcripts from the beads. Perform cDNA synthesis with a custom primer targeting the adapter, or perform direct RNA sequencing.

Day -n

Prepare a 5'-phosphorylated adapter oligo. This can be stored at -20°C indefinitely.

Note

A tested functional adapter oligo is oTA526, though you can use another if desired. For example, if your nanopore run or other downstream step is already using a specific sequencing primer, it might be good to use the reverse complement of that oligo as the adapter sequence.

oTA526: `CCTAATTCAGGTAACCGGAGGAG`

1. Prepare the following reaction mix for the adapter oligo. This can be scaled up or down as required.

Component	Volume
100 μM oligo	4 μL (400 pmol)
T4 ligase buffer	1 μL
PNK	0.5 μL
Water	4.5 μL

2. Run a phosphorylation program (37°C for 1 hour, 65°C for 20 minutes) on a thermocycler.
3. Cleanup using a DNA spin column. Use the "alternative / oligo" cleanup protocol that adds more IPA to reduce the size cutoff. Elute in 10 μL of 0.1x TE.

Make sure that enough genomics-grade stock solutions are available. These are common buffers shared across many protocols. In particular, you will need less than 1mL of the following *shared*

genomics buffers (page **??**):

- 1M Tris, pH 7.5
- 5M NaCl
- 0.5M EDTA

Day 0: Buffer preparation

- Make sure there is sufficient amounts of the following *shared genomics buffers* (page **??**):
  - 5M NaCl
  - 1M Tris-HCl, pH 7.5
  - 0.5M EDTA, pH 8.0

Ligation and target enrichment

Following RNA isolation, setup a ligation reaction. This reaction can be scaled up depending on the amount of recovered RNA.

1. Setup the following reaction. The components come with the T4 RNA Ligase 1 (NEB #M0204)

Component	Volume
10x T4 RNA ligase buffer	2 μL
50% PEG	9 μL
1 mM ATP	2 μL
T4 RNA ligase I	1 μL
Phosphorylated adapter oligo	100 pmol, $\sim$2 μL
RNA	10 pmol
Water	to 20 μL

2. Incubate at 25°C for 2 hours.
3. While incubating, prepare the following buffers. You either need to make the High EDTA buffer or High EDTA + formamide. Only the formamide-containing buffer needs to be made fresh; others can be stored.
   - Wash/binding buffer (5 mL)

Target	Component	Volume
0.5M NaCl	5M NaCl	500 μL
20 mM Tris	1M Tris, pH 7.5	100 μL
1 mM EDTA	0.5M EDTA	10 μL
Water	DEPC water	4.39 mL

- Option A: High EDTA buffer (500 μL)

Target	Component	Volume
0.5M NaCl	5M NaCl	50 μL
20 mM Tris	1M Tris, pH 7.5	10 μL
4 mM EDTA	0.5M EDTA	4 μL
Water	DEPC water	436 mL

- Option B: High EDTA buffer buffer + 15% formamide (500 μL)

Warning

Formamide is a suspected carcinogen and can damage fertility / fetuses! Don't work with this if you are pregnant, and work with both the 100% formamide buffer preparation and capture steps up to the first wash in the actual fumehood, before moving back to the genomics hood.

Collect used buffer, unused buffer, and the first wash after as chemical waste.

Target	Component	Volume
0.5M NaCl	5M NaCl	50 μL
20 mM Tris	1M Tris, pH 7.5	10 μL
4 mM EDTA	0.5M EDTA	4 μL
15% formamide	formamide	75 μL
Water	DEPC water	361 μL

- Low Salt buffer (5 mL)

Target	Component	Volume
0.15M NaCl	5M NaCl	150 μL
20 mM Tris	1M Tris, pH 7.5	100 μL
1 mM EDTA	0.5M EDTA	10 μL
Water	DEPC water	4.74 mL

- Elution buffer (1 mL)

Target	Component	Volume
10 mM Tris	1M Tris, pH 7.5	10 μL
1 mM EDTA	0.5M EDTA	2 μL
Water	DEPC water	988 mL

4. Add 0.5 μL (50 pmol) of 100 μM biotinylated capture oligos to each sample.

5. Dilute each sample five-fold with high-EDTA buffer, with or without formamide, adding 80 μL to a final volume of 100 μL. The EDTA stops the ligase activity.
    - If using formamide, after mixing well, let the reaction sit at room temperature for 10-15 minutes.
    - If not using formamide, after mixing well, use a thermocycler to heat to 65°C for 5 minutes, followed by a slow ramp back to room temperature.
6. While waiting for capture oligo binding, wash 12.5 μL of MyOne Streptavidin C1 beads per sample. Wash a "master mix" of beads if possible to maintain consistent bead concentration. To wash, adding 10x volume (125 μL) of Wash/Binding buffer, magnetically separating the beads, removing the supernatant, and repeating the wash. After washing twice, resuspend the beads back in 12.5 μL of Wash/Binding buffer per sample.
7. Before beginning wash steps, warm an aliquot of Elution Buffer to 65°C. Keep at 65°C.
8. Add 12.5 μL of washed beads to each sample. Mix well with a pipette, and incubate at room temperature for 10 minutes. If beads settle, re-mix with a pipette.
9. Perform 2x wash steps with 100 μL of Wash/Binding buffer. After the second resuspension in wash buffer, move the beads to a fresh set of PCR tubes.
10. Perform one more wash with 100 μL of Wash/Binding buffer.
11. Wash the beads with 100 μL of Low Salt buffer.
12. After removing the supernatant from the Low Salt wash step, add 4 μL of warm elution buffer to the beads, mixing with pipetting. Once all samples are well mixed, put the tube back on the magnet and move the supernatant to a fresh set of PCR tubes.
13. At this point, you have properly ligated, target-enriched RNA. You should freeze the RNA or proceed to downstream uses, like cDNA synthesis.

cDNA synthesis

If doing cDNA synthesis, you can use the reverse complement of the ligation primer to only make cDNA of properly ligated RNA. You can follow the ProtoScript First Strand cDNA Synthesis kit, whose instructions (for half-reactions, which work fine) are listed here for convenience. This protocol uses 3 of the 4 μL eluted in the previous step, to correct for any losses.

1. Prepare the following reaction mix:

Component	Volume
RNA	3 μL
10 μM rev-com primer	1 μL
M-MuLV reaction mix	5 μL
M-MuLV enzyme mix	1 μL

2. Incubate at 42°C for one hour, followed by inactivation at 80°C for 5 minutes. A thermocycler program exists for this.

3. Store at -20°C or colder. The sample can be used directly in downstream steps (qPCR, etc) as long as the volume of cDNA product does not exceed 10% of the downstream volume.

Bulk RNAseq with Plasmidsaurus

1. Culture until over 100k cells attained. MC used 4 wells of a 24 wells/sample at 14 dpi reprogramming.
2. Aspirate media and wash cells with PBS.
3. If using pooling wells for the same sample, aspirate the first well of PBS and add ~200 uL of Zymo DNA/RNA shield to first well.
4. Pipette up and down to lyse cells, aspirate next well, and add the ~200 uL from the first well to the second well. Continue until all cells have been collected.
5. Transfer 50 uL to a PCR tube.
6. Vortex to break up viscous genomic DNA.
7. Place tubes in a 50 mL conical, padded with kim wipes to avoid tube breakage during shipping.
8. Ship like a regular plasmid sample.

Note

DNA/RNA shield is on the shelf with the agarose dissolving buffer. The mixture will become increasingly viscous, so take care to avoid bubbles.

SingleShot RNA isolation and Reverse Transcription

This protocol for RNA isolation uses the Bio-Rad SingleShot Cell Lysis Kit[64] and Maxima H Minus Reverse Transcriptase[65].

Materials need for RNA isolation

Solution	Location
SingleShot Cell Lysis Buffer	All reagents located in kit in -20°C freezer (Sven) in original Bio-Rad packaging.
Proteinase K solution	-20°C freezer (Sven)
DNase Solution	-20°C freezer (Sven)

[64] https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000112808.pdf

[65] https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0012047_TS_Maxima_H_Minus_Reverse_Transcriptase_2000U_UG.pdf

Materials needed for cDNA synthesis

Solution	Location
Oligo(dT) $_{18}$	-20°C freezer (Anna)
dNTP mix	-20°C freezer (Anna)
DEPC-treated Water	inside genomics hood
5x RT Buffer	-20°C freezer (Sven)
Maxima H Minus Reverse Transcriptase	-20°C freezer (Sven)
Thermo Scientific RiboLock RNase Inhibitor	-20°C freezer (Sven)

Other materials:

- RNase-free surface decontaminant
- Filter tips for pipettes
- PBS
- adherent cells in a 96-well plate
- PCR tubes
- Thermocycler

Important

Since cells are being lysed, work does not need to be in the BSC. However, once the cells are lysed, extra precaution should be taken to prevent RNase contamination.

Dedicated RNAse-free tubes and tips should be used, and all pipettes and working surfaces should be wiped down with the RNase-free surface decontaminant spray. Only use the genomics hood for these steps.

Use the genomics hood. There are tubes on the shelf to the right of the genomics hood that are RNase-free.

RNA isolation using SingleShot Cell Lysis

1. Thaw SingleShot Cell Lysis Buffer, Proteinase K, and DNase on ice.
2. Prepare Cell Lysis Master Mix on ice according to the number of wells to be isolated. It is recommended to make enough master mix for your number of wells plus 5%

Component	volume per well, µL	volume 96-well, µL
SingleShot Cell Lysis Buffer	48	4608
Proteinase K solution	1	96
DNase solution	1	96

3. Aspirate media from cells, and wash with 125 μL of room temperature PBS.
4. Remove PBS
5. Add 50 μL of prepared SingleShot master mix to each well
6. Incubate without agitation at room temperature for 10 minutes.

Important

Do not incubate for longer than 20 minutes at room temperature

Important

Do not mix the cells with SingleShot master mix by pipetting

7. After 10 minutes, transfer the mastermix solution from the plate to a PCR tube. Multichannel pipettes work well here as PCR tubes have the same spacing as a 96-well plate.
8. Immediately transfer to thermocycler. Incubate at 37°C for 5 minutes followed by 75°C for 5 minutes for heat inactivation of cell lysis.

Note

Cell lysate can be stored on ice up to 4 hours, at -20°C for 2 months, and -80°C for 12 months.

cDNA synthesis using Maxima H Minus Reverse Transcriptase

Important

Continue to work with RNase-free equipment and in an RNase-free area.

9. Thaw on ice the Oligo(dT) $_{18}$, dNTP mix, 5x RT Buffer, Maxima H Minus Reverse Transcriptase (RT), and the RiboLock RNase Inhibitor.
10. Prepare **RT master mix 1**. Up to 9 μL of cell lysate from the SingleShot lysis kit can be used per reaction. AMB has used the maximum and obtained satisfactory results.

Component	volume per reaction, µL
Oligo(dT) $_{18}$	1
dNTP mix	1
DEPC-treated water	12.5 - (μL of lysate to be used, max of 9 μL lysate)
total	at least 5.5 μL if maximum 9 μL of lysate and 1 μL of RT is to be used

11. Determine how many reactions you are running. Normally, run one reaction per lysate. Additionally, pick lysates to perform no-reverse-transcriptase controls (e.g. one per condition) and add an extra reaction for those.

Note

For example, if you have nine lysates across three conditions, you might want to setup 12 reactions; one for each of the nine lysates, and three additional reactions for a lysate from each of the conditions for no-RT.

12. Add $X = 14.5\mu\text{L} - (\mu\text{L of lysate})$ of RT master mix 1 to a new PCR tube for each reaction.
13. Add up to 9 μL of a cell lysate to a PCR tube containing RT master mix 1. Mix gently.
14. Incubate lysate + master mix 1 at 65°C for 5 minutes, then cool on ice to melt secondary structures of RNA.
15. Prepare RT master mix 2. 0.5-2 μL of RT may be used per reaction (50-200 Units). AMB has used 1 μL and obtained satisfactory results. If using different amount than 1 μL, you may need to adjust the water amount in step 10.

Component	volume per reaction, µL
5x RT Buffer	4
Maxima H Minus Reverse Transcriptase	1 - can be varieed between 0.5 and 2
Thermo Scientific RiboLock RNase Inhibitor	0.5
total	5.5 μL

15. For each of the no-RT controls, prepare a control master mix that replaces the Reverse Transcriptase with Elga water.
16. Add 5.5 μL of RT master mix 2 to each PCR tube. Mix gently. If doing a no RT control, use the no-RT master mix.
17. Transfer to thermocycler, incubate at 50°C for 30 minutes, followed by 85°C for 5 minutes for heat inactivation.
18. Proceed to qPCR or freeze for future use.

Note

cDNA can be stored at -20°C for 1 week and -80°C for longer. Avoid freeze/thaw cycles.

Test PCRs

Before amplifying an entire DNA library for sequencing, we often perform a **test PCR**, amplifying a fraction of the library to estimate the number of amplification cycles required for the remainder. This helps ensure that we do not over-amplify the library, which would reduce library complexity and bias it towards shorter fragments.

Test PCRs have three key variables that depend on the upstream genomics technique:

- **The desired amount of product**. A common sequencing minimum is 15 μL of a 2-nM library. For a ~500-bp library, this is 5 ng. Normally, you want to amplify to reach 50-500 ng per library. This variable sets the number of test cycles that need to be performed. Our protocols for each genomics technique suggest cycle counts for the test PCR based on expected yield (and previous results).
- **Choice of primers** based on how the library was constructed. Libraries created via adaptor ligation use one set (`ligation_fwd` and `ligation_rev`); libraries created via Tn5 transposition use another set (`tagmentation_fwd` and `tagmentation_rev`). Other methods might have custom primers. These primers bind to a common sequence after the unique sample indexes, so you can use the same ones for all your samples.

Primer	Sequence
`ligation_fw(`	AATGATACGGCGACCACCGAGATCTACACTATAGCCTA-CACTCTTTCCCTACACGACGCTCTTCCGATC*T
`ligation_rev`	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTG-GAGTTCAGACGTGTGCTCTTCCGATC*T
`tagmentatio`	AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCG-GCAGCGTCAGATGTG*T
`tagmentatio`	CAAGCAGAAGACGGCATACGAGATCGAGTAAT-GTCTCGTGGGCTCGGAGATGTG*T

*T is an internal phosphorothioate.

- **Ratio between input volumes**. The suggested protocol uses 3 μL of pre-amplification library as input for the test PCR. Typical upstream elution volumes vary from 25 to 50 μL. The ratio between the volume used in the test PCR and the volume used in the real PCR is important; it defines how the optimal cycle count is converted from the test to the real amplification.

Protocol

1. Thaw the primers (Sven –20°C, "Library prep reagents" box) at room temperature.
2. Prepare the PCR Master Mix using the NEBNext Ultra II DNA Library Prep Kit (NEB E7645[66]). You will need 27 μL of mix per reaction; prepare 10% excess. You can use either undiluted 100 μM primers or diluted 10 μM primers.

   PCR Master Mix (27 μL / reaction)

Component	Volume (100 µM primers)	Volume (10 µM primers)
2x NEBNext Ultra II Q5 Master Mix	15 μL	15 μL
DEPC-treated water	11.85 μL	10.5 μL
forward primer	0.075 μL	0.75 μL
reverse primer	0.075 μL	0.75 μL

[66] https://www.neb.com/en-us/products/e7645-nebnext-ultra-ii-dna-library-prep-kit-for-illumina

> **Note**
>
> We use 1/4 of the amount of primers that the NEBNext protocol suggests. Empirically, this works great! Pick the dilution that makes sense for the number of samples you are running. For example, use the diluted primers if you only have 3-4 conditions.

3. In fresh PCR tubes, add 3 μL of the pre-amplification library per condition to 27 μL of PCR Master Mix. There will be one tube per library.

> **Note**
>
> It is fine if your input samples contain streptavidin or other magnetic beads. They do not interfere with the PCR or downstream steps.

4. Set up the thermocycler protocol as written below (`lib_prep/test_pcr`). Decide on the number of cycles you wish to test, generally N, N+2, and N+4. Rather than preparing 3 tubes for each reaction, there are holds in the thermocycler protocol after N and N+2 cycles during which sub-samples are removed (explained below).

Temp (°C)	Time (MM:SS)	Description
72	5:00	Polymerase activation
98	0:30	Initial denaturation
–	–	*N cycles of:*
98	0:10	Denaturation
65	1:15	Extension
–	–	*[end cycle]*
42	Hold	Remove 7 μL Qubit sample and 7 μL gel sample
–	–	*2 cycles of:*
98	0:10	Denaturation
65	1:15	Extension
–	–	*[end cycle]*
42	Hold	Remove 7 μL gel sample
–	–	*2 cycles of:*
98	0:10	Denaturation
65	1:15	Extension
–	–	*[end cycle]*
42	Hold	Remove 7 μL gel sample

5. Run the thermocycler protocol. While the initial cycles of the PCR are running (~30 min), prepare a normal DNA gel (e.g., 1.5% agarose with ethidium bromide) and four sets of fresh PCR tubes (4 new tubes per reaction). For **three** of the sets of tubes, pipet 3 μL of Orange Loading Dye in each tube.

> **Note**
>
> We use Orange Loading Dye rather than Purple because the dye overlaps less with the DNA fragments we expect from the PCR. This makes it easier to visualize bands in the imager. Orange Loading Dye is stored at room temperature in or next to the genomics hood. **Pipet carefully**, as the dye sticks to the pipette tips.

6. When the hold step is reached, **do not hit enter to continue**. Instead, press "Stop" then "Lid open", and briefly take the tubes out. Use the multi-channel P10 to take a 7-μL subsample, combining it with the pre-prepared 3 μL of Orange Loading Dye for the gel. For the Qubit subsamples, pipet the solution into empty tubes.

> **Note**
>
> Promptly take the subsamples to avoid leaving the thermocycler sitting at the hold step.

7. Place the tubes back in the thermocycler and close the lid. Press "Lid close", then "Resume", and finally "Enter" to continue past the hold. Repeat the subsampling at the next hold and at the end of the protocol.
8. Once the test PCR is complete, run the three subsamples per reaction on the prepared DNA gel. It is convenient to group the three subsamples per condition together, rather than grouping by cycle count.
    - After removing the first subsamples, you can start loading the gel (if you have many samples). Just don't forget to return to remove the next subsample at the second hold!
    - Load the entire sample into the gel, pipetting carefully to ensure the solution stays in the well.
    - Use 1.8 μL of Apex DNA Ladder II (Genesee Scientific 42-431[67]) or NEB 1 kb Plus DNA Ladder (NEB N3200[68]), since the expected bands are <1 kb.
9. When the gel has finished running, image it on both on our imager and the ChemiDoc in the Niles lab for better quantification.
10. While waiting on the gel to run, clean up the final set of subsamples (without loading dye) using SPRI beads (stored in a bottle at room temperature in the genomics hood).

> **Note**
>
> You can alternatively use Ampure beads instead of SPRI beads if you plan to use these to clean up your final amplified libraries. Allow the Ampure beads to equilibrate to room temperature beforehand.

[67] https://www.geneseesci.com/product/apex-quantitative-dna-ladders-i-ii-iii/?sku=42-431
[68] https://www.geneseesci.com/product/apex-quantitative-dna-ladders-i-ii-iii/?sku=42-431

a. Vortex the beads well, at least 30 seconds. Aliquot the total amount of beads needed into a 1.7-mL Eppendorf.

b. Add 0.9x (6.3 μL) of beads to each 7-μL Qubit subsample and mix well via pipetting.

c. Incubate at room temperature for 15 minutes.

d. While waiting, prepare fresh 80% ethanol in water, enough for 400 μL per sample. Use the ethanol bottle labeled "genomics" in the flammables cabinet.

e. Place tubes on the magnetic rack and discard the supernatant.

f. Wash twice with 200 μL of 80% ethanol without disturbing the beads: keep the tubes on the magnet and do not pipet to resuspend.

g. Discard the second wash and start at 5-minute timer. Remove residual ethanol from the bottom and sides of the tube using a P10 pipet.

h. Air-dry the beads (i.e., with the tube caps open) until they transition from shiny to matte in appearance, not longer than the 5-minute timer. An over-dried bead pellet will look cracked rather than smooth.

i. Add 20 μL of 0.1x TE to the tubes to elute the DNA. Mix well via pipetting and incubate off-magnet for 2 minutes at room temperature.

j. Place the tubes back on the magnet and transfer the supernatant to new PCR tubes.

11. Quantify the cleaned-up samples on the Qubit in the BMC. Follow the *Qubit protocol* (page **??**).

Determine the optimal cycle counts

Use the data from the gel and the Qubit to determine the number of cycles for amplifying each full library. Use the calculations below to convert test PCR cycle counts from the optimal conditions to "real" PCR cycle counts. Since the Qubit measurement can saturate, prefer the gel quantification results over the Qubit ones if in conflict.

This calculation should be performed for each library (sample) individually, meaning that the cycle counts for the "real" PCR may vary across samples. This helps ensure that the final libraries end up with similar total DNA amounts.

Gel-based quantification

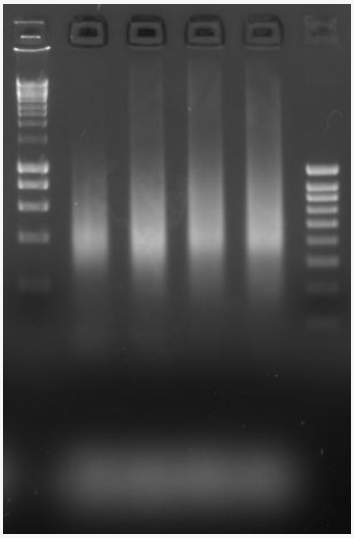
1. Identify the optimal band on the gel.

   Call the test PCR cycle count corresponding to this band N_{test}.

   Look for a band with a visible fragment smear that is not over-amplified. Depending on genomics method, you may see smears corresponding to mononucleosome, dinucleosome, and/or trinucleosome fragments.

Example

In the example below, something between the first and second lanes appears optimal, since the reaction saturates in the third and fourth lanes. Here, the cycle counts were 12, 14, 16, 18, so we'll say $N_{test} = 13$.



2. Calculate the volume of the pre-amplification library in the gel subsample, V_{test}:

$$V_{test} = V_{input} \times \frac{V_{subsample}}{V_{reaction}}$$

where:

- V_{input} is the volume of the pre-amplification library used as input to the test PCR reaction, here 3 μL as written in step 3 above
- $V_{reaction}$ is the total volume of the test PCR reaction, here 30 μL
- $V_{subsample}$ is volume of the test PCR reaction subsampled for the gel, here 7 μL

Example

For the amounts suggested in the protocol above, we have:

$$V_{test} = 3\ \mu\text{L} \times \frac{7\ \mu\text{L}}{30\ \mu\text{L}} = 0.7\ \mu\text{L}$$

3. Compute the relative cycle count ΔN for the "real" PCR compared to the test PCR.

This is related to the ratio of the input volumes for the test PCR (V_{test}) and the "real" PCR (V_{real}). As you increase the amount of starting material, you'll need fewer cycles to obtain the same amount of product. For instance, if you start with twice the starting material, you'll need one fewer cycle, since the product doubles each cycle in a PCR. More generally, the

change in amount of product for the "real" PCR compared to the test PCR is set by the ratio of input volumes, which must be compensated for by a change in cycle count:

$$\frac{V_{test}}{V_{real}} = 2^{\Delta N}$$

which gives:

$$\Delta N = \log_2(\frac{V_{test}}{V_{real}})$$

Example

If the total pre-amplification library volume was 23 μL and we'll use the entire remaining volume in the "real" PCR, we have:

$$\begin{aligned}\Delta N &= \log_2(\frac{0.7}{23-3}) \\ &= -4.84\end{aligned}$$

4. Finally, combine these values to find the cycle count for the "real" PCR, N_{real}:

$$\begin{aligned}N_{real} &= N_{test} + \Delta N \\ &= N_{test} + \log_2(\frac{V_{input} \times \frac{V_{subsample}}{V_{reaction}}}{V_{real}}) \\ &= N_{test} + \log_2(\frac{V_{input}\ V_{subsample}}{V_{real}\ V_{reaction}})\end{aligned}$$

Example

Continuing the example above, we have:

$$\begin{aligned}N_{real} &= N_{test} + \Delta N \\ &= 13 - 4.84 \\ &= 8.16 \\ &\approx 9 \text{ cycles}\end{aligned}$$

While we don't want to excessively over-amplify, diluting the final library is better than undershooting, which would then require an additional PCR step before submission. So we'll round up to **9 cycles** for the "real" PCR.

Qubit quantification

The calculation with the Qubit data is similar in concept, except it uses masses instead of volumes.

1. Calculate the final mass of the test PCR subsample used in the Qubit reaction, m_{test}.

This is the volume of the cleanup elution (from step 10 in the above protocol) times the measured Qubit concentration, accounting for the dilution factor:

$$m_{test} = V_{cleanup} \times C_{Qubit} \times \text{dilution factor}$$

Example

Let's say the Qubit measured a concentration of 10 ng/mL when the sample was diluted 1:200. Following the protocol above, we eluted the cleanup of the Qubit subsample in 20 μL. Since ng/mL is equivalent to pg/μL, we have:

$$\begin{aligned} m_{test} &= 20\ \mu\text{L} \times 10\ \text{pg}/\mu\text{L} \times 200 \\ &= 40,000\ \text{pg} \\ &= 40\ \text{ng} \end{aligned}$$

2. Compute the expected mass of the "real" PCR, if the same cycle count is used.

   This is the final mass of the test PCR subsample scaled by the change in input volume. Use the volume of the pre-amplification library in the gel subsample (V_{test}) calculated in step 2 of the gel quantification.

$$m_{expected} = m_{test} \times \frac{V_{real}}{V_{test}}$$

Example

Using the same example, with $V_{test} = 0.7\ \mu$L as before and 20 μL as the input to the "real" PCR, we find

$$\begin{aligned} m_{expected} &= 40\ \text{ng} \times \frac{20\ \mu\text{L}}{0.7\ \mu\text{L}} \\ &= 1143\ \text{ng} \end{aligned}$$

3. Determine the target mass for the "real" PCR, m_{real}.

   This will depend on the genomics technique but is typically 50-500 ng. Note that the BMC sequencing minimum for a mid-yield sequencer is 15 μL of a 2-nM library, which is 5 ng for a library with an average fragment size of ~500 bp. This means there will be plenty of extra of each library, in case they need to be re-sequenced later (or something goes wrong with library pooling).

4. Compute the relative cycle count ΔN for the "real" PCR compared to the test PCR.

   This is set by the ratio of expected and target masses, which must be compensated for by a change in cycle count:

$$\frac{m_{real}}{m_{expected}} = 2^{\Delta N}$$

which gives:

$$\Delta N = \log_2(\frac{m_{real}}{m_{expected}})$$

Example

If we want the final library to be 100 ng, we'll need to reduce the cycle count for the "real" PCR:

$$\begin{aligned}\Delta N &= \log_2(\frac{100 \text{ ng}}{1143 \text{ ng}}) \\ &= -3.51\end{aligned}$$

5. Finally, the cycle count for the "real" PCR (N_{real}) is simply:

$$N_{real} = N_{test} + \Delta N$$

where N_{test} is the cycle count for the test PCR subsample that was cleaned up for Qubit.

Example

If the Qubit subsample was taken after 12 cycles of the test PCR, then for the "real" PCR we should use

$$\begin{aligned}N_{real} &= 12 - 3.51 \\ &= 8.49 \\ &\approx 9 \text{ cycles}\end{aligned}$$

The Qubit quantification should agree with the gel quantification for the same test PCR. Additionally, the cycle count for the "real" PCR should match expected values in the genomics protocol.

qPCR for library quantification

To quantify genomics libraries (i.e., measure concentration), we perform qPCR on two dilutions of each sample. We use the NEBNext Library Quant Kit (NEB product code todo). This protocol is adapted from the one provided with the kit `here`.

0. If opening a new kit, combine 500 μL primer

TODO

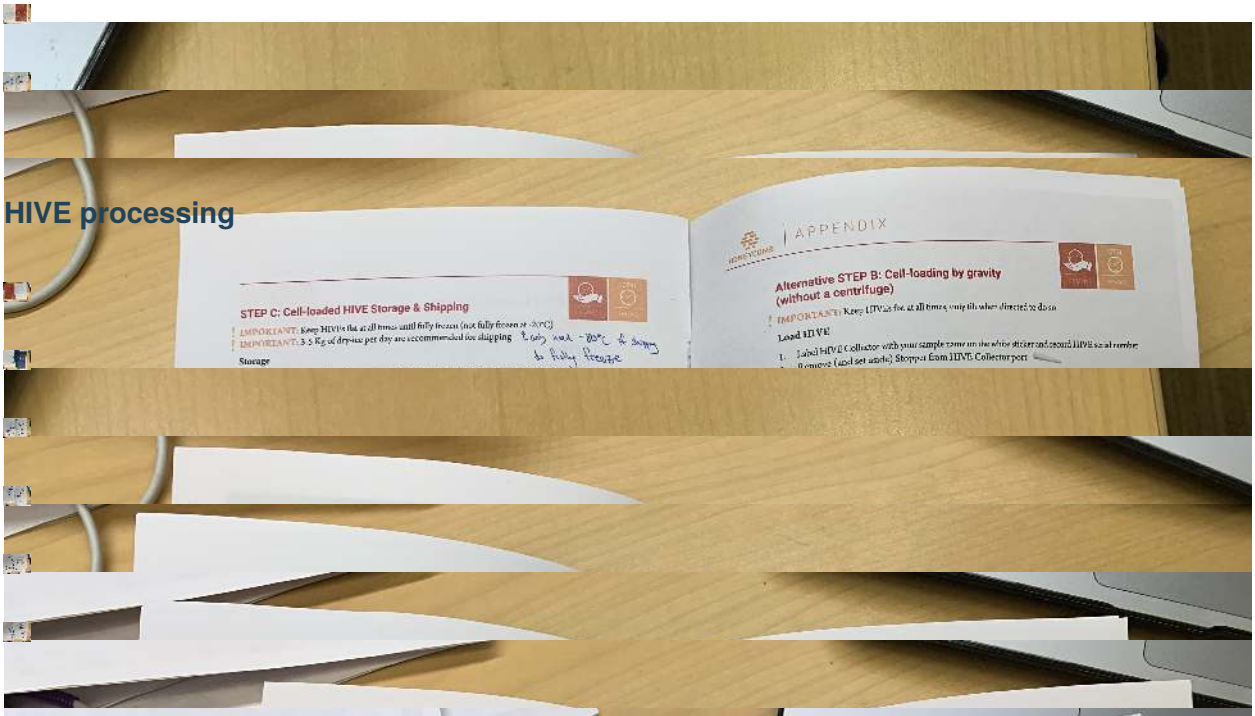
TODO

HIVE scRNA-seq

See

This protocol is from the HIVE protocols page: [**?**]. There are only some added annotated notes.

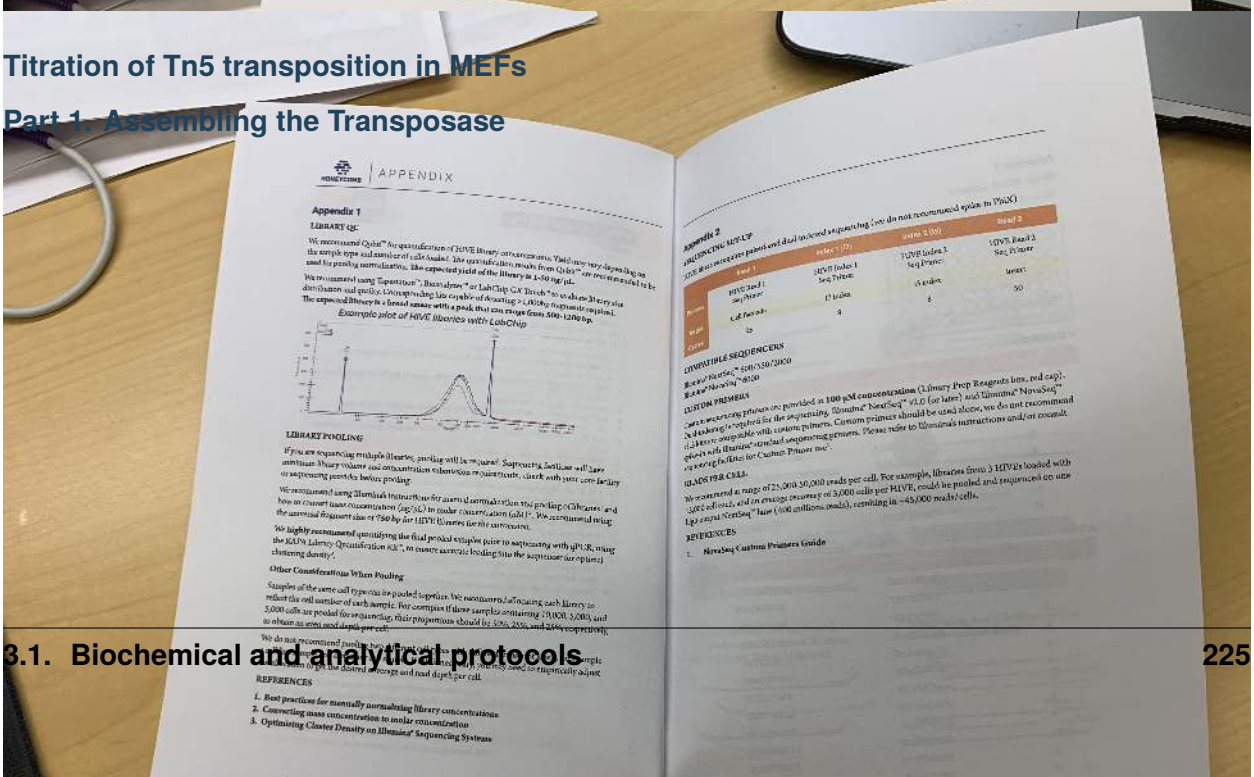
Sample capture



HIVE processing

Titration of Tn5 transposition in MEFs

Part 1: Assembling the Transposase



1. Re-suspend primers to 100uM stock using

An-
neal-
ing
Buffer
(40mM
Tris-
HCl
(pH8.0
50mM
NaCl)

2. Mix the following :

   o Primer A + Mosaic Rev (10uL + 10uL)

   o Primer B + Mosaic rev (10uL + 10uL)

3. Anneal using the following settings:

Temperature	Time
95°C	5 minutes
Cool to 65°C	-0.1°C/second
65°C	5 minutes
Cool to 4°C	-0.1°C/second

4. In a PCR tube, mix the Following (scaled just enough for small scale titration + 10%).

Components	Diagenode Tn5	InHouse
Primer A-Mosaic	1uL	2.5uL
Primer B-Mosaic	1uL	2.5uL
Tn5	2uL	5uL

5. Vortex briefly and incubate at 23°C for 30 minutes in a thermocycler.

Part 1.1 Prepping the Tn5 concentrations from Assembled Tn5 transposome per Transposition reaction (50uL)

1. Prep 3 tubes for each Tn5 variant, one for each concentration to be tested.
2. Follow the example Transposition mix composition below good for 20 rxns, scale according to # of transposition reactions. Adjust Tn5 volume for desired concentration to be tested.

Component	Volume
2X TD buffer (recipe below)	500uL
PBS	330uL
UltraPure dH2O	100uL
1% Digitonin	10uL
10% Tween-20	10uL
Assembled Tn5 (diluted)	20uL
Total	1mL

Recipe for 2X TD buffer

Component	Final Concentration	Volume for 100mL
1M Tris-HCl pH 7.6	20 mM	2 ml
1M MgCl2	10 mM	1 ml
Dimethyl Formamide	20%	20 ml
Sterile water	NA	Bring up to 100 ml

Part 2. Transposition Reaction of Isolated nuclei

1. Resuspend the isolated nuclear pellet in 50 μL of Transposition Mix (Pre-made from Part 1.1) by pipetting up and down six times. Transposition Mix should be made fresh each time and mixed thoroughly prior to use.
2. Incubate reaction at 37 °C for 30 min in a thermomixer with 1,000 rpm mixing.
3. Remove the tubes from the thermomixer, and immediately terminate the transposition reaction by adding 250 μL (five volumes) of DNA Binding Buffer from the DNA Clean and Concentrator-5 Kit and mix well by pipetting or inversion.
4. Pulse centrifuge to collect solution in the bottom of the tube.
5. Clean up the transposition reaction using the DNA Clean and Concentrator-5 Kit. Transfer each sample, mixed with the DNA Binding Buffer, to a Zymo-Spin Column in a collection tube. Centrifuge at RT for 30 s at 10,000g, and discard the flowthrough.
6. Add 200 μL of DNA Wash Buffer to the column, and centrifuge at RT for 30 s at 10,000g.
7. Repeat this wash for a total of two wash steps.
8. Perform a final 'dry spin' after the second wash step to remove any traces of residual wash buffer from the column membrane. To do this, remove any flowthrough from the collection tube and

centrifuge the column and collection tube at RT for 1 min at >13,000g.

9. Transfer the column to a clean prelabeled 1.5 mL LoBind tube. Pipette 21 μL of Elution Buffer directly onto the column membrane, and wait for 1 min.
10. Centrifuge the column at RT for 1 min at 13,000g to elute the DNA. This elution volume typically results in 20 μL of product.

    Pause Point: This can be Stored at -20C for as long as necessary or processed immediately for Barcode amplification followed by sequencing.

Nuclei Isolation for Downstream Genomics Applications

Nuclear Isolation

Estimated time

1 - 1.5 hrs depending on the number of samples

1. Prior to starting, make the Lysis Buffer and Wash Buffer and keep them on ice. Be sure to use freshly made Lysis Buffer and Wash Buffer each time – see recipe at the bottom.
2. Obtain 50,000 cells for MEFs by trypsin and count accordingly.

- *Optional*: if using a spike-in: Add 1000 cells from HEK293Ts to each MEF tube.

3. Pellet at 500xg for 5 mins at 4C. Aspirate supernatant gently using P1000.
4. Resuspend the cell pellet in 50 μL of Lysis Buffer by pipetting up and down three times.
5. Incubate on ice for 3 min. If lysing multiple samples, make sure that all samples are lysed for the same total amount of time by proceeding to Step 7 after 3 min.
6. Add 1 mL of Wash Buffer to dilute the lysis reagents. Invert the tube five times to mix.
7. Pellet nuclei at 500g for 10 min at 4°C in a fixed-angle microcentrifuge. Orient the tubes in a consistent fashion so that the pellet will end up in the same location.
8. STOPPING POINT: Pelleted nuclei can be flash frozen, fixed or immediately processed for downstream genomic applications like ATAC-seq, ChIP-seq, or Chromatin conformation capture.

Recipes

Stock solutions

Make sure you have enough of the following stock solutions. These are often shared with other recipes.

1M Tris pH 7.5 stock solution:

Component	Concentration	g / L	Amount / 10 mL
Tris-HCl	1 M	157.64	1.57g
Elga water	to 9 mL		
NaOH	to pH 7.5		~140 uL of 12 N NaOH
Elga water	to 10 mL		

5M NaCl stock solution:

Component	Concentration	g / L	Amount / 10 mL
NaCl	5 M	292.21	2.92g
Elga water	to 10 mL		

1M magnesium chloride stock solution:

Component	Concentration	g / L	Amount / 10 mL
MgCl2	1 M	95.21	0.952 g
Elga water	to 10 mL		

Resuspension Buffer (RSB)- 100mL, scale as needed

Component	Final Concentration	Amount / 100 mL
1 M Tris, pH 7.5	10 mM	1 mL
5 M NaCl	10 mM	200 μL
1M MgCl2	3 mM	300 μL
Elga water		98.5 mL

Cell Lysis Buffer per 50 uL, scale as needed

Component	Volume
Cold RSB (recipe above)	48.5uL
10% (wt/vol) IGEPAL-CA360	0.5uL
10% (wt/vol) Tween-20	0.5uL
1% (wt/vol) digitonin	0.5uL
Total volume	50uL

Wash Buffer per 1000 uL, scale as needed

Stock solutions	Volume
Cold RSB (recipe above)	990uL
10% (wt/vol) Tween-20	10uL

ATAC-see

Warning

ATAC-see is a very inefficient procedure, and gives effectively useless results compared to background, even in flow cytometry.

Transposase buffer preparation

Estimated time

1.5 hours.

1. Order the following primers:

Primer name	Scale	Purification	5' modification	Sequence
Tn5ME_rev	25nmo	DSL	Phosphate	*CTGTCTCTTATACACATCT*
Tn5ME_A	50nmo	HPLC	Cy5	*TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAG*
Tn5ME_B	50nmo	HPLC	Cy5	*GTCTCGTGGGCTCGGAGATGTG-TATAAGAGACAG*

2. Resuspend each primer separately to 100 uM (the standard stock dilution, nmol * 10 uL).
3. Prepare 1:1 solutions of Tn5ME_rev / Tn5ME_A (A+rev) and Tn5ME_rev / Tn5ME_B (B+rev). Each primer is now at 50 uM.
4. Assemble these double stranded oligos by heating to 95C for 5 minutes, followed by slow cooling to room temperature. To do this on a thermocycler, use a program that does ends after the denaturation step, instead of ending in a hold step.
5. Prepare 5 uM stock solution of Tn5/Cy5. Prepare this either from freshly purified Tn5 in dialysis buffer:

Component	Volume fraction
A+rev primers	0.125
B+rev primers	0.125
Glycerol	0.4
Dialysis buffer	0.12
50 uM Tn5	0.1
DI H2O	0.13

If Tn5 is already stored in a 50% glycerol/50% 2x dialysis stock solution at 20 uM, use the following recipe:

Component	Volume fraction
A+rev primers	0.125
B+rev primers	0.125
Glycerol	0.25
20 uM Tn5+Glycerol	0.25
DI H2O	0.25

ATAC-see

We need the following prepared buffers:

- **Nuclear permeabilization buffer**:

Component	Concentration	Amount/100 mL final
Tris-Cl	10 mM	0.1576 g
NaCl	10 mM	0.058 g
MgCl2(anhydrous)	3 mM	0.0287 g
Igepal CA-630	0.01%	10 uL
HCl	to pH 7.4	

- **Wash buffer**:

Component	Concentration	Amount/100 mL final
PBS	Base	
SDS	0.01%	.01 g
EDTA	50 mM	1.461 g

- **2x TD buffer** (from this nature paper[69]):

[69] https://www.nature.com/articles/nprot.2013.118

Component	Concentration	Amount/L final
Tris	20 mM	3.264 g
MgCl2	10 m	0.95 g
DI H2O	main solvent	
Acetic acid	to pH 7.6, before DMF addition	
Dimethylformamide(DMF)	20% (v/v)	

1. After cell fixation, permeabilize cells with lysis buffer for 10 minutes at room temperature.
2. Wash with PBS twice.
3. Prepare transpose mixture:

Component	Volume fraction
2x TD buffer	0.5
5 mM assembled Tn5	0.02
DI H2O	0.48

4. Add 50 uL of transpose mixture to cells to be transposed. Place the cells in a humid 37C box for 30 minutes.
5. For plated cells, wash with wash buffer three times, for 15 minutes each at 55C. For suspended cells, wash twice.
6. Add PBS media to cells and image/flow the resulting cells.

Magnetic bead clean-up for genomics libraries

(Adapted from USC Epigenome Center)

Note

This protocol describes two methods to clean up libraries following various adapter ligations. Most recently, I (Katie) used this workflow to clean up samples from 10X 3'RNAseq before sequencing. 01/23/23 - NW did not update b/c the HIVE bead clean up is different

Warning

INCOMPLETE! Part of 10X Genomics workflow; the rest of the protocol needs to be added. This section separates DNA by size in preparation for sequencing.

Uses

- Clean-up Samples or Libraries (buffers, salts, NTPs, or other undesirable fragments)
- Condense / Concentrate into lower volume

Determine Bead Volume to use by size range you wish to recover:

- To recover ALL nucleic acids regardless of size use bead volume of 1.8x sample volume.
- To recover nucleic acids >50bp - use bead volume of 1.5x sample volume.
- To remove Primer-dimers & recover everything >100 bp - use bead volume of 1.0x sample vol.
- To remove Adaptor-dimers & recover everything >200bp - use bead volume of 0.8x sample vol.
- To remove very HMW bands, see the *2-step* (page **??**) protocol modification below.

Preparations:

- Locate a strong rare-earth-metals magnet
- Bring AMPure XP Beads to RT (from 4°C), vortex vigorously to mix
- If using a plate or strip-tubes & trough add volume of beads needed + 300-500μL extra
- Mix fresh EtOH at 75%.
  - For large double stranded DNA can use 70%
  - For very small &/or single stranded RNA use 80%

Basic Bead Clean Protocol:

1. Measure volume of samples.
2. Calculate bead volume for desired size range
3. Add beads to sample & mix well – if some splashes onto tube lid or sides do a quick pulse centrifuge to collect at bottom of tube.
4. Incubate 5 m @ RT.
5. Place tube on magnet to condense beads into pellet (1 - 15 minutes, depending on magnet strength)

Important

Keep beads pelleted throughout washes!

6. Draw off supernatant & discard
7. Wash with 200μL of fresh 70%-80% EtOH 2x.
8. Draw off EtOH & discard each time
9. After 2nd wash allow residual EtOH to evaporate
10. Before pellet overdrys & starts to "crack" or "flake off" elute in volume of reagent appropriate for next step in protocol (typically diH2O or EB).
11. Mix well, pellet beads on magnet, draw off supernatant with your sample
12. Pipette into new, labeled tube.

2-step "Big" bead clean to remove large fragments >~350bp.

1st clean:

1. 0.7x Bead:lib vol, incubate 5m, pellet beads with everything >350bp.
2. Transfer soup into clean tube containing 2nd bead volume.

2nd clean:

1. 1.1x Bead:original lib vol, for total ratio of 1.8x PEG+NaCl to original lib vol.
2. Mix well & incubate up to 15m, pellet beads well (8-10 min), EtOH wash 2x
3. Allow to air dry & elute back into desired vol of diH2O or EB.

Recipes

- *Shared genomics buffers* (page **??**)

Sequencing technologies

"Last generation" sequencing

The original sequencing technology that you may be familiar with is Sanger sequencing. Sanger sequencing works by running a normal PCR reaction for a single cycle, starting from a primer binding site and using a low percentage of fluorescent, chain-terminating modified nucleotides. The molar fraction of modified nucleotides is chosen so that, across an entire population of plasmids, the chain reaction stops, on average, at every base pair for some member of the population.

This results in a mixture of PCR products of different lengths. Every product terminates with a fluorescent base pair. Then, the mixture is run through a chromatography column that separates

the fragments based on length. The resulting fluorescent measurements as a function of column running time is the `ab1` file that you load into Snapgene.

Next generation sequencing

Next generation sequencing (NGS) is also a "sequencing by synthesis" method that uses fluorescent base pairs, but it measures the data in a fundamentally different way. Instead of measuring a single species for ~1000 base pairs, like Sanger sequencing, NGS uses a patterned flow cell that reads millions to billions of DNA molecules simultaneously.

However, NGS is a "short-read" sequencing method because each of the molecules are not read in full; only the outer ~50-100bp edges are sequenced. One company, Illumina, invented this technique, but other companies like Element Biosciences have innovated on the design. Element (AVITI Cloudbreak sequencer) is currently the most cost-effective up to about a billion reads, whereas Illumina (NovaSeq X Plus sequencer) is the most cost-effective for billions of reads.

There is a lot of quality control that goes into these libraries, but the convenient thing is that, as the user, you are only responsible for providing a library (i.e., a mixture) of DNA molecules that contain known DNA anchors at the end. Core facilities (like the BioMicro Center at MIT) are responsible for actually loading and running the chip.

The high-level process of what the core facility does is as follows:

1. Facility staff perform additional QC on your sample to confirm library concentrations, low contamination, etc.
2. Facility staff load your library onto a "flow lane" of a "flow chip". Loading binds individual DNA molecules to binding sites on the patterned flow chip.
3. In the sequencer, the bound DNA molecules are amplified, leading to small clusters of identical DNA molecules, spread spatially along the chip.
4. Primers and fluorescent bases, added in successive wash/binding steps in the sequencer, allow sequences of each cluster to be read out base-by-base.

Long-read sequencing

Long-read sequencing is (currently) dominated by nanopore technology. These methods sequence DNA by forcing individual DNA or RNA molecules through a small pore. A known adapter sequence is still needed to bind a DNA fragment to the pore to start sequencing.

Given a library with a known sequence, these methods allow for sequencing of the entire fragment length, using a flowcell with thousands of nanopores that measure the electrical properties of each base as it passes through the pore.

These techniques are more expensive per-read than short-read sequencing, but give full-length sequence information that is especially important for examining long, spliced transcripts and other complicated fragments.

3.1.2 Staining and labeling

Homemade EU click

1 rxn = 1 single 24-well (250 μL EU or 175 μL click mix).

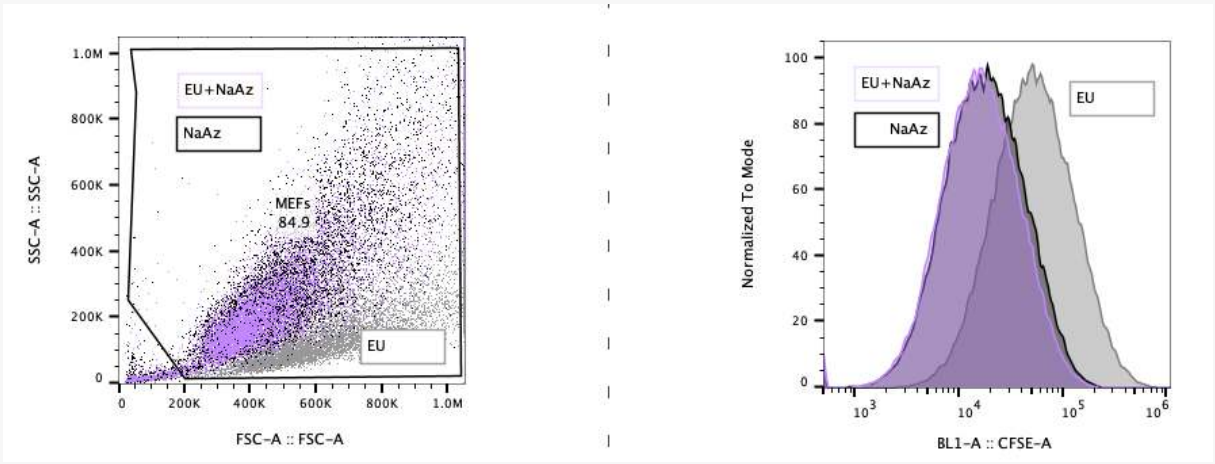
Note that ascorbic acid should be made fresh and **EU will likely be the limiting reagent.**

Warning

Click chemistry is known to diminish fluorescence due to ROS that are produced during the Cu-catalyzed azide-alkyne cycloaddition[70]. Have not tested but the ThermoFisher Click-iT Plus EdU kit[71] supposedly fixes this due to "the use of the picolyl azide combined with a copper protectant is the basis of the upgraded Click-iT Plus EdU technology, which achieves the same sensitive, reliable detection of cell proliferation as the original Click-iT EdU assay while also preserving the fluorescence of GFP, RFP."

Important

The NaN3 is important to include in any single color controls! We observe that presence of EU doesn't change CFSE only control signal but any azide addition (with or without EU) significantly changes FSC/SSC and CFSE signal. The following image shows the effects of azide addition on 1 dpi CFSE-labeled MEFs flowed at 4 dpi.



Stock solutions

- Main solutions

[70] https://doi.org/10.1002/biot.201400026

[71] https://www.thermofisher.com/us/en/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-70/click-it-plus-edu-proliferation-kits.html

Stock solutions	Stock concentration	How to make	Final concentration
EU (100X)	100 mM	5 mg EU + 186 μL **DMSO** (74 rxns)	1 mM
CuSO4.5H2O (100X)	200 mM	250 mg CuSO4.5H2O + 5 mL H2O (2,800 rxns)	2 mM
Ascorbic acid (10X) **MAKE FRESH**	200 mg/ml	120 mg Ascorbic acid + 600 μL H2O (34 rxns)	20 mg/mL

- Main azide solutions

Stock solutions	Stock concentration	How to make	Final concentration
NaN3 (use to make 500X soln)	4 M	250 mg NaN3 + 961 μL H2O	–
NaN3 (500X)	4 mM	1 μL of 4 M NaN3 + 1,000 μL H2O (2,800 rxns)	8 μM
Sulfo-Cy5 azide (500X)	4 mM	5 mg Sulfo-Cy5 azide + 1,638 μL H2O (4,600 rxns)	8 μM
BDP TR azide (500X)	4 mM	5 mg BDP TR azide + 2,469 μL **DMSO** (7,000 rxns)	8 μM

- Misc/wash solutions

Stock solutions	How to make
0.5% Tween-20 in PBS	50 mL PBS + 250 μL Tween-20. **Add Tween-20 to 1 mL PBS first to mix easier!**
3.7% PFA in PBS	44 mL PBS + 5.7 mL 32% PFA
(*Optional*) 0.1 μg/mL DAPI in PBS	Dilute 0.1 mg/mL DAPI (1,000X stock in -20/Olaf) into PBS

Note

The 200 mg/mL ascorbic acid (10X) should be made fresh day of. It takes a while to dissolve so start it early and vortex often.

Warning

Azides are toxic! Always use azides in the fume hood and do not breathe it in! Azide waste should be collected in the satellite accumulation area in the fume hood.

Add EU to cells

1. In BSC hood, add 2.5 μL of 100 mM EU (100X) to 250 μL of complete growth medium (DMEM/FBS or N3 with no neurotrophics) per 24-well. For a full 24-well plate, 60 μL into 6 mL (90 μL into 9 mL if need more for single color controls).
2. Incubate cells for 1 hour at 37°C.

Dissociate

3. Remove media, wash with PBS, trypsinize, and spin down in eppendorfs.

Note

We did not notice a difference but you can precoat with 7.5% BSA/PBS and/or use low-retention eppendorfs. For 7.5% BSA/PBS precoating, just pipette up and down (no need to incubate).

Fix cells

4. Add 100 μL of 3.7% PFA/PBS to each Eppendorf and mix well. Let sit 15 at RT in the dark (covered).
5. Add 1 mL PBS and spin cold at 4°C to pellet cells.
6. Remove supernatant.

Permeabilize

7. Add 100 μL 0.5% Tween/PBS. Let sit 15 minutes at RT in the dark (covered).
8. Wash by adding 900 μL PBS and spin down at 4°C.

(*Alt to step 8*) Just add 175 μL of label mix to cells.

Note

Other protocols use 0.5% Triton X-100, but comparing Tween-20 and Triton X-100 we observed that the pellets permeabilized with Triton disappeared after the click step.

Important

Any immunostaining should be done at this point, before the click reaction. Staining after the click reaction results in high levels of non-specific staining and no detectable specific signals.

Prepare click/label mix

9. Make label mix just before use by combining (in this order: precipitate is formed after addition of CuSO4 to PBS, this is dissolved after addition of ascorbate):
    - The following mix is enough for a full 24-well plate: 4,500 μL $\sim$ 25 rxns (OR 1,000 μL for NaN3 ctrls)

Solution	Volume (for NaN3 ctrls)
PBS	4,000 μL (or 888 μL)
CuSO4 (100X, 200 mM)	45 μL (or 10 μL)
Azide (500X, 4 mM)	9 μL (or 2 μL)
Ascorbate (10X, 200 mg/mL)	450 μL (or 100 μL)

Label and wash

10. Add 175 μL click/label mix per tube and incubate cells 30 min at RT with label mix on rotator, protected from light, at room temp.
11. Wash by adding 900 μL PBS and spin down at 4°C.
12. (*Optional*) Incubate cells 5 minutes with 175 μL of 0.1 μg/mL DAPI/PBS if you want to detect nuclei in flow
13. Wash by adding 900 μL PBS and spin down at 4°C.
14. Analyze by flow.

Note

All spins are performed at ~500 rcf for 5 min. Our centrifuge follows RCF = 1e-4*[rpm]^2 + 4e-2*[rpm] - 6e1, where **2200 rpm = 512 rcf**. It is recommended to perform all spins at 4°C once the cells have been fixed to prevent pellet loss.

Immunofluorescent Staining

Solutions Required

Stock solutions	How to make
4% PFA in PBS	Pre-made, found in Olaf **OR** 3 mL PBS + 1 mL 16%PFA from an ampoule **OR** 8.75 mL PBS + 1.25 mL 32% PFA
0.5% Tween-20 in PBS	50 mL PBS + 250 μL Tween-20. **Add Tween-20 to 1 mL PBS first to mix easier!**
0.1% Tween-20 in PBS	40 mL PBS + 10 mL 0.5% Tween/PBS
25% FBS in PBS (5X)	37.5 mL PBS + 12.5 mL FBS
Blocking Solution	40 mL 0.5% Tween/PBS + 10 mL 25% FBS in PBS

- **Blocking Solution**: 5% FBS, 0.1% Tween in PBS

Adherent Cell Staining

Expected time: 3 days

- Day 1: Fix cells, permeabilize overnight
- Day 2: Incubate with primary antibody overnight
- Day 3: Incubate with secondary antibody + image

1. Remove media and add cold 4% paraformaldehyde (PFA) *(in fume hood)*
2. Incubate cells in 4% PFA for 1 hour at 4°C
3. Remove 4% PFA *(in fume hood)*
4. Wash cells 3 times with cold PBS
    - *Cells may be left in PBS overnight at 4°C*
5. **If staining nuclear proteins**, change solution and incubate cells in 0.5% PBS/Tween for 1 hour at room temperature to overnight at 4°C to permeabilize

Note

Overnight is preferred for the permeabilization step

Tip

You can also combine the permeabilization and blocking step at the same time. Use 0.5% Tween and 5% FBS in PBS solution for either 1 hour at room temperature or overnight at 4°C.

6. Change solution and incubate cells in blocking solution for 1 hr at room temperature or overnight at 4°C
    - *Cells may be left in blocking solution overnight at 4°C*
7. Change solution and incubate cells in primary antibody (diluted in blocking solution) overnight at 4°C
8. Change solution and wash cells with 0.1% Tween/PBS quickly three times.
9. Incubate cells in 0.1% Tween/PBS for 20 min
    - *Cells may be left in 0.1% Tween/PBS overnight at 4°C*
10. Change solution and incubate cells in secondary antibody for 30 min - 1 hr at room temperature or overnight at 4°C (secondary antibody is diluted 1:300-500 in blocking solution)
    - *You can incubate your cells in secondary antibody overnight at 4°C*
    - If staining with DAPI or Hoechst, this can be added to the secondary antibody mixture.
11. Change solution and wash cells with 0.1% Tween/PBS quickly three times.
12. If doing a DNA stain, add Hoechst or DAPI diluted in PBS for 10 minutes at room temp, then wash three times with PBS
13. Either store in PBS or remove solution and add vectashield solution. Your cells can stay in the solution at 4°C for a long time before you image them, just make sure to parafilm the sides to the liquid does not evaporate

Note

1. Never remove all of the media from the cells-they will dry and absorb antibody in a non-specific way, thereby skewing your results
2. Unless otherwise noted, everything is performed at room temp
3. Keep your antibodies on ice at all times
4. Generally, **50 μL/96-well** for all steps is good (can go higher for PBS wash steps). AMB has used as low as 35 μL/96-well with comparable results.

5. If mounting, try to avoid bubbles in the final step. Leave some solution still on your slides so the cells do not dry and carefully dispense 2-3 drops of the solution, very carefully putting the coverslip on your slide
6. Be very gentle with neuronal cultures, they are not very adherent. Do not use the aspirates, manually aspirate with a P200.

Cell Staining for Flow

General notes:

- Cells are usually stained in U- or V-bottom 96-well plates but they can be stained in any container (e.g. test tubes, Eppendorf tubes, polystyrene round-bottom tubes, cluster tubes).
- Antibody dilutions will require optimization.
- Primary antibodies are generally good at 1:500 dilution, Secondary antibodies ~1:1000, Live/Dead (e.g. Zombie) ~1:1000.

Tip

It is recommended to stain with ice-cold reagents/solutions and at 4°C, since low temperature prevents modulation and internalization of surface antigens which can produce a loss of flores-cence intensity.

Expected time: 1-2 days (only 2 if overnight primary antibody)

1. Perform any Live/Dead stains prior to dissociation and fixing
2. Dissociate cells (trypsin or DNase/Papain if MNs) and spin down
3. Remove supernatant and incubate cells in 3.7-4% paraformaldehyde (PFA) *(in fume hood, at room temperature)* for 15 min
    - EU protocol calls for 3.7% PFA, AMB uses this for antibody staining as well.
4. Add 1 mL PBS and spin cold at 4°C to pellet cells
5. Aspirate solution and incubate cells in 0.5% Tween/PBS for 15 min at room temperature to permeabilize

    Note

    Permeabilized cells do not pellet as well. Be careful not to aspirate the cell pellet when aspirating solution after centrifugation.

6. Add 1 mL PBS and spin cold at 4°C to pellet cells
7. Aspirate solution and incubate cells in 100 μL primary antibody (diluted in blocking solution) for at least 1 hr in the rotator at 4°C

> **Tip**
>
> Some antibodies (e.g. Ki67) work better overnight in the rotator at 4°C

8. Add 1 mL 3% FBS (diluted in PBS) and spin cold at 4°C to pellet cells

> **Note**
>
> AMB has found using FBS in wash buffer improves cell yield.

9. Aspirate solution and incubate cells in 100 μL secondary antibody (diluted in blocking solution) for 30 min in the rotator at 4°C **in the dark**
10. Add 1 mL 3% FBS (diluted in PBS) and spin cold at 4°C to pellet cells
11. If doing a DNA stain, add Hoechst or DAPI diluted in PBS for 10 minutes at room temp, then wash with PBS

> **Note**
>
> All spins are performed at ~500 rcf for 5 min. Our cold Eppendorf centrifuge follows RCF = 1e-4*[rpm]^2 + 4e-2*[rpm] - 6e1, where **2200 rpm = 512 rcf**. It is recommended to perform all spins at 4°C once the cells have been fixed to prevent pellet loss.

Alkaline Phosphatase Staining

This staining can be used to identify pluripotency in cells. If pluripotent, cells will turn visibly pink and can also be viewed in CH3 on the Keyence.

> **Note**
>
> The lab has ImmPACT® Vector® Red Substrate Kit, Alkaline Phosphatase (AP) (SK-5105) as of July 2025.

Solutions Required

Kit components	Quantity in working solution
immPACT Vector Red Diluent	5 mL
immPACT Vector Red Reagent 1	2 drops (about 80 uL)
immPACT Vector Red Reagent 2	2 drops (about 60 uL)

Procedure

1. Either in BSC or in cells and media waste, aspirate media from wells.
2. Add 4% PFA and incubate at room temp for 2-15 minutes. MC has tested both without noticeable difference.
3. Aspirate PFA and wash 1x with PBS.
4. Prepare working solution by adding the three kit components in the quantities listed. Do this immediately before use.
5. Add enough stain to cover each well. MC has used 500 uL for a 24 well and 40 uL for a 96 well. Let incubate at room temperature wrapped in foil for 20-30 minutes. MC has tested both without noticeable difference.
6. Aspirate stain solution and wash 1x with PBS.
7. Add fresh PBS to each well and image.

CellTrace Dye Labeling

CFSE and other CellTrace dyes are used to measure relative proliferation rates of cells. These dyes are amine-reactive and cell permeable and thus react with proteins within the cell. As cells divide, the dye is split between the daughter cells and thus diluted through cell division. Lower CFSE signal is indicative of more cell divisions, relative to a reference population.

In our lab, we use CFSE and other CellTrace dyes to measure relative proliferation rates of reprogramming populations. Cells are stained the day after transduction of reprogramming factors (1 day post-infection, 1 dpi). However, this protocol may be applied to any cell population where relative proliferation rates are of interest.

It is possible to stain cells in suspension or on the plate. In suspension uses less dye and may lead to more even staining. The drawback is that with some of the dyes (e.g. CFSE) you start to lose resolution at 4 dpi in NILDDRR conditions.

Note

CFSE (500 μg) is the cheapest by far but NW has observed it may dilute out faster than CTV and CTFR. Specifically, NILDDRR at 4 dpi will be closer to the negative control in CFSE than with other dyes.

CellTrace dyes available in lab

Dye	Attune Channel (excitation/emission)	Stock Concentration	How to Make
CFSE	BL1 (495/519)	10 mM (1000x)	Add 9 or 90 μL of DMSO directly to vial of CFSE. For the Cell Trace Kits, add 9 μL. For the 500 μg vials, add 90 μL of DMSO.
Cell Trace Violet (CTV)	VL1 (405/450)	5 mM (1000x)	Add 20 μL of DMSO directly to vial of CTV Cell Proliferation Kit.
Cell Trace Far Red	RL1 (630/661)	1 mM (1000x)	Add 20 μL of DMSO directly to vial of Cell Trace Far Red Cell Proliferation Kit.

Other materials needed:

- PBS
- DMEM + 10% FBS
- Adherent cells to stain

Important

Always include a negative (unstained) control. When adding + DDRR, you want to know when you're starting to lose signal.

Note

For MEFs and reprogramming experiments, you may want to work in **24-well plates or larger** to have enough cells for flow quantification (>10,000 cells). A typical 96-well of MEFs NIL at 4 dpi will have ~1-10k cells and NIL + DDRR at 4 dpi will have ~10-100k cells. Be careful to remember if you're doing 6F you will need even more. However, results are normally consistent with 96-wells if careful.

Dilute Cell Trace Dye to working concentration

1. If available, use an already resuspended vial of desired CellTrace dye. If unavailable, or not enough available, resuspend dye in DMSO according to the above table.
    - One entire 24-well plate will require 6 mL of working concentration CellTrace Dye, so you will need 6 μL of stock dye per plate being stained.
2. Dilute stock solution of dye into PBS to make a working solution. Stocks are at 1,000x concentration.

Wash cells and add CellTrace

3. Working in a BSC, aspirate the media and wash with PBS to remove media proteins.
4. Aspirate PBS and replace with working solution of CellTrace. For control cells that do not get stained with CellTrace, incubate in PBS + 1:1,000 DMSO or just PBS.
    - For the CellTrace staining a "half" well volume may be used (e.g. 1 mL per well of 6-well plate, 0.5 mL in a 12-well, etc.)
5. Incubate the cells in CellTrace in the incubator (37°C) for 30 minutes.

Remove CellTrace and wash

6. After incubation, aspirate the CellTrace solution and wash once with FBS containing media.
7. Return cells to incubator and treat cells according to your experiment. For reprogramming, cells are collected at 4 dpi and assayed via flow cytometry.

Halo-tag Labeling

Cell expressing the Halo protein or a Halo-tagged fusion protein can be labeled live, without fixation using Halo ligands ordered from Promega. This protocol for labeling of live cells with Halo ligand is also found on the Promega website[72] in the "Focus on Imaging Technical Manual."

[72] https://www.promega.com/products/protein-detection/protein-labeling/janelia-fluor-halotag-ligands/?catNum=GA1110#protocols

Halo-tag cell permeable dyes available in lab

Dye	Attune Channel (excitation/emission max)	Stock concentration	How to Make
Janelia Fluor 549	YL1 (549/571)	200 μM (1000x)	Dissolve 1 vial of JF549 in 37.8 μL of DMSO. Mix thoroughly by pipetting. Make 1 μL aliquots in 1.7 mL centrifuge tubes and freeze in -20°C.
Janelia Fluor 646	RL1 (646/664)	200 μM (1000x)	Dissolve 1 vial of JF646 in 35.5 μL of DMSO. Mix thoroughly by pipetting. Make 1 μL aliquots in 1.7 mL centrifuge tubes and freeze in -20°C.

Note

Halo ligand is aliquoted and frozen to avoid multiple freeze-thaw cycles, as recommended by Promega.

Note

The Promega documentation recommends diluting the stock solutions to 200x at time of use (a working stock) and replacing one fifth of the media on the cells at time of labeling. AMB has skipped this step, diluted straight to 1000x, and added to cells and it works well.

Other materials needed:

- Appropriate culture medium
- Adherent cells to stain

Halo ligand staining

1. Working in a BSC hood, dilute resuspended Halo ligand in appropriate culture medium 1:1000 to making the Halo-working solution.
2. Aspirate media from adherent cells and replace media with the Halo-working solution.
    - For the Halo staining a “half” well volume may be used (e.g. 1 mL per well of 6-well plate, 0.5 mL in a 12-well, etc.)
3. Incubate the cells in Halo-working solution in the incubator (37°C) for 15 minutes to overnight.

Note

AMB has tested 15 minutes, 30 minutes, and 1 hour. All have worked well with no noticeable loss-of-signal in shorter incubation times.

4. Remove Halo-working solution and proceed to downstream application (imaging, flow, etc.). No wash required.

Live nuclear staining using Hoechst

Materials

Stock solutions	Working solution
Hoechst 33342 Solution[73] (20 mM)	Dilute 1:10,000 in media

Protocol

Estimated time

30 min total

1. Remove media and add Hoechst working solution, diluted using the media of the cells in culture. A half-volume (e.g., 50 μL/well in a 96-well plate) can be used.
2. Incubate cells at 37°C for 20 min.
3. Aspirate the Hoechst solution and replace with appropriate culture media or PBS.
4. Image stained nuclei using the Keyence microscope or analyze cells by flow on the Attune.

Snap-tag Labeling

Cell expressing the Snap protein or a Snap-tagged fusion protein can be labeled live, without fixation using Snap ligands ordered from NEB. This protocol for labeling of live cells with Snap ligand is also found on the NEB website[74].

[73] https://www.thermofisher.com/order/catalog/product/62249
[74] https://www.neb.com/protocols/0001/01/01/cellular-labeling-s9105

Snap-tag cell permeable dyes available in lab

Dye	**Attune Channel** (excitation/emission max)	**Stock concentration**	**How to Make**
Snap-Cell 430	VL1 (421/444)	1 mM (200x)	Dissolve 1 vial of Snap-ligand in 50 μL of DMSO. Mix thoroughly by pipetting.
Snap-Cell TMR-Star	YL1 (554/580)	0.6 mM (200x)	Dissolve 1 vial of Snap-ligand in 50 μL of DMSO. Mix thoroughly by pipetting.
Snap-Cell 647-SIR	RL1 (645/661)	0.6 mM (200x)	Dissolve 1 vial of Snap-ligand in 50 μL of DMSO. Mix thoroughly by pipetting.

Note

Resuspended Snap-ligand is kept frozen in the -20°C freezer and has been used after several freeze/thaw cycles with no apparent detriment.

Note

The NEB website recommends the above stock concentrations as 200x. Greater dilutions can been used and labeling is still sufficient. AMB has used 400x dilution (2.5 μL dye / mL media) to label and had detectable signal. Greater dilutions may also reduce the amount of background staining.

Other materials needed:

- DMEM + 10% FBS
- Adherent cells to stain

Add Snap ligand to cells

1. Working in a BSC hood, dilute resuspended Snap ligand in an FBS containing medium 1:200 to making the Snap-working solution. Greater dilutions can be used for high-abundance proteins.
2. Aspirate media from adherent cells and replace media with the Snap-working solution.
    - For the Snap staining a “half” well volume may be used (e.g. 1 mL per well of 6-well plate, 0.5 mL in a 12-well, etc.)
3. Incubate the cells in Snap-working solution in the incubator (37°C) for 30 minutes.

Wash and destain ligand

4. After 30 minutes, remove the Snap-working solution and wash 3 times with FBS containing medium.
5. On the third wash, leave the media on the cells and incubate at 37°C for 30 minutes to allow unreacted ligand to diffuse out.
6. Remove final wash media and proceed to downstream application (imaging, flow, etc.)

Note

If you got high background signal, you can dilute resuspended Snap ligand in an FBS containing medium 1:500-1:1000

3.1.3 Imaging and analysis

CellBaum User's Guide

A practical guide to CellBaum for those who want to live track cells but know absolutely nothing about computers.

Important

You can use CellBaum either on your computer or on a cluster. The instructions for the MIT Supercloud are **deprecated**, as we no longer use this cluster.

Local Usage

Getting started

1. Make sure you have CellProfiler[75], Fiji[76], and Conda installed on your computer. Conda is needed for some of the dependency installation. We recommend Miniconda[77]; do not let it install Python into your PATH.
2. Using the Fiji plugin manager, install MIST, a Fiji stitching plugin.
3. Clone the CellBaum[78] repo, either e.g. with VSCode or via a terminal (remember, the `$` just marks the terminal ready for input, you don't type it):

```
$ git clone https://github.com/GallowayLabMIT/CellBaum.git
```

4. Start a new branch for your own work.
5. Follow the instructions in the `README.md` file. This involves running `conda env create --prefix ./cenv --file env.yml`. There are suggestions in the README on making this faster.
6. Download all the timelapse images to your computer so they can be accessed quickly. This can be done by right clicking on the folder and selecting "Always keep on this device". After you are done analyzing the images you can remove them from your computer by right clicking on the folder and selecting "Free up space".
7. Complete the `cellbaum_config.yml` file for your specific experiment. This can be done using VS Code. See more detailed instructions below.
8. Open up a terminal in the CellBaum folder. You may open the terminal using VS Code.
9. Activate the virtual environment:

```
$ conda activate ./cenv
```

[75] https://cellprofiler.org/releases
[76] https://fiji.sc/
[77] https://docs.conda.io/projects/miniconda/en/latest/
[78] https://github.com/GallowayLabMIT/CellBaum

10. To do a dry-run the CellBaum pipline, type the following in the terminal. This is to double check that everything is configured correctly:

```
$ snakemake --dry-run
```

11. To run CellBaum, type the following into the terminal. To assign a set number of cores on your computer, add the `-j#` flag after snakemake. For example, to use 7 cores on your computer to run CellBaum, you could run:

```
$ snakemake --j7
```

12. Let CellBaum run. This may take many hours. The typical order of jobs is:
    1. folder merging (if needed, e.g. you stopped and re-started the timelapse)
    2. grayscaling
    3. find focus (if z-stacking was used)
    4. stitching (if stitching was used)
    5. run Cell Profiler pipeline to collect cell data and locations (nuclei_masking.cppipe)
    6. btracking (for more information on Bayesian Tracking see GitHub[79] and their User's Guide[80])
    7. Add cell data to HDF tracks file

Tip

If you want to stop CellBaum from running, hit ctrl+C while in the terminal and CellBaum will stop running after it finishes all the jobs it is currently running.

If you are too impatient to wait for CellBaum to finish running or it is stuck on the btracking step, you can kill the terminal. However, if you kill the terminal, you will need to unlock it before you can run again:

```
$ snakemake --unlock
```

You may also need to use the rerun-incomplete flag after unlocking if you stopped CellBaum partway through a job such as grayscaling:

```
$ snakemake -j7 --rerun-incomplete
```

CellBaum Config File

If you have not completed a config file previously, copy the `cellbaum_config.yml.template` file and remove ".template" from the file name. This will be your config file. If you have a previous config file, you may simply edit that one.

Complete the config file with the following information:

[79] https://github.com/quantumjot/BayesianTracker
[80] https://btrack.readthedocs.io/en/latest/

- directories
  - Put the path on your computer to each item.
  - data_dir = Keyence data folder
  - cp_dir = CellProfiler app
  - fiji_dir = Fiji app
  - pipe_dir = pipeline for CellBaum (in CellBaum repository)
  - output_dir = where you want to save all output files
  - log_dir = where to save logs, usually this can be in an output_dir/Logs folder
  - cell_config = btrack config file, under the models folder in CellBaum repository
  - See examples below:

```
data_dir: C:\Users\ChemeGrad2020\Massachusetts Institute of Technology\
↪GallowayLab-Timelapse - Brittany\2022.12.16_Hb9-Activation\
cp_dir: C:\Program Files\CellProfiler
fiji_dir: C:\Users\ChemeGrad2020\Documents\Fiji.app
pipe_dir: C:\Users\ChemeGrad2020\Documents\GitHub\CellBaum\cp_files
output_dir: C:\Users\ChemeGrad2020\Documents\CellBaum_Analysis\2022.12.
↪16_Hb9-Activation
log_dir: C:\Users\ChemeGrad2020\Documents\CellBaum_Analysis\2022.12.16_
↪Hb9-Activation\Logs
cell_config: C:\Users\ChemeGrad2020\Documents\GitHub\CellBaum\models\
↪cell_config.json
```

- folder_merging_needed
  - false if all your images are in a single folder from the Keyence
  - true if you need to merge two or more folders, for example you re-started the timelapse on the Keyence.
  - If true, you must include list under folders_to_merge
- gray_channels
  - list of channels you want to grayscale and which grayscale method to use.
  - 'max' uses the RGB channel in which the maximum pixel value occurs
  - 'avg' uses the average of all three RGB channels
  - using a list of 3 ratios lets you choose the weight of each RGB channel
  - For example:

```
gray_channels:
    CH2: max
    CH3: max
```

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```
    CH4: avg
    Overlay: avg
```

- focus_finding_needed
  - True if you used z-stacking during your timelapse.
  - If true, you also need to include focus_channels.
- image_regex
  - A regular expression that extracts data from image names such as time, well, stitching position, z-stack, and channel. You can check if your regular expression fits your file names using https://regex101.com/. For example, your regular expression could be:

```
image_regex: (?P<prefix>.*)_(?P<time>T\d{4})_(?P<well>XY\d{2})_(?P
↪<position>\d{5})_Z(?P<stack>\d{3})_(?P<channel>.*)\.tif
```

- focus_channels
  - List of channels for which you want to find the most in-focus z-slice. For example:

```
focus_channels:
 - CH2
 - CH3
 - CH4
 - Overlay
```

- example_image_name
  - An example name of your image to check your regular expression above. You can just copy paste a file name of an image from your data folder:

```
example_image_name: 10x_T0001_XY01_00001_Z001_CH2.tif
```

- pre_stitch_correction_needed
  - True if you want to make some kind of correction to your images before stitching. For example you could apply a threshold filter to the image and reduce noise.
  - If doing pre-stitch corrections, you will need to update the img_processing.cppipe in CellProfiler to make the desired pre-stitch corrections to your images.
- stitching / prefix / template
  - Needs to be included if you used stitching during your timelapse.
  - grid_width and grid_height are how many images are in each dimension of your stitching grid.
  - Prefix is a list of which channels you want to stitch.
  - Template is which channel in the list you want to use as a template (zero indexed!). It is probably best to use a channel with the most "information" as a template.

  - Example usage:

```
stitching:
    grid_width: 3
    grid_height: 4
    Prefix:
    - CH2
    - CH3
    - CH4
    - Overlay
    Template: 3
```

- minsize/maxsize
  - The expected minimum and maximum size of objects/nuclei being tracked (pixel diameter). For example:

```
minsize: 7
maxsize: 45
```

- Update_method
  - Which method to use when tracking objects (EXACT or APPROXIMATE). It is recommended to start with the default EXACT. This option considers all possible combinations of linking objects, so it can be slow for very large datasets. If there are issues converging to switch to APPROXIMATE.
  - In general, for cell datasets with <1000 cells per time point we recommend EXACT. If you have larger datasets, we recommend APPROXIMATE.
  - Example usage:

```
Update_method: APPROXIMATE
```

- Max_search_radius
  - The maximum search radius for the tracking algorithm in isotropic unit of the data. This parameter can be used to prevent very large displacements when linking objects.
  - Example usage:

```
Max_search_radius: 100
```

- Volume
  - An estimate of the dimensions of the imaging volume, used to define the edges of the field of view for generating hypotheses and labeling tracks as lost.
  - This is pretty much the dimensions of the stitched images in pixels.
  - It is recommend to use 'auto' which automatically calculates these values based on the size of the images.
  - Example usage:

```
Volume: auto
```

  - OR:

```
Volume:
    x:
    - 0
    - 4577
    y:
    - 0
    - 5427
    z:
    - 0
    - 2
```

- Step_size
  - Not really sure what this does. It doesn't seem to affect anything. You can just put like 100 or something:

```
Step_size: 100
```

- CP_Data_Keep
  - List of which CellProfiler data to keep, or you can just put 'all' for simplicity:

```
CP_Data_Keep: 'all'
```

- time_interval_to_track
  - This feature is useful if the btracker isn't converging you want to change the parameters in the btracker config file (models/cell_config.json).
  - Choose which time points to track. For example, if you have 181 time points and you want to see if you can get it to converge on the subset of time points from 0 to 50 you could put:

```
time_interval_to_track:
    min: 0
    max: 50
```

Getting the btracker to Converge

The best way to make sure CellBaum will converge is by carefully choosing the conditions of your timelapse. Two recommendations are:

1. Adding a population of dark cells and/or seeding cells sparsely will help with tracking. Many tracking issues are due to simply way too many cells in the field of view.

2. Imaging more frequently can help when cells move around a lot. However, you may start worrying about photo bleaching or computer storage space restrictions if you image too frequently.

Ensure your nuclei masking pipeline works well with your stitched images.

- It is required that you always re-do the CellProfiler pipeline nuclei_masking.cppipe for your specific images AFTER they have been stitched.
- You will probably need to stop CellBaum partway through in order to update the pipeline and use stitched images.
- Stitching will affect the RegEx used in your CellProfiler pipeline.
- You may use different channels between experiments, nuclei will be different size ranges, there may be weird background luminance, etc.

Change the btrack model parameters (models/cell_config.json)

- You can read more about the btrack config models here[81].
- The output of the tracking is very sensitive to the choice of parameter values. The global optimization step can take a very long time to complete if you have a poor choice of model parameters.
- Changing the Motion Model probably won't help much.
- Brittany has found that increasing the values for `lambda_link` and `lambda_branch` helped most with converging.

MIT Supercloud Usage

Getting Access to the MIT Supercloud

1. Follow the directions on the MIT Supercloud website[82] for getting access to the MIT supercloud.
2. First you will need to fill out the Account Request Form.
3. Then you will need to send an email to supercloud@mit.edu and CC Katie.
4. Then you might have to do some other setup stuff. Ask Chris for help and then add those instructions to this protocol.

Hint

You may use this as a template for your email:

Hello,

I have submitted an account request form so I can get access to the MIT Supercloud for my research project. I will be using the Supercloud to analyze and generate data from time-lapse

[81] https://btrack.readthedocs.io/en/latest/user_guide/configuration.html#
[82] https://supercloud.mit.edu/requesting-account

microscopy images. I have Cc'd my PI, Katie Galloway, so she can confirm that I will need access for my work.

Thanks,

Tony Stark

Upload Timelapse Files from the Keyence to the Supercloud

1. Open a terminal **in your Keyence Timelapse Ondrive folder** (shift + right click then choose Open Terminal).
2. Use rclone to copy your experiment folder to the supercloud. Rclone uses the following command to copy files:

```
$ rclone copy (--dry-run) (--progress) source:sourcepath dest:destpath
```

3. It is recommended to first do a dry run to ensure everything will transfer correctly:

```
$ ~/downloads/rclone-v1.60.1-windows-amd64/rclone-v1.60.1-windows-amd64/
↪rclone.exe copy --dry-run 2022.12.16_Hb9-Activation supercloud-blende:/
↪home/gridsan/blende/galloway_shared/data/2022.12.16_Hb9-Activation
```

4. Then when you are ready to transfer the files, include the progress flag to see upload progress:

```
$ ~/downloads/rclone-v1.60.1-windows-amd64/rclone-v1.60.1-windows-amd64/
↪rclone.exe copy --progress 2022.12.16_Hb9-Activation supercloud-blende:/
↪home/gridsan/blende/galloway_shared/data/2022.12.16_Hb9-Activation
```

Note

rclone is safe to interrupt at any point.

On the Keyence computer, rclone isn't in the Path, so you need to do the whole path for rclone.exe in the Downloads folder.

Remember to include add supercloud-blende: (or your equivalent) before the dest path to specify you are using a remote path.

It can take several hours for all the files to transfer to the supercloud.

Using the MIT Supercloud

1. Open powershell.
2. In the terminal type:

```
$ ssh supercloud
```

3. You may need to type your password. If you don't want to type your password every time you can use ssh-add to use an agent to control authentification.
4. Navigate to the CellBaum folder:

```
$ cd ~/galloway_shared/CellBaum
```

5. Do a git pull to update CellBaum:

```
$ git pull
```

6. If you have your own branch in CellBaum, switch to your branch. For example:

```
$ git checkout KTRs
```

7. Update the supercloud_config.yml file for your specific timelapse. You can update the file in VS Code on your computer, push it to GitHub, and then do git pull on the supercloud. Or you can use nano:

```
$ nano supercloud_config.yml
```

8. You might also need to update the snakefile to use the supercloud_config.yml instead of the cellbaum_config.yml file:

```
$ nano snakefile

# Then change

configfile: "cellbaum_config.yml"

# to

configfile: "supercloud_config.yml"
```

9. Update any CellProfiler pipelines that are used. You can copy files to the supercloud via scp[83], or you can git push the file from your computer and then git pull from the supercloud.
10. To run CellBaum you need to run the submission bash script:

```
$ LLsub sub_script.sh
```

11. To see what is running, you can type:

```
$ LLstat
```

12. When you want to leave the supercloud, just type exit:

```
$ exit
```

[83] https://supercloud.mit.edu/accessing-and-transferring-data-and-files

13. You can view the files on the supercloud using the Powershell and ls. Or you can log into the online web portal: https://txe1-portal.mit.edu/

Supercloud Config

This config file is virtually the same as the cellbaum_config.yml file, except you will want to change all the directories to locations on the supercloud. For example:

```
data_dir: /home/gridsan/blende/galloway_shared/data/2022.12.16_Hb9-Activation/
↪2022.12.16_Hb9-Activation
cp_dir: /home/gridsan/blende/galloway_shared/cluster_infrastructure/conda_envs/
↪cellbaum/bin/
fiji_dir: /home/gridsan/blende/galloway_shared/bin/Fiji.app
pipe_dir: /home/gridsan/blende/galloway_shared/CellBaum/cp_files
output_dir: /home/gridsan/blende/galloway_shared/data/2022.12.16_Hb9-Activation/
↪CellBaum_results
log_dir: /home/gridsan/blende/galloway_shared/data/2022.12.16_Hb9-Activation/
↪CellBaum_results/Logs
cell_config: /home/gridsan/blende/galloway_shared/CellBaum/models/cell_config.
↪json
```

Sample Prep and Confocal Imaging at the Koch Microscopy core

This protocol aims to guide users on how to prepare samples for imaging using the Leica Sp8 confocal microscope at the Koch Institute. This protocol is only supplemental to the mandatory training provided by KI microscopy core and would only cover important tips and tricks learned over trial and error on sample preparation and cell-specific imaging process that have proven to improve the confocal imaging acquisition.

Confocal access and Experimental planning:

Note

Before starting make sure you have undergone or have requested for training to access the Leica Sp8 microscope. The training will cover mostly the instructions on how to operate the hardware and software components of the microscope, as well as, practical advise on lasers based on your specific fluorophore needs.

1. Carefully plan your Fluorophore-conjugated secondary Abs, (for example the Cy7 detection is weak unless your signal is very bright) and use no more than 1:10000 for both Hoechst and DAPI for counterstaining, as these channels tends to be very bright. If in doubt consult Jeff for Fluorophore-Laser compatibility.
2. All cells to be used for confocal imaging have to be cultured on glass-bottom plates. Before plating your cells, try to position them as close to the center as possible, leaving at least 2 outer rows and columns blank. If imaging Hb9::GFP cells, have 1 well close to the edge to be used as background control (uninfected, non-treated and unstained). This is because

Hb9::GFPs tend to be very bright causing halo and grainy effect on higher magnifications, so you need to use this well for black balance correction.

3. Make sure you coat the glass bottom plate appropriately (i.e. Laminin - Neurons and Intermediate 4dpi cells, Matrigel- mature neurons, or gelatin- MEFs) so that cells will adhere all throughout the fixation, permeabilization and washes.
4. During storage or transport to KI, always cover the plate with foil and do not let wells dry.

Note

Filter-sterilize the PBS and gelatin to be used to have less debris at high-resolution imaging.

Confocal imaging

1. Book a time at iLabs and take your sample to KI for confocal imaging
2. Go through the proper log-ins in the Leica computer and turn on the microscope in proper sequence (will be instructed during training).
3. Open LasX software, load a configuration (this will be set-up during training) or start a new one, if using different lasers than in your training.
4. Once inside the LasX software, navigate to the Acquisition panel on the Left-hand side and make sure to use 1024x1024 Image format and Line average of 2.
5. You can switch on the lasers you need (this will be instructed during training) or the lasers are automatically turned on if you've loaded a configuration. Use only a minimum of 1 for Laser Power during the start-up, this can be modified as needed in Step 9 or with consultation with Jeff.
6. Once the lasers are turned on, you can start setting up your plate in the scope. Begin by selecting the desired magnification (I've only used 100x) then add 2 drops of Oil into the objective. Select the plate adapter for the sample holder (or slide adapter, if needed), then place your plate into adapter. Make sure all sides of the plate are evenly pushed into the adapter before placing it in the microscope.
7. Start navigating into your target wells using the joysticks. Use the focus to find an initial Z-stack of cells.
8. Go back to the software and press Live at the bottom left. The Image panel on the right should display a Live acquisition of all your target Fluorophores (lasers). Don't worry if nothing shows up right away, this usually takes a few minutes to load.
9. Refine the quality of the acquisition depending on your target fluorophore by tuning the Laser Power. Less is always recommended to avoid artefacts.
10. To capture several stacks, press Fast Live and navigate to the Z-stack option, turn the Z-stack knob to either direction and Set the start and end-point of the Z-stacks to be captured.
11. Press Start to begin Image acquisition. More Z-stacks and channel sequences takes longer time.

Saving and Export of files

1. Navigate to the Open Projects Panel in LasX and verify that the image-sets per well that you have acquired are all displayed. You can either save all of them in one go or save them individually.
2. To save and export, right click either the Entire Project or the Individual image acquisitions and click Export. It should redirect you to a file path in the D-drive of the microscopy core, where you can save files in a folder under your name.
3. This can later on be accessed and analyzed using one of the Workstations in the Core.

Image segmentation and particle analysis in ImageJ

Warning

CellProfiler has become the preferred software for image segmentation and quantification.

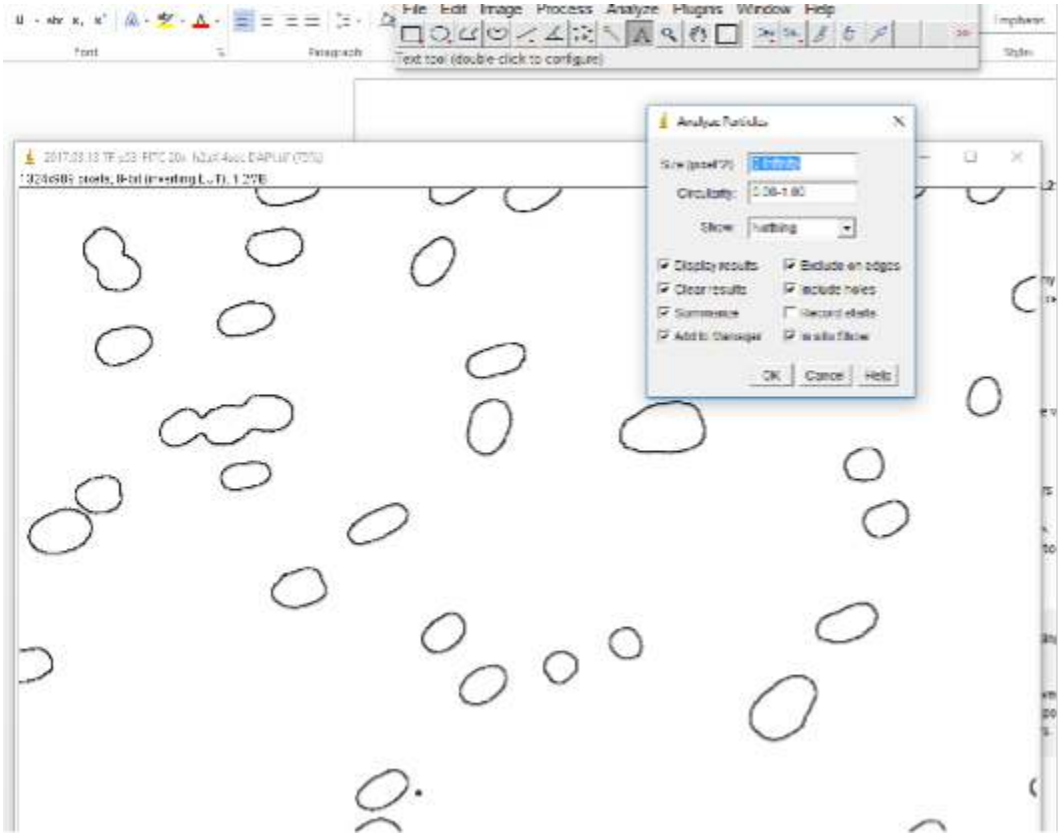
This protocol describes how to use DAPI images to make a nuclear mask and quantify nuclear staining intensity.

Important

To use this process, images need to be collected from the same field with DAPI and any other desired channel (e.g., green, red). Thus, this protocol only works for fixed cells. Potentially, live cell imagining with an H2B-Cerulean could allow nuclear image analysis with this method.

Defining regions of interest (ROI)

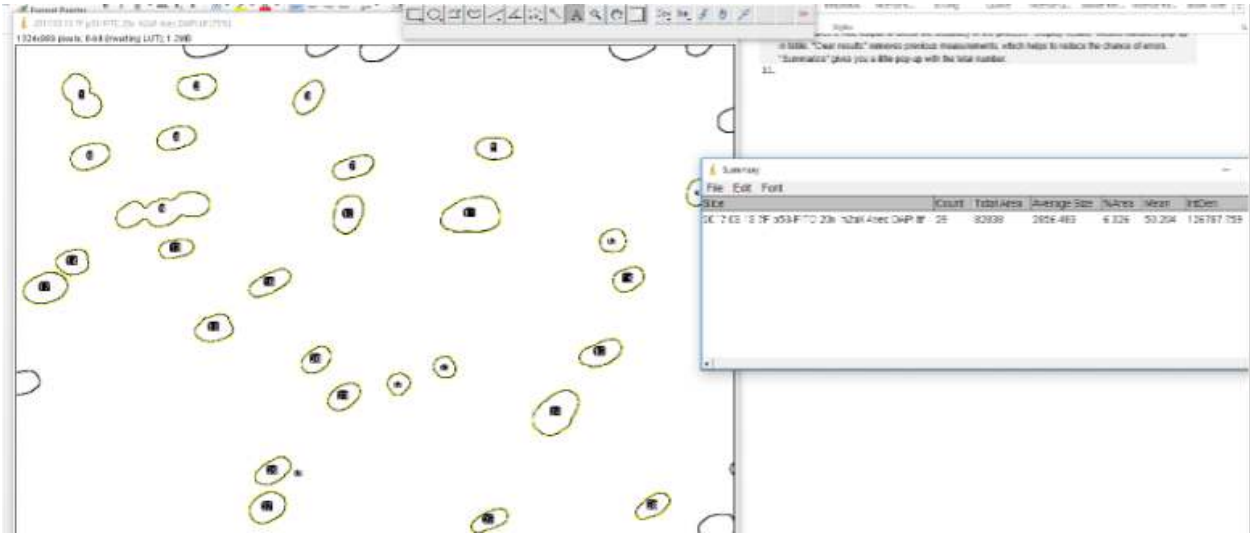
1. Since we are trying to look at the nucleus, the nucleus is our ROI. To define this region we will use the DAPI stain to build a mask.
2. Open the DAPI image for your sample.
3. Change the format to 8-bit. Image > Type > 8-bit.
4. Adjust the threshold (`Ctrl-Shift-T` or *Image -> Adjust -> Threshold*) until each nucleus appears bright enough to be distinguished from the background.
5. Then delineate the nuclei by tracing: *Process -> Find Edges*. If nuclei are well separated, this should work well. If not, there is a program/plug-in called *Watershed* that can be used to resolve merged nuclei segments.
6. Then use Analyze Particles function: *Analyze -> Analyze Particles*. The pixel size can be changed to exclude particles that are too small or big or not circle enough or too circular.
7. The ROIs should now be labeled with numbers to indicate which is which. If you only want to count numbers of nuclei, the *Summary* window will tell you how many. In the case below, it counts 29. However, we can see that #9 is 3 nuclei. That's why the *Watershed* program

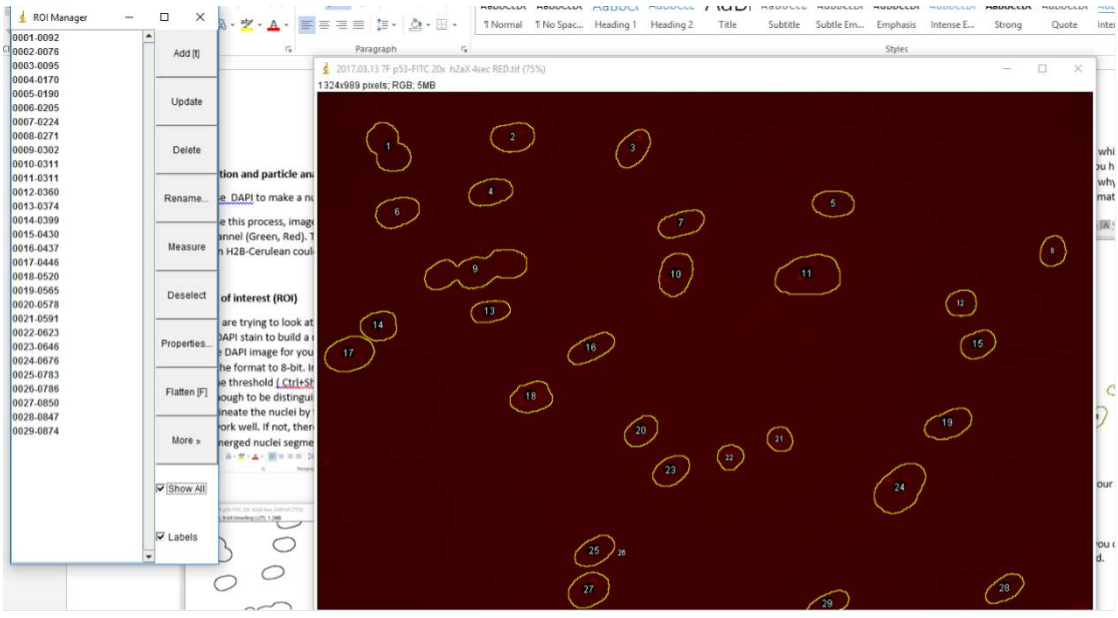


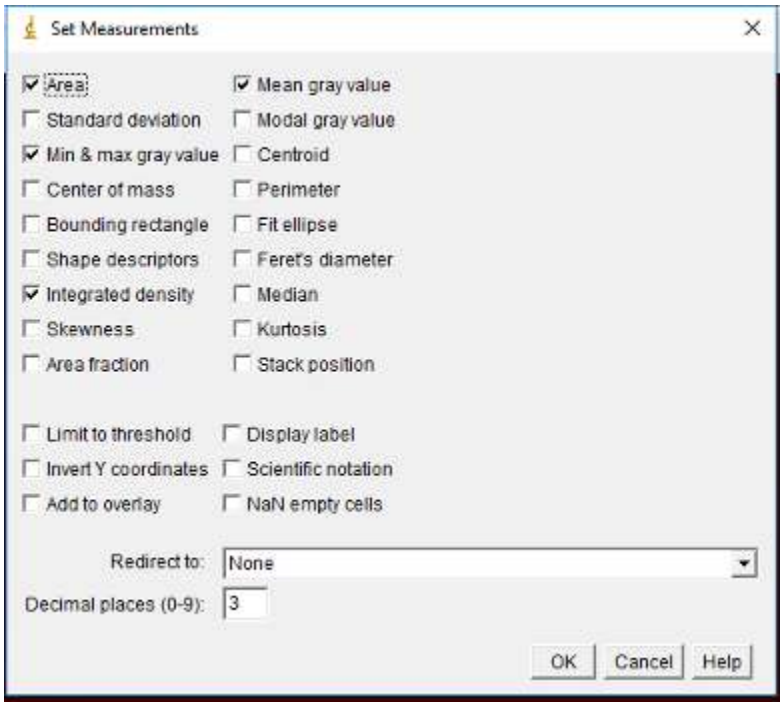
may be useful and necessary. Still this process gives us a good estimate for generally well separated nuclei.

Now that we have our regions of interest, it is time to get our other images with our staining.

8. Open the RED/GREEN or other image.
9. GO to *Analyze -> Tools -> ROI Manager*. In the ROI Manager, you can click on the different numbered ROIs to view them in the image you just opened.
10. If you would like to get the intensity for each ROI, then simply go to *Analyze -> Set Measurements*. Make sure *Area*, *Min & max gray value* and *Mean gray value*, and *Integrated density* are selected. Then click *OK*.
11. Next go to back to the ROI Manager and make sure all the desired regions are selected. You can deselect any that are problematic (too small, overlapping nuclei, etc). Then click on *Measure* and a window with the measurements will pop-up.
12. Save this as a tab delimited file to open in Excel later. The area will be useful for normalizing the intensity for size of the nucleus.




ROI Manager
0001-0092
0002-0076
0003-0095
0004-0170
0005-0190
0006-0205
0007-0224
0008-0271
0009-0302
0010-0311
0011-0311
0012-0360
0013-0374
0014-0399
0015-0430
0016-0437
0017-0446
0018-0520
0019-0565
0020-0578
0021-0591
0022-0623
0023-0646
0024-0676
0025-0783
0026-0786
0027-0850
0028-0847
0029-0874
Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
Show All
Labels
1324x989 pixels; RGB; 5MB



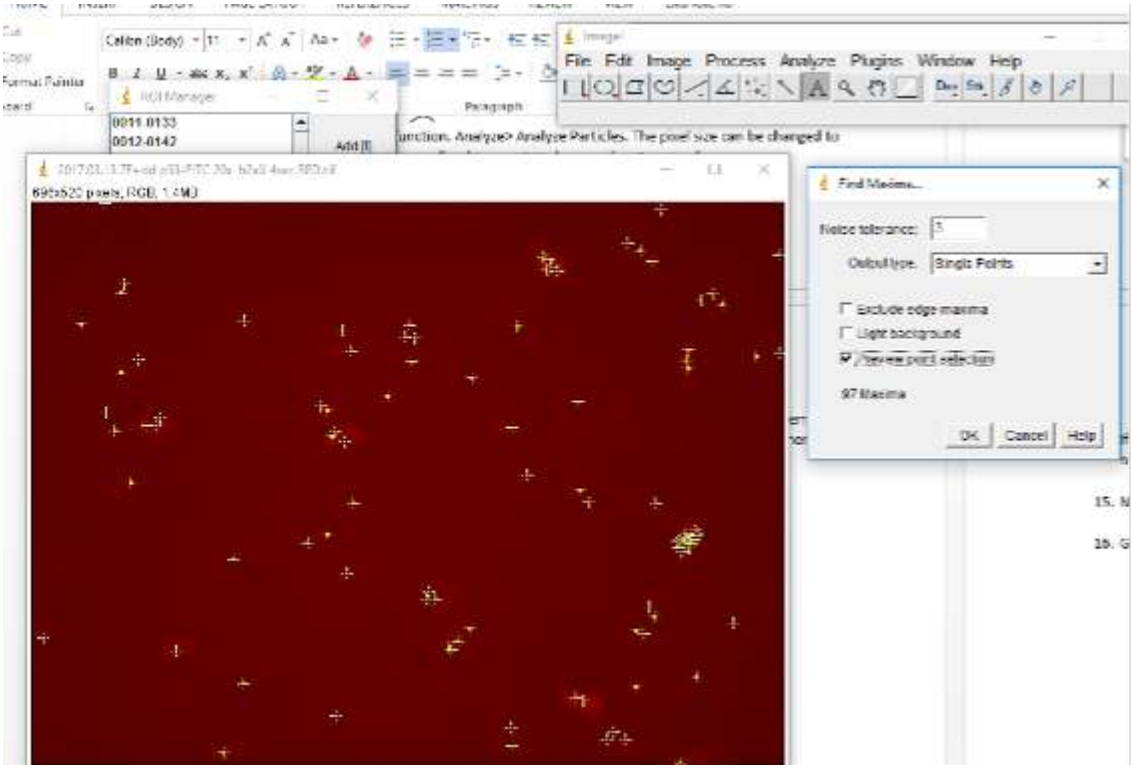
Results

File Edit Font Results

	Area	Mean	Min	Max	IntDen	RawIntDen
1	4172	19.818	18	24	82681	82681
2	2958	21.396	19	36	63288.000	63288.000
3	2638	22.130	19	50	58378.000	58378.000
4	2654	21.017	19	28	55778.000	55778.000
5	2517	21.005	20	24	52870.000	52870.000
6	2985	20.826	18	29	62167.000	62167.000
7	2645	22.363	20	29	59150.000	59150.000
8	1790	20.426	19	28	36563.000	36563.000
9	7444	21.161	19	33	157520.000	157520.000
10	3316	22.606	21	40	74960.000	74960.000
11	6094	22.284	20	51	135797.000	135797.000

Quantifying nuclear-localized foci

13. If instead of quantifying total intensity across the nucleus, you want to quantify foci in the nucleus, you will process the DAPI image as previous described.
14. Next open your stained image for foci counting.
15. Go to *Process -> Find Maxima*. Toggle with noise tolerance setting to get good detection of foci. The higher the noise tolerance the stronger the signal has to be to be detected.

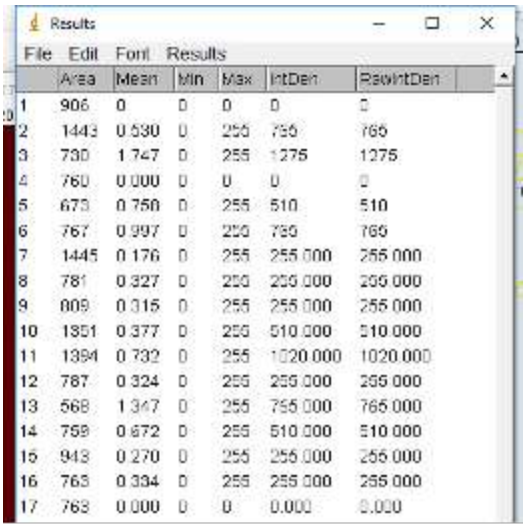


16. Now go to ROI Manager and click on *Measure*.
17. The *RawIntDen* tells you how many foci in each ROI (e.g. it sums the 255 values (code for black dots) within each ROI). To determine the number of foci per region, simply divide by 255. Save this file in excel for further processing.

Reference: http://microscopy.duke.edu/HOWTO/countfoci.html

Merging images in ImageJ

1. Create a *Merged* folder inside the folder where your images are.
2. Open the files for the various channels (e.g., DAPI, red, green) you want to merge, then do *Image -> Color -> Merge*.
3. Make image any adjustments (e.g., brightness, contrast).
4. Save the new merged image as a `.jpeg`.



	Area	Mean	Min	Max	IntDen	RawIntDen
1	906	0	0	0	0	0
2	1443	0.530	0	255	765	765
3	730	1.747	0	255	1275	1275
4	760	0.000	0	0	0	0
5	673	0.758	0	255	510	510
6	767	0.997	0	255	765	765
7	1445	0.176	0	255	255.000	255.000
8	781	0.327	0	255	255.000	255.000
9	809	0.315	0	255	255.000	255.000
10	1351	0.377	0	255	510.000	510.000
11	1394	0.732	0	255	1020.000	1020.000
12	787	0.324	0	255	255.000	255.000
13	568	1.347	0	255	765.000	765.000
14	759	0.672	0	255	510.000	510.000
15	943	0.270	0	255	255.000	255.000
16	763	0.334	0	255	255.000	255.000
17	763	0.000	0	0	0.000	0.000

Image analysis using the KI workstation - Nemo

Important

Before starting make sure you have been trained by the core staff about Workstation access. This protocol is supplemental only to training provided by the core staff, and aimed mainly for basic 3D reconstruction using Z-stack images. More advanced analysis can be consulted from the core staff.

This protocol aims guide users how to perform basic image processing, specifically 3D reconstruction from Z-stacks, using softwares installed at the KI Microscopy core workstation – Nemo. At the time of my training, this workstation has 3 different softwares installed: Fiji, LasX and Imaris. All images acquired in Lecia Sp8, can be processed and analyzed by either of 3 softwares, 3D reconstruction from Z-stacks can be done better using LasX (Leica software) and Imaris. For simplicity, I've used the LasX 3D function to acquire Volume data, but use Imaris 3D visualization function for the representative reconstructed images.

LasX 3D Image Viewer

1. Log-in to the Workstation with your MIT credentials.
2. Access the images acquired in Leica Sp8 by navigating into the D- drive.
3. Double click on your Project. It should automatically open in the LasX software (the same software used for Image acquisition in the Sp8).
4. On the Left-hand side, you can see your Project acquisitions, with the subfolders of the different wells you imaged. Click on the desired "condition" or "well" to display the acquired images and stacks in the viewing panel.
5. Select on the image displayed on DAPI or Hoechst channel and click the Browse Workflow. Under this, you can click the "Z" tab. Check to make sure it is displaying all the stack that you've captured.

6. On the Tool list, select 3D model. Adjust the parameters of 3D options accordingly. A preview will be display on the right-hand side.
7. Once satisfied with the 3D model, you can click on the Measurement tab to obtain 3D metrics including Volume.

Imaris

Imaris software is handy to use for obtaining the representative 3D image because it has a built-in function that can focus on only 1 nucleus per field.

1. Open the Imaris software and stage your LasX project. This has `.lif` file extension and you need to convert this to Imaris file extension `.ims`.
2. Once converted, you can double click to open into Imaris.
3. The loaded image is automatically Rendered in 3D, focus on the nuclei that you want to represent and crop around it.
4. The cropped image will display the metrics such as Volume and Area which you can confirm from the data measured from LasX software.
5. The displayed image can be saved as .png using the Export button.
6. The 3D reconstructed image using Imaris is used mainly for representative image visualization and not for data processing.

3.1.4 Other biochemical and analytical protocols

Droplet Digital PCR (ddPCR)

This protocol describes how to run ddPCR copy number variability assays on the BioRad QX200[84] system, following instructions from the STRAIGHT-IN *Nature Protocols* paper [**?**]. See also this presentation[85] from A.B.A. on the SharePoint.

Design

The presentation above and resources from BioRad[86] contain useful information for designing new assays.

Each assay consists of a primer set and a corresponding probe. The probes are in one of two fluorescence channels, HEX or FAM, so each reaction can measure up to two targets. To detect copy number, one of the targets is typically a reference gene on the genome. For human cells, we use *RPP30*, which has a copy number of two in normal diploid cells. However, HEK293T cells have unstable genomes and are often aneuploid—we've measured the copy number for *RPP30* as 2.7, but this and other genes may be unstable.

In lab, we currently have primers and probes (assays) for the following targets:

Probe	Target
HEX	*RPP30* (human reference gene)
FAM	*mRuby2*, *mScarletI*
	PuroR, *BleoR*
	AmpR, pUC19 backbone

Note that the instrument runs a set of 8 reactions (1 strip of PCR tubes) at a time, so aim to run multiples of 8 reactions to optimize consumable use. Include a negative control (e.g., unedited cells) and a positive control (e.g., cells with a known copy number of your target) in each set of reactions, especially for new assays.

Protocol

1. Extract high-quality genomic DNA from your cells, e.g., using the Qiagen DNeasy Blood and Tissue Kit[87]. Nanodrop to measure concentration and confirm purity.
2. Obtain 20X assay mixes that include a probe and matching primer set.

   We make these in 100 μL aliquots, which is enough for ~90 reactions. They are stored in the shared ddPCR box in Olaf (-20°C) and should be thawed on ice.

   20X Assay Mix:

[84] https://www.bio-rad.com/en-us/life-science/digital-pcr/qx200-droplet-digital-pcr-system
[85] https://mitprod.sharepoint.com/:p:/s/GallowayLab/EaPOh1lNzpZHpF5xqf2SNscBnhMz82q7pHYbfiS__mnsvw?e=1zoBkN
[86] https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf
[87] https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit

Component	Amount (µL)
Probe (100 μM)	5
Fwd primer (100 μM)	18
Rev primer (100 μM)	18
IDTE buffer	59
Total	**100**

3. Prepare the PCR reaction mixes for each condition on ice.

   We prepare 22 μL per condition so that 20 μL can be easily loaded into the machine without bubbles. The following table shows amounts for one reaction and for 8.8 reactions (a full set plus extra), which is useful for making a master mix for 8 samples. The 2X ddPCR Supermix is the ddPCR Supermix for Probes (no dUTP)[88] from BioRad, and is stored in Sven (-20°C).

   Reaction Mix:

Component	1X	8.8X
2X ddPCR Supermix	11	96.8
20X HEX Assay	1.1	9.68
20X FAM Assay	1.1	9.68
HindIII	0.55	4.84
gDNA	3	
Elga water	5.25	46.2
Total	**22 μL**	

   If making a master mix for several samples, combine 19 μL MM with 3 μL of sample gDNA. The amount of gDNA to add to each reaction depends on the expected copy number of the gene. For low copy targets, aim to add ~100-200 ng of gDNA to each reaction. Scale down this amount for high-copy targets (e.g., PiggyBac-integrated cargoes). Dilute gDNA samples in Elga water to 3 μL per reaction for easy math/pipetting.

4. Bring your reactions and supplies over to the Weiss lab, where the ddPCR machine is located. We buy our own consumables for the instrument and store them in the Weiss lab, so you should also bring over pipettes (P200 and P20 for accuracy) and tips.

5. Turn on the plate sealer to allow it to come to temp. Remove the large block from the machine first.

6. Load the cartridge for creating droplets:

   1. Pipet 20 μL of each reaction into the wells in the middle channel of the cartridge, ensuring there are no bubbles. For unused wells, add 20 μL water.

   2. Pipet 70 μL Droplet Generation Oil per well into the bottom channel of the cartridge. For wells corresponding to those without sample, you can add water rather than oil.

   3. Place the cartridge into the white holder and cover it with the gasket (stretchy rust-colored material), being careful not to spill.

[88] https://www.bio-rad.com/en-us/life-science/digital-pcr/digital-pcr-supermixes/ddpcr-supermix-for-probes-no-dutp

4. Load the cartridge into the QX200 Droplet Generator machine (see picture). When loaded correctly, the middle green light will turn on.

Fig. 1: ddPCR Droplet Generator correctly loaded (middle green light) with cartridge. The cartridge (clear) is in the cartridge holder (white) and covered by a gasket (rust-colored).

7. Run the machine. Droplets will be generated in ~2 minutes. Wells should be cloudy if droplets have been formed correctly. If you have more than 8 reactions, repeat the droplet generation steps with additional, fresh cartridges.
8. Transfer the droplets from the cartridge to a 96-well plate.
    - Pipet up the entire well volume (70 μL) from the top channel of the cartridge. Place your pipette tip at the center-bottom of the well to ensure you get the full volume.
    - Dispense the droplets into a well of the 96-well plate, going slowly to not disturb the droplets. Place your pipette tip on the side of the well when dispensing, and avoid bubbles as usual (though this is not critical at this step).
    - Tip: Transfer droplets from one cartridge into one column of the 96-well plate for easy plate layouts.
    - Tip: Leave the cartridge in the white holder while pipetting. After transfer to the 96-well plate is complete, you can throw away the cartridge.
9. Seal the 96-well plate. Place the block, plate, and piece of foil in the PX1 PCR Plate Sealer (see picture) and run. This takes ~10 seconds.
10. Load the sealed plate in the thermocycler and run the `ddPCR_DEMO` protocol. This takes ~2 hours to run.

Note

After running the ddPCR reaction in the thermocycler, the plate can be stored at 4°C for up to a

Fig. 2: ddPCR Plate Sealer with block, plate, and foil ready to run.

few days before analysis. Typically, however, we run the analysis on the same day.

Analyze the droplets using the QX200 Droplet Reader:

11. Remove the plate from the thermocycler and turn on the QX200 Droplet reader (switch on the backside)
12. Load the plate oriented correctly into the QX200 Droplet Reader with the metal plate cover on top with the latches clasped down.

Fig. 3: Loading the plate into the Droplet Reader.

13. Open the QX Manager software (username: admin, password: on post-it on computer)
14. If the three lights on the front the of machine are green, you are ready to start a new experiment by selecting the plate icon button in the top left.

15. Fill out the three input tabs for experimental information:

1. Plate information
    - Create a plate/experiment name
    - IMPORTANT: select “ddPCR Supermix for Probes (No dUTP)” for Supermix
    - Select to acquire wells by columns or rows
2. Well selection
    - Select wells to analyze
3. Well information
    - Select “Experiment type” (for this example a Copy Number Analysis was selected)
    - Input “Sample Description” for each well for easier identification
    - Input target information for each well (the dye and gene for the probe); multiple wells can be selected if the targets are shared

In this example, we were interested in *mRuby2* copy number in hiPSCs with *RPP30* as a reference gene. We use the HEX probe in Channel 2 for *RPP30* (with 2 copies in the genome) and the FAM probe in Channel 1 for *mRuby2*.

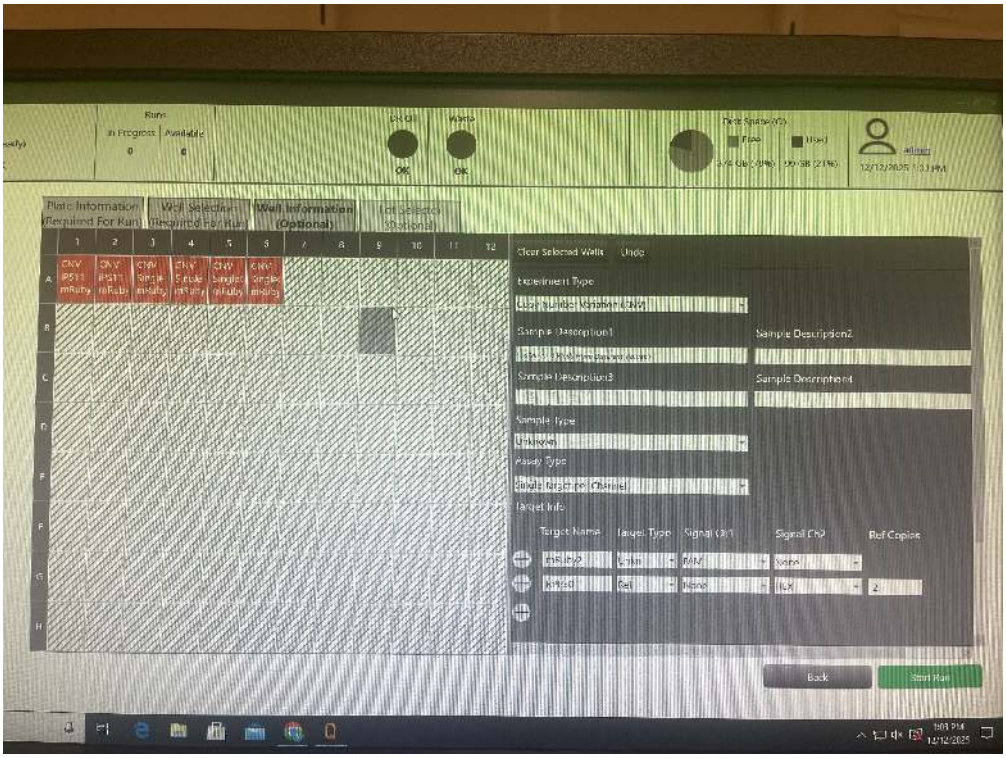


Fig. 4: Example inputting sample target information in software.

16. Run the experiment. The machine will take ~10 minutes to analyze 8 wells.

Note

A live analysis window can be opened to monitor the progress. It can be helpful to watch the "Event Count", i.e., the number of droplets analyzed. We are targeting >10,000 droplets per well to ensure sufficient droplet generation and good data quality.

17. The experiment data will be automatically saved to the "ddPCR data" file on the computer. Copy your `.ddpcr` file to a flashdrive and transfer the file to the lab data folder at `instruments/data/Weiss.lab.ddPCR`.
18. Remove and dispose of the plate from the machine and turn off the QX200 Droplet Reader.

Note

It is possible to perform analyses in the BioRad software, but it may be preferable to perform your own calculations directly on the raw droplet data in Python. The `.ddpcr` file contains all the relevant data and any metadata you inputted using the software, and you can use the `load_ddpcr` function in `rushd` to load this info into a Pandas DataFrame.

References

HCR DNA-FISH

Prepare necessary buffers as described in *FISH buffers* (page **??**)

Estimated time

2 days

Fixation

1. Steps 1-5 of *Immunofluorescent Staining* (page **??**)

Hybridization

1. Preheat Hybridization Buffer (HYB) to 37 degrees Celsius
2. Incubate cells at room temperature for 30 minutes in 500 uL of 20% glycerol in PBS
3. Flash freeze the cells
    a. Place coverslips in liquid nitrogen for 30 seconds
    b. Remove and let thaw for 1 minute

Warning

Liquid Nitrogen can shatter the coverslip; be careful when freezing

4. Incubate cells at RT for 20 mins in 500 uL of 20% glycerol in PBS
5. Repeat Step 4
6. Incubate cells at RT for 5 mins in 500 uL of .1 N HCl
7. Rinse cells at RT with 500 uL of 2X SSC
    a. Repeat x2
8. Incubate cells at 37 degrees C in 500 uL of HYB for 30 mins
9. Remove HYB and add 100 uL of probe+HYB mixture
10. Incubate at 78 degrees C for 5 mins
11. Incubate **overnight** at 37 degrees C in a humid environment

Probe Preperation

1. Preheat Hybridization Buffer (HYB) to 37 degrees Celsius
2. Dilute probe stock with HYB to 10 pmol per 100 uL per reaction
3. Heat probe+HYB mixture at 70 degrees C for 5 mins
4. Chill on ice until use

Post-Hybridization

1. Preheat Hybridization Buffer (HYB) to 37 degrees Celsius
2. Preheat Probe Wash Buffer to 37 degrees C
3. Remove probe+HYB, then wash with 500 uL of Probe Wash Buffer
    a. Repeat
4. Wash at RT with 500 uL of 5X SSCT for 5 mins
    a. Repeat
5. Wash at RT with 500 uL of PBS for 5 mins
6. Proceed to imaging

HCR RNA-FISH

Prepare necessary buffers as described in *FISH buffers* (page **??**). The total amount needed per sample (without technical replicates) is:

Buffer	Total amount per sample
4% PFA	100 μL
0.5% Tween	100 μL
Hybridization buffer	270 μL
Wash buffer	200 μL
Amplification buffer	270 μL
5x SSCT	600 μL

Note

All spins are performed at ~500 rcf for 4 min. Our centrifuge follows RCF = 1e-4*[rpm]^2 + 4e-2*[rpm] - 6e1, where **2200 rpm = 512 rcf**. It is recommended to perform all spins at 4°C once the cells have been fixed to prevent pellet loss.

Warning

Both PFA (used in the fixation) and Formamide (used in the hybridization and wash buffers) are hazardous, so all steps must be performed in the fume hood.

Day 1

Estimated time

3-4 hours

1. Dissociate cells and spin down. Resuspend in PBS and transfer to a 96-well V-bottom plate.
2. Spin down and aspirate PBS. Resusepnd in 100 uL of 4% PFA and incubate for 15 min at room temp.
3. Add 150 uL of PBS, spin down, and aspirate.
4. Resuspend in 100 uL of 0.5% Tween, and incubate for 15 min at room temp.
5. Add 150 uL of PBS, spin down, and aspirate.
6. Add 200 uL of probe hybridization buffer for 30 min at 37 degrees C.
7. Prepare probe solution by adding 1 uL of 1 uM stock to 70 uL of probe hybridization buffer (per sample) at 37 degrees C.
8. Spin down with reduced deceleration, and aspirate hybridization buffer.

Important

The hybridization buffer is viscous, so the deceleration setting on the centrifuge should be lowered to 5 to get a good pellet.

9. Add 70 uL of the prepared probe solution, and incubate samples **overnight (12-16 h**) at 37 degrees C.

Day 2

Estimated time

2 hours

1. Spin down at reduced deceleration, and aspirate probe solution.

Important

The hybridization buffer is viscous, so the deceleration setting on the centrifuge should be lowered to 5 to get a good pellet.

2. Resuspend in 200 uL of probe wash buffer, and incubate for 15 min at 37 degrees C.
3. Spin down, and aspirate wash buffer.
4. Resuspend in 200 uL of 5X SSCT, and incubate for 5 minutes at room temp.
5. Spin down, and aspirate 5X SSCT.
6. Pre-amplify samples in 200 uL of amplification buffer for 30 min at room temp. While waiting, begin *Hairpin Preparation*:
    a. Prepare 9 pmol each of hairpin h1 and h2 by snap cooling 3 uL of 3 uM stock (per sample) at 95C for 90 seconds. Snap cool each individual hairpin in **separate tubes**.
    b. Cool hairpins in the dark at room temperature for 30 minutes.
    c. Prepare hairpin solution by adding h1 and h2 hairpins to 70 uL amplification buffer (per sample).
7. Spin down, and aspirate amplification buffer.
8. Add 70 uL uL of the hairpin/amplification buffer solution, and incubate samples **overnight (12-16 h)** in the dark at room temperature.

Day 3

Estimated time

1 hr

1. Spin down, and aspirate hairpin solution.
2. Resuspend in 200 uL of 5X SSCT, and incubate for 30 min in the dark at room temp.
3. Spin down, and aspirate 5X SSCT.
4. Resuspend in 200 uL of 5X SSCT, and incubate for 5 min in the dark at room temp.
5. Spin down, and aspirate 5X SSCT.
6. Resuspend in 220 uL of PBS. Store at 4 degrees C in the dark until flow cytometry.

KAPA SYBR® FAST qPCR

Protocol

Any existing qPCR assay performed efficiently using standard cycling conditions may be converted to a fast qPCR assay with KAPA SYBR FAST qPCR Kits. Typically, re-optimization of reaction parameters is required.

qPCR is performed at the MIT BioMicro Center on a Roche LightCycler 480 using SYBR Green MasterMix. Roche's documentation is here.

Tip

To do qPCR with HEK293T RNA, use about one million cells as input to the Monarch Total RNA Miniprep Kit and elute into at least 50 μL. Be sure to do the optional "on-column" DNase I treatment to prevent contamination with genomic or plasmid DNA. cDNA can then be prepared using the ProtoScript First Strand cDNA Synthesis Kit with oligo-dT primers. Include a control without reverse transcriptase to ensure qPCR amplification is not coming from residual DNA.

1. Master Mix Preparation

1. Ensure all reaction components are properly thawed and mixed.
2. Prepare a master mix containing the appropriate column of all reaction components common to all or a subset of reactions to be performed.
3. Always include a No Template Control (NTC) to allow for detection of contamination of reaction components.

> **Tip**
>
> If applicable, include a water control and a no reverse transcriptase control.

4. Calculate the required volume of each component based on the following tables:

Component	ROX	No ROX	Final conc.
PCR-grade water	N/A		
KAPA SYBR FAST qPCR Master Mix (2X) Universal (order from BioMicro Center when reservation is made)	10 μL	10 μL	1X
10 μM forward primer	0.4 μL	0.4 μL	200 nM
10 μM reverse primer	0.4 μL	0.4 μL	200 nM
Template DNA	As required	As required	<20 ng
50X ROX High/Low (as required)	0.4 μL	--	1X

2. Reaction Setup

1. Transfer the appropriate volumes of qPCR master mix, template, and primers to each well of a PCR plate/tube(s).
2. Cap or seal the reaction plate/tube(s) and centrifuge briefly. The BioMicro Center has a centrifuge for this.

3. qPCR

1. If applicable, select fast mode on the instrument.
2. Confirm that the qPCR protocol to be used conforms to the following parameters:

Detection Format	Block Type	Reaction Volume	
SYBR Green	96 well	10 - 25 μL	
	384 well	3 - 20 μL	
Program Name	**Cycles**	**Analysis Mode**	
Pre-incubation	1	None	
Amplification	40	Quantification	
Melting Curve	1	Melting Curves	
Cooling	1	None	
Program Name	**Target (°C)**	**Acquisition Mode**	**Hold** (hh:mm:ss)
Pre-incubation	95	None	00:03:00
Amplification	95	None	00:00:10
	Primer dependent	None	00:00:20
	72	Single	00:00:01
Melting curve	95	None	00:00:05
	65	None	00:01:00
	97	Continuous	5-10 acq/ °C
Cooling	40	None	00:00:10

4. Data Analysis

1. Data analysis is dependent on experimental design. Refer to the instrument guidelines for more information on how to perform the appropriate data analysis.

Tip

For analysis of HEK293T RNA, the following primer pairs can be used for normalization ActB: **CTACCTCATGAAGATCCTCACC** and **AGTTGAAGGTAGTTTCGTGGAT** GAPDH: **GTATCGTGGAAGGACTCATGAC** and **ACCACCTTCTTGATGTCATCAT**

Psoralen-qPCR supercoiling assay

Warning

This protocol is deprecated. It did not work well with our original UV crosslinker, and could not measure plasmids.

Note

This protocol was used for the experiments described in Babos and Galloway, Cell Stem Cell, 2019 for measuring DNA supercoiling using psoralen intercalation as a metric of negative supercoiling. Psoralen preferentially incorporates into underwound DNA (negatively supercoiled DNA).

Experiment setup

1. Prepare half of a 6-well **plate** per replicate. For conditions in triplicate, this means **1.5 6-well plates** per condition!
2. Treat cells according to your experiment.
3. If you want to arrest the cell cycle prior to measurement, treat the cells with 1 μM aphidicolin for 1.5 to 2 hours prior to cell harvest and DNA extraction.

Chemical	Concentration	Molar mass	mg / mL
Aphidicolin	1 μM	338.48 g/mol	

Single cell qPCR

Single-cell acquisition

Estimated time

3-5 minutes per cell.

Important

All work should be performed with RNase free reagents!

1. Cells should be plated on the lids of electrophysiology dishes to prevent interferences of dish sides with micromanipulator angle of approach.
2. Label PCR tube strips with numbers to correspond to the number of the cell picked.
3. Add 5 ul of Cells Direct 2X rxn (Cells Direct One-step qRT-PCR kit , Life, Cat# 11753-100) mix to each PCR tube.
4. Pull glass micropipettes with the machine in the Chow lab. Pipettes pulled with 1st T=61.2 °C and 2nd T=47 °C is most optimal.

TODO

Specify glass micropipette diameter and describe machine used in this step.

5. Obtain dry ice in a cooler box for immediate freezing of cell following acquisition.
6. Setup fluorescence on an inverted microscope, imaging at 40X.
7. Identify isolated GFP+ cells with neuronal morphology using bright field and FITC.
8. Use RNAse inhibitor (~3 ul per 30 cells picked) to be placed in each glass micropipette prior to use in single-cell picking.

9. Place pipette in micromanipulator. Position so that the tip comes into the field of view. Careful not to break the tip by lowering the Z position too far.
10. Carefully move the tip to the edge of the cell and aspirate, scooping the cell off the dish and retrieving as much of the cell body and dendrites as possible.
11. Crush pipette tip into 5 ul of 2X Rxn mix to capture cell in solution.
12. Immediately place on dry ice to freeze. Keep on dry ice until ready to store in -80 °C freezer.

Note

Cells dissociate from plate more easily with greater amount of time out of incubator. Average time to acquire a single cell runs from 3-5 minutes.

Lysis, reverse transcription (RT), and specific target amplification (STA)

1. Prepare primer mix
    a. Each primer stock should be 200 uM. (e.g. If primer is 40.4 nM, add 202 uL of RNAse free water (DEPC treated or other).
    b. For bookkeeping it is helpful to label each primer with a letter and keep in order (e.g A-Z then AA- AZ).
    c. Make 20uM stock of forward and reverse primer together (e. g "A F+R" for gene A.) For 250 ul F+R mix, add 25 ul F and 25 ul R and 200 ul of RNAse free water .
    d. For 200 nM (each primer at 200 nM) RT and STA primer mix of 1 mL, add 10 ul of each 20 uM F+R mix and bring to 1000 uL. For 48 genes (480 ul of primer), add 520 ul RNAse free water.

Estimated time

1 hour for steps 2 through 7

2. Remove cells from -80°C and immediately place on ice.
3. Move quickly to 50 oC water bath for 15 sec defrost, then place back on ice.
4. Make RT/STA mix:

Component	Volume/ 1rxn	Volume / 100 rxn
Superscript III/Platinum Taq	0.2 uL	20 uL
200 uM Primer Mix	2.5 uL	250 uL
RNAse-free water	1.3 uL	130 uL
Total	*4 uL*	*400 uL*

5. Add 4 ul of RT/STA mix per tube.

6. Spin down samples in minifuge to collect all liquid.
7. Place in thermocycler.
8. Run thermocycler program (1 hr and 55 min)

#	Time	Temperature (°C)	Name
1	15 min	50	Reverse transcription
2	2 min	95	Initial denature
3•		Cycle 4&5 18 times	Cycle
4	15 sec	95	Denature
5	4 min	60	Annealing and elongation
6	Hold	4	Chill, end

9. Spin down samples in minifuge to collect all liquid. Steps i- n = 1 hr and 40 min)
10. Make Exonuclease I and SAP (Shrimp Alkaline Phosphotase) mix:

Component	Volume/ 1rxn	Volume / 100 rxn
Exonuclease I	0.5 uL	50 uL
SAP	1.0 uL	100 uL
RNAse-free water	2.5 uL	250 uL
Total	*4 uL*	*400 uL*

11. Add 4 ul of Exonuclease I SAP mix per tube.
12. Place in thermocycler, running the following program:

#	Time	Temperature (°C)	Name
1	20 min	37	Digest primers, dNTPs
2	15 min	80	Denature
3	Hold	4	Chill, end

13. Store @ -20 oC.

Single-cell qPCR on Fluidigm Biomark

Estimated time

2 hours for steps 1 through 8

1. Defrost samples on ice.
2. Dilute 5x by adding 52 ul of H20 to 13 ul of sample to make 65 ul of single-cell amplified cDNA (13 ul is standard volume size following above procedure).

3. Make Sso Fast Evagreen super mix (Bio-rad) with 20X DNA binding dye (Fluidigm, green cap):

Component	Volume/ 1rxn	Volume / 96 rxn
Sso Fast Evagreen super mix, low ROX	2.5 uL	480 uL
20X DNA binding dye loading reagant	0.25 uL	48 uL
RNAse-free water	2.75 uL	264 uL
Total	*5.5 uL*	*528 uL*

4. Mix samples in 96-well qPCR plate. Place samples in well corresponding to number to order correctly (e.g. Sample 1= A1, Sample 2= A2... Sample 13= B1, Sample 14= B2...Sample25= C1 etc).

i. Sso Fast/DNA binding Dye mix.....5.5uL/well

ii. Single-cell STA cDNA samples.....4.5 ul/well

5. Cover with qPCR sticky lid. Vortex lightly to mix. Spin down in plate spinner and place on ice.

6. Prepare assays with 2X loading reagent (Fluidigm), F+R primers, and water.

Component	Volume/ 1rxn	Volume / 4 rxn
2X Assay Loading reagent	2.5 uL	10 uL
20 uM F+R for each assay	0.25 uL	5 uL
RNAse-free water	2.25 uL	5 uL
Total	*5.0 uL*	*20 uL*

7. Add 5 uL of each mix to each inlet. Place samples in well corresponding to number to order correctly (e.g. Assay 1 (Gene A)= A1, Assay 2 (Gene B)= A2... etc).

8. Cover assay lids with qPCR sticky lid. Vortex lightly to mix. Spin down in plate spinner and place on ice.

9. PRIME Chip: Add fluid to accumulators by depressing springs and O-rings and injecting control line fluid. Tilting the plate will ensure the fluid goes into the accumulators. Run "Prime" script on Fluidigm Mixer (HX for 96.96 and MX for 48.48.)

Note

Special thin tips are to be used on this device compatible with loading 384-well plates. Using the 96-well plate spacing for tips more compatible with 96 well plates assays and sample preparation and requires using the Chip pipetting map to quickly and correctly load the assays and sample inlets. It is recommended to aspirate 5.2 uL and dispense 5 uL to avoid introducing air bubbles. By preparing extra assay and sample mix in the 96 well plate, air bubbles should be avoided using this pipetting method.

10. Use electronic multi-channel pipette to add 5 uL to each well.

11. Following addition of all samples and assays, follow the detailed protocol for loading the Chip on the IFC mixer and then running the Chip on the Biomark HD. (See below). Note: The Camera requires time to turn on (~20 min) make sure to turn on early. Also, loading the Chip takes ~ 1 hr 20 min and running the chip takes ~1 hr and 30 min.

Pipetting Map for 48.48 Pipetting Map for 96.96

Note

Consult Chip Pipetting Map (PN 68000130, REV B) to optimize efficiency of set-up for pipetting with multi-channel pipette. More detail on Page 5 and 6. (ref: http://openwetware.org/images/5/52/Fluidigm_96.96_RT-_PCR_Quick_Reference_.pdf)

Western Blot

Lysis

Materials:

- 10X Cell Lysis Buffer from CST
- 200mM PMSF (200X concentrated)
- Cell Scraper
- Blunt 21 gauge needles
- Lock-joint syringe
- Cells to lyse

Note

- If 10X cell lysis buffer will be continually used, it is recommended that the 10x buffer be kept at 4°C for 1-2 weeks. For longer periods of time, buffer should be stored at -20°C. Aliquoting of 10x buffer is recommended if many small experiments are to be performed.
- PMSF is unstable in water and should be added to lysis buffers or other aqueous solutions just prior to use. Typically, a final concentration of 1 mM provides sufficient protease protection. Store lyophilized at RT for 24 months protected from direct light. Once in solution, PMSF can be stored at -20ºC for up to 3 months, protected from light.

Protocol:

Important

All reagents and lysates must be kept cold. In addition, **keep plates on ice** during lysis step.

1. Cool microcentrifuge to 4°C.

2. Dilute 10X Cell Lysis Buffer to a 1X solution using Elga water.
    - Can use either 10X lysis buffer or RIPA in -20°C "Western Reagents" box.
3. Chill 1X buffer on ice and add PMSF just prior to use.
4. Wash plate with ice-cold PBS to remove residual media.
5. Add 400 uL of 1X lysis buffer to the 10cm dish of cells.

Solution	Vol for 1x10cm	Vol for 3x10cm
ELGA water	358 μL	1,170 μL
10X cell lysis buffer	40 μL	130 μL
200 mM PMSF	2 μL	6.5 μL

Note

BAD has also used 6-well plates to make cell lysate instead of 10cm dishes. Volume of 1x lysis buffer is scaled by surface area, so 68uL of lysis buffer is required per 6-well.

6. Tilt to coat bottom of dish, then incubate on ice for 5 minutes.
7. Scrape cells with a cell scraper and transfer lysed solution to a small centrifuge tube.
8. Use 21 gauge needles to shear the cells 5-6 times (change out needle so 1 needle/sample).
9. Spin extract 10-12 minutes at 14,000 x g (~11,500 rpm) in the 4°C microcentrifuge.
10. Remove supernatant for use and aliquot.

- You should have ~500-600 μL per 10cm dish (or about 70-80 uL per 6-well).
- **~50-60 μL/aliquot is good because you can load 25 μL/well (12.5 μL lysate + 12.5 μL 2X Laemmli)**
  so 50 μL will give enough for 4 wells (i.e. 4 antibodies).
- BAD uses 20-25 uL/aliquot for lysates from a 6-well since 12.5 uL/well (6.25 uL lysate + 6.25 uL 2X Laemmli) has be sufficient for detecting proteins.
- Also make a 2-3uL aliquot to measure protein concentration in a Bradford assay.

Tip

- Use prepared lysates as quickly as possible, and store for as short a time as possible.
- Store lysates at -80°C for as long as possible. For lysates that will need to be kept around long term, transfer freshly prepared tubes to an available -80°C freezer to prevent degradation.
- Lysates have a shorter shelf life when stored at -20°C; long-term storage at this temperature is not recommended. CST recommends that lysates are stored at -20°C for no longer than 3 months.

- Minimize your freeze/thaw cycles as much as possible. Instead, aliquot into smaller volumes.
- BAD has seen a drastic reduction in detected protein (especially phosphorylated proteins) after thawing from -80°C for a second time. Only thaw an aliquot from -80°C once.

Lysis for phosphatase treatment

Note

This protocol for phosphatase treatment of cell lysate requires further troubleshooting.

In the normal RIPA or CST Lysis Buffer, the Na3VO4 (Sodium Orthovanadate) and EDTA components can inhibit lambda protein phosphatase (NEB #P0753). Therefore, a different lysis buffer lacking these components is required.

Lambda Phosphatase-compatible lysis buffer:

1 M Tris-HCl stock solution, adjust to pH7.5

Component	Final concentration	Amount Needed
Tris-HCl	1M	0.394 g
DI Water		2.5 mL

5 M NaCl stock solution

Component	Final concentration	Amount Needed
NaCl	5M	0.731 g
DI Water		2.5 mL

200 mM (200x) DTT (dithiothreitol) stock solution (aliquot at store at -20°C)

Component	Final concentration	Amount Needed
DTT	200 mM	25 mg
DI Water		810 μL

Warning

DTT stock should be collected as a separate waste stream.

Lysis Buffer (aliquot and store at -20°C)

Component	Final concentration	Amount Needed
1M Tris-HCl	50 mM	250 μL
5M NaCl	150 mM	150 μL
Triton X-100	1%	50 μL
DI Water		To 5 mL

Right before use add 200x DTT and 200x PMSF to this lysis buffer. Lyse cells following above protocol.

Phosphatase Treatment

1. After measuring protein concentration using Bradford Assay (see protocol below), dilute samples to equal concentration using the phosphatase-compatible lysis buffer with PMSF and DTT.
2. To each sample, add 10x buffer for PMP and 10x MnCL2 to reach a 1x concentration.
3. Split each sample into 2 equal volumes: 1 for non-treated, 1 for phosphatase treated.
4. Add phosphatase to reach ~16 U/μL (stock from NEB is 400 U/μL) in samples designated for phosphatase treatment.
5. Incubate for 30 minutes at 30°C.
6. Mix 1:1 with laemelli buffer.
7. Incubate for 5 minutes at 95°C for 5 minutes to denature proteins.
8. Continue on to loading and running the gel protocol as usual.

Note

Verify the protocol worked by blotting for a phosphorylated protein (such as ppERK) and comparing the - and + phosphatase samples.

Bradford Assay

Introduction

The Bradford Assay is used for protein concentration quantification. It utilizes Coomassie Brilliant Blue G-250 dye which binds to proteins and shifts the maximum absorption from 470nm to 595nm. This absorption at 595nm can be measured using the NanoDrop. The protein concentration can be estimated by creating a standard curve and using Beer's law.

Materials

- Bradford Assay Reagent
- BSA Standard (2 mg/mL)
- Lysis Buffer
- Lysate Samples

Important

Ensure that the Bradford assay kit is compatible with the lysis buffer.

For example, the Bradford assay should be compatible with RIPA lysis buffer but is not compatible with the CST Cell Lysis Buffer due to high amounts of Triton.

See Bradford Assay Documentation[89] for the full list of compatible reagents.

BAD has actually seen that neither RIPA or CST lysis buffer are compatible with Bradford assay without additional dilution, despite what the documentation says.

Protocol

1. Remove Bradford Protein Assay Reagent from the refrigerator and gently mix by inverting the bottle several times.
2. Aliquot the required volume of the dye reagent and allow it to equilibrate to room temperature before use.
3. Prepare diluted BSA protein standards. Be sure to dilute the supplied BSA standard (2000 ug/mL) in the same buffer as the test samples (e.g. 1x lysis buffer). Use the tables below as a guide for preparing a set of protein standards.

For a working range of 100 to 1500 ug/mL:

Tube #	Buffer/Diluent Vol. (uL)	BSA Standard (2 mg/mL) Vol. (uL)	Final Protein Conc.
Blank	80	0	0
1	0	80	2000 ug/mL
2	20	60	1500 ug/mL
3	40	40	1000 ug/mL
4	50	30	750 ug/mL
5	60	20	500 ug/mL
6	70	10	250 ug/mL
7	75	5	125 ug/mL
8	79	1	25 ug/mL

For a working range of 1 to 25 ug/mL:

[89] https://geneseesci.com/shop-online/product-doc/18-442?doc_id=1

Tube #	Buffer/Diluent Vol. (uL)	BSA Standard (2 mg/mL) Vol. (uL)	Final Protein Conc.
Blank	800	0	0
1	790	10	25 ug/mL
2	792	8	20 ug/mL
3	794	6	15 ug/mL
4	796	4	10 ug/mL
5	798	2	5 ug/mL
6	799	1	2.5 ug/mL
7	799.5	0.5	1.25 ug/mL

Tip

- BAD dilutes the BSA standard in water. In order for the lysate to be in the 25-2000 ug/mL range, it needs to be diluted 10-20x. Because the RIPA and CST lysis buffers are not compatible with the Bradford assay, they also need to be diluted further so the components do not interfere with the assay.
- BAD recommends diluting lysates by 10x in water. This has worked well previously with the BSA standards also diluted in water.
- BAD also recommends making 2-3 wells of each condition to have standard and sample measurements in duplicate/triplicate.

4. Combine each standard and unknown sample with the Bradford Reagent.
    - For a working range of 100-1500 ug/mL, pipette 10 uL of each standard or unknown sample into a 96-well. Add 200 uL of the Bradford Protein Assay Reagent and mix well.
    - For a working range of 1-25 ug/mL, pipette 100 uL of each standard or unknown sample into a 96-well and add 100 uL of the Bradford Protein Assay Reagent and mix well.
5. Incubate at room temperature for 10 minutes.
6. Proceed to measure absorbance at 595nm. This can be done with the NanoDrop (*not recommended*), or borrowing the Coley Lab plate reader.
7. NanoDrop instructions:
    a. Select the Proteins tab and then Bradford Assay.
    b. Enter the concentrations of each BSA standard and select the number of replicates.
    c. Measure the absorbance of of each BSA standard as directed by the NanoDrop to construct the standard curve.
    d. Measure the absorbance of each sample. The NanoDrop will automatically calculate the protein concentration for you based on the standard curve.
8. Plate reader instructions:

a. (*Recommended*) Contact someone in the Coley lab to ensure no one else is using the plate reader at the desired time.

b. Turn on the plate reader by flipping the switch on the back of the instrument.

c. On the computer, open the Magellan Pro application.

d. Select "Start Measurement" and then click the Next button (with green triangle) in the lower right of the screen.

e. Select "Use Predefined Method". The saved method for the Bradford assays is located under Magellan / mth / 595nm_Bradford.mth.

f. Put the plate in the plate reader and close the door. The plate holder can be opened and closed either using the software, or by using the eject button on top of the instrument.

g. Click start, and the plate reader will measure the whole plate.

h. Copy the data to Excel. This can be done by selecting the well layout in the software and directly copy/pasting into Excel, or clicking Edit + Copy to Excel from the ribbon at the top of the page.

i. You can save the Excel file to a folder on the Desktop (e.g. Desktop/Galloway/Brittany/experiment1) and email it to yourself.

j. When you are finished, take your plate out of the plate reader, turn it off, and close the software.

Note

Sometimes the Magellan Pro software can't find the instrument. If this happens, try the troubleshooting below. If this doesn't work, contact the Coley lab for help.

1. Open the "Device Manager" application.
2. Under libusbK USB Devices, right click on Tecan Device and select update driver.
3. Browse computer, and select Tecan i1000Pro Device from the available list.
4. Sometimes it will then ask you to restart the computer.
5. Turn the plate reader off and on again.
6. You may have to repeat these steps several times before it will work.

1. Dispose of the Bradford reagent in the proper waste container.
2. Analyze the results.

   a. Subtract the average 595nm measurement for the Blank replicates from the measurements of all other individual standards and samples.

   b. Create a standard curve by plotting the average Blank-corrected standard curve measurements on the y-axis and known concentrations on the x-axis.

   c. Fit a line to the standard curve. A quadratic or best-fit curve will be more accurate than a purely linear fit.

d. Use a non-linear solver to solve for the concentrations of each sample based on the fitted equation.

e. Remember to account for the initial dilution of the lysate samples in water in the final calculation.

Note

- BAD's MEF lysate concentrations have ranged from 2,500 ug/mL (NIL) to 20,000 ug/mL (NIL RIDD).
- In order to load equal volumes and total amounts of protein for a Western Blot, BAD dilutes all samples to the lowest concentration using more lysis buffer right before mixing with laemmlli buffer and denaturing the samples.

Protein Gel Casting

Modified for a Western Blot from this protocol[90].

Required stock solutions

- **40% Acrylamide stock solution**: Solution of monomers for gel polymerization.

  We find it cheaper to buy premixed 40% stock solution, with a acrylamide:bis-acrylamide ratio of 29:1 (3.3%). Stocks with a 37.5:1 ratio also work, and are typically used for resolving larger proteins.

- **3x bis-Tris gel buffer**: Ion buffer used in gel casting.

Component	Concentration	g/L to final concentration
bis-Tris	1 M	209.242
HCl	Add to pH 6.5-6.8	

- **10% APS**: One of the polymerization initiators. Only a small quantity needs to be prepared; each gel only requires 35 uL. Make fresh each time by dissolving in water. This must be made fresh.

Component	Concentration	g/L to final concentration
Ammonium persulfate	10%	100 (For example: 10mg/100uL or 100mg/1mL)

[90] https://gallowaylabmit.github.io/protocols/en/latest/protocols/protein_production/bis_tris_protein_gels.html

Casting protocol

Warning

The acrylamide monomers used here are toxic. Read the SDS[91].

Perform polymerization steps with a lab coat in a fume hood, and collect rinse waste in a waste container.

1. Prepare 1X resolving and stacking buffers. These buffers can be stored in the refrigerator for several weeks. Recipes given here for enough for 2 0.75mm gels.

   Resolving buffer: ~3 mL per gel (6.5 mL total). Final acrylamide concentration depends on desired protein size:

Protein Size	Gel %	Vol 40% Acrylamide Stock	Vol DI Water	Vol 3x bis-Tris gel buffer
4-40 kDa	20%	3.25 mL	1.05 mL	2.2 mL
12-45 kDa	15%	2.44 mL	1.86 mL	2.2 mL
10-70 kDa	12.5%	2.03 mL	2.27 mL	2.2 mL
15-100 kDa	10%	1.63 mL	2.67 mL	2.2 mL
25-100 kDa	8%	1.30 mL	3.00 mL	2.2 mL

   Stacking buffer: ~1.2 mL per gel (2.5 mL total):

Component	Volume	Final concentration
3x bis-Tris gel buffer	0.83 mL	1x
40% Acrylamide stock	0.32 mL	5%
DI water	1.36 mL	
Bromophenol blue		50 uL (enough to give color which helps when loading)

Gel casting setup

In-lab, we have the ability to cast two gels simultaneous; this is recommended even if you only need one, so that you have a backup in case of pouring mishaps. Our gel runner also requires two poured gels to properly seal. We use this BioRad gel casting setup and gel runner[92].

1. Locate two 0.75mm spacer plates and two short glass plates.
2. Use ethanol and a Kimwipe to clean both glass surfaces. It is important that these are very clean.

[91] https://www.fishersci.com/store/msds?partNumber=BP14081&productDescription=ACRYLAMIDE%3ABISACRYLAMIDE+29%3A1&vendorId=VN00033897&countryCode=US&language=en

[92] https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/10007296D.pdf

3. Assemble them in the green alignment device. Make sure the bottom of the glass plates align well by using a flat surface.
4. Lock the two gels into the transparent gel pouring device. If leaking is an issue, you can add a parafilm layer between the glass molds and the grey gaskets.

Resolving gel

Tip

The resolving gel can polymerize within a just minute or two, especially at higher percentages of acrylamide. Therefore, pour the gel quickly using a P1000 pipette.

It is best to pour the gel from the edges of the gel mold to avoid bubbles.

Get ~10 mL isopropyl alcohol (IPA) ready before pouring the resolving gel to help keep the gel interface straight and level.

1. Prepare fresh 10% APS. 1 gel requires ~35 uL so if making 2 gels, prepare ~100 μL (10 mg).
2. Measure 6.5 mL of **1X resolving buffer** per gel to pour.
3. Add 50 uL of **10% APS** per gel, mix well.
4. Add 20 uL **TEMED**, mixing quickly (don't pipette mix, just flip it x3 manually to mix).
5. Pour both gels to the resolving gel height (3 mL per gel, 1,000 μL at a time).
6. Ideally there shouldn't be bubbles, but if so, lightly tap and tilt the gel to remove
7. Once done pouring, quickly but carefully fill the remaining height with IPA, making sure the gel-water interface stays undisturbed. This is to ensure the resolving-stacking interface is straight and level.
8. Wait for the polymerization reaction to finish (noticeable by a change in refractive index).
9. Drain the IPA by tilting the gel past 90 degrees, and wicking away with a Kimwipe.

Stacking gel

1. Measure 2.5 mL of **1X stacking buffer** to pour.
2. Add 20 uL of **10% APS**, mix well.
3. Add 10 uL **TEMED**, mixing quickly (don't pipette mix, just flip it x3 manually to mix).
4. Add 1,000 μL of stacking gel into each gel.
5. Insert the comb into the top very carefully, one edge at a time to avoid bubbles. The stacking gel will overflow.
6. If any bubbles, pop comb slightly up near problem area and use remaining buffer to fill before closing again.
7. Wait for the stacking gel to polymerize.

8. Rinse with water or IPA (evaporates faster) to remove unpolymerized acrylamide.
9. If removing the combs prior to storage, slowly remove the comb, ensuring that wells are not broken.

Loading and Running the Gel

Modified for a Western Blot from this[93] protocol.

Solutions required

- **20x MES-SDS running buffer stock solution**: Suitable for separating proteins with a molecular weight less than 75 kDa.

  It is also generally cheaper to order this as a pre-mixed 20x stock solution. If you need to make it yourself, the recipe is:

Component	Final concentration	g/L to final concentration
MES	1 M	195.2 g
Tris	1 M	121.13 g
EDTA	20 mM	5.845 g
SDS	2%	N/A

- **200x running buffer reductant**: Ensures that the gel remains under reducing conditions when run. Add directly to 1x running buffer before filling the gel tank. Dissolve sodium bisulfite in DI water.

> **Note**
>
> Dilute sodium bisulfite solution loses effectiveness in ~2 days so spike in fresh each time.
>
> This helps because although β-mercaptoethanol in the Laemmli buffer is a strong reductant that prevents crosslinking via reduction of disulfide bonds, over time it can degrade.

Component	Final concentration	g/L to final concentration
Sodium bisulfite	1 M	104.061 g

- **200 mM Tris-HCl stock**: Dissolve components in DI water.

Component	Concentration	g/L to final concentration
Tris-HCl	200 mM	31.52 g
NaOH	Add to pH 6.8	

[93] https://gallowaylabmit.github.io/protocols/en/latest/protocols/protein_production/denaturing_protein_gel.html

- **20% SDS stock**: At low temperatures, the SDS may fall out of solution. Therefore, warm in a water bath to dissolve. Mix well before transferring.

Component	Concentration	To make final concentration
Sodium dodecyl sulphate	20%	2g / 10 mL DI water

- **0.1% bromophenol blue**: 1 mg / mL
- **2x Loading Buffer (Laemmli Buffer)**: Used to denature and solubilize protein samples. Can be stored at 4°C.

Component	Final concentration	Volume
200 mM Tris-HCl stock	100 mM	5 mL
Glycerol	20%	2 mL
20% SDS stock	4%	2 mL
0.1% bromophenol blue stock	0.01%	1 mL
2-mercaptoethanol	10%	1.1 mL

Warning

2-mercaptoethanol smells awful; always add it inside a fume hood.

2-mercaptoethanol is hazardous waste and should be disposed of in the proper waste container.

Running procedure

1. Add **2x Laemmli Buffer** to an equal volume of lysate in PCR tubes. 50-60 μL is good for ~4 lane (need 12.5 μL lysate/lane) This is recommended unless the online antibody datasheet indicates that non-reducing and non-denaturing conditions should be used.
2. Use a PCR machine to reduce and denature the lysate samples at 95°C for 5 minutes (use 4°C hold at end to keep cold).
3. Dilute enough **20x MES-SDS running buffer** to fill the gel tank, adding fresh **200x running buffer reductant** if a gel has not been recently run.
4. Place a prepared bis-Tris protein gel in the gel-runner. Fill both chambers with the prepared 1x MES-SDS running buffer. Fill the inner chamber to the top of the stacking gel, and the outside chamber to the top of the resolving gel. You will need about 1 liter of the 1x MES-SDS running buffer.
5. Carefully load equal amounts of protein samples, including 5 μL of a protein ladder, into the wells of the gel. Each well can be loaded with a maximum of 25 uL. 10-30 ug of total protein from cell lysate is generally used unless further optimization is needed for the desired protein(s).
    - The protein ladder is in the -20°C fridge in the restriction enzyme ice box

> **Note**
>
> BAD has used 15-20ug of protein per well.
>
> BAD tried freezing Laemmli buffer-denatured lysate at -20°C and it worked for Western

> **Tip**
>
> Choose an asymmetric loading pattern so if the gel is flipped over, you will still know the order of your samples.

> **Warning**
>
> The glass gel holders have directionality! If your gel isn't reaching 30 mA check that the open side is facing inwards.

6. Run the gels at constant current, about 30 mA (~43V) per mini-gel for approximately 125 minutes. The dye band runs around 3-5 kDa, so it is typically ok to run the dye band to the bottom of the gel unless very small proteins are of interest.
    - Rinse gel holder and runner with water to help reduce smell

> **Note**
>
> BAL has run the gel for up to 180 minutes and found this helps separate out some of the larger proteins such as pERK which has bands at both 42 and 44 kDa.

7. Pour DI water into a plastic tray (tip box lid), about half a centimeter deep.
8. Very carefully separate the gel plates without breaking the gel. The gel will stick to one side or the other.
9. With a razor blade, cut off the stacking portion of the gel while still on glass.
10. Invert the plate/gel over the water and "convince" the gel to fall into the dish. It can help to put the gel and plate into the water and let the solution help the gel release. Using a green gel scraper can also help with this process.
11. Place the gel on a rocker for 2-5 minutes to remove excess free proteins.

Coomassie Staining

Solutions required

- **Coomassie staining dye**: When preparing this dye, pour the 10% methanol first, using it to dissolve the R-250. Then, add water. Add the glacial acetic acid last to prevent aggregation.

Component	Final concentration	Amount per 1 liter
Coomassie R-250	0.2% (2g/L)	2g
Methanol	10%	100 mL
Water	80%	800 mL
Acetic acid	10%	100 mL

- **10% Acetic Acid**: Used as a destain solution.

> **Warning**
>
> Do not microwave pure acetic acid.

Procedure

1. Drain the water from the dish without dropping your gel in the sink, and cover with ~0.5 cm of Coomassie staining dye.
2. Place the gel in stain in the microwave and microwave on high until the solution just begins to boil (this step greatly accelerates the procedure and allows you to see you bands in a minute or so). This only takes 20-30 seconds in the microwave.
3. Remove from the microwave and place on a rocker for a few minutes. Once you see the gel filled with Coomassie, it's done.
4. Drain the Coomassie and cover the gel with water, rock for about 5 minutes, drain.
5. Cover with **10%** acetic acid, place a couple folded Kim-wipes over the gel, and microwave again until the solution begins to boil (20-30 seconds).
6. Remove from microwave and rock to remove Coomassie not bound to protein. If there is excess stain, replace the 10% acetic acid and Kim-wipes and continue to rock until the gel is clear with dark purple protein bands.

Transferring the protein from the gel to the membrane

> **Tip**
>
> Proteins in the gel can be transferred to a membrane using the iBlot2. It is recommended to watch this iBlot2 tutorial video[94] to learn how to use this device. The iBlot2 manual[95] is another helpful resource for learning to use the iBlot2 and contains more detailed instructions.

1. Open the lid of the iBlot2 device using the latch. Ensure the blotting surface is clean. Wipe down electrical contacts.

[94] https://www.youtube.com/watch?v=g7DT5xGheCE
[95] https://tools.thermofisher.com/content/sfs/manuals/iblot2_device_man.pdf

2. Unseal the iBlot™ 2 Transfer Stack.
3. **Separate the Top Stack and set it to one side of the bench with the transfer gel layer facing up.**
   Keep the Bottom Stack in the transparent plastic tray. Top and bottom stacks are divided by a separator. IMPORTANT: Ensure the membrane is not stuck to the separator and is with the bottom stack.
4. Place the Bottom Stack with the plastic tray directly on the blotting surface.
5. Ensure there are no bubbles between the membrane and the transfer stack. Remove any trapped air bubbles using a roller such as a plastic conical.
6. Carefully separate the glass plates surrounding the gel so the gel is left on one of glass plates. Using a razor blade, cut off the stacking region of the gel. Then carefully remove the gel from the glass into a dish filled with 1 cm of DI water.
7. Place the pre-run gels on the transfer membrane of the Bottom stack. Note: two gels can fit onto a single membrane.
8. Use a roller to remove any air bubbles between the gel and the membrane.
9. Soak the iBlot Filter Paper from the transfer stack in a clean container of deionized water.
10. Place the presoaked iBlot Filter Paper on the pre-run gel. Use a roller to remove any air bubbles between the filter paper and gel.
11. Remove and discard the white plastic separator from the Top stack.
12. Take the Top Stack from the bench and place it on top of the presoaked filter paper with the copper electrode facing up (and transfer gel layer facing down). Remove any air-bubbles using a roller.
13. Place the iBlot™ 2 Absorbent Pad on top of the iBlot™ 2 Transfer Stack such that the electrical contacts are aligned with the corresponding electrical contacts on the blotting surface of the iBlot™ 2 Gel Transfer Device.
14. Use the Blotting Roller to flatten any protrusions in the transfer stack.
15. After assembling the iBlot™ 2 Gel Transfer Stack, perform blotting within 10-15 minutes of assembling the stacks with the gel.
16. Gently close the iBlot™ 2 Gel Transfer Device lid by pressing down with two hands on the sides of the lid. Make sure the latch is secure. Do not forcibly push the lid when closing, because it can cause the transfer stack or metal contacts to shift out of position.

> **Note**
>
> If the iBlot2 device says the transfer stack is not detected, try opening and closing the lid until you are able to start the program. Cleaning the electrical contacts before and after each use may help with this issue.

17. Ensure that the correct Method is selected.

Method	Voltage	Default Run Time	Run Time Limit
P0	20 V for 1 min 23 V for 4 min 25 V for 2 min	7 min	13 min
P1	25 V	6 min	10 min
P2	23 V	6 min	11 min
P3	20 V	7 min	13 min
P4	15 V	7 min	16 min
P5	10 V	7 min	25 min

Note

See page 17 of the iBlot2 manual[96] for more detailed information about running parameters.

Transfer protocol P0 with default run time has worked previously when blotting for Beta-actin, WT-p53, and RAS.

For proteins from 30 to 150 kDa method P0 for a 7 minute run time is recommended. For proteins >150 kDa methods P0 or P3 with a run time of 8-10 min is recommended.

BAD has used a transfer protocol of 23 V for 3 min and 20 V for 2 min to detect p53DD (~11 kDa) and WT p53 (53 kDa).

18. Touch the Start icon on the screen to begin the transfer.
19. At the end of the transfer, the current automatically shuts off and the iBlot™ 2 Gel Transfer Device signals the end of transfer with repeated beeping sounds, and a message on the digital display.
20. Touch the Done icon to stop the beeping.
21. To obtain good transfer and detection results, open the device and disassemble the stack within 30 minutes of ending the blotting procedure.
22. Open the lid of the iBlot™ 2 Gel Transfer Device.
23. Discard the iBlot™ 2 Absorbent Pad and Top Stack.
24. Carefully remove and discard the gel and filter paper. Remove the transfer membrane from the stack.
25. If needed, cut the membrane with a razor blade or scissors to separate the regions corresponding to each gel.

[96] https://tools.thermofisher.com/content/sfs/manuals/iblot2_device_man.pdf

Antibody Staining

Solutions required

- **10X Tris-Buffered Saline (TBS)**: Add ~450 mL of DI water to dissolve the Tris and NaCl. Adjust to a pH of 7.6. Then add the remaining DI water to reach a final volume of 500 mL.

> **Note**
>
> Took ~8-9 mL 12N HCl to get to pH ~ 7.6

Component	Final concentration	Amount Needed
Tris-base	200 mM	12 g
NaCl	1500 mM	44 g
DI Water		To 500 mL

- **10% Tween20**:

Component	Final concentration	Amount Needed
Tween20	10%	1 mL
DI Water		9 mL

> **Note**
>
> Larger volumes of Tween20 are easier to measure because it is very viscous.

- **1x Tris-Buffered Saline / Tween (TBST)**:

Component	Final concentration	Amount Needed (50 mL)	Amount Needed (1 L)
10X TBS	1X	5 mL	100 mL
10% Tween20	0.1%	0.5 mL	10 mL (or 1 mL Tween-20)
DI Water		To 50 mL	890 mL (or 900 mL)

- **Blocking Buffer**:

Component	Final concentration	Amount Needed
Milk Powder	5%	2.5 g
10% Tween20	0.1%	0.5 mL
10x TBS	1X	5 mL
DI Water		To 50 mL

- **10% Blocking Buffer**: For diluting primary and secondary antibodies.

Component	Final concentration	Amount Needed
Blocking Buffer	10%	5 mL
1x TBST (TBS/0.1% Tween-20)		45 mL

Staining Procedure

> **Note**
>
> A 10 cm dish works well for the wash steps.

1. Wash the membrane with DI water for 5 minutes using agitation.
2. Block the membrane with blocking solution for 30-60 minutes at room temperature with agitation. Alternatively, block overnight at 2-8°C. (NW does 60 min at RT).
3. Incubate the membrane with 4 mL/10 cm of primary antibody diluted (at manufacturer's recommended dilution) in 10% blocking solution overnight at 2-8°C.
4. Wash the membrane 3 times for 10 minutes each in TBST using agitation to remove any unbound primary antibody.
5. Incubate blot with 4 mL/10 cm of secondary antibody HRP-conjugate at a 1:10,000 dilution (or at the manufacturer's recommended dilution) for 30 minutes to 1 hour at room temperature using agitation. (NW does 1 hr at RT)

> **Note**
>
> BAD recommends starting with 1:20k for 1°Ab and 1:50k for 2°Ab. BAD uses 1:50k for both 1° and 2° for beta-actin. This is because the SuperSignal West Femto Substrate works better with very diluted antibodies.

6. Wash the membrane 6 times for 5 minutes each in TBST to remove any unbound secondary antibody conjugate. It is crucial to thoroughly wash the membrane after incubation with the HRP enzyme conjugate.
7. Prepare the SuperSignal West Femto Substrate[97] working solution by mixing equal parts of

[97] https://www.thermofisher.com/order/catalog/product/34094

the Substrate and Stable Peroxide components (e.g. 5 mL substrate with 5 mL stable peroxide). Use a sufficient volume (~2-3 mL/10 cm) to ensure the blot is completely wetted with the substrate and does not become dry.

Note

The working solution is stable for up to 6-8 hours at room temperature.

8. Incubate the membrane with the substrate working solution for 5 minutes.
9. Remove the blot from the working solution and place it in a labeled, clear plastic bag, and remove excess liquid with an absorbent tissue.
10. Image the blot using chemiluminescence. The membrane does not need to be removed from the clear plastic bag for imaging. The Niles Lab in BE has a ChemiDoc Imaging System that they let us use, and images can be transferred using a USB flash drive.
11. Blot quantification can be done using the Gel Analyzer tool[98] in ImageJ.

Note

Use colorimetric for a black/white photo that you can merge with the chemiluminescence photo

Note

NW uses optimal auto-rapid as default

Note

BAD has used a fluorescent secondary antibody instead of chemiluminescence since the ChemiDoc Imaging System can detect various fluorescent channels. This method seems to be slightly less sensitive and requires a high concentration of antibody for protein detection. Anti-rabbit secondary antibodies seem to bind to the protein ladder. Keep blots protected from light after adding the secondary antibody during staining. Online protocols suggest that it is important to make sure the membrane is dry before imaging.

BAD has been able to re-probe a fluorescent blot for chemiluminescence. You can rewet the membrane by first using 70% ethanol and then washing with TBST because the PVDF membrane does not absorb aqueous solutions uniformly unless prewet. Once rewet, you can proceed to re-probe the membrane.

[98] https://alfresco.uclouvain.be/alfresco/service/guest/streamDownload/workspace/SpacesStore/62eef827-f095-4bfd-b607-e0688df2317c/ImageJ%20-%20western%20blot%20quantification.pdf?a=true&guest=true

3.2 Cloning protocols

3.2.1 Cloning Workflow Overview

Molecular cloning is the process by which we construct synthetic DNA sequences, aka how we build plasmids in lab. (Since, alas, we do not have cheap, multi-kilobase-scale DNA printer. Perhaps someday soon!) The broad outline of the workflow is described below and links to specific, relevant protocols at each step. You might also want to check out the *tips, tricks, and troubleshooting guide* (page **??**).

Step 1: Generate DNA fragments

To begin, we first must generate sufficient copies of the DNA fragments we want to stitch together, possibly with defined 5' and 3' ends. Typically, these fragments are generated from existing sequences in lab, though they can also be ordered from DNA synthesis companies.

Methods of DNA fragment generation include:

- *PCR* (page **??**), with or without new ends added by primer overhangs
- *Restriction digest* (page **??**)
- *Annealing oligos* (page **??**) synthesized by an external company (e.g., Azenta/Genewiz)
- Ordering a long dsDNA sequence from an external company (e.g., gBlock from Azenta/Genewiz or Twist)
- *Prepping* (page **??**) an existing plasmid to use directly as a fragment (e.g., pPV plasmid for our *Golden Gate cloning scheme* (page **??**))

Step 2: Assemble plasmids

Next, fragments are assembled into plasmids, circular pieces of DNA typically on the order of 1-15 kilobases. Plasmids are the primary means by which we deliver DNA (or produce viruses to deliver DNA) to mammalian cells. Assembly occurs in a biochemical reaction in a tube (i.e., *in vitro*, for everything we do currently), and efficiencies of these reactions vary widely.

Methods of plasmid assembly include:

- *Ligation* (page **??**)
- *Gibson Assemby (aka Hifi)* (page **??**)
- *Gateway Assemby* (page **??**)
- *Golden Gate Assembly* (page **??**)

Step 3: Transform bacteria

The product of the assembly reaction is then *transformed* (page **??**) into bacteria. This serves two functions:

1. To select for correctly formed products via antibiotic or other selection methods.

Bacteria are plated on agar containing antibiotic(s) for which the desired plasmid confers resistance. Non-transformed bacteria and bacteria transformed with assembly products (or original reactant plasmids) containing the wrong resistance marker die.

Another selection method is the ccdB protein: when expressed from a plasmid, this protein kills the host cell. When you design cloning such that the ccdB expression sequence is removed in your desired plasmid, you can select against cells harboring the original plasmid or other incorrect assembly products. This strategy is used in Gateway cloning.

2. To generate single clones of bacteria harboring plasmid products for screening.

   Transformed bacteria are plated at a density sparse enough to allow single clones to be isolated. This may mean scaling down the reaction for highly efficient assemblies, or scaling up the reaction (e.g., using more cells, increasing the reaction volume) for very inefficient reactions. That way, you can propagate a single, correct plasmid and isolate it from other products.

Step 4: Screen colonies

Next, single colonies are screened to determine whether they contain the desired plasmid. While the selection step eliminates many incorrect plasmid products, it may not be possible by selection alone to identify correct clones (e.g., when a plasmid used as a template for generating fragments has the same resistance marker as the desired plasmid, or when there are multiple fragments to assemble).

The standard approach for screening colonies is *colony PCR* (page **??**), since the tiny amount of plasmid DNA present in a small bacterial colony is sufficient to generate results. Other screening approaches, such as plasmid digestion, typically require larger volumes of DNA and are therefore more common as a secondary confirmation metric. Of course, screening at this stage can be skipped, but this is only advisable for extremely efficient reactions (such as Golden Gate assembly of pShip plasmids without internal cut sites), or if reagent/sequencing/time costs are not an issue.

As colonies are screened, a portion of the colony is often reserved to enable multiple bacterial cultures to be grown easily. For colony PCR, this involves streaking the implement used to pick the colony (e.g., a toothpick or pipette tip) on a fresh agar plate. Alternatively, for large colonies, a portion of the colony may be picked for screening, leaving a portion remaining on the plate for later use.

Step 5: Purify plasmid DNA

To obtain sufficient quantities of the putatively correct plasmid for use or further confirmation, we next inoculate a liquid culture from the colony of interest (e.g., from the original plate or from a streaked plate generated during screening). After growth for 12-16 hours, the plasmid DNA can be harvested from the bacterial culture using a *plasmid prep* (page **??**) kit. Purified plasmid DNA should be assessed on the Nanodrop (or similar machine) to determine concentration and confirm purity.

Step 6: Sequence

The recent widespread availability of low-cost whole-plasmid sequencing has unmasked lurking issues in plasmid preps, such as mutations in backbone regions, plasmid dimerization, and the presence of additional "piggy backing" plasmids—which may not be readily identifiable by Sanger sequencing and often lead to headaches down the line. Thus, it's usually a good idea to whole-plasmid sequence DNA vital to experimental workflows.

However, at close to one-fourth the cost of whole-plasmid sequencing, traditional Sanger sequencing remains useful in some circumstances. These cases include:

- Plasmids with small inserts or edits to existing plasmids that are fully covered by two or fewer ~800-1000-bp reads
- Intermediate plasmids where mutations outside of the sequenced region will not be passed on in the next cloning step
- As a screening step before whole-plasmid sequencing of candidates from inefficient or error-prone assemblies

Step 7: Glycerol stock

Once plasmids are sequence-confirmed, we want to save the bacterial clone harboring the plasmid so that we can purify more of it later. Bacteria streaked on agar plates only save well at 4°C for on the order of ~1 month; for longer-term storage, we make a *glycerol stock* (page **??**) (in triplicate) that is added to our lab's shared set of stocks. To allow others in lab—and eventually the wider scientific community, upon publication—to access our plasmids, the sequence should be added to our existing *plasmid database* (page **??**) and assigned a `pKG` number for reference.

Tip

Don't wait too long to stock plasmids! Stocking a giant batch all at once often feels tedious, while stocking a few at a time may be more manageable. Also, you don't want the bacteria on the agar plate to become unrecoverable. If this does happen, or you are otherwise unable to grow the bacterial clone harboring the plasmid, you can re-transform the plasmid according to the steps below.

Starting with an assembled plasmid

To make a stock of a plasmid for which you only have a DNA prep (e.g., obtained from a collaborator, the original agar plate dried out), simply transform (or re-transform) the plasmid directly—you do not need to reassemble it. This means you can skip Steps 1 and 2 above, beginning directly with Step 3.

Step 3: *Transform* (page **??**) chemically competent cells with 0.5 μL plasmid DNA.

Tip

The transformation should be highly efficient since all the DNA is (presumably) the correct plasmid product. You may want to dilute your plasmid DNA or scale down

the volume of cells to avoid overcrowding of the colonies the next day. Be careful not to overgrow such that the colonies merge!

Step 4: It is usually okay to skip the initial screening step if your plasmid DNA is pure. Instead, pick 1-2 colonies directly into liquid cultures and shake overnight at 30ºC. The next day, save ~2 uL to inoculate a new culture or streak onto a fresh plate.

Steps 5-7: Purify, sequence, and stock your plasmid as described above.

Cloning Workflow Timeline

The timeline from cloning design to sequence-confirmed product is typically ~4 days, since several steps require time for bacterial growth. The sequence can be accelerated in a pinch, with greater probability of error, reduced efficiency, and additional reagent consumption.

Typical Timeline

Day 1: Generate fragments, assemble plasmids, transform bacteria (*incubate overnight*)

Note that some assembly reactions may have much greater efficiency if run overnight before transformation (e.g., Gateway or an difficult Golden Gate).

Additionally, it is common for the fragment generation step to take several days if the reactions have a long run-time and require troubleshooting (e.g., a tricky PCR).

Day 2: Screen colonies, start liquid cultures of candidates (*shake overnight*)

If plates are streaked with colonies in the morning, cultures can usually be started from them in the evening. Otherwise, the streaked plates may need to incubate overnight before starting liquid cultures of candidate clones.

Day 3: Purify plasmid DNA, send for sequencing (*typically next-day turn-around*)

Same-day sequencing results are sometimes possible for sequencing orders submitted for 9/10 am pickup; results usually are ready around 6pm. Otherwise, sequencing picked up at the afternoon or evening cutoffs generate results sometime the next day (except when submitting on Saturday).

Day 4: Assess sequencing results, start liquid cultures for glycerol stock (*shake overnight*)

At this point, you can use the purified DNA in the next cloning step or in a tissue culture experiment. Note that some experiments require high DNA concentrations or high purity, so you may want to start a culture for a *midiprep* (page **??**).

Day 5: Make glycerol stock

Accelerated Timeline

Warning

This timeline is not recommended, since it is more error-prone and uses more reagents.

Day 1: Generate fragments, assemble plasmids, transform bacteria (*incubate overnight*)

Even incubating at 37°C, it is difficult to see colonies faster than 12-16 hours.

Day 2 (am): Screen colonies, start liquid cultures (*shake at 37°C for ~8 hours*)

Start a liquid culture directly for each picked colony; after screening, discard cultures for incorrect clones.

Day 2 (pm): Miniprep, send for Sanger sequencing (*by 7pm pickup*)

Note that plasmid preps will likely have low concentrations after growing for an abbreviated period. You may want to start new cultures to grow overnight and prep again after the sequencing results come in on Day 3.

Day 3: Assess sequencing results

Sequencing results may be ready before 6am, or not until 2pm—plan experiments accordingly.

3.2.2 Colony PCR

Colony PCR is one method to screen colonies after transformation to identify clones harboring the correct plasmid product. (See the Cloning Workflow Overview *Step 4: Screen colonies* (page **??**) for more.)

Materials

- Agar plate with colonies
- Fresh agar plate (with the same antibiotic)
- Taq 2X Master Mix[99]
- Elga water
- Two primers, each diluted to 10 μM (typical working stock)
- PCR strip tubes, one tube per colony to be picked
- Toothpicks

Protocol

1. **Identify a primer pair** to amplify a region unique to your desired plasmid product (i.e., distinct in length from any reactants or incorrect products).
    - It is usually convenient to choose two primers in the plasmid backbone, amplifying the entire new insert. For entry vectors or `pShip` plasmids, `oSEQ043` and `oSEQ044` work well.
    - Amplified regions (amplicons) can be large; regions up to 4 kb or more can amplify sufficiently. This allows the entire insert to be amplified, which helps verify that all fragments are present. However, the reaction takes much longer to run for large amplicons.
    - For small inserts, or for inserts that vary only slightly in size from the original backbone vector, primer pairs should instead be chosen to amplify *as short a region as possible* that still captures the expected differences. This helps make small size differences more apparent when analyzing the results.
    - Be sure to choose a primer pair with compatible annealing temperatures. Avoid primers with Tm's that differ by more than 5ºC.
2. **Label colonies** to screen on your agar plate (e.g., circle, point an arrow) and number each colony.
    - Choose colonies that you can isolate with confidence, i.e., not touching or too close to other colonies.
    - The number of colonies to pick is determined by the estimated specificity of the assembly reaction. For reactions that rarely produce incorrect products (e.g., Gateway with proper antibiotic selection), fewer colonies will be sufficient (2-3). For reactions that often produce incorrect products (e.g., oligo annealing and ligation), more colonies will likely need to be screened ($\geq$6).

[99] https://www.neb.com/en-us/protocols/2012/09/13/protocol-for-taq-2x-master-mix-m0270

3. **Prepare a fresh agar plate** by drawing many small regions, one for each colony to pick, on the bottom of the plate (a simple grid works well). Label each region with a colony number.
4. **Make the reaction mix** according to the calculations below, scaling by the number of colonies to pick. Pipet the reaction mix into PCR strip tubes, 15 μL per tube and one tube per colony.

Reagent	1x (1 colony)	8x
Elga water	6	48
Primer 1	0.75	6
Primer 2	0.75	6
Taq 2X MM	7.5	60
Total	**15 μL**	**120 μL**

> **Note**
>
> The reaction will run well with slightly more or less than 15 μL per colony. Therefore, rather than making extra mix to account for pipetting loss when screening many colonies, you can instead pipet 14-14.5 μL per tube.

5. **Pick colonies** one at a time under a flame: Touch the end of a sterile toothpick (or pipette tip) to the colony, swirl the toothpick in the reaction mix of one of the PCR strip tubes, then streak the wet toothpick onto the corresponding region of the prepared fresh agar plate. Discard the toothpick and close the tube.
6. **Run the PCR reaction** by placing the tubes in a thermocycler, using the program from the *PCR protocol* (page **??**). Be sure to set the proper extension time (1 minute/kb).
7. **Visualize the amplicons** via *gel electrophoresis* (page **??**), using the parameters below. Note colonies with the correct amplicon band size.
    - **For amplicons >500 bp:** Use a 1% gel (100 mg agarose per 10 mL 1xTAE buffer) and run at 100 V for 25 min.
    - **For amplicons <500 bp:** Use a 2% gel and run at 90 V for 30-40 min.
    - Load 5 μL of reaction mix. Note that the reaction mix already contains loading dye, so no additional dye needs to be added.
8. **Incubate the streaked plate** overnight at 30°C. The plate can be used to inoculate cultures for *plasmid preps* (page **??**) and *glycerol stocks* (page **??**). To start cultures from the streaked plate on the same day, instead grow for ~6 hours at 37°C (bacterial growth doesn't need to be visible yet). Once the plate has visible growth, wrap it in parafilm and store at 4°C for up to ~4 weeks.

For hard-to-amplify templates

If your desired amplicon is GC-rich or contains hard-to-amplify sequences, try adding GC Enhancer (10% of final volume) to the reaction.

Reagent	1 (per colony, µL)	8× (µL)
Elga water	3.75	30
GC Enhancer	1.25	10
Primer 1	0.625	5
Primer 2	0.625	5
Taq 2× MM	6.25	50
Total	**12.5**	**90**

3.2.3 Restriction digest

Restriction digest refers to the cutting of DNA fragments using restriction enzymes, which recognize particular DNA sequences (~4-6 bp). We have a number of restriction enzymes in lab; a current list can be found here[100]. Most, if not all, of these enzymes have optimal activity in the NEB rCutSmart Buffer, but you can check this NEB chart[101] for details.

1. Combine the following in a PCR strip tube:

Reagent	Amount	Notes
DNA	~1 μg	
rCutSmart buffer	5 μL	
Enzyme(s)	1 μL each	
rSAP (*optional*)	1 μL	Dephosphorylates 5' DNA ends to prevent re-ligation of digested product
Elga water	X μL	Add Elga water to reach total volume
Total	**50 μL**	

2. Incubate the mixture at 37°C in the water bath for 1 hour.

> **Tip**
>
> If you'd like to run a digestion but can't come back in an hour, you can incubate the mixture in a thermocycler at 37°C for 1 hour followed by 20 min at 65°C (or 80°C, check[102] the inactivation temperature for your enzyme). This way, you don't over-digest, which can lead to off-target cutting.

3. *Gel extract* (page **??**) your desired digestion product.

> **Note**
>
> Depending on your use-case for the product, you may be able to skip the gel extraction step and instead purify the digestion directly. This will increase the concentration of the final product but may affect downstream assembly reactions.

[100] https://mitprod.sharepoint.com/:t:/s/GallowayLab/EafvG6wxMYtIldd_kYfRWuUBgfdTJmFjVKERHH2ouqYQIw?e=asZyuv
[101] https://www.neb.com/en-us/tools-and-resources/usage-guidelines/nebuffer-performance-chart-with-restriction-enzymes
[102] https://www.neb.com/en-us/tools-and-resources/usage-guidelines/nebuffer-performance-chart-with-restriction-enzymes

3.2.4 DNA cleanup

When generating DNA fragments via an enzymatic reaction (e.g., PCR, restriction digest), we need to purify the resulting product and remove the leftover enzyme and buffer. That way, the product can be easily used in downstream cloning reactions.

To do so, we use the NEB Monarch® Spin PCR & DNA Cleanup Kit (NEB T1130[103]). Actually, we source the components separately:

- **Buffer BZ** (NEB T1115[104]): Binding buffer, add 72 mL isopropanol (or volume indicated on bottle) when first opened; not required when using agarose dissolving buffer
- **Spin S1A columns** (NEB T2037L[105]): 5-μg binding capacity, not the same as the columns for the miniprep kit
- **DNA wash buffer**: made in-house, mix from stock as needed (*recipe* (page **??**))

1. Add the appropriate volume of Buffer BZ to your sample and pipet to mix. Note that the max volume in a PCR strip tube is 250 μL—transfer to a 0.6-mL Eppendorf tube if necessary.
    - **For larger fragments** (>50 bp dsDNA): Use 5 volumes of buffer (e.g., 125 μL buffer into 25 μL sample)
    - **For smaller fragments and oligos** (dsDNA 11-50 bp, ssDNA 16-200 nt): Use 2 volumes of buffer (e.g., 50 μL buffer into 25 μL sample)

> **Important**
>
> If your sample already contains agarose dissolving buffer, you do **not** need to add Buffer BZ.

2. Transfer the solution into a Spin S1A column placed in a collection tube.
3. Spin at max speed (16,000xg) for 1 minute in a benchtop centrifuge.
4. Aspirate the flowthrough into the miniprep waste.

> **Warning**
>
> Do not add the flowthrough into the media/cells waste! If this occurs, talk to the EHS rep. Add bleach to the affected flask in the fume hood and allow fumes to disperse. A black precipitate may form; dispose of the solids in the biowaste and drain the liquid in the sink.

5. Add 200 μL of DNA wash buffer to the column. Spin at max speed for 1 minute.
6. Repeat the wash step for a total of 2-5 washes.
    - When cleaning up a reaction directly, 2 washes is sufficient.

[103] https://www.neb.com/en-us/products/t1130-monarch-spin-pcr-and-dna-cleanup-kit-5-ug
[104] https://www.neb.com/en-us/products/t1114-monarch-buffer-bz
[105] https://www.neb.com/en-us/products/t2037-monarch-spin-columns-s1a-and-tubes

- When cleaning up a fragment extracted from a gel, additional washes help remove contaminants from the agarose dissolving buffer.
- Aspirate the flowthrough into the miniprep waste after every 3 washes. This prevents the solution from touching the column (max fill is 800 μL).

7. Aspirate the flowthrough into the miniprep waste.
8. Spin at max speed for 1 minute. This dry spin helps remove traces of buffer from the column.
9. Transfer the column to a fresh 1.7-mL tube.
10. Add 5-20 μL of Elga water to the column. Pipet the liquid directly onto the filter without touching it with the pipette tip. Incubate at room temperature for at least 1 minute.

> **Tip**
>
> Pre-warm Elga water in a 55ºC water bath for better elution.

11. Spin at max speed for 1 minute to elute the DNA.
12. To quantify yield, measure 1.5-2 uL of sample on the Nanodrop.
    - Concentrations <20 ng/uL are unreliable measurements. However, the product can likely still be used in a downstream reaction if the spectrum has the correct shape.
    - The A260/280 ratio should be 1.80-2.00. Lower values indicate protein contamination.
    - The A260/230 ratio should be >2.00 (>1.80 is okay). Lower values indicate salt contamination from the buffers. Additional washes can reduce this contamination.

3.2.5 Gel electrophoresis and extraction of DNA

Gel electrophoresis

Gel electrophoresis is a technique used to separate DNA fragments by size. DNA is negatively charged, so when an electric field is applied, DNA fragments will migrate towards the positive electrode. Smaller fragments migrate faster than larger ones, allowing for size-based separation.

1. To prepare a 1% agarose gel by weight, mix 0.5 g (500 mg) of agarose powder per 50 mL of 1X TAE buffer.

- A 1% agarose gel is appropriate for fragments >500 bp. For shorter fragments, increase the amount of agarose (up to 3% for ~100 bp fragments).
- We have 3 mold sizes, so you should scale the volume accordingly: 30 mL for 1-comb, 50 mL for 2-comb, and 100 mL for mega gels.
- For gels with a higher percentage of agarose, increase the total volume, as the thicker solution will be harder to pour into the mold.

2. Heat the mixture in a microwave until the agarose is completely dissolved (1 min, longer for >1% agarose gels). Place a kimwipe in the mouth of the flask during microwaving to reduce splatter.

> **Warning**
>
> The microwaved flask will be hot! Use the pot holders to remove the flask from the microwave.

3. Allow the solution to cool until you can touch the flask (~1 min).
4. Add a nucleic acid stain to the agarose. Typically, this is 1 drop of ethidium bromide from the dropper bottle (2 drops for mega gels). For RNA gels, use SYBR™ Safe (10,000X, pipet 5 μL per 50 mL gel). Swirl the flask to mix.

> **Warning**
>
> Ethidium bromide is a carcinogen. Avoid spilling it on your skin. Dispose of empty dropper bottles with trace liquid into the appropriate solid waste bucket.

5. Place a gel mold into the holder and tighten to seal the edges. Use a level to check that the mold is not tilted.
6. Pour the gel into the mold and add the desired combs to create the wells. The small wells can hold up to ~15 μL of sample, while the larger wells can hold up to ~50 μL (assuming sufficient gel height).
7. Rinse the flask to remove traces of leftover gel.
8. Let the gel solidify at room temperature for at least 25 minutes.

9. Carefully remove the combs by lifting straight up to keep the wells intact. Remove the mold with the gel from the holder.
10. Place the gel (still in the mold) into the gel runner. Orient it such that the wells are closer to the black electrode.

> **Tip**
>
> A helpful way to remember the gel orientation is "run to red", as DNA will migrate to the positive (red) electrode when the electric field is applied.

11. Add 1X TAE buffer to the gel runner so that the surface of the gel is fully covered (i.e., buffer fills the wells). Do not fill above the max fill line on the runner.
12. Prepare your samples by mixing them with 6X Loading Dye. For larger samples, add 10 μL of dye per 50 μL sample. For samples of only 1-2 μL (e.g., checking a PCR product), add 0.5-1 μL dye and mix on a piece of parafilm.

> **Note**
>
> We typically use purple loading dye, since this comes with many of the restriction enzymes we order. However, for nicer looking images (e.g., for publication), use orange loading dye. The orange dye interferes less with visualization of the DNA.

13. Load your samples with dye into the wells. Carefully place the pipette tip in the well and dispense the liquid slowly, going only to the first stop. Avoid pipetting air into the well, as bubbles can cause your sample to float out of the well.
14. Load DNA ladder into at least one well per row of the gel. For small wells, use ~2 μL ladder; for large wells use ~5 μL. We have two different DNA ladders:
    - Apex DNA ladder I (Genesee Scientific 42-428[106]): bands at 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, and 10,000 bp.
    - Apex DNA ladder II (Genesee Scientific 42-431[107]): bands at 100, 200, 300, 400, 500, 600, 700, 800, and 1,000 bp.
15. Securely place the lid on the gel runner, aligning the red and black electrodes. Plug the wires into the machine and unplug unused runners.
16. Run 1% agarose gels at 100 V for 25 minutes (set the voltage and time settings; the current will adjust accordingly). For gels with a higher percentage of agarose (or small products), run at 90 V for 30-40 minutes. The goal is to obtain enough separation of the ladder bands to identify your product while not letting the shortest fragments run off the end of the gel.
17. When the gel is finished running, press the stop button on the machine. Carefully remove the lid by lifting straight up (to avoid bending the metal prongs).

[106] https://www.geneseesci.com/product/apex-quantitative-dna-ladders-i-ii-iii/?sku=42-428
[107] https://www.geneseesci.com/product/apex-quantitative-dna-ladders-i-ii-iii/?sku=42-431

18. Lift the gel out of the runner and tilt to drain off the buffer. Place your finger at the lowest point to prevent the gel from sliding off the mold.
19. Place the gel (still in the mold) on the UV imager. Lower the UV shield and turn on the light to view by eye. Alternatively, with the light off, lift up the UV shield and place the black box over the light source. Then turn on the light and align your phone camera with the circular filter at the top, capturing an image of the gel below. Turn off the light source as soon as you finish.

> **Warning**
>
> Don't look directly at the UV light source! Always use the shield when viewing by eye. Additionally, if manipulating the gel with the light on, wear a blue lab coat to protect your skin from the UV rays.

> **Warning**
>
> Don't touch your phone with gloves on! Remove one glove when taking a picture of the gel.

20. Clean up: Dispose of the gel in the solid waste bin, since it contains ethidium bromide. Rinse off the gel mold and place it back in the appropriate spot to dry. Wipe off the UV imager with ethanol. Set the lid back on the gel runner to slow evaporation of the buffer.

> **Note**
>
> To take publication-quality images (required for quantification), use the ChemiDoc MP Imaging System (BioRad 12003154[108]) located in the Niles Lab (16-378). Bring a USB drive to save the images.

Gel extraction

Gel electrophoresis can also be used to select and purify DNA fragments by size.

1. Run your reaction (PCR or restriction digest) at a 50-μL scale. You'll need a lot of product, as much will be lost during the extraction process.
2. Perform gel electrophoresis as described above. Load the **entire volume** of your reaction into a single well, if possible, with the appropriate volume of 6X Loading Dye (e.g., 10 μL Loading Dye plus 50 μL reaction).

> **Note**

[108] https://www.bio-rad.com/en-us/product/chemidoc-mp-imaging-system?ID=NINJ8ZE8Z

Since you will be running a larger volume, you should use the gel comb that creates larger wells (6 wells in a row for the small gels). Also, be careful to adequately fill the mold with agarose so that the well is deep enough to hold the entire volume.

3. After the gel is finished running, use the UV imager to identify the desired band on the gel. Using a razor blade, cut out a fragment of gel containing your product. Do your best to cut the **smallest** gel fragment that includes your product. Cut the gel while still in the mold; don't cut it directly on the backlight.

Warning

To avoid UV exposure while cutting your gel at the imager, wear a blue lab coat (if you are not already wearing long sleeves).

4. Place the gel fragment into a 1.7-mL tube. Clean up the gel/imager as described above.
5. Weigh the fragment (tare the scale on an empty tube first) and record the weight in mg.
6. Add agarose dissolving buffer (Fisher Scientific 50-444-175[109]) to the tube. Use a 3:1 ratio of buffer in μL to gel fragment in mg (e.g., add 300 μL buffer for a 100 mg gel fragment).

Tip

Cut your gel carefully so that you use less than 400 μL gel dissolving buffer, as larger volumes will introduce more contaminants and reduce the quality of DNA prep. In many cases, using a standard 250 μL of gel dissolving buffer works well for a reasonably sized gel fragment.

7. Incubate in the water bath at 56ºC for ~10 minutes, or until the gel looks dissolved.
8. Follow the *DNA cleanup protocol* (page **??**) to purify DNA from this solution. You do *not* need to add binding buffer in the first step, and you should perform 4+ washes to improve purity.
9. To confirm effective purification, measure the concentration of the final product on the Nanodrop. Concentrations below ~20 ng/μL are unreliable measurements and indicate poor purification, but the product may still be used in downstream reactions at lower efficiency.

[109] https://www.fishersci.com/shop/products/adb-agarose-disslvg-buff-100ml/50444175

3.2.6 Gibson assembly

Gibson assembly a larger DNA fragment or plasmid from overlapping fragments. During a Gibson reaction, an exonuclease chews back the 5' ends of each fragment. The complimentary ends can then anneal, with nucleotides added by a DNA polymerase to fill in gaps in the annealed fragments. Finally, a ligase repairs nicks to generate one contiguous fragment or plasmid.

Since this method makes use of an exonuclease, it is important that **each fragment is longer than 200 bp**. Additionally, because the reaction takes place at 50ºC, the overlapping sequences should have a melting temperature greater than 50ºC. (The SnapGene tool will warn you if this is not the case.) Practically, this means the fragments should **overlap by at least 20 bp**.

Protocol

1. Design the assembly. Online tools such as NEB Builder[110] and built-in tools in SnapGene are helpful for doing this.
2. Generate fragments by either *PCR* (page **??**) followed by DpnI digest, or by *restriction digest* (page **??**) of a target template. Measure the concentrations of fragments via Nanodrop.

> **Note**
>
> The DpnI digest step for PCR fragments is not strictly necessary, but it reduces the number of background colonies from leftover template plasmid. This is relevant if the template plasmid and your final plasmid have the same antibiotic resistance.

3. Calculate volumes of each fragment required for the Gibson reaction. NEBioCalculator[111] is a helpful tool. Generally, 150 ng of the vector backbone fragment should be used. For inserts, a 2:1 molar ratio (insert : backbone) is usually successful, although a 3:1 ratio may be used for smaller fragments.
4. Set up a reaction with the calculated volumes of each fragment and 2X NEB HiFi assembly mix. The master mix is 2X by volume, so add an amount equal to the sum of all backbone and insert volumes used.

> **Hint**
>
> If your backbone vector is 5 kb at a concentration of 50 ng/μL, you need to add 3 μL to the reaction. If your insert is 1 kb at a concentration of 30 ng/μL, you need to add 2 μL (2:1 molar ratio) to the reaction. Finally, the sum of vector and insert volumes is 5 μL, so add 5 μL of the 2X master mix.

5. Incubate at 50°C for 15 minutes (2-3 fragments) or for 60 minutes (4-6 fragments).
6. *Transform competent cells* (page **??**) with 2-5 μL of the reaction.

[110] http://nebuilder.neb.com/
[111] https://nebiocalculator.neb.com/#!/ligation

7. Store unused Gibson products at -20°C.

3.2.7 Glycerol stocking

After successfully sequencing a bacterial plasmid, we stock it for long-term storage. Bacterial plates keep well for ~1 month at 4ºC, but may dry out or be contaminated if stored for longer.

Note

This protocol describes how to stock plasmids in the lab's shared pKG database. For personal glycerol stocks (e.g., plasmids waiting to be tested in TC before adding to the database), one vial per plasmid is sufficient. These can be stored in personal boxes in the -80ºC.

1. Add the plasmid to the database in Quartzy. See instructions on *this page* (page **??**).

   Important

   Add plasmids to the database before stocking! That way, you don't run into issues with overlapping numbering if someone else is also stocking around the same time.

2. Aliquot 4 mL of LB media with the correct antibiotic into a culture tube.
3. Using a toothpick or pipette tip, pick the bacterial clone to stock from an agar plate into the liquid culture.
4. Shake at 30ºC overnight.
5. The next day, label three cryovials per stock with the pKG#, the date, your initials, and a short description of the plasmid. Labels can be printed using the LabBot "Print Labels" app on Slack. (If it doesn't show up, type "/labels" in the Slack search bar.) You must be connected to the MIT wifi for this app to work.
6. Add 2 mL of 60% glycerol to the 4 mL of culture. Mix well by pipetting.
7. Aliquot 1.5 mL per vial of the resulting mixture into the three labeled cryovials.

   Note

   Avoid overfilling the cryovials, as this makes them hard to open. The maximum capacity is 1.8 mL to allow room for expansion upon freezing.

8. Store the cyrovials in the designated pKG boxes at -80ºC. Put one cryovial per box.

3.2.8 Golden Gate assembly

Golden Gate assembly allows us to create a construct using Type IIS restriction enzymes (REs). These enzymes can generate custom connector sequences (short single-stranded "sticky ends"), enable scarless assembly, and enhance reaction efficiency through multiple rounds of digestion and ligation.

Beyond the general reaction protocol, in lab we have a custom scheme (described *below* (page **??**)) of connector sequences and library of plasmid parts that can be modularly combined. This allows for easy, rapid assembly of single and multiple transcription units.

Protocol

This protocol is derived from the standard NEB protocol (here[112] and here[113]).

1. **Design the assembly.** Use the lab's *custom Golden Gate scheme* (page **??**), if possible. Otherwise, online tools such as NEB Golden Gate[114] are available for designing primers, choosing connector sequences, etc.
2. **Mix the Golden Gate reaction.** The volumes below are for a 10 μL reaction, but it is possible to scale down to 5 μL for efficient assemblies (e.g., pShips from pPV plasmids). If performing many assemblies in parallel, it can be helpful to make a mastermix with buffer, ligase, RE, water, and any shared fragments.

Component	Amount (µL)	Notes
75 ng/fragment	X	
10X T4 DNA ligase buffer	1	
T4 DNA ligase (400 U/μL)	1.25	500 U, see note below
Type IIS RE	0.5	increase to 1 μL if >10 inserts
Water	to 10	

Note

Make sure to use 500 U of T4 DNA ligase. NEB sells 400 and 2,000 U/μL T4 DNA ligase so scale the volume accordingly.

Tip

Gel extract PCR fragments before using them in Golden Gate assemblies. This removes primers and incomplete fragments, which can interfere with the assembly. Alternatively, pre-digest the PCR product with DpnI, rSAP, and the Type IIS RE to be used in the reaction (just spike these in and incubate at 37°C for 1 hour, like a

[112] https://www.neb.com/protocols/2018/06/05/golden-gate-24-fragment-assembly-protocol
[113] https://www.neb.com/-/media/nebus/files/manuals/manuale1601.pdf
[114] https://goldengate.neb.com/

typical *DpnI digest* (page **??**)). Then, use a 2:1 molar ratio of insert : backbone in the reaction. Note that reactions using PCR fragments are usually less efficient.

Tip

For plasmid fragments (e.g., pPVs), the reaction often works with 1 μL of each plasmid, as long as the concentration is ~50-150 ng/μL (this simplifies pipetting).

3. **Run the reaction on a thermocycler.** Almost always use the recommended protocol for 2-10 inserts.

# Inserts	Assembly protocol	Estimated time
1	5 min at 37°C (cloning) or 1 hr at 37°C (library preparation) -> 5 min at 60°C	10 min - 1 hr
2-10	(1 min at 37°C -> 1 min at 16°C) x 30 -> 5 min at 60°C	~1.5 hrs
11-20	(5 min at 37°C -> 5 min at 16°C) x 30 -> 5 min at 60°C	~5.5 hrs

4. *Transform competent cells* (page **??**) with 2-5 μL of the Golden Gate mixture. For very inefficient reactions, transform 100 μL of competent cells with the entire 10-μL reaction.
5. Store unused Golden Gate assembly products at -20°C.

For difficult assemblies

When fragments contain internal Type IIS RE cut sites, the Golden Gate assembly reaction will be less efficient. Ensure that the sticky ends from the internal cut sites are not the same as the connector sequences used in your assembly, otherwise the reaction will produce incorrect products. Assemblies with one internal cut site will typically work, particularly with the modification below.

On the other hand, **assemblies with multiple internal cut sites are likely to fail**. Look for a domesticated sequence (often labeled ".d" in the plasmid database) to use instead, or domesticate it yourself by mutating a base in the RE recognition site. Choose a synonymous codon to maintain the same amino acid sequence (if applicable). In general, it is worth domesticating sequences that you will use more than once in cloning (almost everything).

Assembly protocol with additional ligation

1. Mix a 10-μL assembly reaction as described above (step 2).
2. Run the reaction with the modified thermocycler protocol below. Note that increasing the number of cycles likely will *not* improve efficiency.

Step	Temperature (ºC)	Time
1. Digestion	37	1 min
2. Ligation	16	1 min
x 30 cycles		
Additional ligation	16	1 hr
Enzyme inactivation	80	20 min

3. Store assemblies at 4ºC until proceeding to the next step. (Or, cool to 4ºC for ~5 min and leave at room temp short-term.)
4. Spike in additional 10X T4 DNA ligase buffer and T4 ligase.

Component	Amount (µL)
Assembly mix	10
10X T4 DNA ligase buffer	1.25
T4 DNA ligase (400 U/μL)	1.25
Total	**12.5**

5. Ligate for 1 hour at 16ºC or overnight at room temperature.
6. *Transform* (page **??**) 100 μL of competent cells with 10 μL of the final assembly. To further increase efficiency, incubate the transformation on ice for 1 hour instead of 10 minutes.

"Diluted" PaqCI reaction

The glycerol in the PaqCI enzyme solution can interfere with the reaction. If you are getting few colonies in PaqCI Golden Gate assemblies, try a "diluted" reaction: increase the total reaction volume (with water and buffer) while keeping the amounts of the other reactants the same.

Component	Amount (µL)
75 ng/fragment	X
10X T4 DNA ligase buffer	**2**
T4 DNA ligase (400 U/μL)	1.25
PaqCI	0.5
Water	to **20**

In-house Golden Gate assembly scheme

In lab, we use a custom, hierarchical Golden Gate assembly scheme to modularly swap parts in single- and multi-transcription unit vectors. This is analogous to other MoClo (modular cloning) schemes.

Level 0: pPV

First, we create a library of **"part vector" plasmids (pPVs)** encoding individual genetic parts, such as promoters, coding sequences, and polyadenylation signals. Often, we use Gibson assembly to generate the pPVs, but other assembly methods (including Golden Gate, just not with this scheme) also work. pPV plasmids are equivalent to "Level 0" plasmids in other MoClo schemes. These vectors have **ampicillin resistance**. The important point here is that this library of parts is reusable! We only need to create each pPV once, then it can be used in many downstream assembly reactions.

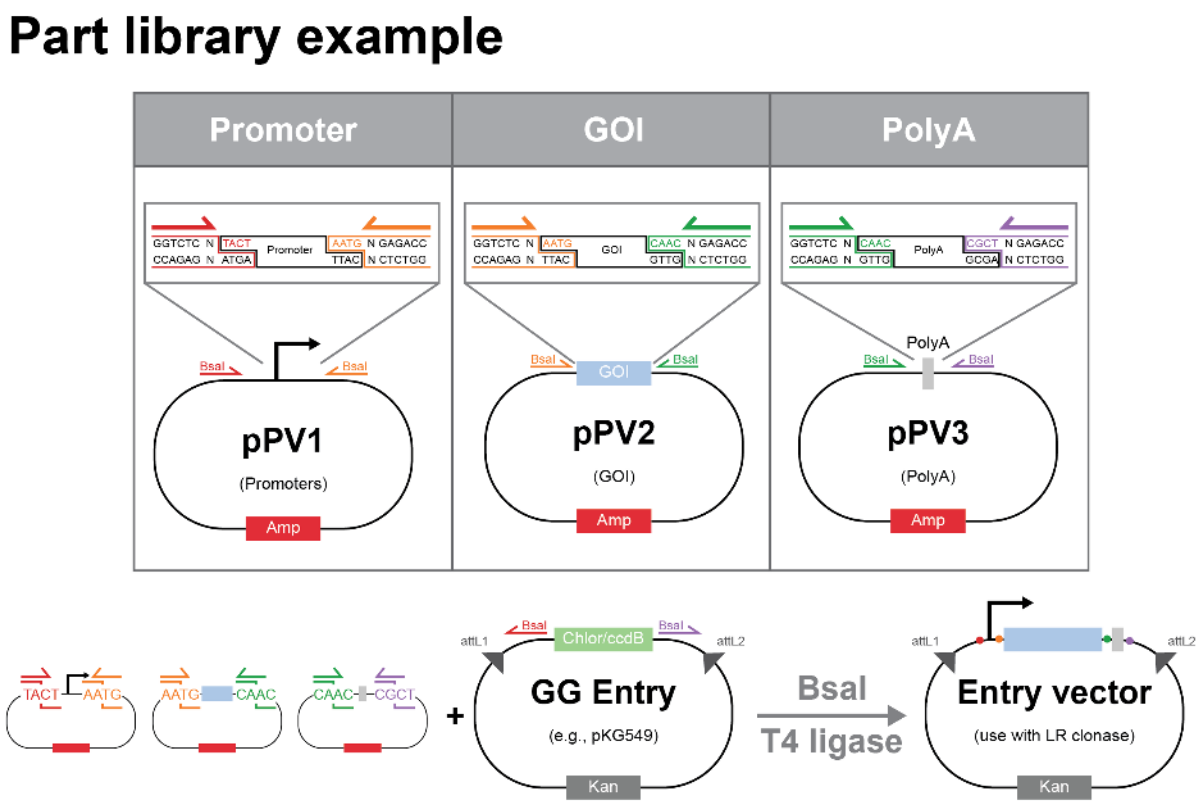
Level 1: pShip

Next, we use the pPVs to assemble **single transcription units (TUs)** using a Golden Gate reaction with **BsaI**. The resulting plasmids are **pShip** plasmids, which contain **kanamycin resistance**. These are equivalent to "Level 1" plasmids in other MoClo schemes.

This assembly requires at least 4 plasmids:

1. pPV1: promoter
2. pPV2: coding sequence aka gene of interest (GOI)
3. pPV3: polyadenylation signal
4. The vector backbone

The vector backbone (aka pPV0) includes plasmid backbone components like the origin of replication and antibiotic resistance cassette. It also includes sequences that flank the pPV inserts for downstream cloning steps. Note that this assembly reaction removes a cassette containing chloramphenicol resistance and the ccdB protein from the original pPV0 vector. This reduces background colonies containing the uncut vector backbone plasmid.



The vector backbones we use are usually one of the following:

- **pKG0549:** GG Entry vector, flanks pPV inserts with both PaqCI sites for downstream Golden Gate reactions and attL sites for Gateway assembly
- **pKG1117:** Janus, flanks pPV inserts with PaqCI sites such that the resulting pShip can be used to generate a single-TU expression vector
- **pKG1118-21:** Multi Janus, flanks pPV inserts with PaqCI sites such that the resulting pShip can be included in a multi-TU expression vector

Additionally, each pPV plasmid can be replaced by multiple plasmids, where the first contains the left pPV connector sequence, the last contains the right pPV connector sequence, and the middle connector sequences are complementary. For example, you can use both a pPV3a plasmid and a pPV3b plasmid in place of a pPV3 plasmid. See the list of connector sequences below.

Level 2: Expression vector

Finally, single or multiple transcription units can be combined into an **expression vector** using a Golden Gate reaction with **PaqCI**. The final plasmid contains additional sequences necessary for downstream delivery to mammalian cells. This includes ITRs for PiggyBac integration, LTRs and other sequences for virus production, or selection cassettes for landing pad integration. The resulting plasmids have **ampicillin resistance** and are equivalent to "Level 2" plasmids in other MoClo schemes.

Single TU assemblies require only two plasmids:

1. pShip (from pKG0549 GG Entry or pKG1117 Janus backbone)
2. Harbor plasmid: the vector backbone containing appropriate PaqCI sites and connector sequences

Multi-TU assemblies require 4 plasmids:

1. pShip.USF or pShip.USI: upstream TU in forward or inverted orientation
2. Spacer plasmid: intergenic sequence between the TUs
3. pShip.DSF or pShip.DSI: downstream TU in forward or inverted orientation
4. Harbor plasmid: the vector backbone containing appropriate PaqCI sites and connector sequences

The **Harbor plasmids** are conceptually similar to the Janus and Multi Janus vectors. They include plasmid backbone components and a chloramphenicol and ccdB cassette that is removed during assembly to reduce background. Additionally, they contain the relevant auxiliary sequences for downstream delivery to mammalian cells.

Common Harbor plasmids include the following:

- **pKG0893:** pLentiX1-Harbor, 3rd-generation lentiviral vector backbone
- **pKG4859:** pPB-Harbor, for PiggyBac integration with the (corrected) long ITRs
- **pKG3560:** Rogi2 landing pad Harbor, for integrating at the Bxb1-GT recombination site
- **pKG2765:** STRAIGHT-IN Harbor, for integrating at the Bxb1-GT landing pad (EF1a promoter trap, excise with Cre)
- **pKG2812:** STRAIGHT-IN Harbor, for integrating at the Bxb1-GA landing pad (EF1a promoter trap, excise with Flp)

The resulting expression vector is usually named based on the final vector backbone, e.g., pLentiX1 or pPB.

List of connector sequences

Golden Gate pPV Cloning Scheme (BsaI)

pPV0	TACT	pPV1	AATG	pPV2			CAAC	pPV3									CGCT	pPV0
				pPV2a	ACAA	pPV2b		pPV3a			AGGA	pPV3b						
								pPV3a1	GACC	pPV3a2		pPV3bx	CCCG	pPV3by	ACCC	pPV3bz		

Example 1			
Left Overhang	Insert	Right Overhang	Description
CGCT	pPV0	TACT	backbone
TACT	pPV1	AATG	promoter
AATG	pPV2a	ACAA	5'UTR
ACAA	pPV2b	CAAC	GOI
CAAC	pPV3a1	GACC	3'UTR part 1
GACC	pPV3a2	AGGA	3'UTR part 2
AGGA	pPV3b	CGCT	PAS

Example 2			
Left Overhang	Insert	Right Overhang	Description
CGCT	pPV0	TACT	backbone
TACT	pPV1	AATG	promoter
AATG	pPV2	CAAC	GOI
CAAC	pPV3a	AGGA	PAS
AGGA	pPV3bx	CCCG	att-prom.-att
CCCG	pPV3by	ACCC	GOI (reporter)
ACCC	pPV3bz	CGCT	PAS

Example 3			
Left Overhang	Insert	Right Overhang	Description
CGCT	pPV0	TACT	backbone
TACT	pPV1	AATG	promoter
AATG	pPV2	CAAC	GOI
CAAC	pPV3	CGCT	PAS

Upstream	Overhang	Downstream
pPV0	TACT	pPV1
pPV1	AATG	pPV2,2a
pPV2a	ACAA	pPV2b
pPV2,2b	CAAC	pPV3,3a,3a1
pPV3a1	GACC	pPV3a2
pPV3a,3a2	AGGA	pPV3b,3bx
pPV3bx	CCCG	pPV3by
pPV3by	ACCC	pPV3bz
pPV3,3b,3bz	CGCT	pPV0

Golden Gate MultiJanus Cloning Scheme (PaqCI)

Harbor	TACT	Ship.US	CGAA	Spacer	CTTA	Ship.DS	CGCT	Harbor

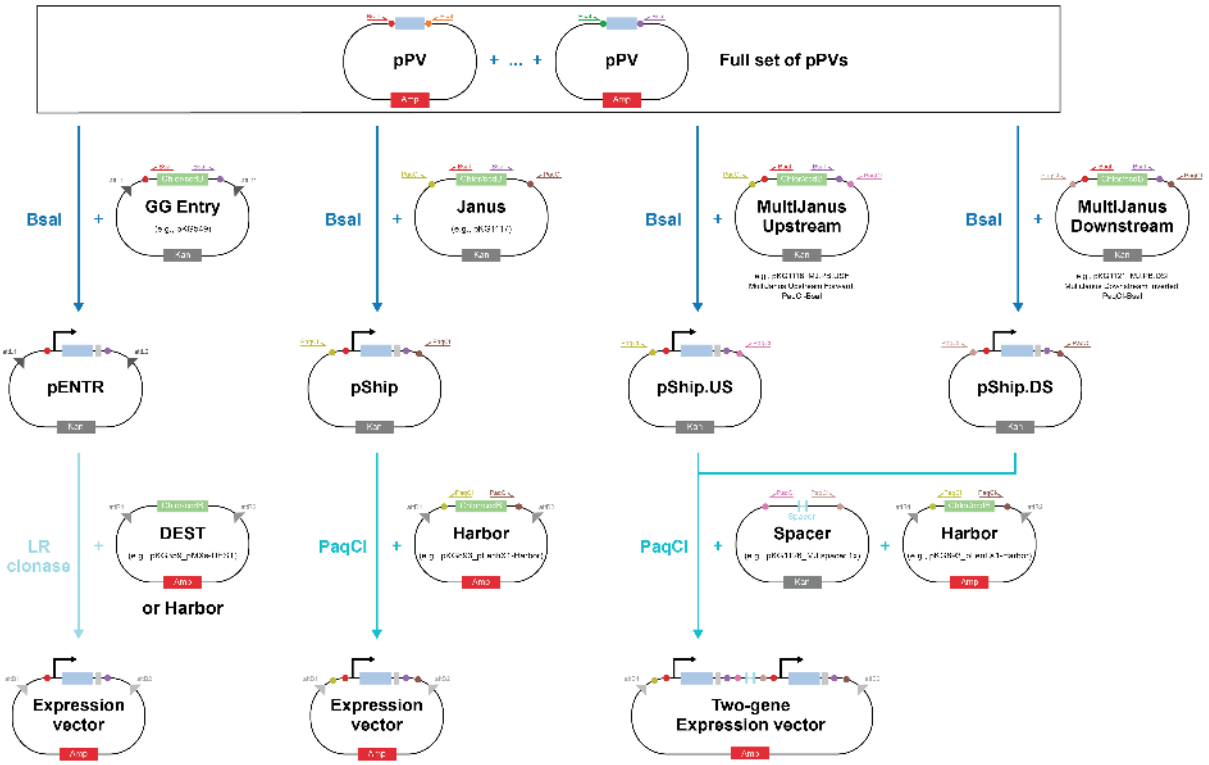
Upstream	Overhang	Downstream
Harbor	TACT	Ship.US
Ship.US	CGAA	Spacer
Spacer	CTTA	Ship.DS
Ship.DS	CGCT	Harbor

Overhang sequences are all written 5' to 3' for the sense (top) strand.

"Ship.US" refers to the transcription unit formed by a Golden Gate reaction (BsaI) with pPVs and MultiJanus Upstream Forward/Inverted.
"Ship.DS" refers to the transcription unit formed by a Golden Gate reaction (BsaI) with pPVs and MultiJanus Downstream Forward/Inverted.

Schematic of entire Golden Gate workflow

Full Golden Gate expression vector assembly



3.2.9 Ligation assembly

Estimated time

At least 1 hour, or overnight for best efficiency

Ligation[115] creates phosphodiester bonds between different DNA fragments to generate a larger linear fragment or plasmid. Ligation reactions can be performed with *restriction digest* (page **??**) products and/or *oligos* (page **??**) that contain compatible sticky ends.

Protocol

1. **Design the assembly** such that each fragment has compatible sticky ends (~4 bp sequences of single-stranded DNA at the 5' and 3' ends). Sticky ends allow for DNA fragments to bind each other for efficient ligation. Built-in tools in SnapGene are helpful for designing these.
2. **Generate the fragments** for ligation. Perform a *restriction digest* (page **??**) of the recipient vector as well as of any desired inserts to create fragments with sticky ends. If ligating using oligos, *anneal and phosphorylate* (page **??**) oligos prior to assembly, as 5' phosphorylation is required for proper ligation.

Tip

Performing the vector digest with rSAP to dephosphorylate the backbone can reduce background colonies and increase efficiency when assembling using oligos.

Similarly, gel extraction of digests can also be useful to decrease background ligation products.

3. Mix the reaction.

   Ligation with general digest products: Mix 150 ng of digested vector backbone and desired inserts in a 1:3 molar ratio (backbone : insert). NEBioCalculator[116] is very useful for calculating required masses for ligation reactions based on vector and insert lengths (a spreadsheet can also be helpful for quickly calculating the required masses and volumes for multiple fragments).

Reagent	Amount (µL)
Vector digest (150 ng)	X
Insert(s) (1:3 molar ratio)	Y
T4 ligase buffer	1
T4 ligase enzyme	0.5
Water	to 10

   Ligation with oligos only: Use 1 μL of oligos that were separately phosphorylated and annealed, or 1 μL of a 1:10 dilution of oligos where these steps were performed

[115] https://en.wikipedia.org/wiki/Ligation_(molecular_biology)
[116] https://nebiocalculator.neb.com/#!/ligation

simultaneously. Additionally, multiple annealed oligo fragments with compatible sticky ends can be assembled to create longer fragments for insertion. Add 1 μL of each set of oligos.

Reagent	Amount (µL)
Vector digest (150 ng)	X
Oligos (annealed and phosphorlyated)	1 each set
T4 ligase buffer	1
T4 ligase enzyme	0.5
Water	to 10

4. Incubate at 16ºC for one hour or at room temperature overnight for best efficiency.
5. *Transform competent cells* (page **??**) with at least 5 μL of the ligation product.
6. Store unused ligation products at -20°C.

Example

You want to replace microRNA target sites that are between two restriction enzyme sites, AvrII and HindIII, that are on the 5' and 3' ends respectively of the region of interest on your plasmid. To swap out the sites, you choose to do a ligation reaction with oligos.

To be compatible with the vector, you design your new target site oligos with a 5' AvrII sticky end on the top strand and a 5' HindIII sticky end on the bottom strand. You anneal and phosphorylate these oligos and then perform a digest of the template with AvrII and HindIII, ending up with a concentration of 75 ng/μL.

A quick calculation shows that 2 μL of digest contains the 150 ng of DNA template required for the reaction. You then perform a ligation reaction by mixing the digest product with the oligos as detailed above and incubating at room temperature overnight.

3.2.10 LR cloning

Estimated time

1 hour to overnight for best efficiency

Protocol

1. Combine 50-150 ng of entry plasmid and 150 ng of destination plasmid. Dilute with 1X TE buffer to 4 μL total.
2. Thaw LR Clonase II enzyme mix (Fisher Scientific 11-791-100[117]) on ice for two minutes. Vortex the enzyme mix briefly.
3. Add 1 μL clonase mix to the reaction.
4. Incubate the reaction at room temperature for 1 hour to overnight.
5. *Optional, if transforming immediately:* Add 0.5 μL Proteinase K to each sample to terminate the reaction. Incubate at 37°C for 10 minutes.
6. Transform competent cells with the reaction product.

Expected results

An efficient 5-μL reaction will produce >5000 colonies, if the entire volume is transformed and plated.

Warning

LR clonase does expire, leading to weird fragments or an unreacted backbone. Make sure to pay attention to the expiration date (~1 year from receipt).

[117] https://www.fishersci.com/shop/products/invitrogen-clonase-gateway-lr-clonase-ii-enzyme-mix-2/11791100

3.2.11 Oligo phosphorylation and annealing

This protocol describes how to anneal and phosphorylate oligos (short single-stranded DNA sequences), usually to add short sequences to plasmids via ligation cloning. This could be useful to make new microRNA target sites or CRISPR guide RNAs, for instance.

Oligo phosphorylation

1. Order desalted oligos (e.g., from Azenta/Genewiz) and reconstitute as usual to 100 μM.
2. Combine the following in a PCR tube:

Reagent	Amount
Oligo (**100 μM**)	1 μL
10X T4 DNA ligase buffer	1 μL
T4 PNK (polynucleotide kinase)	0.5 μL
Water	7.5 μL
Total	**10 μL**

Important

Use the *original 100 μM stock* of oligos, not a diluted working stock, in the reaction.

Note

The PNK buffer that comes with the PNK enzyme does not include ATP, so T4 ligase buffer is used instead. If you want to use the PNK buffer, also add 1 mM ATP.

3. Run the reaction on a thermocycler at 37°C for 1 hr, then heat inactivate the PNK at 65°C for 20 min.
4. Phosphorylated oligos can be stored at -20°C.

Oligo annealing

1. Combine 1 μL of each phosphorylated oligo (i.e., forward and reverse oligos from the reaction above) in a PCR tube with 18 μL water, for a total volume of 20 μL.
2. In a thermocycler, heat at 95°C for 3 min then cool to room temperature over ~30-60 min. There should be a temperature step function on the thermocycler, e.g., cool by 0.1°C every second.
3. Store annealed oligo fragments at -20°C so they remain annealed.

Important

Make sure you design your fragments to have sticky ends appropriate for your ligation!

Combined oligo phosphorylation and annealing

To save time, the phosphorylation and annealing steps can be combined as follows:

1. Order desalted oligos (e.g., from Azenta/Genewiz) and reconstitute as usual to 100 μM.
2. Combine the following in a PCR tube:

Reagent	Amount
Forward oligo (100 μM)	1 μL
Reverse oligo (100 μM)	1 μL
10X T4 DNA ligase buffer	2 μL
T4 PNK	1 μL
Water	15 μL
Total	**20 μL**

3. Run the reaction on a thermocycler: 37°C for 1 hr, heat at 95°C for 3 min, then cool to room temperature over ~30-60 min. There should be a temperature step function on the thermocycler, e.g., cool by 0.1°C every second.
4. Store phosphorylated and annealed oligo fragments at -20°C.

Note

When combined protocol is used, a 1:10 dilution of annealed oligos may be more effective in a downstream ligation reaction.

Note

Measuring the concentration of annealed oligos on a Nanodrop can be done, but is not necessary. 1 μL of the final annealed and phosphorlyated product (~0.5 μM) is typically relevant for ligation asssemblies.

3.2.12 Polymerase chain reaction (PCR)

Polymerase chain reaction, or PCR, is a method to amplify sequences from template DNA using custom short oligonucleotides (primers). The primers can additionally add short new sequences to the 5' or 3' ends of the DNA product.

The identity of the polymerase used in the reaction determines the speed and fidelity of product generation. In lab, we have the following polymerases:

- **Q5**: relatively efficient with high fidelity, recommended for generating fragments for cloning
- **PrimeSTAR**: extremely fast with slight compromises in fidelity, recommended for difficult-to-amplify sequences
- **Taq**: slow with lower fidelity, recommended for screening bacterial colonies (*colony PCR* (page **??**)) or other large-batch screening
- **KOD Xtreme**: also good for difficult-to-amplify sequences

Protocols for each polymerase are below. After running the reaction, *confirm and purify* (page **??**) the product. See also the *Determining annealing temperatures* (page **??**) and *Troubleshooting* (page **??**) sections.

Q5

We use NEB Q5® High-Fidelity DNA Polymerase (NEB M0491[118]).

Reaction mix:

Reagent	Amount (µL)	Notes
DNA template	1	Dilute the DNA template to ~5 ng/μL to add ~5 ng
Primer 1	1.25	Use 10 μM primers diluted from stocks
Primer 2	1.25	Use 10 μM primers diluted from stocks
Q5 5X mix	5	Thaw working aliquot from small cold block in Anna (-20°C)
dNTPs	0.5	10 mM, thaw working aliquot from Anna (-20°C)
Q5 polymerase	0.25	Stored in small cold block in Anna (-20°C) — keep cold!
GC enhancer (*optional*)	5	Use for difficult or high GC templates
Elga water	15.75 (10.75)	Without (with) GC enhancer
Total	**25**	

Thermocycler protocol:

[118] https://www.neb.com/en-us/products/m0491-q5-high-fidelity-dna-polymerase

Step	Temperature (ºC)	Time
Initial denaturation	98	30 sec
1. Denaturation	98	10 sec
2. Annealing	Ta	10 sec
3. Extension	72	30 sec/kb
x 25 cycles		
Final extension	72	2 min

Note

There is no need to set at 4ºC infinte hold at the end of the reaction. DNA is quite stable, so it is fine to sit at room temp overnight. A 4ºC infinte hold decreases the lifetime of the thermocycler. For longer-term storage, keep at 4ºC or -20ºC.

Source: NEB Q5® High-Fidelity DNA Polymerase[119]

PrimeSTAR

We use Takara PrimeSTAR® Max DNA Polymerase Ver.2 (Takara R047A[120]).

Note

Most reactions will run well at the default Ta of 55ºC. You can change the Ta if you need to optimize the reaction.

Reaction mix:

Reagent	Amount (µL)	Notes
DNA template	1	Dilute the DNA template to ~5 ng/μL to add ~5 ng
Primer 1	1.25	Use 10 μM primers diluted from stocks
Primer 2	1.25	Use 10 μM primers diluted from stocks
PrimeSTAR Max 2X Premix	12.5	Stored in small cold block in Anna (-20ºC)
Elga water	9	
Total	**25**	

Thermocycler protocol:

[119] https://www.neb.com/en-us/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491
[120] https://www.takarabio.com/products/pcr/high-fidelity-pcr/primestar-max-dna-polymerase?catalog=R047A

Step	Temperature (ºC)	Time
Initial denaturation	98	30 sec
1. Denaturation	98	10 sec
2. Annealing	55	5 sec
3. Extension	68	5 sec/kb
x 30 cycles		
Final extension	68	2 min

You can also use the two-step reaction for enhanced specificity (less off-target binding):

Step	Temperature (ºC)	Time
Initial denaturation	98	30 sec
1. Denaturation	98	10 sec
2. Extension	68	5 sec/kb
x 30 cycles		
Final extension	68	2 min

Source: Takara PrimeSTAR® Max DNA Polymerase Ver.2[121]

Tip

You can scale down the reaction volume to 10 μL and increase the cycle number to 60 to increase the total amount of DNA. At smaller scales, PrimeSTAR can be cheaper than other polymerases!

Tip

In a pinch, you can use a glycerol stock as template for a PrimeSTAR reaction. This has worked well for DSP.

Taq

We use Apex Taq RED Master Mix, 2X (Genesee Scientific 42-138B[122]). If you are using Taq for a colony PCR, see the *full protocol* (page **??**) for steps beyond running the reaction itself.

Reaction mix:

[121] https://www.takarabio.com/documents/User%20Manual/R047S/R047S_R047A_DS.pdf
[122] https://www.geneseesci.com/product/apex-taq-red-master-mix-2x-1-5mm-mgcl2-final-conc/?sku=42-138B

Reagent	Amount (µL)	Notes
Primer 1	0.75	Use 10 μM primers diluted from stocks
Primer 2	0.75	Use 10 μM primers diluted from stocks
Taq 2X MM	7.5	Thaw from Anna (-20ºC)
Elga water	6	
Total	**15**	Scale up as needed, 1X per colony

Thermocycler protocol:

Step	Temperature (ºC)	Time
Initial denaturation	95	3 min
1. Denaturation	95	30 sec
2. Annealing	Ta	30 sec
3. Extension	72	1 min/kb
x 30 cycles		
Final extension	72	5 min

Source: Apex Taq RED DNA Polymerase Master Mix Kit[123]

KOD Xtreme

We use KOD Xtreme Hot Start DNA Polymerase (Sigma Aldrich 71975-3[124]). This polymerase is very good for amplifying difficult templates (e.g., CAG promoter, transcription factor coding sequences, GC-rich sequences). However, it is relatively expensive, so you might want to attempt a PCR with a different polymerase first.

Reaction mix:

Reagent	Amount (µL)	Notes
DNA template	1	Use template that is ~100-500 ng/μL
Primer 1	1	Use 10 μM primers diluted from stocks
Primer 2	1	Use 10 μM primers diluted from stocks
2x Buffer	10	Thaw from box in Anna (-20ºC)
dNTPs	4	Thaw working aliquot from box in Anna (-20ºC)
KOD Polymerase	0.4	Stored in small box in Anna (-20ºC) — keep cold!
Elga water	3.5	
Total	**20.9**	

Thermocycler protocol:

[123] https://geneseesci.asset.akeneo.cloud/Technical_Documents/media/42138b20xtaqmastermixred15mm022022.pdf
[124] https://www.sigmaaldrich.com/US/en/product/mm/71975m

Step	Temperature (ºC)	Time
Initial denaturation	94	2 min
1. Denaturation	98	10 sec
2. Annealing	Ta (or 61)	30 sec
3. Extension	68	1 min/kb
x 30 cycles		
Final extension	68	2 min

Source: KOD Xtreme PCR Protocols and Guides[125]

Confirm and purify

DpnI digest

If you plan to use your PCR product in a cloning reaction, it is helpful to perform a DpnI digestion on your PCR product before purification. DnpI is a restriction enzyme that recognizes dam methylation, which is found only on cell-derived DNA—i.e., your plasmid template, but not the newly synthesized DNA from the PCR. This chops up any of the original template from the reaction, which is particularly useful if the template plasmid has the same antibiotic resistance as the product of your downstream assembly reaction. This can be performed in parallel with gel electrophoresis (below).

1. Add 0.5 μL DpnI per 25 uL PCR directly to the PCR tube.
2. Pipet up and down or flick the tube to mix.
3. Incubate at 37ºC for 1 hour in the water bath. Avoid prolonged incubation, as the enzyme may promiscuously cut non-methylated GATC sites in your PCR product.
4. Immediately proceed to purification (below) or store short-term at 4ºC.

Confirm

To check that your PCR reaction was successful, visualize the product(s) using gel electrophoresis. The gel can help you confirm that you have the correct number of products (usually just one) of the correct length. This can be performed in parallel with a DpnI digestion (above).

Follow the steps in the *gel electrophoresis protocol* (page **??**), with the following parameters:

- Combine ~2 μL of your PCR product with 0.5-1 μL of 6X Loading Dye. This can be done in a small droplet on a piece of parafilm.
- Use a comb with small wells.
- **For amplicons >500 bp:** Use a 1% gel (100 mg agarose per 10 mL 1xTAE buffer) and run at 100 V for 25 minutes.
- **For amplicons <500 bp:** Use a 2-3% gel and run at 90 V for 30-40 minutes.

[125] https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/203/182/pr3366en-ms.pdf

Purify

If the gel shows a single band at the size you expect for your PCR product, you can purify the remaining product directly. Follow the steps in the *DNA cleanup protocol* (page **??**).

Sometimes, PCR reactions will result in off-target amplification, which will likely cause problems in downstream cloning steps. If this is the case, you can run the *entire volume* of the PCR product on a gel and cut out the band of the correct size. See the *gel extraction protocol* (page **??**) for details.

Calculating annealing temperatures

To run the PCR, you'll need an annealing temperature (Ta) specific to your primers. The NEB Tm Calculator[126] provides one estimate, but **calculating the annealing temperature from the melting temperatures provided by SnapGene is better**.

1. Find the melting temperature (Tm) calculated by SnapGene for your forward primer.

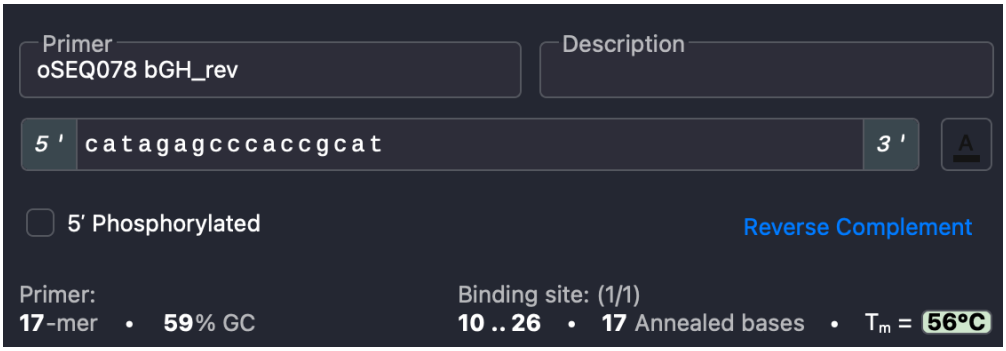


Fig. 5: The SnapGene Tm estimate is on the right side of the primer window. Click the boxed number for more information on SnapGene's calculations.

2. Adjust this value based on the polymerase type. For Taq, subtract 0-5ºC. For Q5 and others, add ~10ºC.

   > *"When PCR is performed using a traditional polymerase such as Taq, the optimal annealing temperature for the PCR reaction is about 0–5°C below the primer Tm values."*
   >
   > *"We recommend that when a polymerase with a dsDNA-binding domain is used, the annealing temperature for the PCR reaction should be about 6–12°C (for Phusion or Phire or Q5 polymerase) . . . above the primer Tm values calculated by our software."*

3. Calculate the adjusted Tm for the reverse primer. Choose the *lower* of these values as the Ta for the reaction.

Tip

[126] https://tmcalculator.neb.com/#!/main

The easiest way to troubleshoot a PCR is to change the annealing temperature: If you obtained no product (no band on the gel), try decreasing the Ta by a few degrees. This increases the amplification while reducing specificity.

PCR variations

Touchdown PCR

One method to PCR a difficult product is to start at a high annealing temperature (Ta) and "touchdown" by progressively decreasing the Ta. This way, the most specific product is amplified first, followed by less specific (but more efficient) ones.

Annealing temperature at each cycle:

Cycle #	Ta
1	72°C
2	71°C
3	70°C
4	69°C
...	...
n	Predicted Ta
Repeat last cycle (30-n) times	Predicted Ta

Note

You could go even lower than the predicted Ta (we've gone down to 57°C as a robust protocol).

Source: https://www.nature.com/articles/nprot.2008.133

Two-phase PCR

When using primers with long overhangs, the Tm when the primer initially binds to the template DNA (at the 5' end only) will be much lower than the Tm when the entire primer binds to newly synthesized DNA in subsequent cycles. To enable initial amplification but retain specificity overall, you can perform a two-phase PCR.

1. In the first phase (cycles 1-5), use the Ta calculated from the Tm of the primer sequence that binds to the template (without the overhang, i.e., the 3' end complementary to the template DNA).
2. In the second phase (cycles 6-30), increase the Ta to the value calculated for the entire primer, as most amplification at this point will occur by binding to newly synthesized DNA.

Temperature gradient PCR

When no amplification occurs, a common troubleshooting tip is to reduce the Ta by a few degrees. However, if this fails, or if it causes significant off-target bands, a more efficient way to identify the optimal Ta is by running a temperature gradient.

To do so, mix a 50-μL reaction (2x volumes above) and split it into 4 tubes of 12.5 μL each. Using the large thermocyclers, set the temperature at the annealing step to be a gradient across the sample plate. Each column will have a different temperature. Then, place your 4 tubes in different columns to simultaneously run reactions at 4 Ta's.

Because you've scaled down the volume, the concentration of purified product from one of these PCRs will be low. If reactions at multiple Ta's produce strong bands on the gel, you can combine these in downstream purification steps. Otherwise, set up a new 25-μL (or 50-μL, if the band is still faint) reaction with the optimal Ta.

Overlap extension PCR

It is possible to combine two overlapping fragments into a single larger fragment via PCR. This is useful for reducing the number of fragments added to a Gibson assembly. To do so, perform a PCR with the following parameters:

- Template: add 5 ng of each fragment
- Forward primer: use a forward primer that binds to the 5' end of the 5' fragment (i.e., the forward primer used in the initial PCR for a first PCR fragment)
- Reverse primer: use a reverse primer that binds to the 5' end of the 3' fragment (i.e., the reverse primer used in the initial PCR for a second PCR fragment)
- Polymerase: if applicable, the polymerase used to amplify the initial fragments
- Ta: determined as usual for the primer pair; if the Tm's of the primers are quite different, you may need to optimize the Ta using the strategies above

This works because the initial fragments will bind to each other in their overlapping region during the first annealing step. Then, each fragment can be extended by the polymerase, using the other fragment as a template. This generates full-length product in the first few cycles of the reaction. The added primers amplify this product in later cycles.

Troubleshooting

No product (no band on the gel)

- Re-dilute your template and/or primers
- Optimize the annealing temperature, using one or more of the strategies above
- Increase the extension time by 5-10 seconds
- Add GC enhancer to the reaction
- For long amplicons, split the reaction into two (don't forget to include overlap in these fragments as needed)

- If nothing works, try the KOD Xtreme polymerase—very expensive but very effective!

Off-target bands or smear on the gel

- Increase the annealing temperature
- Decrease the extension time (especially relevant if you were running a longer PCR simultaneously)
- Remove GC enhancer from the reaction
- Use a different template—look for one without known off-target binding sites or other potentially similar sequences
- Scale up the reaction to 50 μL and *gel extract* (page **??**) the correct band
- If the concentration is very low after gel extracting, use that product as the template in a new reaction

3.2.13 Plasmid prepping

To obtain plasmids for cloning steps or use in tissue culture, we amplify them in bacteria and purify the resulting plasmid DNA. We can prep at several scales (mini, midi, or maxi, even larger versions also exist) depending on the culture volume.

Miniprep

This protocol is adapted from instructions for the NEB Monarch® Spin Plasmid Miniprep Kit (NEB T1110L[127]). **Minipreps yield ~40 uL of DNA at ~100-400 ng/uL.** High copy, large plasmids like those with the LentiX backbone can reach ~400 ng/uL, while smaller plasmids like pPVs may remain <100 ng/uL.

When receiving a new kit: store RNase A in Anna -20°C in the dedicated box, and the remaining reagents at room temp.

When opening a new bottle of buffer:

- Buffer B1: add RNase A (1 vial per bottle, stored in Anna -20°C in a dedicated box), then store at 4°C
- Buffer BZ: add 18 mL isopropanol
- Buffer WZ: add 104 mL ethanol

1. Under a flame, aliquot 3-5 mL of LB media with antibiotic matching the plasmid antibiotic resistance. Use 14-mL culture tubes, placing the cap on securely but not airtight (the bacteria need aeration to grow).
2. Pick a single colony or dab a glycerol stock with a toothpick or pipette tip. Place the toothpick or pipette tip in the liquid culture.
3. Let the bacteria grow in the shaker overnight, ideally 12-16 hours and no longer than 24 hours.

> **Note**
>
> Grow viral plasmids in 30°C to reduce the chance of recombination. Non-viral plasmids may be grown at 37°C for better yield.

4. Spin the liquid cultures at 4800xg (max speed in bucket rotors) for 5 minutes, using the large centrifuge by shakers.
5. Pour or aspirate the supernatant into a cells/media waste flask.

> **Warning**
>
> Do not add media/cells into the miniprep waste or vice versa! If this occurs, talk to the EHS rep. Add bleach to the affected flask in the fume hood and allow fumes to disperse.

[127] https://www.neb.com/en-us/products/t1110-monarch-spin-plasmid-miniprep-kit

A black precipitate may form; dispose of the solids in the biowaste and drain the liquid in the sink.

6. Resuspend the cell pellet in 200 uL Buffer B1 (stored in the 4ºC deli fridge) and transfer to a 1.7-mL tube.
7. Add 200 uL Buffer B2 and gently invert 5-6 times to lyse the cells. Incubate at room temp for 1 minute.
8. Add 400 uL Buffer B3 and invert until uniformly yellow (no pink). Incubate for 2 minutes at room temp.

Note

MC adds 250 uL of B2 and immediately after addition starts a time for 90 seconds, inverts for 30 seconds, and adds 500 uL of B3 at 90 seconds.

9. Spin down on a benchtop centrifuge at max speed (16,000xg) for 5 minutes.
10. While samples are spinning, place a filter column in a collection tube for each sample.

Important

Be sure to use the provided Monarch **Spin S2D columns**, not the Spin S1A ones that come with the PCR cleanup kit. The binding capacity of the Spin S2D column is 20-100 ug, but only 5 ug for the Spin S1A columns.

11. Transfer the lysate to the column. Discard the pellet.
12. Spin the lysate at max speed for 1 minute.
13. Aspirate the lysate from the collection tube into the miniprep waste.
14. Add 200 uL of Buffer BZ (Wash 1).
15. Spin at max speed for 1 minute.
16. Add 400 uL of Buffer WZ (Wash 2).
17. Aspirate the flow-through from the collection tube into the miniprep waste.
18. Spin at max speed for 1 minute. This dry spin helps remove traces of buffer from the column.
19. Transfer the column to a fresh 1.7-mL tube.
20. Add 40 uL of Elga water to the column and incubate for at least 1 minute at room temp.

> **Tip**
>
> Pre-warm Elga water in a 55ºC water bath for better elution.

21. Spin at max speed for 1 minute to elute the DNA.
22. To quantify yield, measure 1.5-2 uL of sample on the Nanodrop.
    - Concentrations <20 ng/uL are unreliable and indicate a poor prep.
    - The A260/280 ratio should be 1.80-2.00. Lower values indicate protein contamination.
    - The A260/230 ratio should be >2.00 (>1.80 is okay). Lower values indicate salt contamination from the buffers used in the kit.

Midiprep

This protocol is adapted from instructions for the QIAGEN Plasmid Plus Midi Kit (Qiagen 12945[128]), following the **high-yield protocol** (suitable for high-copy plasmids, which applies to most of ours). **Midipreps yield ~200 uL of DNA at ~500-2,000 ng/uL.** Midipreps are preferred for transfections with large volumes of DNA (e.g., for virus production) because the kit better removes endotoxins (bacterial outer membrane components that induce an immune response in mammalian cells).

When opening a new bottle of buffer:

- Buffer P1: add RNase A and LyseBlue reagent (1 vial each per bottle), then store at 4ºC
- Buffer PE: add 40 mL ethanol (confirm volume on bottle)

1. Under a flame, aliquot 40-50 mL of LB media with antibiotic matching the plasmid antibiotic resistance. Use a 250-mL flask for optimal aeration of the culture (125-mL flasks can be used in a pinch).
2. Pick a single colony or dab a glycerol stock with a toothpick or pipette tip. Place the toothpick or pipette tip in the liquid culture.
3. Let the bacteria grow in the shaker overnight, typically ~24 hours.

> **Note**
>
> Grow viral plasmids in 30ºC to reduce the chance of recombination. Non-viral plasmids may be grown at 37ºC for better yield.

4. Transfer the culture to a 50-mL conical and spin at 4800xg (max speed in bucket rotors) for 10-15 minutes, using the large centrifuge by shakers.

[128] https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-plus-kits?catno=12945

Note

The midiprep protocol recommends spinning at 6000xg and 4ºC for 15 minutes, but KL has had successful preps with the parameters written above.

Important

If spinning at 6000xg, the fixed rotor is required (stored in the cabinet below the centrifuge). To avoid spilling culture into the rotor, fill the 50-mL conical with **up to 40 mL** of culture.

5. Pour off the supernatant into one of the empty flasks.
6. Safely dispose of the supernatant and disinfect your glassware: Add bleach to a final concentration of 10% to the supernatant, wait 20 minutes, and pour down the sink. To the remaining empty flasks, add enough 10% bleach to cover the surfaces, swirl, and wait 20 minutes. Then, thoroughly rinse the disinfected flasks and place them next to the Elga machine to be dishwashed.

Note

Collecting the supernatant (cells/media) waste in flasks and bleaching it yourself keeps the aspirator waste from filling up quickly!

While waiting for the flasks to disinfect, keep them at your bench rather than in the sink, if possible. This keeps the sinks clear for others to use.

7. Resuspend the cell pellet in 4 mL of Buffer P1 (stored at 4ºC in the deli fridge).
8. Add 4 mL of Buffer P2 and gently invert 5-6 times to lyse cells. Incubate at room temp for 3 minutes. The suspension should turn blue and will become viscous.
9. While you wait, place a large filter column in a new 50-mL conical for each sample.
10. Add 4 mL Buffer S3 and mix 4-6 times, or until the lysate is completely colorless.
11. Transfer the lysate to the filter column (pouring is fine) and incubate for 10 minutes.
12. During incubation, place the small spin columns on the vacuum manifold and add tube extenders. Connect the vacuum manifold to the miniprep aspirator.
13. Gently insert a plunger into the filter column to filter the lysate into the 50-mL conical.
14. Add 2 mL Buffer BB to the cleared lysate and invert 4-6 times.
15. Transfer the solution to the spin column + tube extender on the vacuum manifold. Turn on the vacuum to draw all the liquid through.

Troubleshooting the vacuum manifold

Sometimes, the vacuum pump is not strong enough to effectively filter the solution through the column.

- Double check all the tubing connections and ensure caps seal unused spots on the manifold.
- If the solution is not flowing through, you can try the other miniprep vacuum pump, or directly connect each column to the aspirator tube.
- Alternatively, you can add 700 uL of lysate to the spin column at a time and centrifuge at max speed (16,000xg) for 1 minute, aspirating the supernatant from the collection tube after each spin.

16. With the vacuum still on, remove the tube extender and add 700 uL Buffer ETR to the spin column.
17. Once all the liquid flows through, add 700 uL Buffer PE and allow the vacuum to draw all the liquid through the column.
18. Remove the spin column from the vacuum manifold and transfer to a collection tube. Spin at max speed (16,000xg) for 1 minute. This dry spin helps remove traces of buffer from the column.
19. Transfer the column to a fresh 1.7-mL tube.
20. Add 200 uL of Buffer EB and incubate at room temp for at least 2 minutes.

Note

Elga water can also be used to elute the DNA. However, Buffer EB (10 mM Tris-Cl, pH 8.5) helps stabilize the DNA for long-term use. Since Buffer EB doesn't contain EDTA, it shouldn't interfere with downstream reactions, if you later use the midiprep for cloning.

Tip

Pre-warm Buffer EB in a 55ºC water bath for better elution.

21. Spin at max speed for 1 minute to elute the DNA.
22. To quantify yield, measure 1.5-2 uL of sample on the Nanodrop.
    - Concentrations <200 ng/uL indicate a poor midiprep, but the DNA is still fine to use.
    - The A260/280 ratio should be 1.80-2.00. Lower values indicate protein contamination.
    - The A260/230 ratio should be >2.00 (>1.80 is okay). Lower values indicate salt contamination from the buffers used in the kit.
23. Store midiprepped plasmids at -20ºC for best long-term stability.

Maxiprep

This protocol is adapted from instructions for the QIAGEN Plasmid Plus Maxi Kit (Qiagen 12963[129]), following the **high-yield protocol** (suitable for high-copy plasmids, which applies to most of ours). **Maxipreps yield ~400 uL of DNA at ~500-2,000 ug/uL.** This protocol is essentially identical to midipreps, just with larger volumes. We typically only maxiprep the packaging and envelope plasmids for viral production, which require sequencing after every prep given the likelihood of recombination.

When opening a new bottle of buffer:

- Buffer P1: add RNase A and LyseBlue reagent (1 vial each per bottle), then store at 4ºC
- Buffer PE: add 24 mL ethanol (confirm volume on bottle)

1. Under a flame, aliquot 100 mL of LB media with antibiotic matching the plasmid antibiotic resistance. Use a 500-mL flask for optimal aeration of the culture.
2. Pick a single colony or dab a glycerol stock with a toothpick or pipette tip. Place the toothpick or pipette tip in the liquid culture.
3. Let the bacteria grow in the shaker overnight, typically ~24 hours.

> **Note**
>
> Grow viral plasmids in 30ºC to reduce the chance of recombination.

4. Transfer the culture to several 50-mL conicals and spin at 4800xg (max speed in bucket rotors) for 10-15 minutes, using the large centrifuge by shakers.

> **Note**
>
> The midiprep protocol recommends spinning at 6000xg and 4ºC for 15 minutes, but KL has had successful preps with the parameters written above.

> **Important**
>
> If spinning at 6000xg, the fixed rotor is required (stored in the cabinet below the centrifuge). To avoid spilling culture into the rotor, fill the 50-mL conical with **up to 40 mL** of culture.

5. Pour off the supernatant into one of the empty flasks.
6. Safely dispose of the supernatant and disinfect your glassware: Add bleach to a final concentration of 10% to the supernatant, wait 20 minutes, and pour down the sink. To the remaining empty flasks, add enough 10% bleach to cover the surfaces, swirl, and wait 20 minutes.

[129] https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-plus-kits?catno=12963

Then, thoroughly rinse the disinfected flasks and place them next to the Elga machine to be dishwashed.

Note

Collecting the supernatant (cells/media) waste in flasks and bleaching it yourself keeps the aspirator waste from filling up quickly!

While waiting for the flasks to disinfect, keep them at your bench rather than in the sink, if possible. This keeps the sinks clear for others to use.

7. Resuspend the cell pellet in 8 mL of Buffer P1 (stored at 4ºC in the deli fridge). You can split the volume across the conicals, resuspend each, then recombine into a single conical.
8. Add 8 mL of Buffer P2 and gently invert 5-6 times to lyse cells. Incubate at room temp for 3 minutes. The suspension should turn blue and will become viscous.
9. While you wait, place a large filter column in a new 50-mL conical for each sample.
10. Add 8 mL Buffer S3 and mix 4-6 times, or until the lysate is completely colorless.
11. Transfer the lysate to the filter column (pouring is fine) and incubate for 10 minutes.
12. During incubation, place the small spin columns on the vacuum manifold and add tube extenders. Connect the vacuum manifold to the miniprep aspirator.
13. Gently insert a plunger into the filter column to filter the lysate into the 50-mL conical.
14. Add 5 mL Buffer BB to the cleared lysate and invert 4-6 times.
15. Transfer the solution to the spin column + tube extender on the vacuum manifold. Turn on the vacuum to draw all the liquid through.

Troubleshooting the vacuum manifold

Sometimes, the vacuum pump is not strong enough to effectively filter the solution through the column.

- Double check all the tubing connections and ensure caps seal unused spots on the manifold.
- If the solution is not flowing through, you can try the other miniprep vacuum pump, or directly connect each column to the aspirator tube.
- Alternatively, you can add 700 uL of lysate to the spin column at a time and centrifuge at max speed (16,000xg) for 1 minute, aspirating the supernatant from the collection tube after each spin.

16. With the vacuum still on, remove the tube extender and add 700 uL Buffer ETR to the spin column.

17. Once all the liquid flows through, add 700 uL Buffer PE and allow the vacuum to draw all the liquid through the column.
18. Remove the spin column from the vacuum manifold and transfer to a collection tube. Spin at max speed (16,000xg) for 1 minute. This dry spin helps remove traces of buffer from the column.
19. Transfer the column to a fresh 1.7-mL tube.
20. Add 400 uL of Buffer EB and incubate at room temp for at least 2 minutes.

> **Note**
>
> Elga water can also be used to elute the DNA. However, Buffer EB (10 mM Tris-Cl, pH 8.5) helps stabilize the DNA for long-term use.

> **Tip**
>
> Pre-warm Buffer EB in a 55ºC water bath for better elution.

21. Spin at max speed for 1 minute to elute the DNA.
22. To quantify yield, measure 1.5-2 uL of sample on the Nanodrop.
    - Concentrations <200 ng/uL indicate a poor midiprep, but the DNA is still fine to use.
    - The A260/280 ratio should be 1.80-2.00. Lower values indicate protein contamination.
    - The A260/230 ratio should be >2.00 (>1.80 is okay). Lower values indicate salt contamination from the buffers used in the kit.
23. If prepping common stocks of viral packaging and envelope plasmids, dilute the plasmids to 500 ng/uL and aliquot 500 uL each in fresh 1.7-mL tubes. Send each prep for whole plasmid sequencing to confirm the plasmids have not recombined/mutated.
24. Store maxiprepped plasmids at -20ºC for best long-term stability.

3.2.14 Cloning tips, tricks, and troubleshooting

Help! I got no colonies

Troubleshooting assemblies

- Are you sure you added all components to the assembly reaction? When in doubt, set up a new reaction.
- Check out tips on the protocols for each type of assembly to increase reaction efficiency.
- You can PCR amplify the assembly to determine whether any correct product formed. Choose primers that span fragment junctions. (Note that simply running the assembly on a gel will not work, as there is not enough DNA to see bands.)

Troubleshooting transformations

- Double check that the antibiotic in the agar plate you used matches the antibiotic resistance of your plasmid product.
- Be sure to outgrow plasmids containing kanamycin or chloramphenicol resistance before plating. Outgrow in SOC for 1 hour, but not much longer (1.7-mL tubes are not aerated, so the bacteria will eventually die).
- For plasmids containing ccdB (usually alongside chloramphenicol resistance), be sure to transform ccdB resistant bacteria (not NEB Stable cells). This usually applies to destination vectors, Harbor plasmids, and Janus/Multi-Janus vectors (but *not* to Gateway or Golden Gate assemblies that use these as inputs).
- Try some of the other tips in the *transformation protocol* (page **??**) to increase efficiency.
- For plasmids prone to recombination, such as those with the lentiviral backbone (the LTRs, which have very similar sequences, recombine to form backbone-only products) use the following strategies:
  - Perform a short transformation, incubating on ice only the recommended 20 minutes and not longer.
  - Do not include an outgrowth step (this is not required for plasmids with ampicillin resistance).
  - Always grow plates and cultures at 30°C rather than 37°C.

Designing cloning

Adding short sequences

- To make point mutations, order primers with the mutations in the middle of the sequence (flanked by ~8 bp on either side). Typically, it works well to order the forward and reverse primers with complementary sequences and use them with another pair of primers to generate two PCR fragments (forward with mutation + other reverse, reverse with mutation + other forward). These fragments can then be assembled via a Gibson reaction.

- To insert very short sequences (<20 bp), add the sequence to the primer when amplifying a fragment with PCR, then use Gibson assembly. You can also PCR these fragments with new primers to add longer sequences.
- To insert short sequences (<100 bp), try ligation with annealed oligos. Note that you can PCR the backbone to add restriction sites (also add ~6 bp between the RE binding site and the end of the fragment for efficient digestion). For sequences up to ~60-100 bp, you can use two sets of annealed oligos. (More than two likely will be too inefficient to work.)
- To insert sequences 100-200 bp, your best bet is probably Golden Gate cloning from PCR fragments with custom connector sequences. This size of fragment is too long for annealed oligos but too short for Gibson assembly.

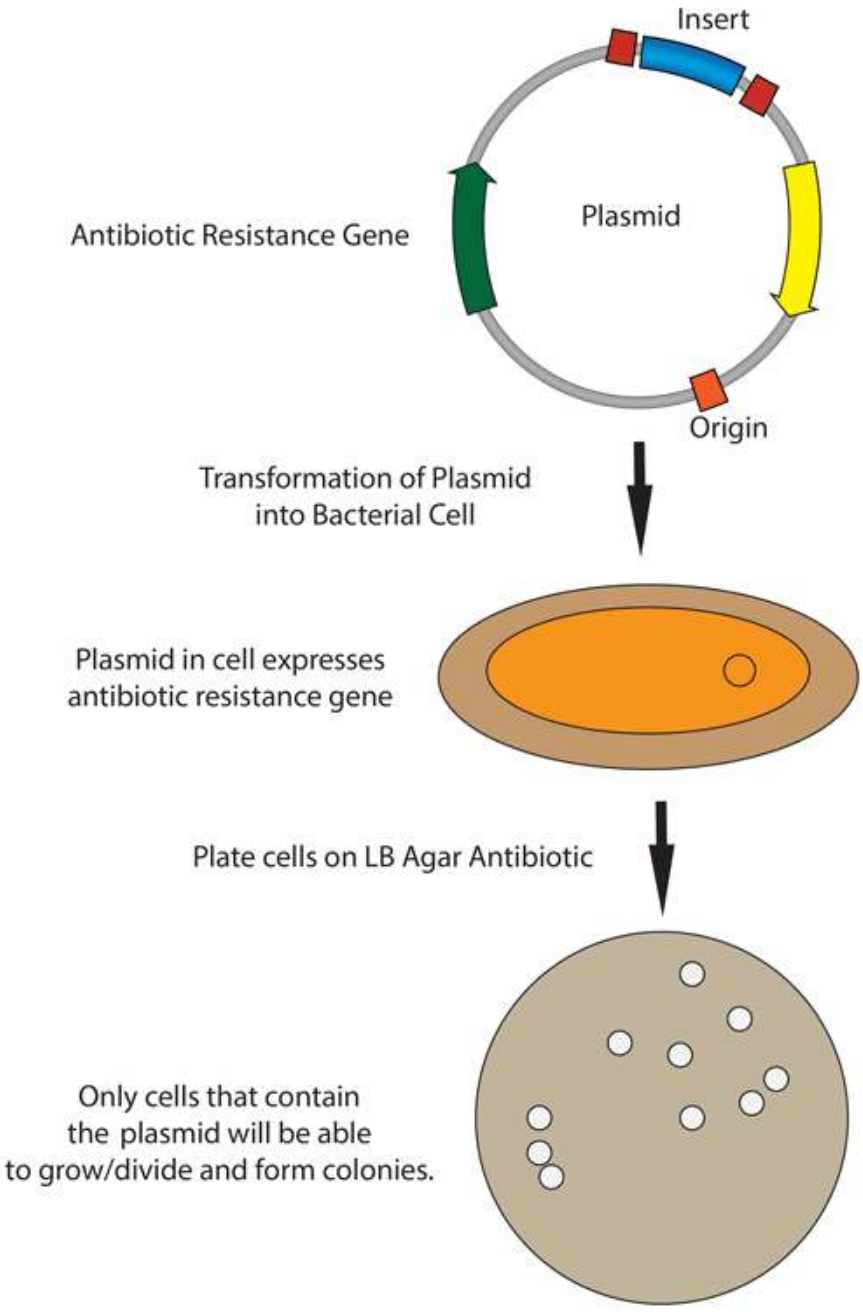
Designing Gibson assemblies

- Choose fragments such that no one fragment contains both the origin of replication and the antibiotic resistance cassette. This reduces background colonies.
- When generating fragments that do not contain an antibiotic resistance cassette (i.e., most fragments that are not the vector backbone), try to choose templates that have a different antibiotic resistance from your final plasmid product. This reduces background colonies.
- Gibson assemblies with >4 fragments will likely be inefficient. To reduce the number of fragments, perform an *overlap extension PCR* (page **??**). This will generate a single fragment from two shorter ones.
- Avoid assembling a single fragment into a plasmid. Instead, generate two fragments to improve efficiency (and probably reduce PCR times!).

3.2.15 Bacterial transformation

Cloning summary

In molecular cloning, we transform DNA of interest into competent bacterial cells. There are several different competence methods that require different transformation methods. After transformation, bacteria that have incorporated the DNA of interest are selected for using various antibiotics.



Transformation of chemically competent bacteria

Estimated time

30 minutes (no outgrowth) or 90 minutes (with 1 hour outgrowth)

1. Thaw chemically competent cells from the -80°C (Elsa) on ice, approximately 10 minutes.
2. Take out an agar plate with the appropriate antibiotic from the 4°C deli fridge and let warm up to room temperature. Optionally, warm to 37°C in the incubator.
3. Mix 1-5 μL of DNA (usually 10 pg – 100 ng) or assembly product into 20-50 μL of competent cells in a 1.7-mL tube. Generally, use NEB Stable cells unless your plasmid product contains ccdB. GENTLY mix by flicking the bottom of the tube with your finger a few times.

Important

Ensure that the total volume of transformed DNA or reaction mixture does not exceed more than 10% of the total volume (i.e., for 50 μL of competent cells, don't use more than 5 μL of DNA or reaction mixture).

Note

Typically, 2-5 μL assembly reaction mixed with 20-50 μL of competent cells generates sufficient colonies. For lower efficiency reactions, use the higher end of this range or more. Transformation efficiencies will be approximately 10-fold lower for assemblies than for an intact control plasmid.

4. Incubate the cell/DNA mixture on ice for 10 min.

Tip

To increase transformation efficiency, you can incubate on ice for 1 hour or more.

5. (*Optional*) Heat shock the mixture by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 sec (45 sec is usually ideal, but this varies by the type of competent cells). Place the tubes back on ice for 2 min.

Note

With the competent cells used in our lab, a heat shock step is not required but may increase transformation efficiency, especially with ccdB/Chlor selection or low efficiency assemblies.

6. To outgrow the bacteria, add 10x volume of LB or SOC media (without antibiotic) to the mixture (e.g., for 30 μL of DNA/cells, add 300 μL SOC). Shake at 37°C for 1 hour.

> **Note**
>
> This outgrowth step allows the bacteria time to express the antibiotic resistance proteins encoded on the plasmid backbone so that they will be able to grow once plated on the antibiotic-containing agar plate. This step is not necessary for Ampicillin resistance but is important for other antibiotic resistances (Kan, Chlor). If skipping the outgrowth step, 300 μL of Elga water can be added to help spread the cell mixture.

7. Spread the transformation mixture onto the pre-warmed agar plate.

   Spread with beads: Before adding the transformation mixture, shake ~20 sterile glass beads onto the agar plate. Add the transformation mixture to the plate and gently shake in a lateral motion (shaking in a circular motion will only spread around the plate edges). Collect used glass beads in the designated, bleach-containing box for disinfection and reuse.

   Spread with a pipette: Using a P1000 pipette, add the transformation mixture to the plate. Holding the pipette as parallel as possible to the plate, gently spread the liquid across the surface. Avoid scratching the agar. This is useful for plating multiple transformations on a single plate—just make sure the liquid from separate transformations don't touch.

> **Note**
>
> If the culture volume is too big, gently collect the cells by centrifugation for 5 minutes at ~200xg and resuspend in a smaller volume of LB or water. If the agar plate doesn't dry adequately before the cells begin dividing, the bacteria will diffuse through the liquid and won't grow in colonies.

8. Incubate plates overnight at 30ºC (to reduce mutation/recombination, important for viral plasmids or those with repeats) or at 37°C (for faster growth).

> **Tip**
>
> For most cloning strategies, 2-5 μL of the reaction mixture is sufficient to add. Ligation reactions are less efficient and may require larger volumes. Usually, using 30 μL of competent cells and 300 μL of SOC media (or Elga water if plating with Ampicillin) yields a sufficient number of bacterial colonies.

3.3 in vitro transcription

3.3.1 Design and preparation of DNA IVT template

We currently have three harbors (pHAs) for IVT:

- pKG01864: contains human beta globin 5' and 3' UTRs

- pKG01834: contains the 5' and 3' UTRs from the Pfizer-BioNTech COVID-19 vaccine
- pKG03198: contains human beta globin 5' and 3' UTRs AND polyA is encoded on the plasmid

Important

When making new DNA IVT templates, use pKG03198! This plasmid already encodes the polyA and the template is generated with a simple MluI digest.

Creating a new IVT template plasmid

pKG03198 is compatible with existing pPV2s for BsaI golden gate cloning, as is pKG01864.

If a desired CDS is not available, you can either 1) make a pPV2 or 2) clone directly into pKG01864 via Gibson. If proceeding with option 2, the plasmid backbone can be PCR amplified with the following primers:

VECTOR-FWD: 5'-ctcgagctcaagcttcgaattcac-3'

VECTOR-REV: 5'-gaccggtagcgtgcttt-3'

Generating IVT linear DNA template from an IVT template plasmid

Our IVT platform relies either on MluI digestion (pKG03198 backbone) or on PCR amplification (pKG01864 or pKG01834 backbone) using a universal primer pair and a plasmid harboring the CDS of interest as template to generate the IVT template. The forward and reverse primers bind to the 5' and 3' beta globin UTR, respectively. The forward and reverse primers also encode the T7 polymerase promoter and the polyA tail, respectively.

PCR-amplified template

1. If a PCR-generated linear template already exists, skip to step 4. Else, perform a small-scale PCR on a new IVT template to verify desired amplification and product specificity:

Component	Amount
5x Q5 buffer	5
dNTP mix	0.4 μL
CleanCap AG F-primer (10 μM)	0.2 μL
R-primer (10 μM)	0.2 μL
Plasmid Template DNA (1 ng/μL)	1.0 μL
Q5 polymerase	0.1 μL
Water	to 20 μL

Important

Make sure you're using the correct primer pairs for a given plasmid IVT template backbone!

2. Perform PCR using 57 C annealing temperature, adjust extension time based on length of linear template.
3. Cleanup the PCR product and run an aliquot on a gel to validate the desired product size was amplified.

Note

This template can now serve as a "master" PCR template for future PCR amplifications. This is advantageous for tricky PCR amplicons and essential for those that contain internal primer binding sites (such as pKG02007, which harbors Cas9), as the annealing temperature can be increased due to the lengthened primer/template homology.

4. Perform the PCR outlined in step 1, substituting the Plasmid Template DNA with the PCR-generated linear template DNA. Scale-up accordingly to generate the amount of linear IVT template needed for your IVT reaction. Store unused linear PCR template in the IVT box and use as needed.

MluI digested template

Note

You should get about 1 μg (100 ng/μL, 10 μL) of IVT template using this method which is good for 3 IVT reactions (each IVT gives ~50-60 μg so at 100ng/96-well, this should give modRNA for ~15-18 plates)

1. Combine the following in a PCR strip tube:

Reagent	Amount	Notes
DNA IVT plasmid	~1 μg	
rCutSmart buffer	5 μL	
MluI	1 μL	
rSAP	1 μL	Dephosphorylates 5' DNA ends to prevent re-ligation of digested product
Elga water	X μL	Add Elga water to reach total volume
Total	**50 μL**	

2. Incubate the mixture at 37°C in the water bath for 1 hour.

3. PCR clean-up the linearized template DNA and use for downstream IVT reactions. Elute in ~10 μL.

Important

As of 2023.06.04: use pKG01864. The Pfizer-BioNTech template (pKG01834) contains two point mutations in the 3' UTR, presumably from using 293T genomic DNA for cloning. We've witnessed poor expression in primary cells (both MEFs and human adult fibroblasts) relative to the beta-globin UTRs. If anyone would like to fix these and characterize (undergrad project?), that could prove useful!

3.3.2 circRNA synthesis

This procedure outlines circRNA synthesis based on Engineering circular RNA for enhanced protein production[130].

Generating IVT linear DNA template from plasmid

Our IVT platform relies on PCR amplification using a universal primer pair and a plasmid harboring the CDS of interest as template to generate the IVT template. The forward and reverse primers bind to the T7 promoter and terminator, respectively. They also contain a polyA tail to promote non-circularized RNA degradation by RNase R.

Note

Alternatively, one could encode these elements on the plasmid and generate IVT template via restriction digestion. Although obviating the need for PCR and using an expensive oligo, the downside of this method is variable polyA tail length due to truncation of long repeat A sequences in E. coli. There is a report of reducing these recombination events, if someone is feeling ambitious and would like to build/test this :)

1. If a PCR-generated linear template already exists, skip to step 4. Else, perform a small-scale PCR on a new IVT template to verify desired amplification and product specificity:

Component	Amount
PrimeSTARv2 (2X)	12.5 μL
Water	9.5 μL
circRNA_fwd (10 μM)	1 μL
circRNA_rev (10 μM)	1 μL
Plasmid Template DNA (5 ng/μL)	1 μL

Note

circRNA_fwd: 5' AAAAAAAAAAAAAAAAAAAAAAAAAAAAGGCCAGTGAATTGTAATACGACTCACTATAGGG 3' circRNA_rev: 5' TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAAAAAACCCCTCAAGACCCGTTTAGAGGC 3'

Note

For certain sequences, Nat found that Q5 with GC enhancer and a 2-step anneal temperature (5 cycles at 70°C, 25 cycles at 72°C) worked better.

1. Perform 2-step PCR, adjust extension time based on length of linear template. 98°C for 10s, 68°C for X s, x35 cycles.

[130] https://www.nature.com/articles/s41587-022-01393-0

2. Gel extract PCR product

Note

This template can now serve as a "master" PCR template for future PCR amplifications. This is advantageous for tricky PCR amplicons and essential for those that contain internal primer binding sites (such as pKG02007, which harbors Cas9), as the annealing temperature can be increased due to the lengthened primer/template homology.

3. Perform the PCR outlined in step 1, substituting the Plasmid Template DNA with the PCR-generated linear template DNA. Scale-up accordingly to generate the amount of linear IVT template needed for your IVT reaction. Store unused linear PCR template in the IVT box and use as needed.

Important

Nat found that a good clean band (no smearing) is important for efficient circRNA IVT

IVT reaction for circRNA

The protocol for IVT is adapted from the NEB HiScribe protocol[131] and Engineering circular RNA for enhanced protein production[132].

1. Thaw the necessary kit components on ice and microfuge to collect solutions to tube bottoms.
2. Assemble the IVT reaction at room temperature in the following order (total volume = 20 μL):

Note

Wear a blue lab coat and wipe down area and pipets before starting. Use barrier tips when handling any of the reagents (nucleosides, buffers, enzymes).

1. Thaw the necessary kit components, gently invert to mix, and microfuge to collect solutions to tube bottoms.

Note

Thaw reaction buffer, DTT, and dNTPs at room temp, then make a master mix of 11 μL per tube. Then add template, water, and enzymes separately.

2. Assemble the IVT reaction at room temperature in the following order (total volume = 20 μL):

[131] https://www.neb.com/en-us/protocols/0001/01/01/standard-rna-synthesis-e2040
[132] https://www.nature.com/articles/s41587-022-01393-0

Component	Amount
10X T7 IVT rxn buffer	2 μL
ATP (100 mM)	2 μL
GTP (100 mM)	2 μL
UTP (100 mM)	2 μL
CTP (100 mM)	2 μL
DTT (0.1 M)	1 μL
Template ($\sim$1 μg)	X μL
Nuclease-free water	6 - X μL
ECIPP	1 μL
T7 RNAP mix	2 μL
Total	20 μL

1. Gently mix reactions, microfuge, and incubate at 37°C in a mixing thermoblock overnight at 1,000 rpm (12-16h)

Note

The reaction should be cloudy if successful. The ECIPP is a pyrophosphatase to counter pyrophosphates which will precipitate with magnesium and decrease IVT efficiency but even with the ECIPP, it will precipitate and look cloudy if successful.

2. Add 30 μL nuclease-free water (to $\sim$50 μL), add 2 μL DNase I, and incubate at 37°C for 30 min.
3. Purify circRNA with NEB Monarch Spin RNA Cleanup Kit[133] following manual provided in kit. 50 μL elution with nuclease-free water should give >1,500 ng/μL.
4. Keep the purified circRNA on ice and Nanodrop to determine concentration (for a 20 μL reaction, one should expect $\sim$90-100 micrograms of RNA).
5. Digest purified circRNA with 1 U RNaseR (20 U/μL) per of μg RNA add 2 μL DNase I, and incubate at 37°C for 30 min. If circRNA is $\sim$1,500 ng/μL, 13 μL should be about 20 μg circRNA.

Component	Amount	Amount if < 1.2µg/µL
10X RNase R rxn buffer	2 μL	3 μL
circRNA (20 μg)	X μL ($\sim$15ish)	Y μL ($\sim$20ish)
RNaseR (20 U/μL)	1 μL	1 μL
Nuclease-free water	17 - X μL	26-Y
Total	20 μL	30 μL

6. Repeat step 3 to clean up RNA and elute in 50 μL nuclease-free water
7. QC by sumitting samples to BMC AATI Fragment Analyzer[134]. It's $15/sample for 5 μL and

[133] https://www.neb.com/en-us/protocols/2025/01/10/monarch-spin-rna-cleanup-kit-protocol
[134] https://bmcwiki.mit.edu/index.php/BioMicroCenter:Advanced_analytical_Fragment_analyzer

the Nano kit can handle 5 to 500 ng/μl (25 to 2500 ng) or Pico RNA 0.05 to 5 ng/μl (0.25 to 25 ng). I submit 0.5 μL circRNA + 4.5 μL nuclease-free water.

Note

TODO: Add image of QC

3.3.3 modRNA synthesis

This procedure outlines modRNA synthesis with Cap 1 structure, where IVT and capping is conducted simultaneously using CleanCap AG reagent[135]. Just be certain that your IVT template contains an AG directly downstream of the T7 promoter element: 5' TAATACGACTCACTATA **AG** 3'

You could also use Anti-Reverse Cap Analog (ARCA)[136], but this workflow has fallen out of favor since the development of CleanCap, as ARCA 1) reduces yield of capped mRNA and 2) contaminates the product with uncapped mRNA, which elicits an immunogenic response.

Important

Please be careful to avoid degradation from RNase[137].

Perform these steps in the genomics hood, wipe down all surfaces with the RNase surface decontaminant, and use a new pair of gloves sprayed with the decontaminant each time you enter/exit the lab.

Also, use fresh barrier tips from inside the genomics hood when handling any of the reagents (nucleosides, buffers, enzymes).

IVT reaction

The protocol for IVT is adapted from the NEB HiScribe protocol[138].

1. Thaw the necessary kit components on ice and microfuge to collect solutions to tube bottoms.
2. Assemble the IVT reaction at room temperature in the following order (total volume = 20 μL):

Component	Amount
nuclease-free water	10.8 - X μL (for 20 μL final volume)
10X T7 IVT reaction buffer	2.0 μL
ATP (100 mM)	1.2 μL (6 mM final)
CTP (100 mM)	1.0 μL (5 mM final)
GTP (100 mM)	1.0 μL (5 mM final)
m1ψ-UTP (100 mM)	1.0 μL (4 mM final)
CleanCap AG (100 mM)	0.8 μL (4 mM final)
Template DNA	100 ng, X μL
Pyrophosphatase (U)	0.2 μL
T7 RNA Polymerase Mix	2.0 μL

[135] https://www.neb.com/protocols/2019/09/23/co-transcriptional-capping-using-cleancap-reagent-ag-from-trilink-and-hiscribe
[136] https://www.neb.com/products/s1411-3-o-me-m7g5ppp5g-rna-cap-structure-analog#Product%20Information
[137] https://www.neb.com/tools-and-resources/usage-guidelines/avoiding-ribonuclease-contamination
[138] https://www.neb.com/protocols/2021/10/12/mrna-synthesis-protocol-with-modified-nucleotides-using-the-hiscribe-t7-mrna-kit-wi

Note

If performing multiple IVTs, one can prepare a mastermix. Dilute each template DNA to 5 μL total volume, prepare mastermix using 5.8 μL Nuclease-free water (based on one 20 μL reaction), and distribute 15 μL of master mix to each 5 μL DNA template tube.

3. Gently mix reactions, microfuge, and incubate at 37 C in a thermoblock for 2-4 hours (longer incubation times recommended for transcripts >3 kb).
4. Optional: post IVT, you can treat you sample with DNase I if your application cannot tolerate residual amounts of DNA template. Add nuclease-free water to 50 μL, add 1 μL DNase I, and incubate at 37 C for 15 min.
5. Purify modRNA with NEB Monarch RNA Cleanup Kit[139] following manual provided in kit.
6. Keep the purified modRNA on ice to perform quality control analysis. Nanodrop to determine concentration (for a 20 μL reaction, one should expect ~50 micrograms of RNA).
7. Perform gel electrophoresis to confirm the full-length product was synthesized. Add nanodropped samples to a new PCR tube containing 8 μL of NEB 2X RNA Loading Dye (blue reagent), incubate at 90 C for 3 minutes, and immediately place on ice for 2 minutes. This is to denature RNA secondary structure so it can be resolved on a native agarose gel.
8. In parallel, prep a ssRNA ladder[140] to serve as a standard.
9. Run your denatured RNA samples on a gel to confirm product size.

Important

Ethidium bromide does not stain ssRNA very well, prepare your gel using SYBR safe!

10. Aliquot modRNA into single-use tubes (scale appropriately for your experiment) and store in -80 C. We have a box for synthesized modRNA in Nokk.

Note

I have observed no reduction in product quality multiple months after storing. Although I have witnessed some sublimination which leads to more concentrated RNA samples.

IVT using MluI-digested pKG3198 (polyA encoded on plasmid)

The protocol for IVT is adapted from the NEB HiScribe protocol[141].

[139] https://www.neb.com/products/t2050-monarch-rna-cleanup-kit-500-ug#Protocols,%20Manuals%20&%20Usage
[140] https://www.neb.com/products/n0362-ssrna-ladder#Product%20Information
[141] https://www.neb.com/protocols/2021/10/12/mrna-synthesis-protocol-with-modified-nucleotides-using-the-hiscribe-t7-mrna-kit-wi

Note

Wear a blue lab coat and wipe down area and pipets before starting. Use barrier tips when handling any of the reagents (nucleosides, buffers, enzymes).

1. Thaw the necessary kit components, gently invert to mix, and microfuge to collect solutions to tube bottoms.

Note

Thaw DTT, dNTPs, and CleanCap at room temp, then make a master mix of 7.8 μL per tube. Then add template, water, and enzymes separately.

2. Assemble the IVT reaction at room temperature in the following order (total volume = 20 μL):

Component	Amount
10X T7 IVT rxn buffer	2 μL
DTT	1 μL
GTP	1 μL
CTP	1 μL
ATP	1 μL
m1ψ (100 mM)	1 μL
CleanCap Reagent AG	0.8 μL
Template (~300-400 ng)	X μL
Nuclease-free water	9.2 - X μL
ECIPP	1 μL
T7 RNAP mix	2 μL
Total	20 μL

3. Gently mix reactions, microfuge, and incubate at 37°C in a thermoblock for 2-4 hours (longer incubation times recommended for transcripts >3 kb).

Note

The reaction should be cloudy if successful. The ECIPP is a pyrophosphatase to counter pyrophosphates which will precipitate with magnesium and decrease IVT efficiency but even with the ECIPP, it will precipitate and look cloudy if successful.

4. Optional: post IVT, you can treat you sample with DNase I if your application cannot tolerate residual amounts of DNA template. Add 30 μL nuclease-free water (to ~50 μL), add 2 μL DNase I, and incubate at 37°C for 30 min.

5. Purify modRNA with NEB Monarch RNA Cleanup Kit[142] following manual provided in kit. 60 μL elution with nuclease-free water should give ~800 ng/μL.

[142] https://www.neb.com/products/t2050-monarch-rna-cleanup-kit-500-ug#Protocols,%20Manuals%20&%20Usage

6. Keep the purified modRNA on ice to perform quality control analysis. Nanodrop to determine concentration (for a 20 μL reaction, one should expect ~50-60 micrograms of RNA).
7. Perform gel electrophoresis to confirm the full-length product was synthesized. Add nanodropped samples to a new PCR tube containing 8 μL of NEB 2X RNA Loading Dye (blue reagent), incubate at 90 C for 3 minutes, and immediately place on ice for 2 minutes. This is to denature RNA secondary structure so it can be resolved on a native agarose gel.

Note

You want >500 ng RNA to resolve on SYBR safe gel. You can put 8 μL NEB RNA loading dye / PCR-tube prior to nanodrop and then add the nanodrop sample directly to the loading dye.

8. In parallel, prep a ssRNA ladder[143] to serve as a standard. Use 2 μL ssRNA + 8 μL RNA loading dye.
9. Run your denatured RNA samples on a gel to confirm product size.

Important

Ethidium bromide does not stain ssRNA very well, prepare your gel using SYBR safe!

10. Aliquot modRNA into single-use tubes (scale appropriately for your experiment) and store in -80 C. We have a box for synthesized modRNA in Nokk. If ~2,400 ng aliquots, you'll have ~20 aliquots.

Note

I have observed no reduction in product quality multiple months after storing. Although I have witnessed some sublimination which leads to more concentrated RNA samples.

[143] https://www.neb.com/products/n0362-ssrna-ladder#Product%20Information

3.3.4 mRNA transfection

We have used Thermo Lipofectamine MessengerMax[144] and Milenyibiotec StemMACS[145] reagents with good success in both immortalized cell lines and primary cells.

For each, we adapted the protocol from the manufacturer's protocol.

MessengerMax transfection protocol

1. Seed cells to be 70-90% confluent at time of transfection.

Note

Amounts given are for transfecting a 96-well with 100 ng of mRNA. If you would like to transfect more mRNA, adjust the amount of MessengerMax (2:1 ratio of reagent to mRNA, i.e., .2 μL MessengerMax per 100 ng mRNA) Scale up amounts of mRNA, reagent, and KO-DMEM solutions 5x for a 24-well (25 μL total volume) and 12.5x (125 μL total volume) for a 6-well.

1. Dilute .2 μL MessengerMax Reagent in 4.8 μL opti-mem, mix well, and incubate 10 minutes at room temperature.
2. Dilute 100 ng mRNA in opti-mem to a total volume of 5 μL, and add to the MessengerMax/opti-mem mixture.
3. Incubate for 5 minutes at room temperature.
4. Add the 10 μL of mRNA-lipid complex to desired 96 well.

Note

KO-DMEM can also be used instead of opti-mem media. KO DMEM will have lower efficiency.

StemMACS transfection protocol

1. Seed cells to be 70% confluent at time of transfection.

Note

Amounts given are for transfecting a 96-well with 100 ng of mRNA. If you would like to transfect more mRNA, adjust the amount of StemMACS (3:1 ratio of reagent to mRNA, i.e., .3 μL StemMACS per 100 ng mRNA) Scale up amounts of mRNA, reagent, and KO-DMEM solutions 5x for a 24-well (25 μL total volume) and 10x (100 μL total volume) for a 6-well (their recommendations are different from MessengerMax)

[144] https://www.thermofisher.com/order/catalog/product/LMRNA001?gclid=Cj0KCQiAm5ycBhCXARIsAPldzoUQKASct6CpctkEyzxfPpO
dj1xPJys2Td34cCGrxLcwaAseqEALw_wcB&ef_id=Cj0KCQiAm5ycBhCXARIsAPldzoUQKASct6CpctkEyzxfPpObolE2SZKRp_
dj1xPJys2Td34cCGrxLcwaAseqEALw_wcB:G:s&s_kwcid=AL!3652!3!535167329917!!!g!!!398236708!127976663191&
cid=bid_clb_tfx_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!535167329917!!!g!!

[145] https://www.miltenyibiotec.com/US-en/products/stemmacs-mrna-transfection-kit.html#130-132-949

1. Add .3 μL StemMACS to 4.7 μL of StemMACS Transfection Buffer (in 4 C TC fridge) and mix well.
2. Dilute 100 ng of mRNA in StemMACS Transfection Buffer to a total volume of 5 μL.
3. Add the diluted StemMACS reagent/buffer solution to the diluted mRNA and incubate for 20 minutes at room temperature.
4. Add the 10 μL of mRNA-lipid complex to desired 96 well.

Note

If performing multiple mRNA transfections, media change 4 hours post-transfection to reduce cytotoxicity.

3.4 Protein production

3.4.1 Protein gel casting

The recipes and procedures on this page were originally posted here[146].

Required stock solutions

- **40% Acrylamide stock solution**: Solution of monomers for gel polymerization.

  We find it cheaper to buy premixed 40% stock solution, with a acrylamide:bis-acrylamide ratio of 29:1 (3.3%). Stocks with a 37.5:1 ratio also work, and are typically used for resolving larger proteins.

- **3x bis-Tris gel buffer**: Ion buffer used in gel casting.

Component	Concentration	g/L to final concentration
bis-Tris	1 M	209.242
HCl	Add to pH 6.5-6.8	

- **10% APS**: One of the polymerization initiators. Only a small quantity needs to be prepared; each gel only requires 25 uL.

Component	Concentration	g/L to final concentration
Ammonium persulfate	10%	100

[146] https://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best

Casting protocol

Warning

The acrylamide monomers used here are toxic. Read the SDS[147].

Perform polymerization steps with a lab coat in a fume hood, and collect rinse waste in a waste container.

1. Prepare 1X resolving and stacking buffers. These buffers can be stored in the refrigerator for several weeks. Recipes given here for enough for 2 0.75mm gels.

   Resolving buffer: ~3 mL per gel (6.5 mL total):

Component	Volume	Final concentration
3x bis-Tris gel buffer	2.2 mL	1x
40% Acrylamide stock	1.3 mL	8%
DI water	3 mL	

   Stacking buffer: ~1.2 mL per gel (2.5 mL total):

Component	Volume	Final concentration
3x bis-Tris gel buffer	0.83 mL	1x
40% Acrylamide stock	0.32 mL	5%
DI water	1.36 mL	
Bromophenol blue	50 uL	(enough to give color)

Gel casting setup

In-lab, we have the ability to cast two gels simultaneous; this is recommended even if you only need one, so that you have a backup in case of pouring mishaps. Our gel runner also requires two poured gels to properly seal.

Gels can be stored in zip-lock bags in the fridge for at least several days.

1. Locate two 0.75mm spacer plates and two short glass plates.
2. Use ethanol and a Kimwipe to clean both glass surfaces.
3. Assemble them in the green alignment device.
4. Lock the two gels into the transparent gel pouring device.

[147] https://www.fishersci.com/store/msds?partNumber=BP14081&productDescription=ACRYLAMIDE%3ABISACRYLAMIDE+29%3A1&vendorId=VN00033897&countryCode=US&language=en

Resolving gel

1. Measure 6.5 mL of **1x resolving buffer** per gel to pour.
2. Add 50 uL of **10% APS** per gel, mix well.
3. Add 20 uL **TEMED**, mixing quickly. Pour both gels to the resolving gel height (3 mL per gel). Lightly tap and tilt the gel to remove any bubbles.
4. Once done pouring, quickly but carefully fill the remaining height with water, making sure the gel-water interface stays undisturbed.
5. Wait for the polymerization reaction to finish (noticeable by a change in refractive index).
6. Drain the water by tilting the gel past 90 degrees, and wicking away with a Kimwipe.

Stacking gel

1. Measure 2.5 mL of **1x stacking buffer** to pour.
2. Add 20 uL of **10% APS**, mix well.
3. Add 10 uL **TEMED**, mixing quickly. Fill the top of the gels until just before overflowing. Insert the comb into the top, letting it rest on the spacers.
4. Wait for the stacking gel to polymerize.
5. Rinse with water to remove unpolymerized acrylamide.
6. If removing the combs prior to storage, slowly remove the comb, ensuring that wells are not broken.

3.4.2 Chitin-binding protein purification

Warning

This protocol was attempted for Tn5 purification. This was unsuccessful, though possibly not because of this protocol.

Required solutions

- **Chitin Lysis buffer**:

Component	Concentration	amount/L final volume
DI water	89.8%	898 mL
HEPES	20mM	4.766 g
NaCl	800 mM	46.762 g
EDTA	1 mM	0.292 g
Triton-X100	0.2%	2 mL
Glycerol	10%	100 mL
KOH	to pH 7.2	

- **Chitin cleavage buffer**:

Component	Concentration	amount/L final volume
Chitin lysis buffer	Base	898 mL
DTT	100 mM	15.423 g

- **Chitin stripping buffer**:

Component	Concentration	amount/L final volume
Chitin lysis buffer	Base	898 mL
SDS	1%	10 g

- **2x Dialysis buffer**:

Component	Concentration	amount/L final volume
DI water	79.8%	798 mL
HEPES	100mM	23.83 g
NaCl	200 mM	11.69 g
EDTA	0.2 mM	0.0584 g
DTT	2 mM	0.3085 g
Triton-X100	0.2%	2 mL
Glycerol	20%	200 mL
KOH	to pH 7.2	

- **10% PEI**:

Component	Concentration	amount/10 mL final volume
Branched PEI (50% w/v)	10%	2 mL
DI water	–	8 mL
HCl	–	to pH 7.2

Note

Use *branched* PEI. Any branched PEI should technically work, though the product mentioned in the literature is Sigma P3143[148].

Protocol

1. Resuspend the cell pellet in lysis buffer plus cOmplete protease inhibitor, on ice. For every gram of cell pellet, use 10 mL lysis buffer.
2. Sonicate the resuspended cells, using 5 cycles of 30-second, medium-amplitude 50% duty-cycle sonication.
3. Spin to clarify the lysate using maximum centrifuge speed (14600 xg) for 30 minutes.
4. If the protein of interest has DNA-binding activity, add 1.2 mL/10mL **10% PEI** dropwise while stirring. This should be **branched PEI** as noted above. Centrifuge at maximum speed for 30 minutes to remove bound DNA.
5. Incubate the supernatant with 10 mL chitin resin / 500 mL original culture. Tube rotate for 1 hour at 4C.
6. Wash with 10-20 CVs of lysis buffer.
7. Add cleavage buffer. Incubate at 4C for 48 hours.
8. Elute into separate 1 mL fractions.
9. Proceed to dialysis and concentration.

Dialysis and concentration protocol

Note

Both the dialysis tubing and the protein concentration columns come with a molecular weight cutoff! Make sure that both the tubing and columns are sufficiently sized so that the protein cannot leave through the tubing but also gets concentrated on the column!

1. Prepare dialysis tubing, long enough to hold the desired volume, plus about a 20% headspace. You can look up length calculators, or try it out with water first.

[148] https://www.sigmaaldrich.com/US/en/product/sial/p3143

2. For easy filling, soak the tubing in water to make it more pliable.
3. Clip the bottom of the tubing shut with the dialysis tubing clips.
4. Set the tubing in a clean glass beaker, in case you spill in the next step.
5. Using a seriological pipette, transfer each elution buffer fraction into segment of tubing. Close the tubing with a second dialysis tubing clip on the top.
6. Check that the tubing is watertight, over/inside the clean glass beaker.
7. Transfer the tubing to a large plastic beaker, filled with 2x dialysis buffer. Place the beaker + tubing on a stir plate and let the dialysis proceed at 4C in the deli fridge for around 4-8 hours.
8. Replace the dialysis buffer with fresh buffer, and let it stir for an additional 24 hours.
9. Further dialysis steps can be added on if desired purity not reached.
10. Follow the instructions on the protein concentrator columns to concentrate the resulting Look up length calculators, or try it out with water to determine the correct length.

Regeneration Protocol

1. Wash the resin with 3CVs of stripping buffer.
2. Stop the flow, and soak the resin in stripping solution for 30 minutes.
3. Wash with an additional 10 CVs of stripping buffer.
4. Rinse with 20 CVs of water.
5. Equilibrate with 5 CVs of 20% ethanol.
6. Store the resin in 20% ethanol at 4C.

3.4.3 Denaturing protein gel run and staining

Solutions required

- **20x MES-SDS running buffer stock solution**: Suitable for separating proteins with a molecular weight less than 75 kDa.

  It is also generally cheaper to order this as a pre-mixed 20x stock solution. If you need to make it yourself, the recipe is:

Component	Final concentration	g/L to final concentration
MES	1 M	195.2 g
Tris	1 M	121.13 g
EDTA	20 mM	5.845 g
SDS	2%	N/A

- **200x running buffer reductant**: Ensures that the gel remains under reducing conditions when run. Add directly to 1x running buffer before filling the gel tank.

Component	Final concentration	g/L to final concentration
Sodium bisulfite	1 M	104.061 g

- **200 mM Tris-HCl stock**:

Component	Concentration	g/L to final concentration
Tris-HCl	200 mM	31.52 g
NaOH	Add to pH 6.8	

- **10% SDS stock**: At low temperatures, the SDS may fall out of solution, but will redissolve when mixed with the other ingredients. Mix well before transferring.
- **0.1% bromophenol blue**

Component	amount/50 mL to final concentration
bromophenol blue	50 mg
DI water	50 mL

Note

The bromophenol blue solution will not be blue. It is more brown at this concentration. It will be blue when you add it into other things.

- **2x sample buffer**: Used to denature and solubilize protein samples. Can be stored

Component	Final concentration	Volume / 10 mL
200 mM Tris-HCl stock	100 mM	5 mL
Glycerol	20%	2 mL
10% SDS stock	2%	2 mL
0.1% bromophenol blue stock	0.01%	1 mL

- **Coomassie staining dye**: When preparing this dye, pour the 10% methanol first, using it to dissolve the R-250. Then, add water. Add the glacial acetic acid last to prevent aggregation.

Component	Final concentration
Coomassie R-250	0.2% (2g/L)
Methanol	10%
Acetic acid	10%
Water	80%

- **Coomassie destain solution**

Component	Final concentration
Acetic acid	10%
Water	90%

Running procedure

1. Dilute enough **20x MES-SDS running buffer** to fill the gel tank, adding fresh **200x running buffer reductant** if a gel has not been recently run.
2. Add **2x sample buffer** to the protein sample of interest. Add 2-mercaptoethanol to a final concentration of 1%.

Warning

2-mercaptoethanol smells awful; always add it inside a fume hood.

3. Briefly heat the protein sample to above 80C for one minute, to ensure full protein denaturation.
4. Place a *prepared bis-Tris protein gel* (page **??**) in the gel-runner, and fill both chambers with the prepared 1% MES-SDS running buffer. The combs (with the shorter glass side) should be facing towards the center. Fill the center chamber first, and wait for a minute to check for leaks before filling the outer chamber. If there are leaks, reseat the gels in the runner and repeat.
5. Carefully load samples, including a protein standard.

6. Run the gels at constant current, about 30 mA per mini-gel. The dye band runs around 3-5 kDa, so it is typically ok to run the dye band to the bottom of the gel unless very small proteins are of interest.

Note

If the gel runner cannot reach 30 mA per mini-gel, double check that you oriented the gel properly! The loading side should be facing inward to ensure a full liquid seal.

Staining (Coomassie)

1. Fill a small dish (the top of a pipette tip box works great!) with DI water.
2. Carefully separate the gel plates from each other, without ripping the delicate gel. Invert the gel over the dish of water, and gently move the gel into the dish.
3. Place the gel dish on a rocker for 5 minutes to remove any unbound proteins.
4. Drain the water, and replace it with a roughly half-inch layer of Coomassie stain.
5. Either gently shake, while covered, for several hours, or microwave until the solution just reaches boiling (for accelerated diffusion). If microwaving, cover with Kimwipes to prevent spattering!
6. Drain the Coomassie stain off, replace with DI water, and rock for 5 minutes.
7. Drain the DI water, and replace with destaining solution.
8. Cover the gel with Kimwipes, and either shake, while covered, for several hours or microwave as before.
9. When the destain solution seems substantially blue or purple, replace it with fresh destain solution.

3.4.4 Nanobody-MNase production for GapRUN

This protocol describes how to produce the anti-GFP nanobody-MNase fusion protein required for *GapRUN* (page **??**). It is based on the work of [**?**] and [**?**].

Required materials

In addition to the buffer components listed below, we use the following kits and other components:

- B-Per: bacterial lysis kit (Thermo Fisher 78248)
- EDTA-free protease inhibitors (Thermo Fisher A32965)
- Glutathione spin columns (Thermo Fisher 16106)
- Biotin-tagged thrombin protease (Sigma-Aldrich SAE0147-5KU)
- T1 streptavidin beads (Thermo Fisher 65601)
- 10 kDa MWCO protein concentrator spin columns (Thermo Fisher 88513)

Stock buffer preparation

1. Prepare and check that there is sufficient amounts of the following *shared genomics buffers* (page **??**):

- 1M Tris-HCl, pH 7.5
- 0.5M EDTA, pH 8.0
- 5M NaCl

2. 1 mL of **1M IPTG**:

Component	Concentration	g / L	Amount / 1 mL
IPTG	1 M	238.3	0.238 g
Elga water	to 1 mL		
Sterile filter, 0.22μm filter			

3. 200 mL of **Protein purification buffer**:

Component	Concentration	g / L	Amount / 200 mL
Tris-HCl	125 mM	19.70	3.94 g
NaCl	150 mM	43.83	8.77 g
NaOH	to pH 8.0		
Elga water	to 200 mL		

4. 50 mL of **0.1M K2PO4 (pH 7.0)**:

Component	Concentration	g / L	Amount / 50 mL
K2PO4	100 mM	17.42	0.871 g
HCl	to pH 7.0		
Elga water	to 50 mL		

Day -n

It is best practice to start protein production cultures from single colonies. Either:

a. Transform the plasmid encoding GST-LaG16-MNase (Addgene 170978[149], miniprep from pKG03872) into BL21 (DE3) cells.

b. Or, streak directly from the pKG03873 glycerol stock (GST-LaG16-MNase in BL21 (DE3) cells) onto LB-Amp plates.

Day 0

1. From one colony, start two 5 mL overnight cultures in LB-Amp media.
2. Autoclave four baffled 1L flasks, each filled with 200 mL of freshly prepared LB media.
3. After the flasks have cooled to room temperature (or the following day before starting), spike-in Amp.
4. Prepare fresh **sample buffer**. Sample buffer made within the last month or two can also have fresh β-mercaptoethanol spiked in lieu of making fresh buffer.

Component	Concentration	Amount / 10 mL
50% glycerol	10%	2 mL
1M Tris (pH 7.5)	50 mM	500 μL
0.5M EDTA (pH 8.0)	20 mM	400 μL
10% SDS	2%	2 mL
β-mercaptoethanol	1%	100 μL
Bromophenol blue	to desired color	
Elga water	to 10 mL	5 mL

Day 1

1. Take a sample of the LB-Amp flasks for blanking the NanoDrop.
2. Inoculate each flask (containing 200 mL of media) with 2 mL of the overnight culture.
3. Incubate the flasks at 37C, shaking at 200 rpm, until it reaches an OD600 of 0.5, about two hours.
4. Take a 1.5 mL "pre-induction" sample, spin it down, and resuspend in 300 μL of sample buffer, storing it at -20C for future analysis.

[149] https://addgene.org/170978

5. Induce protein production by adding 200 μL of 1M IPTG to each flask. Lower the incubator temperature to 18C (or, whatever room temperature is) and let flasks grow overnight.

Day 2

1. Measure the OD of the flasks by diluting a sample five-fold. The OD600 should have reached at least 6.0.
2. Take a 500 μL "post-induction" sample, spin it down, and resuspend in 300 μL of sample buffer, storing it at -20C for future analysis.
3. Split the bacterial culture into 50 mL tubes, not exceeding 40 mL per tube (to prevent spillage upon centrifugation in the fixed rotor).
4. Pellet the cells at maximum speed (14000 g) using the fixed-angle rotor. Combine the pellets together and mass them.
5. Prepare 100x protease inhibitor cocktail (1 EDTA-free protease inhibitor tablet dissolved in 500 uL) if there is not enough. The tablet will not fully dissolve: pipette up and down to resuspend settled particles.
6. Prepare 4 mL of complete B-Per (4 mL B-Per buffer, 2 μL lysosome, 2 μL DNAse I, both included in the B-Per kit, plus 40 μL of 100x protease inhibitor cocktail) per 1g of cell mass.
7. Resuspend the pellet in the B-Per. Use a 5 mL serological pipette to fully resuspend the pellet.
8. After incubation at room temperature for 15 minutes, subject the slurry to two freeze thaw cycles to -80C and back to room temperature.
9. Clarify the lysate by spinning at 14000 g in a fixed-angle rotor for 30 minutes at 4C.
10. Take a 50 μL sample of the clarified lysate, dilute it to 300 μL in sample buffer, and store it at -20C for later analysis.
11. Dilute the clarified lysate 2:1 in protein purification buffer.
12. Prepare two 0.2 mL glutathione spin columns, by washing it twice with two column volumes of wash buffer.
13. Load the clarified lysate onto the columns. This takes many, many spins because our vacuum manifold isn't strong enough.

Note

When you spin the columns, don't screw on the caps! Leave the red caps off the columns.

14. Once the lysate has been loaded, wash the columns with successive washes (2 column volumes: 400 μL) until the A280 absorbance drops to background levels. This takes about 10 total washes (20 CVs).
15. Cover the bottom of the columns with parafilm.
16. To each column, combine 100 units of biotin-tagged thrombin protease diluted to 200 μL in wash buffer to each column. Incubate overnight at 4C.

Note

You can likely reduce the amount of protease; I used excess to ensure cleavage worked well.

Day 3

1. Prepare 400μL fresh 1M dithiothreitol (DTT). This is much in excess, but we are limited by balance accuracy.

Component	Concentration	g / L	Amount / 400 μL
DTT	1M	154.25	0.077 g
Elga water	to 400 μL		

Warning

DTT stock should be collected as a separate waste stream.

2. Prepare 5 mL of sterile filtered, PIC-spiked fresh Buffer A:

Component	Concentration	Amount / 5 mL
0.1M K2PO4 (pH 7.0)	20 mM	1 mL
50% glycerol	10%	1 mL
0.5M EDTA (pH 8.0)	0.5 mM	5 μL
1M DTT	1 mM	5 μL
Elga water	to 5 mL	3 mL
0.22 micron filter		
100x PIC		50 μL

2. Spin the columns, collecting "elution 1". Wash with 1 CV (200 μL) for two successive elutions.
3. Combine elutions that have significant A280 absorption: typically, the first two elutions are combined.
4. Wash 60 μL of T1 streptavidin beads three times with PBS. Wash with 200 μL each time.
5. Combine the elutions with the washed streptavidin beads. Rotate the elutions at 4C for 30 minutes.
6. Remove the streptavidin beads with a magnet, and transfer the elution fraction to fresh low-binding tubes.
7. Take a 10 μL sample of the combined elution, dilute to 20 μL in sample buffer, and store at -20C for future analysis.
8. Load the elution fraction to 10 kDa MWCO protein concentrator spin columns, and concentrate to a volume of around 100 μL.

9. Buffer exchange by adding prepared Buffer A. Buffer exchange by adding 100 μL of Buffer A on top, re-concentrating to 100 μL. Repeat this four times.
10. Concentrate the protein to a volume of around 37.5 uL per column. Combine the two volumes to around 75 μL, diluting with Buffer A as needed.
11. Add 75 μL of 100% glycerol on top, to reach 150 μL of purified protein. Aliquot and store at -20C.
12. Confirm the correct protien product by load each of the accumulated samples in sample buffer into a *prepared denaturing protein gel* (page **??**). Look for a single elution band corresponding to the nanobody-MNase.

To benchmark protein activity, proceed to the *GapRUN protocol* (page **??**).

3.4.5 Protein expression

Estimated time

2 days

Protocol

Note

The BL21 strain of *E. coli* is best for protein production. It has several proteases knocked out, among other modifications. There are several sub-strains that can be relevant:

- **BL21**: The classic, useful if your promoter is a standard *E. coli* promoter such as *lac, tac, trc, ParaBAD, PrhaBAD,* or *T5*.
- **BL21(DE3)** : A strain with integrated T7 polymerase. Can be used in place of **BL21** but is required if using the *T7* or *T7-lac* promoters.
- **BL21-RIL** : A strain with extra copies of certain tRNAs, in order to correct for the rarity of some codons in *E. coli*. This can be helpful in upping protein titers, especially when expressing some mammalian proteins.
- **Rosetta2 (DE3)** : A strain with integrated T7 polymerase, plus extra tRNAs and other modifications that make it ideal for protein expression.

1. In the morning, start a **5 mL** culture of your strain, in LB/antibiotic media.
2. Prepare *TB media* (page **??**) in Erlenmeyer flasks. For proper aeration, flasks should be filled to at most 30% the listed volume.
3. Before leaving for the day, incoluate **20 mL** LB/antibiotic cultures with **400 uL** of the 5 mL culture.
4. The following morning, use the NanoDrop to calculate the OD of the overnight cultures. Inoculate large flasks to an OD600 of 0.075 (e.g. three doublings to get to 0.6).

Note

Remember to take a sample of LB/TB media to properly blank the NanoDrop!

Induction to OD600 0.075 means that each 20 mL culture can induce around 1L of TB media, as the overnight OD600 should be around 5-10. If you are inducing more than 1L, you may need to make multiple 20 mL cultures.

Calculate OD's with the simple dilution formula:

$$\text{mL starter culture} = \frac{\text{mL bulk culture} \cdot 0.075}{\text{OD of starter culture}}$$

5. Grow cultures until an OD600 of 0.6. Sample every half hour to an hour. It should take

around 90 minutes to 2 hours to reach and OD of 0.6.

6. Lower the temperature to 30C and induce with 0.5 mM sterile-filtered IPTG.
7. Let the culture grow for 5-6 hours.
8. Harvest cells via centrifugation at 4000xg for 30 minutes. At this point, the cell pellet is immediately ready for further protein purification steps. The cell pellet can also be stored at -20C or -80C.

3.4.6 His-tag protein purification

Warning

This protocol was attempted for Tn5 purification. This was unsuccessful, though possibly not because of this protocol.

Source

The QIAexpressionist is the go-to manual on His-tag purifying proteins.

Required solutions

- **Lysis buffer**: You have options! Check page 113 of the QIAexpressionist.
- **10% PEI**: pH to 7.2

Note

Do not use the cOmplete running and elution buffers with normal Ni-NTA His-tag resin! They contain chelating agents that will remove the nickel ions! cOmplete His-tag resin is resistant to small concentrations of chelating agents.

- **cOmplete running buffer (native)**:

Component	Concentration	g/L final volume
DI water	90%	
HEPES	20mM	4.766 g
NaCl	800 mM	46.762 g
Imidazole	20 mM	1.362 g
EDTA	1 mM	0.292 g
DTT	2 mM	0.3085 g
Glycerol	10%	
NaOH	to pH 7.2	

Note

Perform the pH after all components have been added.

- **Complete elution buffer (native)**:

Component	Concentration	g/L final volume	Purpose
DI water	90%		
HEPES	20mM	4.766 g	Main buffer component
NaCl	800 mM	46.762 g	High salt maintains protein solubility
Imidazole	300 mM	20.42 g	Prevents non-specific binding to column.
EDTA	1 mM	0.292 g	Chelating agent, deactivates proteases and other enzymes.
DTT	2 mM	0.3085 g	Reducing agent, prevents nonspecific cystine crosslinking.
Glycerol	10%		Prevents hydrophobic protein-protein interactions.
NaOH	to pH 7.2		

Note

Perform the pH after all components have been added.

- **NI-NTA running buffer (native)**:

Component	Concentration	g/L final volume	Purpose
DI water	90%		
NaH2PO4	50mM	6.90 g	Main buffer component.
NaCl	800 mM	46.762 g	High salt maintains protein solubility.
Imidazole	15 mM	1.022 g	Prevents non-specific binding to column.
beta-mercaptoethanol	5 mM		Weaker reducing agent, prevents nonspecific cystine crosslinking.
Glycerol	10%		Prevents hydrophobic protein-protein interactions.
NaOH	to pH 8.0		

Note

Perform the pH after all components have been added.

Protocol

1. Resuspend the cell pellet in lysis buffer, on ice. For every gram of cell pellet, use 10 mL lysis buffer.
2. Sonicate the resuspended cells, using 5 cycles of 30-second, medium-amplitude 50% duty-cycle sonication.
3. Spin to clarify the lysate using maximum centrifuge speed (14600 xg) for 30 minutes.
4. If the protein of interest has DNA-binding activity, add 1.2 mL/10mL **10% PEI** dropwise while stirring. Centrifuge at maximum speed for 30 minutes to remove bound DNA.
5. Fill a column with roughly 1.0 mL resin per gram of cell lysate.

> **Note**
>
> When using affinity columns, the volumes required will be listed as Column Volumes (CVs). The amount of resin is the CV. For a gram preparation, this would mean the CV is 1 mL.

5. Equilibrate the column with 3 CVs of running buffer.
6. Flow the clarified cell lysate across the column, collecting the flow-through.
7. Rinse the column with 8 CVs of running buffer, collecting flow-through fractions.
8. Elute with four separate 0.5 CV washes of elution buffer.
9. Run all collected samples on a SDS-page gel to confirm successful purification.

3.5 TC protocols

3.5.1 Induced pluripotent stem cells (iPSCs)

STRAIGHT-IN Landing Pad Creation

STRAIGHT-IN is one method of engineering cell lines by precisely integrating DNA payloads into an hiPSC acceptor line.

Landing Pad Targeting in hiPSCs

The first step is to install a landing pad cassette in the hiPSC genome at a safe harbor locus (e.g. CLYBL, AAVS1) via CRISPR-Cas9 or TALENS-mediated homologous recombination.

Step 1: Landing Pad Targeting

1. Follow standard hiPSC seeding protocol on a laminin coated plate such that the cells will reach ~30% confluency the following day for transfection.
2. With Lipofectamine Stem co-transfect the hiPSCs with a selection of following:
    - Cas9 expression plasmid + sgRNA plasmid targeting the safe harbor locus + HDR donor plasmid + (optional: p53DD modRNA)

- AIO Cas9 & sgRNA plasmid + HDR donor plasmid
- Control HDR donor plasmid only (this will give background fluorescence from the landing pad and an indicator for dilution out of the HDR plasmid)

Note

We have observed p53DD modRNA boosts knock-in efficiency in co-transfection

3. After 4 hours add fresh StemFlex media (iPSC aren't happy in Opti-MEM for long periods)
4. Passage the cells 48 hours post transfection using GCDR (after a passage the cells should lose most plasmid fluorescence due to dilution)

Step 2: Selection and Clonal Isolation

TODO

STRAIGHT-IN Line Procedure

This protocol describes the step-by-step integration of Donor plasmids using the STRAIGHT-IN (Dual) platform to generate genetically modified hiPSC lines. The procedure begins with a STRAIGHT-IN hiPSC acceptor line, derived from either LU99 (from Leiden) or iPS11 backgrounds. We currently possess two types of acceptor lines:

- **Single landing pad lines:** iPS11 background only
- **Dual landing pad lines:** iPS11 at CLYBL and LU99 at either CLYBL or AAVS1

Two landing pad systems are used:

- **Bxb1-GT landing pad**
  - Confers zeocin (bleomycin) resistance upon integration
  - Carries CAG-BFP (bright blue signal in iPS11 Single GT line or iPS11 Dual line) or PGK-BFP (silenced in both LU99 lines)
  - Auxiliary sequences (BFP, BleoR, plasmid backbone) are excised using Cre recombinase
- **Bxb1-GA landing pad**
  - Confers puromycin resistance upon integration
  - Carries CAG-GFP (bright green signal in iPS11 Single GA line) or CAG-mScarlet (bright red signal in iPS11 Dual line) or PGK-mScarlet (silenced in both LU99 lines)
  - Auxiliary sequences (GFP/mScarlet, PuroR, plasmid backbone) are excised using Flp recombinase

Key Reagents (TC Supplies)

- 24-well plates

- rhLaminin-521[150]: aliquots are in Grand Paddie (-20°C) and stocks in Sven (-20°C).
- PBS +/+[151]: we keep the stock in cell culture fridge and in Oaken (4°C).
- PBS -/-
- Gentle Cell Dissociation Reagent[152]
- Penicillin/Streptomycin[153]: aliquots and stocks are in Olaf (-20°C).
- StemFlex medium[154]: aliquots and stocks in Sven (-20°C). It is aliquoted without Penicillin/Streptomycin but once the aliquot of the medium has been thawed, add Penicillin/Streptomycin (1:100). Do not thaw StemFlex medium at 37°C, do it at room temperature or preferably at 4°C overnight.
- Neutralization medium: DMEM/F12[155] + 1% FBS[156]
- RevitaCell Supplement[157]: used at 1:200. Aliquots are in Grand Paddie (-20°C) and stocks in Sven (-20°C).
- OptiMEM[158]: aliquots and stocks are in Oaken (4°C).
- Lipofectamine Stem Transfection Reagent[159]: stocks are in Oaken (4°C).
- Zeocin™ selection reagent[160]: aliquots are in Grand Paddie (-20°C). Stock is at 100 mg/mL and the working solution is 15 μg/mL (1:6666).
- Puromycin[161]: aliquots are in Grand Paddie (-20°C). Stock is at 1 mg/mL and the working solution is 1 μg/mL (1:1000).
- DNeasy Blood and Tissue kit[162]
- STRAIGHT-IN acceptor lines: LU99 CLYBL Dual, LU99 AAVS1 Dual, iPS11 CLYBL Single GT, iPS11 CLYBL Single GA and iPS11 CLYBL Dual

Key Reagents (Plasmids and modRNA)

- eeBxb1 plasmid (pKG3466) or eeBxb1 modRNA (pKG3471). Miniprep fresh plasmid or preferably use modRNA. Frozen aliquots of the modRNA should be stored at -80°C (Elsa) in the box with the rest of the recombinases.
- p53DD modRNA (pKG3199). Frozen aliquots of the modRNA should be stored at -80°C (Elsa) in the box with the recombinases.

[150] https://www.thermofisher.com/order/catalog/product/A29248
[151] https://www.thermofisher.com/order/catalog/product/14040141
[152] https://www.stemcell.com/products/gentle-cell-dissociation-reagent.html
[153] https://www.thermofisher.com/order/catalog/product/15140122
[154] https://www.thermofisher.com/order/catalog/product/A3349401
[155] https://www.thermofisher.com/order/catalog/product/21331020
[156] https://www.geneseesci.com/product/genclone-fetal-bovine-serum/?srsltid=AfmBOor2lSkkxAJVdk6msyt0Qf2OimomtjZPynUMimF1mn
[157] https://www.thermofisher.com/order/catalog/product/A2644501
[158] https://www.thermofisher.com/order/catalog/product/11058021
[159] https://www.thermofisher.com/order/catalog/product/STEM00008
[160] https://www.thermofisher.com/order/catalog/product/R25001
[161] https://www.invivogen.com/puromycin
[162] https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit

- Flp-T2A-BleoR modRNA (pKG3212). Frozen aliquots of the modRNA should be stored at -80°C (Elsa) in the box with the recombinases.
- TAT-Cre protein[163]: aliquots can be found at -80°C (Elsa) in the box with the recombinases.
- Cre modRNA (pKG1779, pKG3213, pKG3214 or pKG3717). Frozen aliquots of the modRNA should be stored at -80°C (Elsa) in the box with the recombinases.
- Donor plasmids containing attB-GT or attB-GA and the desired DNA cargo

General Preparation

Before starting any step, pre-warm all culture media for 15 to 30 minutes at room temperature.

Pre-warm:

- StemFlex (with or without antibiotics)
- Neutralization medium
- OptiMEM

STRAIGHT-IN Integration Protocol

Two Days Before Transfection (d -2)

1. Dilute rhLaminin-521 at 1:20 in PBS +/+.
2. Add 300 μl per well to a 24-well plate (15 μl rhLaminin-521 + 285 μl PBS +/+).
3. Seal with Parafilm and incubate overnight at 4 C.

- Alternatively, coat on the day of splitting and incubate for 2 hours at 37°C.

Day Before Transfection (d -1)

1. Warm the coated plate at 37 C for 10 minutes.
2. Select a hiPSC well that is approximately 80% confluent.
3. Aspirate StemFlex.
4. Rinse once with 1 mL PBS -/-.
5. Rinse again with 1 mL PBS -/-.
6. Add 300 to 500 μl of Gentle Cell Dissociation Reagent.
7. Incubate 5 minutes at 37°C with 5% CO2.
8. Aspirate the dissociation reagent and add 1 mL StemFlex + RevitaCell (1:200).
9. Gently triturate 5 to 10 times to obtain a single cell suspension and transfer to a tube containing 1.5 to 2 mL neutralization medium.
10. Centrifuge at 300g for 3 minutes.

[163] https://www.sigmaaldrich.com/US/en/product/mm/scr508?srsltid=AfmBOorSWM6-h4vYbK-_i-tZ0wl-9yYkrzKFuSHoKG7e7a7mbvmy0fzr

11. Aspirate supernatant and resuspend in 0.5 mL StemFlex.
12. Count cells and seed 100K cells per well (24-well scale) on rhLaminin-521 coated wells. * This number works well for our lines, but can be optimized between 150K-300K.
13. Plate cells and distribute evenly by gentle rocking. Incubate at 37°C and 5% CO2.

Day of Transfection (d0)

1. Confirm cells are approximately 30 to 40% confluent.
   - If too confluent, re-seed.
   - If too sparse, feed and wait until they reach the appropriate density.
2. Prepare transfection mixes. Per well:
   - 600 ng Donor plasmid
   - 400 ng eeBxb1 modRNA
   - 400 ng p53DD modRNA
   - 3 μl Lipofectamine Stem reagent
   - Prepare Lipofectamine master mix based on n + 0.5 wells.
   - Add DNA mix to Lipofectamine mix (not the reverse).
3. Incubate 50 μl Lipofectamine:DNA mix for 10 minutes at room temperature.
4. Aspirate StemFlex, rinse once with PBS -/- and add 0.5 mL OptiMEM.
5. Add 50 μl transfection mix dropwise and gently shake.
6. After 4 hours, add 0.5 mL StemFlex without removing OptiMEM.

Lipofectamine Stem Transfection Overview (24-well format)

Step	Tube	Components	Amount per Well
1	Tube 1	OptiMEMLipofectamine Stem Reagent	22 μL3 μL
2	Tube 2	OptiMEMDNA	25 μL - DNA volume600 ng donor + 400 ng Bxb1 + 400 ng p53DD
3	Add Tube 2 into Tube 1 and mix well		
4	Incubate 10 minutes at room temperature		
5	Rinse cells with PBS -/- and add 0.5 mL OptiMEM		
6	Add 50 μL complex to each well and swirl to distribute		
7	Incubate 4 hours at 37°C with 5% CO_2		
8	Add 0.5 mL warm StemFlex per well and incubate overnight		

One Day After Transfection (d1)

1. Aspirate media, rinse once with PBS -/-, add 0.5 mL StemFlex.
2. Expect some cell death due to plasmid toxicity.
3. Assess fluorescence if applicable.

Two Days After Transfection (d2)

1. Begin antibiotic selection if cells are healthy and above 50% confluent.

- Zeocin 1:6666 for Bxb1 GT
- Puromycin 1:1000 for Bxb1 GA

2. Prepare at least 1.5 mL antibiotic medium for multiple refreshments. Keep at 4°C for up to one week.

Three to Four Days After Transfection (d3 to d4)

1. Rinse once with PBS -/- and add 0.5 mL antibiotic medium.

> **Note**
>
> A lot of cell death during selection is expected.

Five Days After Transfection (d5)

1. Look for clear colonies. If present, stop antibiotic selection.

> **Note**
>
> - Three days of selection are usually sufficient.
> - Include a negative control lacking eeBxb1 and p53DD to confirm full lethality.

2. Replace with 0.5 mL StemFlex without antibiotic.

Six to Eight Days After Transfection (d6 to d8)

1. Refresh medium every other day.
2. Proceed to the STRAIGHT-IN Excision Protocol.

STRAIGHT-IN Excision Protocol

The excision strategy depends on the landing pad:

- **GA landing pad:** excised using Flp recombinase

- **GT landing pad:** excised using Cre recombinase

Each system has two workflow options: A (no replating) and B (replate cells).

Flp-Mediated Excision (GA Landing Pad)

Option A: Direct Transfection (d6 to d8)

1. Once colonies are large and healthy, transfect with Flp-T2A-BleoR modRNA.
2. Per well, use:

- 500 ng Flp-T2A-BleoR modRNA
- 2 μl Lipofectamine Stem

3. Prepare Lipofectamine master mix based on n + 0.5 wells. Add RNA mix to Lipofectamine.
4. Incubate 10 minutes at room temperature.
5. Aspirate medium, rinse with PBS -/-, add 0.5 mL OptiMEM.
6. Add 50 μl Lipofectamine:RNA mix.
7. After 4 hours, add 0.5 mL StemFlex without removing OptiMEM.
8. After another ~2-4 hours, add 0.5 mL StemFlex containing Zeocin (1:6666).

- Selection may also begin the next day if needed.

Option B: Replate and Transfect (d6 to d8)

1. Replate cells into two rhLaminin-521 coated wells, splitting evenly.
2. When wells reach 30 to 40 % confluency, proceed.
3. Transfect only one well with Flp-T2A-BleoR modRNA. Keep the other as a contingency.
4. Follow steps from Option A, starting at step 2.

Cre-Mediated Excision (GT Landing Pad)

Option A: Replate and Use TAT-Cre Protein (d6 to d8)

1. Replate cells into two rhLaminin-521 coated wells.
2. When cells reach 30 to 50% confluency, proceed.
3. Prepare a 0.5 mL StemFlex mixture containing 2.25 μl TAT-Cre (1 μM final).

- Mix the components in a separate protein low-bind/retention tube before adding to cells.

4. Add the mix to one well. Keep the second well as a backup.
5. The next day, rinse with PBS -/- and replace with 0.5 mL StemFlex.
6. Allow cells to grow until confluent, typically 2 days. Refresh every other day.
7. Passage cells and collect samples for gDNA extraction and CNV ddPCR assays for BleoR and EBFP2/EGFP.

- Typical excision efficiency is 50 to 60%.
- Additional rounds of TAT-Cre may further increase excision rates.

Option B: Replate and Use Cre modRNA (d6 to d8)

1. Replate cells into two rhLaminin-521 coated wells, splitting evenly.
2. When wells reach 30 to 40% confluency, proceed.
3. Transfect only one well with Cre modRNA. Keep the other as a contingency.
4. Follow steps from Option A, starting at step 2.
5. The next day, rinse with PBS -/- and replace with 0.5 mL StemFlex.
6. Allow cells to grow until confluent, typically 2-3 days. Refresh every other day.
7. Passage cells and collect samples for gDNA extraction and CNV ddPCR assays for BleoR and EBFP2/EGFP.

- Typical excision efficiency is 30%.
- Additional rounds of Cre modRNA transfection may further increase excision rates.

Culturing Human iPSCs

Reagents

Media

Human iPSCs can be cultured in a range of media. All media come as two components: the basal medium and a 5X or 10X supplement. The supplement is stored at -20°C; the basal medium is stored at 4°C. To make the complete medium, add an entire bottle of supplement to the bottle of basal medium. The complete medium is stored at 4°C (entire bottle) or at -20ºC (40 mL aliquots). Media should be warmed before adding to cells; be sure to warm only an aliquot.

Important

Do not warm the entire media bottle! Instead, aliquot what you will need and only warm that tube. As a bonus, this smaller volume will warm faster.

Media we use:

- mTeSR-Plus[164]: optimized for clump passaging, we mainly use with iPS11 cells
- eTeSR[165]: a variant of mTeSR optimized for single-cell passaging
- StemFlex[166]: good for single-cell passaging, used with STRAIGHT-IN cell lines

Coating

[164] https://www.stemcell.com/products/mtesr-plus.html
[165] https://www.stemcell.com/products/etesr.html
[166] https://www.thermofisher.com/order/catalog/product/A3349401

iPSCs are cultured on plates with specific coatings, either extracellular matrix typically derived from a cancer cell line or a defined, single polymer. Often, a defined matrix is preferred to eliminate lot variability, but these reagents tend to be more expensive. While it is relatively easy to adapt a cell line to a new medium, it takes longer (2 passages) to adapt to a new coating.

- Geltrex[167]: basement membrane derived from murine tumor cells; 50X stored at -20°C in 120 μL aliquots, enough each for one 6-well plate (see *Geltrex Aliquoting* (page **??**) for more information)
- Laminin-521[168]: a defined culture matrix consisting of a single protein that is expressed in human blastocysts; 20X aliquots are stored long-term at -20ºC, and a working aliquot is good at 4ºC for up to 3 months

See coating protocols below for *Geltrex* (page **??**) and *laminin-521* (page **??**).

Dissociation Reagents

There are several dissociation reagents with different use cases.

- ReLeSR[169]: non-enzymatic dissociation of cells in clumps (clump passaging is preferred for regular maintenance to reduce genomic instability); stored at room temperature
- Gentle Cell Dissociation Reagent[170] (GCDR): non-enzymatic dissociation to single cells; preferred over harsher, enzymatic treatments like Accutase; stored at room temperature
- Accutase[171]: enzymatic dissociation to single cells; stored long-term at -20ºC, and working aliquots are stored at 4ºC

See dissociation protocols below for *ReLeSR* (page **??**), *GCDR* (page **??**), and *Accutase* (page **??**).

Survival-promoting small molecules

When thawing iPSCs or passaging them as single cells, it is necessary to add inhibitor(s) to prevent apoptosis and promote cell survival. These compounds should be removed after 24 hours by performing a media change. It is common to observe significant changes in cell morphology with these inhibitors; the cells should return to their typical form a few days after removal. There are several small-molecule formulations that work well:

- ROCK inhibitor[172] (ROCKi, RI): Y-27632, a single chemical inhibitor of ROCK (Rho-associated, coiled-coil containing protein kinase) activity; 1000X aliquots are stored at -20°C, and a working aliquot is kept at 4ºC
- RevitaCell Supplement[173] (RC): a proprietary blend of a ROCK inhibitor and other antioxidant small molecules; 100X aliquots (*can also be used as a 200X solution*) are stored at -20°C, and a working aliquot is kept at 4ºC
- CloneR2[174]: a defined supplement to promote survival, genomic integrity, and differentiation

[167] https://www.thermofisher.com/order/catalog/product/A1413301
[168] https://www.stemcell.com/products/celladhere-laminin-521.html
[169] https://www.stemcell.com/products/relesr.html
[170] https://www.stemcell.com/products/gentle-cell-dissociation-reagent.html
[171] https://www.sigmaaldrich.com/US/en/product/sigma/a6964
[172] https://www.stemcell.com/products/y-27632.html
[173] https://www.thermofisher.com/order/catalog/product/A2644501
[174] https://www.stemcell.com/products/cloner2.html

potential in high-stress culturing conditions like clonal expansion; 10X solution is stored long-term at -20ºC, and working aliquots are stored at 4ºC

Freezing solutions

Like MEFs, iPSCs should be frozen in a solution of 10% DMSO and 90% serum or serum equivalent.

- 10% DMSO + 90% FBS: use a 15 mL aliquot of FBS labeled "iPSC only"
- 10% DMSO + 90% KnockOut Serum Replacement[175]: a more defined, FBS-free serum equivalent with less lot variability

See *freezing protocol* (page **??**) below.

Other Reagents

- DMEM/F12: for coating plates and spinning down cells, use bottle labeled "iPSC only"
- DMEM/F12 + 1% FBS: for spinning down cells; the added protein helps the cells pellet well
- PBS: normal phosphate buffered saline without calcium and magnesium (–/–) for washes; use autoclaved bottle labeled "iPSC only"
- PBS +/+[176]: phosphate buffered saline that includes calcium and magnesium ions; used for Laminin-521 coating; stored at 4ºC
- Penicillin-streptomycin[177] (Pen/Strep, P/S): optional antibiotic to reduce contamination; 100X stored at 4ºC

General Culturing Tips

- Change to fresh media *every other day* to keep the cells healthy. iPSCs are very metabolically active and can easily spontaneously differentiate, so if the media looks very yellow try refreshing with a larger volume.
- If media cannot be changed in two days (e.g., over the weekend), use twice the normal media volume. This should keep the cells healthy for 3 days until the next media change (as long as they are not confluent).
- Some amount of cell death is expected, but regular media changes should keep this to a minimum.
- To maintain optimal cell health, be sure to passage cells before they are 100% confluent. Regular 1:10 passaging can usually be done every 3-4 days.
- iPSCs grow in colonies with a cobblestone-like cell morphology. The edges of the colonies should be smooth, not spiky. However, morphology will change with ROCKi/RevitaCell, but should return to normal 1-2 days after removal. Each cell line has its own normal morphology; be aware of any changes.
- Also look out for large spaces between cells or flat, more transparent cells growing away from a colony. These are signs of differentiation.

[175] https://www.thermofisher.com/order/catalog/product/10828028
[176] https://www.thermofisher.com/order/catalog/product/14040117
[177] https://www.fishersci.com/shop/products/gibco-penicillin-streptomycin-10-000-u-ml-3/15140122

- Be extra attentive with sterile technique (e.g., don fresh gloves before beginning, use separate glass pipette aspirators for different cell lines) to avoid contamination.

Common culturing conditions:

Reagent / Step	iPS11	STRAIGHT-IN
Media	mTeSR-Plus	StemFlex + P/S
Coating	Geltrex	Laminin-521
Dissociation for passaging/freezing	ReLeSR (clumps)	GCDR (single cells)
Survival-promoting addition	ROCKi	RevitaCell

When maintaining iPSCs, it is often useful to keep 1-2 wells in a 6-well plate for iPS11 cells. That way, one well can be used to passage in clumps, while the other can be used to seed for an experiment (1 well is enough for a 96-well plate). For STRAIGHT-IN lines, maintaining ~1 well in a 24-well plate is usually sufficient, if experiments are performed at a smaller scale (only a few wells in a 24-well plate).

Coating plates with Geltrex

Important

Plates require at least 1 hour to coat, so start early or make extras up to a week ahead of time!

Geltrex is stored in aliquots sufficient to coat an entire plate, and once thawed, it must be used immediately. If you do not need an entire plate coated, you can either coat extra wells of a new plate and store that plate at 4ºC or coat your entire plate and use the extra wells later (e.g., for the next passage). Wells with Geltrex + additional DMEM/F12 should still be good as long as the liquid has not evaporated too much and exposed the surface.

1. Thaw Geltrex at 4ºC, on ice (use the designated TC styrofoam for ice), or quickly at room temperature.
    - Thaw one tube (50X, 120 μL aliquot) per 6-well plate to coat. It is recommended to do no more than 2 at a time to avoid unintentional gelling.
    - Flick the tube to mix as it thaws and to speed up the process.
    - While the Geltrex is thawing, prepare other materials in the BSC: 6-well plate(s), 15mL conical, DMEM/F12.
2. Prepare a 15 mL conical with 6 mL DMEM/F12. Immediately once the Geltrex has thawed, pipet it into the prepared 15 mL conical. Pipet 1 mL of the DMEM/F12 mix back into the Geltrex tube to wash out any residual solution and return the liquid to the conical.
3. With a serological pipette, pipet up and down once, then dispense 1 mL per well into the 6-well plate. Rock the plate to coat the entire surface.
4. Incubate the plate at 37ºC for 1 hour to coat.
    - *If using same-day*: Leave the plate in the incubator until use.

- *If preparing ahead*: Add an additional 1-2x volume of DMEM/F12 to each well. Parafilm the plate, then store at 4ºC (Oaken). Plates can be stored at 4ºC for several weeks.

Note

The official recommendation is to store coated plates at 4ºC for up to 2 weeks. As long as the liquid has not evaporated/condensed, the plates should be okay for several weeks longer.

5. To use the plates, ensure they have been pre-warmed in the incubator. Immediately before seeding cells, aspirate the liquid from the coated well(s).

Coating plates with Laminin-521

Important

This protocol uses **human laminin-521** for iPSC culture. This is different from the **Corning mouse laminin** used for human reprogramming (that protocol is *here* (page **??**)).

Materials

- CellAdhere Laminin-521[178] (20X; aliquots stored at -20ºC, working aliquot at 4ºC is good for 3 months)
- PBS +/+[179] (stored at 4ºC)

Important

PBS +/+ is critical as divalent cations (Mg2+, Ca2+) are important for structure and function of laminin-521. Do not use normal, autoclaved PBS!

Protocol

1. If using a new aliquot from -20ºC, thaw laminin-521 at 4°C or on ice.
2. Dilute laminin-521 in sterile PBS +/+ to a final concentration of 5 μg/mL (dilute 1:20). See the table below for helpful volumes. If coating only 1-2 wells, the dilution can be done directly in the well; for larger volumes, using a tube is helpful.
3. Incubate plates for ~2 hours at 37°C.
4. If not using immediately, parafilm the plate and store at 4ºC for up to 4 weeks.
5. Immediately before seeding cells, aspirate the liquid from the coated well(s) *using a P1000 pipette* (not the aspirator) to avoid dislodging the laminin-521.

[178] https://www.stemcell.com/celladhere-laminin-521.html
[179] https://www.thermofisher.com/order/catalog/product/14040117

Culture plate	Surface area per well (cm^2)	Total volume (µL/well)	Laminin-521 (µL/well)	PBS +/+ (µL/well)
6-well	9.4	1,000	50	950
12-well	3.8	500	25	475
24-well	1.9	300	15	285
48-well	0.76	150	7.5	142.5
96-well	0.32	70	3.5	66.5

Thawing

Estimated time

~30 minutes — don't forget to coat plates ahead of time!

Typically, cells dissociated in clumps are frozen such that 1 vial contains 1 well of a 6-well plate. Recovery is variable and depends on clump size, so 1 vial can be thawed into 1-6 wells of a 6-well plate. Decide how many wells to use based on the size of the pellet. ROCKi can be added to improve recovery, but it is not strictly necessary.

For cells dissociated as single cells, 1 vial usually contains 1 well of a 12-well or 24-well plate (this should be labeled on the vial). Recovery is quite high with ROCKi or RevitaCell, so it is recommended to thaw the vial into multiple wells. For instance, a vial containing 1 well from a 24-well plate could be thawed into two new wells of a 24-well plate, split 1/3 and 2/3 into each well. This helps ensure one of the wells will be ready to passage in 2-4 days (optimal).

1. Coat a plate with the appropriate coating. If using a pre-coated plate from the fridge, allow to warm in the incubator.
2. Warm the appropriate volume of media (e.g., 2 mL/well for a 6-well plate) and, if using, DMEM + 1% FBS (5 mL per vial thawed).
3. Remove cryovial(s) of cells from liquid nitrogen and thaw quickly (1-2 minutes) in the 37°C bead bath.

Important

Thaw cells quickly and spin down as soon as all the ice is gone to limit the cells' exposure to DMSO.

4. As soon as cryovial is thawed, transfer the contents to a 15 mL conical using 5 mL serological pipette.
5. Using a P1000 pipette, **SLOWLY** add 1 mL of DMEM/F12 (or warm DMEM/F12 + 1% FBS) to the conical dropwise, shaking the tube every 2-3 drops to evenly mix thawed cells with

DMEM/F12. *Optionally*, pipet 1 mL of DMEM/F12 into the cryovial to remove any residual cells, then add dropwise to conical.

Important

Slow addition of DMEM/F12 is important to prevent osmotic shock to the iPSCs.

6. Using a serological pipette, **GENTLY** add 4 more mL of DMEM/F12 (or warm DMEM/F12 + 1% FBS) dropwise, mixing every 3-5 drops (total tube volume ~6 mL).
7. Spin down cells at 40g (for clumps) or 400g (for single cells) for 4 minutes.

Note

If clumps of cells do not pellet well at 40g, you can try 400g, but cells frozen in these smaller clumps may not recover as well without ROCKi.

8. Aspirate the plate coating and the supernatant from the cell pellet. *Gently* resuspend the cells in the warm culture medium with ROCKi/RevitaCell (if using) and plate.
9. Within 24 hours (i.e., the next day), change to fresh media to remove dead cells and ROCKi/RevitaCell. Then, continue culturing as normal.

Passaging with ReLeSR

Estimated time

~15 minutes — don't forget to coat plates ahead of time!

To passage iPS11 cells for general culture maintenance, use ReLeSR to dissociate cells in clumps. This is less disruptive and may improve the long-term genomic integrity of the iPSCs. For seeding or other applications that require counting, dissociate with Gentle Cell Dissociation Reagent (preferred) or Accutase to achieve a single-cell suspension (see *Dissociating with Gentle Cell Dissociation Reagent (GCDR)* (page **??**) and *Dissociating with Accutase* (page **??**) below).

1. Warm media in the 37ºC bead bath.
2. Aspirate the old media. Gently wash with 1 mL PBS and aspirate.
3. Add 1 mL ReLeSR and incubate at room temperature for 1 minute.
4. Immediately after the 1 minute is up, aspirate to remove the ReLeSR. At this point, *no cells should be lifting off from the plate*.
5. Incubate the empty plate (it essentially has a thin film of liquid) at 37°C for 5-7 minutes.
6. When the plate is done incubating, add 1-3 mL media to each well and tap the plate *for 30-60 seconds* to dislodge the cells.

- Suggestion: Add 0.5-1 mL of media for each new well you're passaging into, e.g., 3 mL media for passaging one well 1:6.

7. Gently pipet up the liquid with a serological and dispense into the prepared wells (with coating aspirated).
   - Use a serological pipette rather than a P1000 to maintain cell clumps.
   - Pipet up and down 1-2 times in the well to resuspend as many cells as possible, since the cells tend to stick to the well. However, don't pipet too much—this will break up the clumps!
8. Add additional media to each well to bring the total volume to the desired amount (e.g., 2 mL). Rock back and forth to mix.
   - Alternatively, dissociated cells from the previous step can be pipetted into a conical to be mixed with fresh media, then transferred from the conical to the new wells.
   - It can also be helpful wash the old well with this additional media before adding to the new well.
   - After dissociation, avoid excessive pipetting to maintain cell clumps.

> **Tip**
>
> Look at the cell clumps under the microscope before putting the plate in the incubator. This can help give you a sense of the clump size to aim for in the future based on how the cells look the next day.

9. Within 24 hours (i.e., the next day), change to fresh media. Then, culture as normal.

Dissociating with Gentle Cell Dissociation Reagent (GCDR)

> **Estimated time**
>
> ~15 minutes — don't forget to coat plates ahead of time!

To seed cells for an experiment, use Gentle Cell Dissociation Reagent (GCDR) to dissociate single cells for counting. GCDR is also used for regular passaging of STRAIGHT-IN lines. GCDR is preferred over Accutase because it is non-enzymatic and gentler on the cells. During seeding, be sure to add a survival-promoting small molecule (ROCKi or RevitaCell).

> **Note**
>
> Addition of ROCKi/RevitaCell leads to cytoskeletal remodeling, so cells will have a spiky morphology the day after passaging. The morphology should return to normal a day or so after ROCKi/RevitaCell is removed.

1. Warm media and DMEM/F12 + 1% FBS (if using) in the 37ºC bead bath.

2. Aspirate old media. Gently wash with 1 mL PBS and aspirate.
3. Add a 0.5-1x well-volume (e.g., 1 mL in a 6-well plate, 100 uL for a 96-well plate) of GCDR and incubate at 37°C for 8-10 minutes until cells ball up on the plate.

> **Note**
>
> Cells are ready to detach when they have balled up/become round, retreating from their neighbors. You will likely NOT observe cells lifting off.

4. Gently pipet up and down to dislodge cells. Add the dissociated cells to a 15 mL conical, optionally with warm DMEM/F12 + 1% FBS, and spin at 400g.
    - Technically, no quenching/diluting step is needed; cells can be spun down in GCDR alone. However, the proteins in DMEM/F12 + 1% FBS can help the cells pellet.
5. Aspirate the media and gently resuspend the cells in a small volume of media with ROCKi/RevitaCell (e.g., 0.5 mL).
6. If seeding cells for an experiment, count cells *as normal* (page **??**).

> **Note**
>
> Counting iPSCs can be difficult! GCDR doesn't always produce perfect single cells; often, clusters of a few cells will remain. Do your best to count accurately.

7. Dilute the cells to the appropriate volume in media with ROCKi/RevitaCell. Aspirate the coating solution and plate cells.
8. Within 24 hours (i.e., the next day), change to fresh media without ROCKi/RevitaCell. Then, proceed with culturing or an experiment as normal.

Dissociating with Accutase

> **Estimated time**
>
> ~15 minutes — don't forget to coat plates ahead of time!

Accutase can be used to dissociate iPSCs to single cells for seeding. However, Gentle Cell Dissociation Reagent (GCDR) is preferred, since Accutase (enzymatic) is harsher on the cells than GCDR (non-enzymatic).

> **Note**
>
> Addition of ROCKi/RevitaCell leads to cytoskeletal remodeling, so cells will have a spiky morphology the day after passaging. The morphology should return to normal a day or so after ROCKi/RevitaCell is removed.

1. Warm media and DMEM/F12 + 1% FBS (if using) in the 37ºC bead bath.
2. Aspirate old media. Gently wash with 1 mL PBS and aspirate.
3. Add a 0.5-1x well-volume (e.g., 1 mL in a 6-well plate, 100 uL for a 96-well plate) of Accutase and incubate at 37°C for 5-7 minutes until cells begin to lift off the plate.
4. Gently pipet up and down to dislodge cells. Add the dissociated cells to a 15 mL conical with DMEM/F12 or warm DMEM/F12 + 1% FBS, and spin at 400g.
    - Accutase must be quenched/diluted before spinning, as it contains enzymes. The proteins in DMEM/F12 + 1% FBS can help the cells pellet.
5. Aspirate the media and gently resuspend the cells in a small volume of media with ROCKi/RevitaCell (e.g., 0.5 mL).
6. If seeding cells for an experiment, count cells *as normal* (page **??**).

> **Note**
>
> Counting iPSCs after dissociation with Accutase may be easier than for GCDR, as it can better break apart clumps/clusters.

7. Dilute the cells to the appropriate volume in media with ROCKi/RevitaCell. Aspirate the coating solution and plate cells.
8. Within 24 hours (i.e., the next day), change to fresh media without ROCKi/RevitaCell. Then, proceed with culturing or an experiment as normal.

Freezing

Appropriate volumes of freezing soluation (10% DMSO + 90% FBS or KOSR):

- 24-well plate: 300-500 μL per well
- 12-well plate: 500 μL per well
- 6-well plate: 0.5-1 mL per well

> **Important**
>
> Be sure to label the cryovials well! It is best practice to include the media and coating, since we have the same lines adapted to different conditions. It is also a good idea to indicate whether the cells are frozen in clumps (ReLeSR) or as single cells (GCDR), in addition to the well size.

1. Dissociate cells *in clumps (ReLeSR)* (page **??**) or *as single cells (GCDR)* (page **??**) as described above, stopping before resuspending in media.
2. Instead, resuspend in an appropriate volume of FBS or KOSR (e.g., 270 μL/well for 24-well or 900 μL/well for 6-well plate) and transfer to a cryovial.

- For cells dissociated with ReLeSR, add FBS or KOSR after incubating the cells with a thin film of ReLeSR for 8-10 minutes. Tap the plate vigorously *for 30-60 seconds* to dislodge the cells. Gently transfer the cells to a cryovial using a serological pipette to avoid disturbing the clumps.
- For cells dissociated with GCDR, after spinning down the cells, resuspend the pellet in FBS or KOSR.

3. Add the appropriate volume of DMSO to the cryovial to achieve a final concentration of 10% DMSO (e.g., 30 μL/well for 24-well or 100 μL/well for 6-well plate).
4. Place the cryovials in the styrofoam boxes in the -80°C freezer to cool slowly overnight.
5. The following day, transfer frozen cryovials to liquid nitrogen for long-term storage.

Transient Transfection of iPSCs

Conceptually, transfection of iPSCs is the same as of other cell types. However, transfections tend to be less efficient, and iPSCs are more sensitive to the transfection reagents. Thus, we do not use PEI; instead, we've found that the FuGENE® HD[180] reagent works well (at least for the iPS11 cell line) and Lipofectamine 3000[181] is a reasonable substitute for plasmid DNA. For mRNA, the Lipofectamine Stem[182] reagent is optimal.

Calculations

For all calculations, you can refer to the iPSC transfection template[183] in the shared projects folder.

Empirically determined reagent amounts to use:

Reagent	Ratio [reagent (µL) : nucleic acid (µg)]
FuGENE® HD[184]	3:1
Lipofectamine 3000[185]	2:1
Lipofectamine Stem[186]	2:1

Note

Not all reagent amounts have been rigorously optimized.

[180] https://fugene.com/wp-content/uploads/2022/12/FuGENE_HD-Users-Guide_2023.pdf
[181] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/lipofectamine3000_protocol.pdf
[182] https://assets.thermofisher.com/TFS-Assets%2FBID%2Fmanuals%2Ftransfection-psc-lipofectamine-stem-mtesr1-protocol.pdf
[183] https://mitprod.sharepoint.com/:x:/s/GallowayLab/ES4uWltgGuFPp4ySG7b3ZbgBSlJ-3WxlB4nHcQb2l2-hSA?e=jClhpT
[184] https://fugene.com/wp-content/uploads/2022/12/FuGENE_HD-Users-Guide_2023.pdf
[185] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/lipofectamine3000_protocol.pdf
[186] https://assets.thermofisher.com/TFS-Assets%2FBID%2Fmanuals%2Ftransfection-psc-lipofectamine-stem-mtesr1-protocol.pdf

Note

These amounts can be scaled to various plate sizes and DNA amounts.

Note

The protocol for Lipofectamine 3000 can be applied to transfection of Jurkat cells in suspension at low efficiency (1-3%).

Protocol

1. One day prior to transfection, seed healthy iPSCs on Geltrex-coated plates with ROCK inhibitor (Ri), at a density equivalent to 100,000 (1e5) cells/well in a 24-well plate.
    - Dissociate iPSCs to single cells for seeding using *Gentle Cell Dissociation Reagent* (page **??**).
    - Addition of Ri is important to promote survival of single cells.
    - The equivalent density for a 96-well plate is 20,000 (2e4) cells/well; for a 6-well plate it is 500,000 (5e5) cells/well.
2. On the day of transfection, first prepare condition mixes of nucleic acid (pDNA or mRNA) diluted in Opti-MEM for a final volume of 5μL (96-well plate), 25μL (24-well plate), or 125μL (6-well plate) per well. These volumes are specified in the transfection template[187].
    - If using Lipofectamine 3000, add P3000 to each condition mix at 2μL/μg DNA.
3. If using Lipofectamine 3000 or Lipofectamine Stem, prepare the reagent master mix. Dilute the appropriate volume of reagent in Opti-MEM for a final volume of 5μL (96-well plate), 25μL (24-well plate), or 125μL (6-well plate) per well.
4. Add the FuGENE® HD reagent or Lipofectamine master mix to each condition mix to form the final complexes. Incubate for 10 minutes at room temperature.
5. Add the final complexes dropwise to the cells, 5μL (96-well plate), 25μL (24-well plate), or 125μL (6-well plate) per well for FuGENE® HD transfections, and twice that amount for Lipofectamine transfections.
6. Gently rock the plate back-and-forth, side-to-side to distribute the complexes. Return the cells to the incubator.
7. After 4-6 hours, media change to fresh mTeSR-Plus to remove the transfection reagent and Ri. Waiting longer than this may compromise cell health.
8. Analyze expression via microscopy or *flow cytometry* (page **??**), or continue with downstream experimental steps.
    - mRNA expression peaks at ~12 hours post-transfection.

[187] https://mitprod.sharepoint.com/:x:/s/GallowayLab/ES4uWltgGuFPp4ySG7b3ZbgBSlJ-3WxlB4nHcQb2l2-hSA?e=jClhpT

- pDNA expression is visible at 24 hours post-transfection (1 dpt) and increases through at least 3 dpt.
- To analyze via flow cytometry, follow the framework for analysis of other cell types, except dissociate in GCDR instead of trypsin.

3.5.2 Other TC Protocols

MEF Isolation Protocol

In reprogramming, we use transgenic mouse reporter lines to easily assess reprogramming yield and efficiency. We have many transgenic lines in house (Hb9::GFP, Oct4::GFP, CX3CR1::GFP, p53-/-) that we use for reprogramming purposes. From these lines we mainly isolate mouse embryonic fibroblasts on day E13.5 to E14.5. The mouse colony manager sets up timed matings as we need and assigns people to take charge of the isolation and other downstream needs.

Isolation protocol

The isolation and embryo processing is done by the two primary people on the MEF isolation. They may also help out with the passaging, freezing and myco testing as needed.

Note

You must have access to the mouse facility in 68N to retrieve the mice. You should also be trained by a senior lab member of how to properly euthanize the mice and transport them back to the lab.

Retrieving the mice

1. Head to the mouse facility with the mouse transport styrofoam (68N sub-basement) and grab all timed mating cages in room 68-050
2. Bring cages to the surgery room and euthanize mice with carbon dioxide and an appropriate secondary method
3. Place carcasses into a small clear plastic bag, then into a larger black bag, and finally into the transport styrofoam
4. Place used cages onto carts outside of the washing station. Remove cage cards and bring them back to the lab with the mice.

Embryo isolation

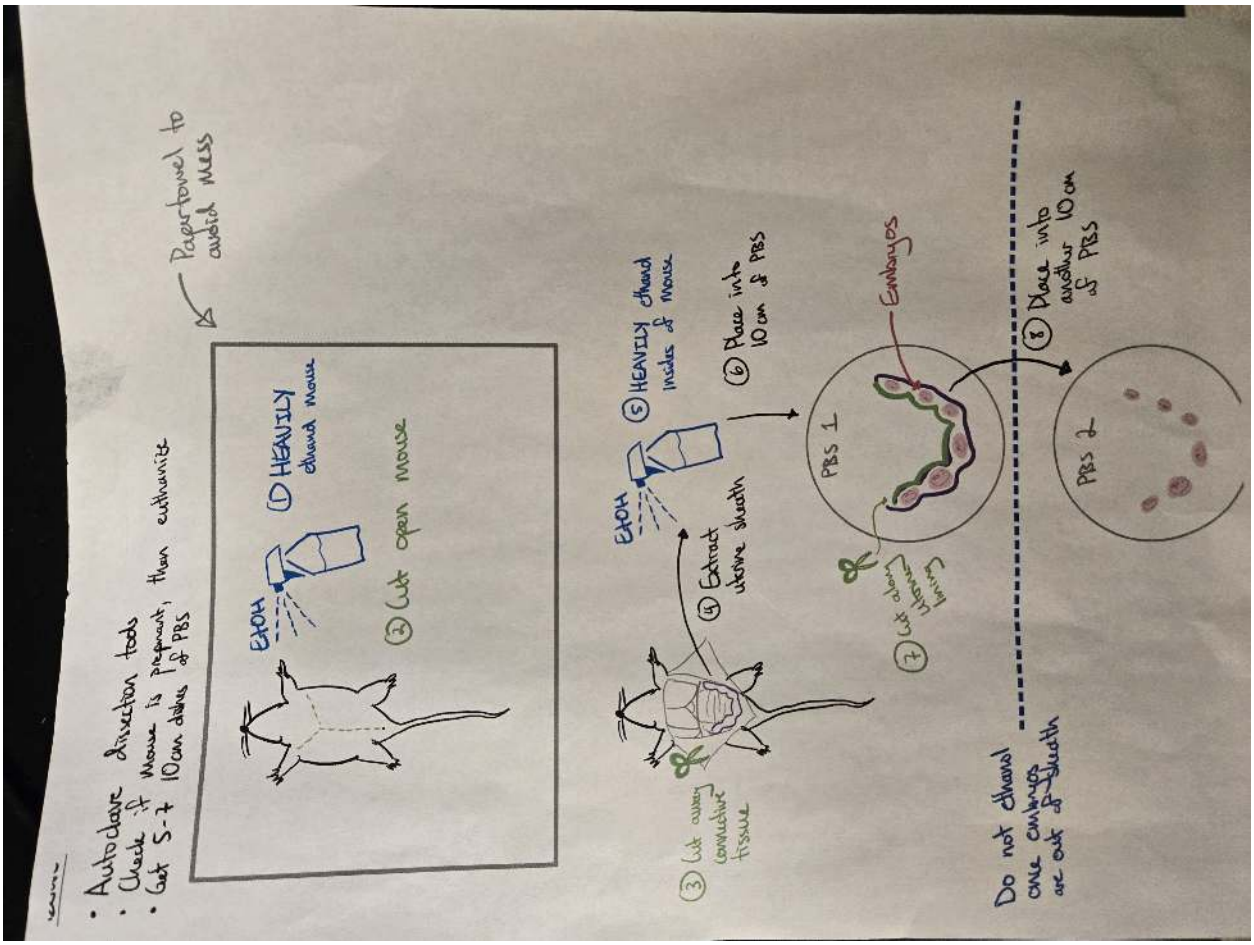
Proceed with all steps in quarantine (66-219) and use autoclaved surgery tools from the metal case. Prepare 10 cm dishes with some sterile PBS in them (usually 3 per mouse).

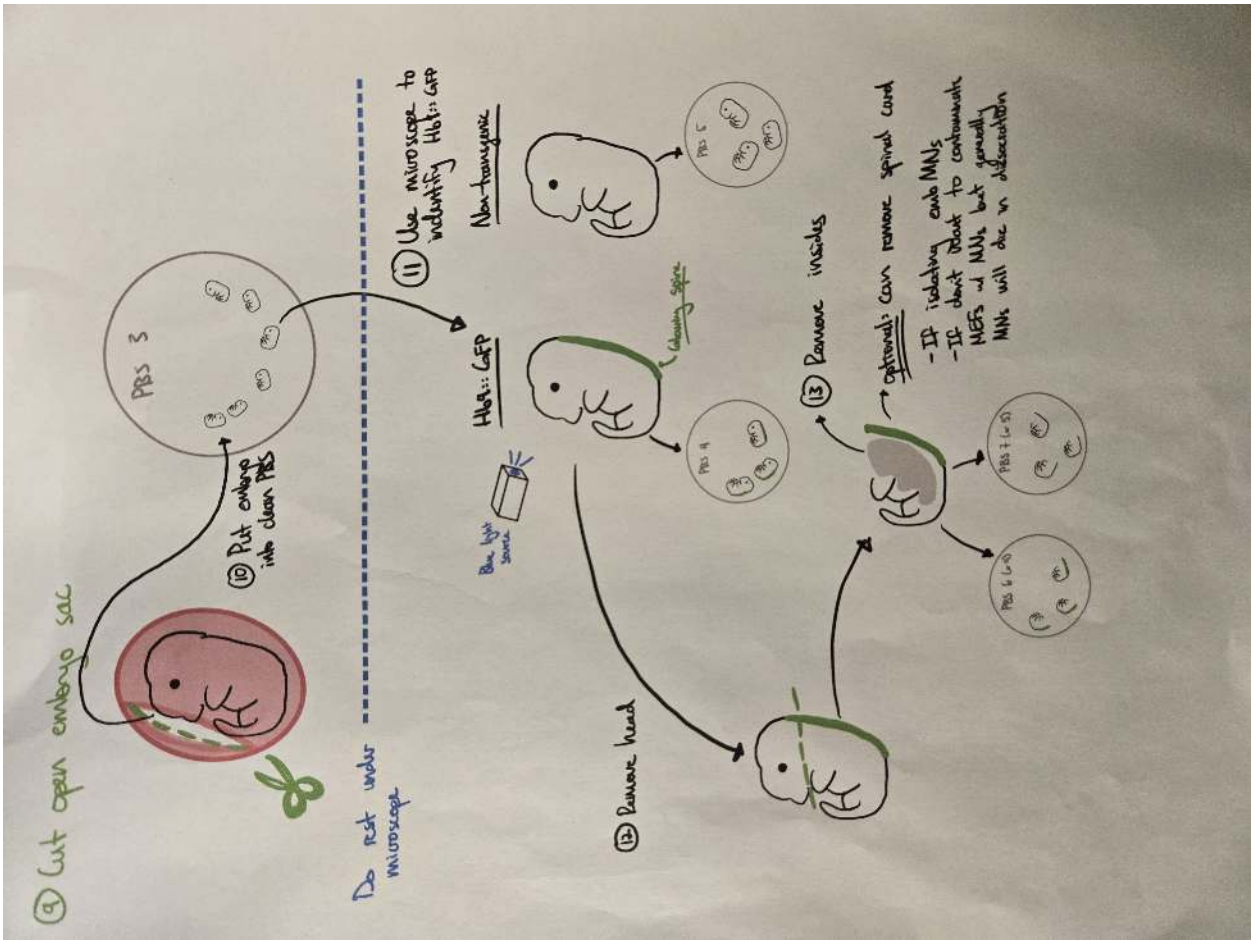
1. Remove mice from the bag and place onto paper towels
2. Heavily spray mice with 70% ethanol and make a “Y” cut along the abdomen. Be careful not to cut the intestines
3. If the mouse is pregnant, pull the uterus with the embryos forward. Heavily ethanol the mouse again.
4. Carefully cut away the uterus from the mouse and place it into a 10 cm dish with PBS.
5. Once the uterus is removed, place the mouse back into the clear plastic bag.

6. Carefully cut the embryos out of the uterine lining. The embryos will still be incased in a smaller sac once removed from the lining. Place them in a separate 10 cm dish.
7. Using scissors or sharper tweezers, remove the final lining around the embryos.
8. If isolating Hb9::GFP embryos, use the blue laser and plastic shield to identify GFP+ embryos. Place the embryos (based on transgenic/NT) into separate 10 cm dishes.
9. Place the dish with the embryos under the dissection microscope. Using 2 pairs of tweezers, carefully remove the head of the embryo and remove all the red insides, leaving behind a "skin suit".
10. Place 2 "skin suits" in one 10 cm dish (with NO PBS) and proceed to downstream processing steps in the hood.

Note

You will need to return the carcasses to the mouse facility and place them in the freezer in the surgery room. Ensure that all carcasses are double bagged and one bag labeled with PI last name.




⑨ Cut open embryo sac
⑩ Put embryo into clean PBS
PBS 3
Do rest under microscope
⑪ Use microscope to identify Hb9::GFP
Hb9:: GFP
Non-transgenic
Blue light source
Columnar Stripe
PBS 4
PBS 5
⑫ Remove head
⑬ Remove insides
Optional: can remove spinal cord
- If isolating endo MNs
- If don't want to contaminate Hb9FS w/ MNs but generally MNs will die in dissociation
PBS 6 (-G)
PBS 7 (-G)

Embryo processing

Complete all steps in the BSC in quarantine (66-219) and with separate quarantine materials. You may use the centrifuge in TC for spin steps.

Note

You will plate 1 embryo per 10 cm dish at the end of processing. The plates MUST be gelatin coated otherwise the MEFs will die. Coat the required number of 10 cm dishes before processing.

1. Coat the required number of 10 cm dishes (one 10 cm dish per embryo)
2. Place the 10 cm dish with 2 embryos into the quarantine hood. Using 2 new razor blades, chop the embryo for 2 minutes.
3. After 2 minutes, add 2 mL of 0.25% Trypsin per 10 cm dish. Chop for an additional 4 minutes.
4. Add 8 mL of DMEM + 10% FBS to each dish. Transfer dish contents to a 15 mL conical.
5. Spin the conical down for 5 minutes at 300-400 g.
6. VERY carefully aspirate the supernatant

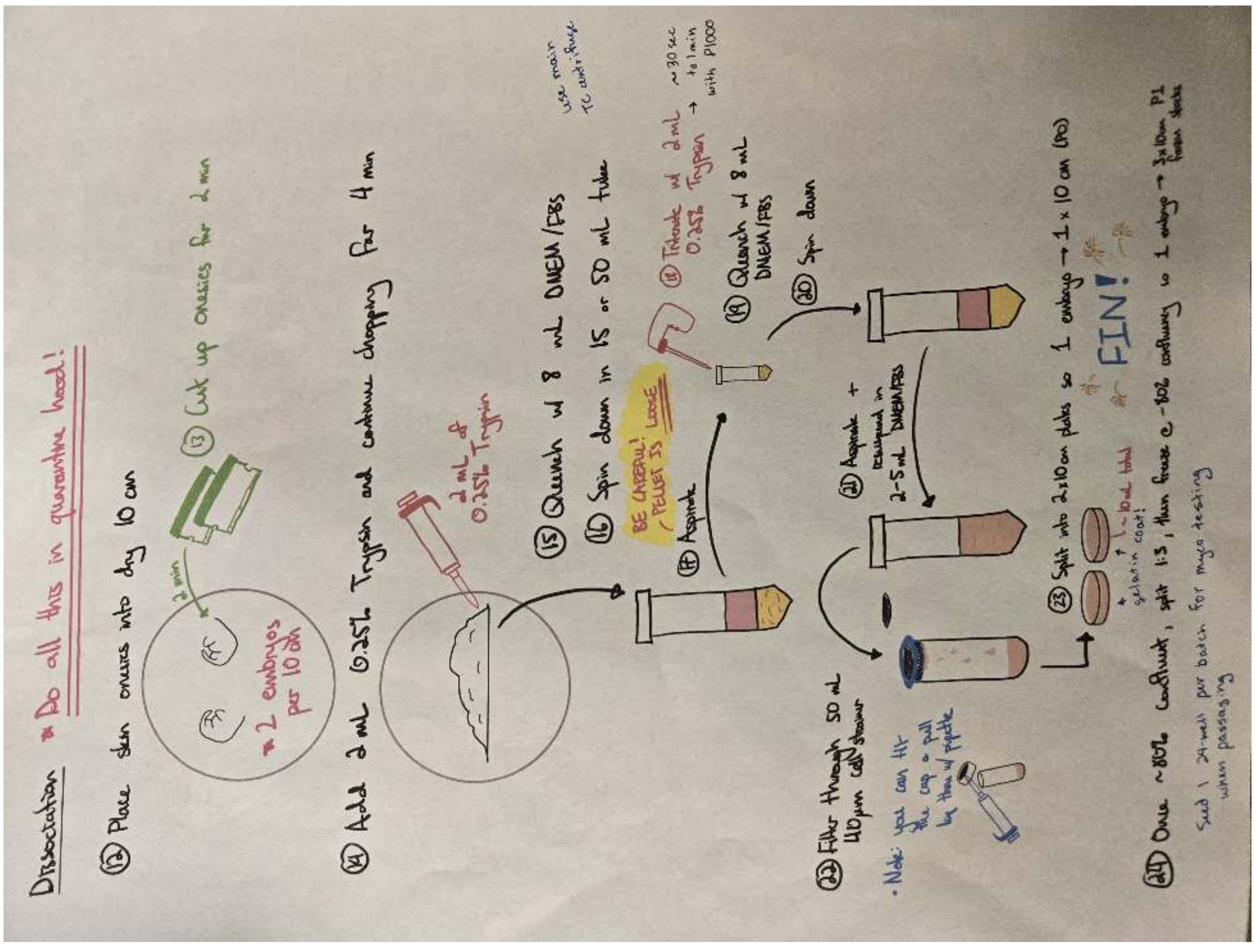
Note

The pellet will be VERY loose. It is important that you are very careful with your aspiration steps otherwise the pellet will be lost. It is ok to leave a little media on top of the pellet to avoid accidentally aspirating it.

7. Resuspend the pellet in 2 mL of 0.25% Trypsin
8. Quench with 8 mL of DMEM + 10% FBS.
9. Spin down for 5 minutes at 300-400 g, aspirate the supernatant and resuspend in 2-5 mL of DMEM + 10% FBS.
10. Place a 40 um strainer over a 50 mL conical. Strain the cell mixture through, using a sterile pestle to help push some of the cells through
11. Wash the strainer using DMEM + 10% FBS. You may find it useful to pull liquid through the opposite side of the strainer with a P1000
12. Spin down the strained mixture for 5 minutes at 300-400 g.
13. Aspirate supernatant and resuspend the pellet to plate onto 10 cm dishes (~10 mL volume per 10 cm dish)

Note

Be sure to label plates depending on which embryos were chopped together (i.e. for each # you will have 2 dishes)


Dissociation * Do all this in quarantine hood!
(12) Place skin onesies into dry 10 cm
2 min
(13) Cut up onesies for 2 min
* 2 embryos per 10 cm
(14) Add 2 mL 0.25% Trypsin and continue chopping for 4 min
2 mL of 0.25% Trypsin
(15) Quench w/ 8 mL DMEM/FBS
(16) Spin down in 15 or 50 mL tube
use main TC centrifuge
BE CAREFUL! PELLET IS LOOSE
(17) Aspirate
(18) Triturate w/ 2 mL 0.25% Trypsin → ~30 sec to 1 min with P1000
(19) Quench w/ 8 mL DMEM/FBS
(20) Spin down
(21) Aspirate + resuspend in 2-5 mL DMEM/FBS
(22) Filter through 50 mL 40 μm cell strainer
• Note: you can tilt the cap & pull liq thru w/ pipette
(23) Split into 2x10 cm plates so 1 embryo → 1 x 10 cm (P0)
gelatin coat!
~10 mL total
FIN!
(24) Once ~80% confluent, split 1:3, then freeze @ ~80% confluency so 1 embryo → 3x10cm P1 frozen stocks
Seed 1 24-well per batch for myco testing when passaging

Maintenance, freezing and testing protocols

The passaging, freezing and myco testing as usually done by the two secondary people on the isolation. This is to help reduce the amount of time one person spends in the hood per isolation. The primary people may also help with these tasks as needed.

Passaging MEFs

MEFs will need to passaged once they reach ~80% confluency (about 3-4 days). Depending on the isolation, certain plates may be ready to passage earlier or later than others.

1. Gelatin coat the required number of 10 cm dishes (three 10 cm dishes per one original 10 cm dish) AND a 24-well plate for myco testing
2. Aspirate media from dishes
3. Wash plates once with sterile PBS
4. Aspirate PBS and add 3 mL of a dilute mixture of PBS and 0.25% Trypsin (either 1:2 or 1:3 works well)
5. Wait 5-8 minutes for cells to start lifting off of the plate. Quench with 3 mL of DMEM + 10% FBS.

> **Note**
>
> When passaging you may combine cells that were chopped together to use less tubes

6. Transfer cells to a 15 mL or 50 mL conical and spin down for 5 minutes at 300-400 g
7. Aspirate supernatant and resuspend pellet in DMEM + 10% FBS
8. Before plating cells on 10 cm dishes, take a small amount of the cell suspension and transfer it to a 24-well for myco testing. Make sure there is at least 0.7 mL of media in the well.
9. Distribute one embryo to three 10 cm plates

Freezing MEFs

MEFs will need to be frozen once they reach ~80% confluency (about 3-4 days since passage). Be sure to print labels for the cryovials before proceeding.

1. Aspirate media from dishes
2. Wash plates once with sterile PBS
3. Aspirate PBS and add 3 mL of a dilute mixture of PBS and 0.25% Trypsin (either 1:2 or 1:3 works well)
4. Wait 5-8 minutes for cells to start lifting off of the plate. Quench with 3 mL of DMEM + 10% FBS.
5. Transfer cells to a 15 mL or 50 mL conical and spin down for 5 minutes at 300-400 g

6. Aspirate supernatant and resuspend pellet in freezing media (10% DMSO + 90% FBS). Resuspend such that each 10 cm plate pellet is about ~1 mL of volume.
7. Distribute cell mixture to the respective labeled cryovial
8. Place cryovials in freezing styrofoams in the -80C
9. The next day, transfer cryovials to the liquid nitrogen in the box labeled "Waiting for myco"

Myco testing MEFs

Myco testing for the MEFs is done according to this procedure. Always add additional media (more than 0.5 mL) back onto the cells in case they need to be retested.

If MEFs come back negative:

1. Negative is defined as a score less than 1
2. Move cyrovials from the "Waiting for myco" box to the correct boxes in the small liquid nitrogen tank
3. Write tube names, initials, and date from the tubes on the MEF stock tracker along with the number of vials added
4. Discard the myco plate

If MEFs come back borderline:

1. Borderline is defined as a score between 1 and 1.2
2. Re-test for myco the next week.
3. If still borderline, bleach vials and discard the myco plate

If MEFs come back positive:

1. Positive is defined as a score above 1.2
2. Bleach all vials and discard the myco plate.

Note

Myco testing at the Koch is relatively expensive ($70 per sample), so we usually only test the MEFs once or twice. We should soon be able to do our own myco testing in lab for $10 per sample

293T Rogi2 single LP V4 (cKG087)

Line summary

V4 Rogi2 dual LP single site integrase line:

locus	pHA for cargo	recombinase	positive selection (gene, drug)	counterselection (gene, drug)
Rogi2 LP	pKG03560	Bxb1 or ee-Bxb1	PuroR, Puromycin	HSV-TK SR39h, GCV or PCV

Note

The Rogi2 v4 is our current version. DO NOT use the pKG02180 donor plasmid, which contains an SV40 promoter which will replicate the plasmid in HEK293Ts. Use pKG03560.

Note

Any Bxb1 or eeBxb1 expression plasmid can be used. Past plasmids used were pKG0570 for Bxb1 and pKG03466 for eeBxb1. The eeBxb1 is a higher efficiency recombinase variant.

Integration protocol

Day -1

Seed cells at 100k per 24 well.

Note

SRK typically plates 200k per 24 well which boosts cell health post-delivery of the plasmids.

Day 0

Co-transfect your donor plasmid and Bxb1 expression plasmid at a 1.5:1 ratio, ie., following our standard PEI protocol.

Note

An example that has worked well for SRK is 850 ng of donor plasmid and 565 ng ofBxb1 expression plasmid per 24-well.

Note

Keep one well with no donor plasmid to be used as a negative control for puromycin selection.

Day 1

Visualize cells to verify efficient transfection and check cell health.

Note

DSP doesn't observe toxicity from PEI/Bxb1 expression, and therefore, does not media change. SRK typically does a media change at this step to improve cell health.

Day 2

Passage entire 24-well to 6-well plate.

Day 3

Verify cells have adhered/look healthy and begin Puromycin selection @ 1 ug/mL (standard concentration).

Note

If cells do not look healthy, wait another 1-2 days to start selection. However, do not wait too long. At maximum, cells should be less than 40% confluent, otherwise selection may not perform as well. Include at least one condition with no integration to be used the monitor the puromycin selection.

Day 6

Check well for survivors. At a minimum, you should see single cells/small colonies with normal morphologies.

Note

Larger cargoes will result in less efficient recombination, and thus, fewer survivors. Depending on your experimental timeline, you can transfect multiple wells with the same condition so you don't have to wait as long for outgrowth.

Day 8

Media change, removing puromycin. DSP recommends a PBS wash to remove cell debris.

Day 9 + n

Monitor cells and passage once confluent. DSP recommends passaging 1:10 at 6-well scale to let the cells recover and dilute out any residual plasmid before proceeding with downstream experiments.

Note

SRK has noticed some outgrowth of non-integrated cell lines over time. Therefore, she recommends maintaining puromycin selection (1 ug/mL) in the media (or at least occasionally adding it) for downstream experiments.

Excision protocol

This is a placeholder until Deon ascertains a robust protocol.

Counter-selection	Concentration	Dilution
GCV	10 μM	1000x

SNAP circuit

This protocol describes the induction of the SNAP circuit in transient transfection in a 96-well plate.

Materials

- Cells
- Plates
- DMEM supplemented with 10% FBS
- PEI
- **Small molecule inducers:**
  - Guanine (*25 mM stock* (page **??**))
  - O6-benzylguanine (*50 mM stock* (page **??**))

  - Solvent controls (e.g., 0.2N NaOH for guanine or DMSO for O6-benzylguanine)

- **Input plasmid:** plasmid expressing SNAPtag conjugated with target protein (e.g., pHAGE-SNAP-tagBFP)
- **Output plasmid:** plasmid expressing reporter gene-ribozyme switch cassette (e.g., pHAGE-UBC-mGreenLantern-p2g6)

Protocol

Day 1: Seed cells

1. Seed the cells at a density of 2.5-4e4 cells/well in a gelatin-coated 96-well plate.
2. Incubate the cells at 37°C overnight.

Day 2: Transfection

1. Transfect 100 μg each of the input and output plasmids per well with PEI by following the *general transfection* (page **??**). To avoid cell death due to toxicity of the small-molecule inducers, reduce cell stress from transfection by using a lower PEI:DNA ratio of 3:1.
2. Incubate the cells at 37°C overnight.

Day 3: Small molecule treatment

1. Dilute the small molecule inducers to the appropriate concentrations in the appropriate culture medium.
   - For both O6-benzylguanine and guanine, no greater than 100 μM should be used to minimize cytotoxicity.
   - Include conditions containing only the inducer solvent as a control (e.g., the same percent volume of DMSO only as in the O6-benzylguanine condition).
2. Remove the media from the wells, being careful not to detach the cells.
3. Add 100 μL/well of the inducer-containing media to the cells.
4. Incubate the cells for 48-72 hours at 37°C.

Note

Be careful when changing the media for HEK293T cells—they easily detach, especially in a 96-well plate.

Day 5-6: Analysis

Analyze the cells via microscopy or *flow cytometry* (page **??**).

Cell Line Creation

Estimated time

~2 weeks for polyclonal; ~ 2 months for monoclonal

While the initial transfection does not take very long, the selection process takes a long time!

Note

These protocols are still in development! While the pDNA-based PiggyBac w/ Puromycin co-integration protocol developed by Sneha seems robust, a markerless PiggyBac protocol leveraging modRNA-based transient selection protocol is currently under exploration.

For CRISPR, Deon has developed a robust modRNA-based delivery method that is capable of single and multiplex HDR-based insertions.

Each method possesses their unique cell plating, transfection, and selection timelines. After the selection period, the methods converge on universal enirichment and single-cell cloning protocols (ie., limiting dilution or cell sorting)

PiggyBac

Day 0

Seed your cells such that they will be around 20-30% confluent on the next day. There is an extended selection outgrowth period required, which means we should start with lower confluence. Recommended rough seeding counts are:

Cell type	Seeding amount (per 24 well)
293T	40k cells
U2-OS	10k cells

Day 1

- Co-transfect the PiggyBac plasmid alongside your plasmid containing PB recognition sites at a 3:1, template:transposase mass ratio.
- If you are doing markerless integration, then co-transfect a plasmid containing your resistance gene and an unused fluorescent marker.

Note

When transfecting, leave some untransfected wells where you just add the KO-DMEM/PEI master mix, without plasmids. This allows you to tell if your selection is working as expected, and

also gives you a proxy for how much PEI-mediated cell death you are seeing.

Day 2

Media change into selection media. Tested concentrations for PiggyBac are:

Cell type	Puro
293T	1.0 μg/mL (10,000x)
U2-OS	0.25 μg/mL (40,000x)

Day 4

Replace the selection media and continue selecting until sufficient cell death occurs.

Note

Puromycin at the given concentration should decidedly kill your untransfected wells. If you do not see sufficient cell death, try using a different aliquot.

Day 7

Passage the remaining cells to 6-well scale.

Day 9

Sort!

CRISPR (Deon)

Day -1

Deon recommends CRISPR at 12-well scale, as this is the smallest scale that yielded a workable number of surviving cells after multiple transfections, CRISPR genotoxicity, and drug selection. The following seeding density permits sufficient transfection efficiency while obviating the need for passaging in between pDNA and modRNA transfections:

Cell type	Seeding amount (per 12 well)
293T	150k cells

Day 0

Transfect the donor plasmid/s containing your cargo flanked by locus-specific homology arms. If targeting two loci, transfect each donor at a 1:1 mass ratio (1 microgram total DNA).

Note

Deon recommends the Mirus LT1 (low-toxicity) DNA transfection reagent. Avoiding PEI-mediated toxicity/cell-death is critical considering the intrinsically low efficiency of HDR-mediated CRISPR editing.

Transfect a separate well with your donor plasmids to monitor plasmid dilution/drug selection and to serve as a negative control for preliminary genotyping analysis.

Day 1

Transfect your cells with 300 ng of Cas9 modRNA and sgRNA/s for your target locus/loci at a 3:1 mass ratio of Cas9 to sgRNA. For Rogi1/2 dual targeting, the amounts are as follows:

RNA species	Amount (ng)
Cas9 modRNA	300 ng
Rogi1 sgRNA	50 ng
Rogi2 sgRNA	50 ng

Day 2

Depending on the health of your cells/confluency, passage onto a 6-well plate. If your cells are not ready for passaging, media change into fresh DMEM+10% FBS to remove the transfection reagents.

Day 3

If you didn't passage on Day 2, passage today onto a 6-well. After checking cells have adhered, media change into selective media corresponding to the drug marker you've integrated.

Days 4-8

media change with fresh Puro-containing media daily, passaging as needed.

Note

In 293Ts, Puromycin (1 ug/mL) should decidedly kill an untransfected control well in ~72 hr. Deon has found 5 days of Puro selection sufficient to obtain a polyclonal CRISPR line.

Day 9 Remove selection and let cells recover/expand. During your next passage, take a small aliquot of cells for genotyping analysis to validate cells within your population contain the desired

insertion (see "Genotyping your line") before proceeding with single-cell cloning/downstream applications.

TALENS (Chris)

Day -1

Seed your cells such that they will be around 30-40% confluent on the next day. There is an extended selection outgrowth period required, which means we should start with lower confluence. Recommended rough seeding counts are:

Cell type	Seeding amount (per 96 well)
293T	12.5k cells
U2-OS	7.5k cells

Day 0

Follow the step for the cell line type to generate

- **PiggyBac**
  - Co-transfect the PiggyBac plasmid alongside your plasmid containing PB recognition sites at a 3:1, template:transposase mass ratio.
- **Crispr**
  - Co-transfect the Crispr/guide plasmid alongside your plasmid containing locus-specific recognition sites at a 1:1 mass ratio.
- **TALEN**
  - Co-transfect the TALEN-R and TALEN-L plasmids alongside your plasmid containing locus-specific recognition sites at a 1:1:1 mass ratio.

Note

When transfecting, leave some untransfected wells where you just add the KO-DMEM/PEI master mix, without plasmids. This allows you to tell if your selection is working as expected, and also gives you a proxy for how much PEI-mediated cell death you are seeing.

Day 1

Media change into selection media. Tested concentrations for PiggyBac are:

Cell type	Puro
293T	1.0 μg/mL (10,000x)
U2-OS	0.25 μg/mL (40,000x)

Day 2-4

Continue selecting the cells until decent cell death occurs, or the cells are too dense.

Note

Puromycin at the given concentration should decidedly kill your untransfected wells. If you do not see sufficient cell death, try using a different aliquot.

Clonal selection or enrichment via limiting dilution

Day 5 - Week 2

Dilute cells into both 96-well plates (one per condition) and onto 24 well plates for outgrowth.

Onto the 96-well plates, hard dilute to ~2 cells per well and use 200 uL of media per well (for 40 cells per mL). The extra media ensures that the plate will not fully evaporate over the next week. If you need a very large fold dilution, it is more accurate to do this as a stepwise dilution in conical tubes (e.g. first a 1000x dilution, then another 1000x dilution).

For the 24-well plates, dilute to the following amount:

Cell type	Seeding amount (per 24 well)
293T	5k cells
U2-OS	2k cells

This reseeding should proceed for about a week, until cells finish outgrowing.

Week 2 + 1 day

After cells have adhered to the 24-well plates, switch back into selection media if some non-integrated cells are visible via microscopy, and maintain it until these cells have died off.

Week 3

At this point, you should see round colonies coming from either outgrowth condition.

If the overall integration percentage is high enough, you can do FACS or the BioMicroCenter single-cell sorting to isolate clonal lines. If not, you can use the microscope to re-pick.

Clonal selection or enrichment via flow sorting

Estimated time

2 hours pre-flow-sort, 20 minutes per sample for flow sorting (min 90 minutes of sort time).

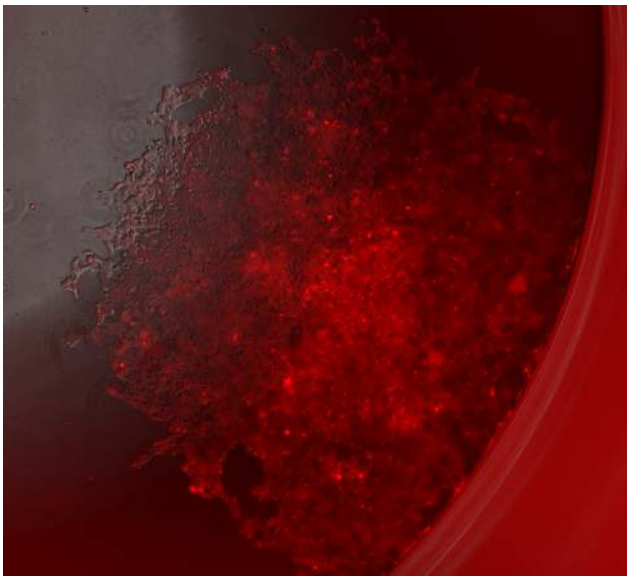


Fig. 6: Example of what outgrowth looks like when grown out from a single-cell dilution. This is one well in a 96-well dilution plate.

Note

This assumes that you are sorting on the Sony in the flow core. You can review the Sony SOP[188]

1. Two to three days before the sort, make sure you have enough cells going for conditioned media collection. A T75 flask of 293Ts or whatever cell type you are using are a good source for this.
2. The day before the sort, make sure you have enough cells to sort. You should have more than a million cells, ideally several million.
3. Prepare your destination tubes and plates. For tubes, if your media does not contain FBS, it is recommended to coat the inside of the tubes with 7.5% BSA solution (put ~1mL in, swirl it around, aspirate). For plates, remember to gelatin coat.
4. Prepare conditioned media. Collect 1-2 day old media from cells, and filter through a 0.22 micron filter. Combine this 1:1 with fresh media.
5. Spin down cells, as if you were passaging. Resuspend the cells and count them.
6. Resuspend cells to a final volume of 2-5 million cells per mL.
7. Add the prepared conditioned media to your tubes and plates to be sorting into.
8. Prep a box to bring with you to flow sorting. You should bring:
    - A P1000 and tips.
    - Gloves

[188] https://docs.google.com/document/d/1toqMY_qnDy0_YDkcEr2ktDJWcteKe0Pj42_scukqT5s/edit?usp=sharing

- Prepared tubes and plates to sort onto.
- An extra plate for aligning, if sorting onto plates.
- Falcon tubes with cell strainer caps.
- Enough ice for how many plates you are sorting onto. Ideally, cells stay directly touching the ice once they are sorted.

9. Bring your stuff to the flow core and sort!

Note

If sorting onto plates, you should update the settings to place 100 cells in well A1, with 1 cell in other wells. This ensures that you will be able to locate the cells during outgrowth.

10. Return your cells to the **quarantine** incubator as quickly as possible.
11. At the end of the week, you likely will need to "top off" media to address evaporation.
12. One week later, you should be able to locate colonies under the microscope.
13. One week after that, passage cells onto 6-well plates.

Repicking

Repicking requires some trial and error using a pipette tip, but can get good enrichment of a target colony relatively quickly.

1. View the well of interest under the Keyence. Mark where the colony is on the top of the plate.
2. Prepare gelatin-coated destination plates. These should typically be 96 well plates. Fill the plates with media.
3. In the BSC, take a P200 tip, set to 50uL and depress the plunger. Scrape the pipette tip in small circles in the target area of the source plate, while slowly withdrawing media to suck up the cells as you scrape them off the bottom.
4. Deposit the 50 uL of cells into the destination plate.
5. Check the scraped regions under the Keyence, repeating if you missed the desired colony. If the media level in the source plate gets too low, just add more media to that well.

Genotyping your line

Diagnostic PCR can be performed to validate the presence of your transgene (random integration, site-specific) and whether it integrated to the desired locus (CRISPR, Landing Pad).

1. design primers specific for your cassette using Primer-BLAST[189]. This tool helps pick primers that anneal only to your target and not other genomic regions.

[189] https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Note

This is a predictive tool, therefore multiple primer pairs may need to be designed and tested.

2. For site-specific integration, design primers to perform "In-Out" PCR, as described here in Figure 3a[190].
3. Isolate genomic DNA from your cell line. When performing routine passaging, spin down an aliquot of cells (50,000-500,000 cells, depending on culturing scale) in a microcentrifuge tube and spin down at 3000 rcf for 5 min.
4. Aspirate media, and re-suspend pellet in 50 μL of Cell Lysis buffer supplemented with 0.5 μL of Proteinase K.

Note

If you're processing multiple samples, you can make a Cell Lysis buffer/Proteinase K mastermix.

5. Transfer the solution to a PCR tube and incubate at 85 C for 45 min.
6. Microfuge the PCR tubes to pellet cell debris and use 1 μL of supernatant as template for 20 μL PCR reaction.

Important

If performing for the first time or you're not planning to sequence verify the amplicons, using Taq colony PCR reagent is sufficient. To validate the sequence junctions for HDR/seamless integration into a LP, use a high-fidelity polymerase (ie., Q5).

7. For transgene-specific primer pairs, include your original vector as a positive control and genomic DNA from untransfected/untransduced cells as a negative control. For site-specific primer pairs, you can only run the negative control (super annoying, I know).

Note

Genotyping can be challenging. Touchdown PCR and nested PCR has helped DSP amplify some tricky genomic DNA segments.

Rogi1 and Rogi2 LPs

We currently have monoclones for v2, v3, and v4. The table below highlights the key attributes of v2 and v3 LP architectures.

[190] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5826598/

Note

The Rogi2 v4 is our current version. Please see the page on Rogi1 and Rogi2 Landing Pads[191] for more information on the v4 LP designs.

V2 Rogi1/Rogi2 dual LP RMCE line:

locus	pHA for cargo	recombinase	positive selection (gene, drug)	counterselection (gene, drug)
Rogi1 LP	pKG01862	Cre	N/A	iCasp9, AP1903
Rogi2 LP	pKG02278	Bxb1	N/A	HSV-TK SR39h, GCV or PCV

V3 Rogi1/Rogi2 dual LP single site integrase line:

locus	pHA for cargo	recombinase	positive selection (gene, drug)	counterselection (gene, drug)
Rogi1 LP	pKG02180	Bxb1	BsdR, Blasticidin	HSV-TK SR39h, GCV or PCV
Rogi2 LP	pKG02181	PhiC31	BleoR, Zeocin	iCasp9, AP1903

RMCE integration of payloads into V2 line

Note

These protocols are still in development! Important variables that need optimization include: 1) the ratio of recombinase to donor plasmid 2) dosing schedule of drug selection/counterselection 3) scale needed to obtain a sufficient number of recombined cells

Day -1

Electroporation of plasmids into MEFs

Warning

This protocol has not been tested!

[191] https://gallowaylabmit.github.io/protocols/en/latest/protocols/tc/other/Rogi1_and_Rogi2_LPs.html

Note

This protocol is specific for electroporation to test settings for the Amaxa II electroporator and plasmid concentrations for MEFs. This is a good starting place for any electroporation protocol including for iPSCs. Settings will differ by cell type.

Overexpression

In order to determine which conditions allow the best efficiency and highest viability for the Amaxa cell electroporator, cells first need to be tested for compatibility with a specific buffer and voltage setting. This is achieved by setting up a matrix experiment with controls as follows:

- MEFs + DNA with no electroporation
- MEFs – DNA with electroporation
- MEFs + 2 μg pMax-GFP with MEF buffer 1, under setting A-23
- MEFs + 2 μg pMax-GFP with MEF buffer 1, under setting T-20
- MEFs + 2 μg pMax-GFP with MEF buffer 2, under setting A-23
- MEFs + 2 μg pMax-GFP with MEF buffer 2, under setting T-20

The procedure for electroporation is as follows:

1. Add 100 μl of Buffer supplement to each buffer type. This reagent will expire in 3 months. Also pre-warm some MEF media and have available.
2. Thaw MEFs from a desired strain, and pass once. After the passage, cells will be ready for electroporation when they reach 50-60% confluency.
3. Grow enough cells so that you have 2 x 10^6 cells for each condition
4. For each condition, have 4 wells of a 6-well culture plate filled with 2 mL of pre-warmed media, and in store in the incubator at 37°C.
5. Trypsinize and count cells.
6. Divide cells into 50 mL tubes so that each tube has 2 x 10^6 cells. Spin cells down and remove media.
7. Resuspend cells in 100 μl of desired MEF buffer, and place slurry in cuvette, making sure all liquid is in the bottom and there are no air bubbles.
8. Call up desired program on the Amaxa electroporator and insert cuvette. It may only be loaded in one direction. Press the "x" button and wait for the program to conclude.
9. Remove cuvette, and in the BSC, add 500 μl of pre-warmed media. Remove entire mixture and add to a 1.5 mL eppendorf tube.
10. Add 150 μl each of the above mixture to 4 wells of the culture plate. Make sure plate is labeled on the bottom, as the lid may get mixed up with another lid.
11. Allow cells to equilibrate in the incubator (37°C, 5% CO_2) for 24 hours, then examine cells under the fluorescent microscope to estimate confluency and percent GFP positive.

12. The experimental parameter that yields the best uptake efficiency and highest confluency will be the program that we use for our experiment. For C57BL/6J, the correct buffer/program mixture is MEF Buffer 1 and T-20.

Plasmid Concentration Experiment

Next, we need to determine the proper amount of overexpression plasmid to add to our cells for our planned experiment. We will standardize this by adding 3, 6, and 10 μg of each to three different sets of cells. We will also have a DNA– control and a pMax-GFP control. Below are our parameters:

- Cells + no DNA with MEF buffer 1, under setting T-20
- Cells + 2 μg pMax-GFP with MEF buffer 1, under setting T-20
- Cells + 3 μg experimental plasmid with MEF buffer 1, under setting T-20
- Cells + 6 μg experimental plasmid with MEF buffer 1, under setting T-20
- Cells + 10 μg experimental plasmid with MEF buffer 1, under setting T-20

The experiment will be run under the same conditions as above, starting at step #1. Since each parameter will have 4 wells, we can perform 4 different tests on them.

1. At 24 hours post-electroporation, from the first well, remove supernatant, but do not dispose of it. Wash cells with 0.5 mL of PBS, remove, but do not dispose of it. Then, trypsinize cells with an appropriate amount of trypsin, incubate at 37°C for 5 min., then save supernatant. This saved supernatant from the trypsin step may be used to count the viable cells left on the plate on the countess. After cells are counted, combine the original, PBS and trypsin supernatants and run on the flow cytometer.
2. At 24 hours post-electroporation, from the second well, remove supernatant and wash well once with 1 ml of PBS. Remove PBS and add 0.5 mL of Trizol to the well and pipette up and down several times to lyse cells. Freeze Trizol/cell mixture immediately.
3. At 48 hours post-electroporation, repeat step 2 with third well.
4. At 96 hours post-electroporation, repeat step 2 with forth well.

Murine Bone Marrow Isolation

This protocol details the isolation of bone marrow from mice along with culturing techniques for bone marrow derived macrophages (BMDMs).

The BMDM differentiation protocol takes 7 days before the cells are fully differentiated into macrophages and can be used for downstream experiments. Make sure to take this into account before planning out experiments.

Materials/Reagents

The dissection is by far the most time consuming part of this isolation. Below are materials that you will need:

Dissection Materials	Isolation/Culturing
Surgical scissors	DMEM + 10% FBS
Tweezers	MCSF1
Scalpels	Pen/Strep
10 mL syringes	40 um cell strainer
25 gauge needles	Pestle

Protocol - Dissection

Estimated time

30 minutes per mouse

1. Sacrifice the mouse and bring it back to 66-219, place down paper towels or pads from the mouse facility and lay out the mouse.
2. Heavily spray the outside of the mouse with 70% ethanol. If a MEF isolation is occurring, allow the embyros to be isolated before moving on to the bone marrow.
3. Cut away the skin from the two hind legs of the mouse. Begin to cut back the muscle with scissors to expose the femur-pelvic joint. See below for image examples.
4. Cut away as much muscle and skin as possible from the hind legs before cutting through the femur-pelvic joint.

Note

It is important that you be very careful when removing the muscle. Mouse bones are very easy to cut with the scissors.

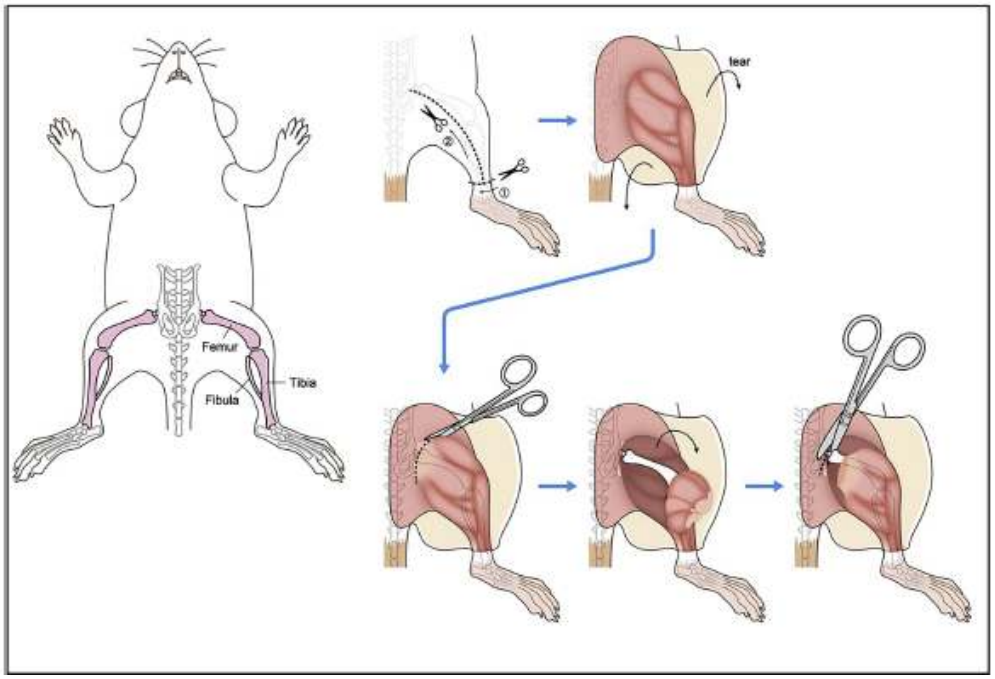
5. Once the legs have been removed from the mouse, return the mouse to the carcass bag.
6. Remove any remaining muscle with the scalpel, being careful to not cut yourself.
7. Once the majority of the muscle has been removed from the bone, place the bones in a petri dish with 1x PBS.

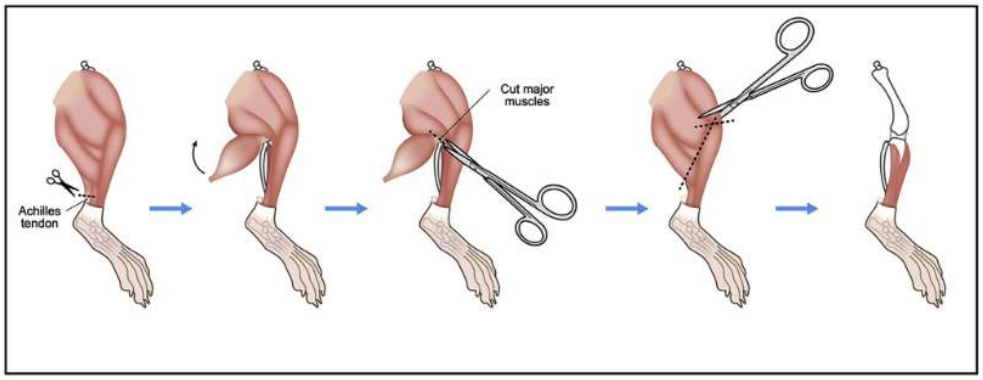
Note

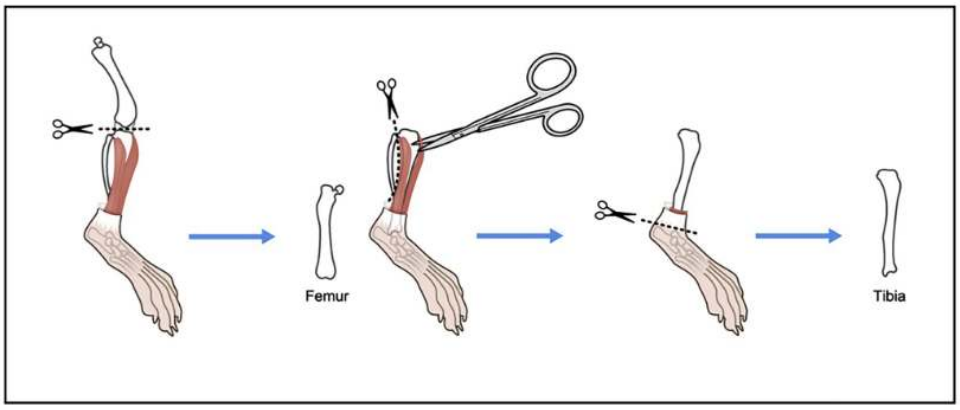
If you will not be harvesting the bone marrow for more than 30 minutes, place the petri dish on ice.

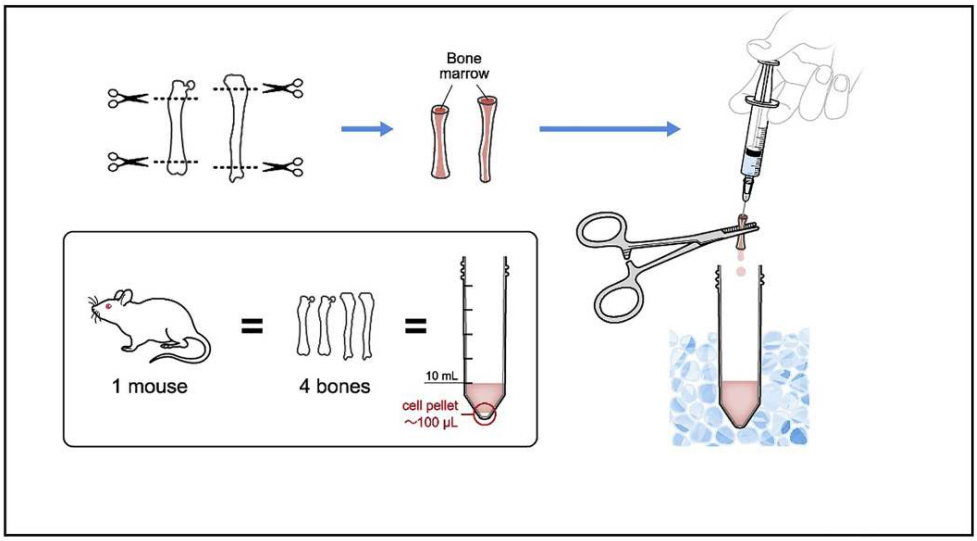
8. Before moving into the hood, try to remove any last bits of muscle that were loosened up with the PBS soak.

Bone Isolation with Images









Protocol - Isolation & Processing

Estimated time

1 hour

1. Bring the petri dish with the bones, tweezers and scissors into the hood
2. You will need to cut off the ends of the femur and tibia to create ends for the bone marrow be washed out of the bones.
    - Pick up the bones using the tweezers and carefully cut just the end of each of the bones.

Note

Be careful when cutting the ends of the bones. Try not to cut too much of the bone and beware of the small bits one bone flying

3. Prepare a 50 mL conical and an aliquot of 1x PBS. The bone marrow will be flushed into this conical.
4. Place the needle onto the end of the syringe and take up 2-3 mL of PBS
5. Pick up the bone with the tweezers and insert the needle into the center of the bone. You should be able to feel a hollow area inside of the bone. Hold the bone over the conical, slowly flush PBS through the bone.

Note

When the bone marrow is flushed from the bone, you should see a red clump expel into the tube. The bone should appear clear/transluscent at this point.

Do NOT flush the PBS too quickly through the bone. It is possible that the PBS will backflow and this will cause liquid to spray outside of the conical.

6. Repeat with the remaining bones.
7. Spin down the cells for 5 mins at 1600g.
8. Aspirate off the PBS and resuspend the cells in DMEM + 10% FBS
9. Place a strainer on another clean 50 mL conical and ready a pestle
10. Prime the strainer with 1 mL of DMEM + 10% FBS and then pass through the resuspended bone marrow
11. Use the pestle to mash the cell suspension through the strainer and wash it through with DMEM + 10% FBS

Note

At this point you can also use a clean pipette tip to pull liquid through the bottom of the strainer if it is clogged

12. If you have a large volume of media, you can spin down the cells for 5 mins at 1600g and resuspend in a smaller volume.
    - If you spun down the cell suspension, aspirate off the media and resuspend into a smaller volume
13. Seed cells into desired plate for downstream experiments. If your desired cell type is BMDMs, culture in DMEM + 10% FBS with 1x Pen/Strep and 10 ng/mL M-CSF.

Note

If you plan to use the cells in TC, be sure to seed a plate of cells for myco testing.

Culturing Tips

Differentiation of bone marrow cells into bone marrow derived macrophages takes 7 days. Bone marrow cells should be cultured in macrophage colony stimulating factor 1 (MCSF1) at a concentration ranging from 1-50 ng/mL. Currently, 10 ng/mL has achieved acceptable yield and purity of BMDMs.

Estimated time

7 days

Differentiation Media	Concentration
DMEM + 10% FBS	N/A
Pen/Strep	1 ng/mL
MCSF1	1-50 ng/mL

Note

MCSF1 aliquots can be found in the -20C TC freezer in the "Microglia Small Molecules" box. Aliquots are at a concentration of 10 kx (i.e. for 10 mL of media, add 10 uL of MCSF1 to achieve 10 ng/mL)

1. Seed cells in differentiation media.

Note

If you are planning to remove the cells from the current plate, be sure to seed them on un-treated plates. These plates are labeled in 66-219. BMDMs are very sticky once fully differentiated and seeding on gelatin coated plates or treated plate will make removal very difficult.

If your experiment is terminal in the current plate, the plate type and coating is less important even though it could impact the transcriptome of the cells.

2. After 4-days, carefully aspirate half of the media from the cells and replenish with freshly made differentiation media.
3. After 7-days of culturing in MCSF, remove the media and perform a PBS wash before replenishing the media.

Note

At 7-days into the differentiation, all BMDMs should be adherent to the plate. Cells that are floating are either masses of dead cells or undifferentiated cells.

Dissociation Methods

Seeding cells that need to be dissociated on un-treated plates is highly recommended. Dissociation protocols differ greatly between different papers and labs.

Option #1: Trypsin

1. Add a 1:1 dilution of trypsin in PBS to the cells

2. Allow cells to incubate for 8-10 minutes until there are visible cells lifting off the plates

> **Note**
>
> It may take a significantly long time to dissociate the BMDMs if they are well adhered, so do not worry if after 10 minutes they are still attached.

3. Add an equal amount of DMEM + 10% FBS to quench the trypsinization and place into a conical.
4. Spin down the cells for 5 mins at 1600 g
5. Aspirate off the media and seed cells for downstream experiments.

Option #2: Scraping

1. Use a cell scraper to gently scrape the bottom of the plate for 2-5 minutes.

> **Note**
>
> Scrape the bottom of the plate in a circular motion. DO NOT just lightly scrape, you will need to push hard to get the cells off.

2. Add additional DMEM + 10% FBS to the plate and pipette up and down, washing the bottom of the plate with the liquid.
3. Place the cell suspension into a conical.
4. Spin down the cells for 5 mins at 1600 g
5. Aspirate off the media and seed cells for downstream experiments

Freezing BMDMs

BMDMs and bone marrow cells can be frozen down for later use. Freezing media should be used to freeze down both cell types. A mixture of 90% FBS and 10% DMSO is preferred.

1. Obtain a cell pellet using the dissociation methods listed above.
2. Resuspend cells in freezing media

> **Note**
>
> It is very important to keep the cell count in the freezing suspension very high as this will help with recovery later. A recommended density if 4x10 ^ 6 cells/mL.

3. Place the cell suspension in a cyrovial.
4. Label the vial and place into the styrofoam boxes in the -80C.
5. After 1-2 days in the -80C, move the cells into liquid nitrogen storage

Murine Splenocyte Isolation

Splenocytes is a blanket term for a large subset of cells that are present within the spleen (T-cells, B-cells, macrophages, fibroblasts, etc.). In this protocol, we are specifically interested in the macrophages. Additional purification methods, such as antibody or bead pull down, could be used to isolate specific cell types.

Splencoyte Dissection

Estimated time

15 minutes per mouse

1. Sacrifice the animal.
2. Generously spray the animal with 70% ethanol.

Note

If a MEF isolation is happening, allow the embyros to be isolated before proceeding to isolate the spleen.

3. Make an incision in the abdomen of the animal and cut about half way up the animal.
4. The spleen will be a long, slender, reddish organ on the left-side of the abdomen, below the stomach (Left-side here is from the point of view of the animal, if the animal is on the back for dissection, it will be on your right). The kidney will also be in this area, but it should have a rounder, bean-like shape.
5. Excise the tissue and remove any additional tissue connected to the organ.
6. Place the spleen into a petri dish with PBS until ready for downstream processing.

Note

If the downstream processing will be more than 10 minutes, keep the petri dish with the spleen on ice.

Splenocyte Isolation/Preparation:

Estimated time

30 minutes

1. Place the spleen into a clean petri dish and using 2 razor blades, mince the spleen into a paste.

2. Once the spleen has been thoroughly minced, add 5-10 mL of DMEM + 10% FBS to the dish
3. Prime a 40 um strainer with 1 mL of DMEM + 10% FBS into a 50 mL conical tube
4. Pipette up the suspension and pass through the primed strainer into the tube.
5. Add additional DMEM + 10 % FBS to the dish to get up any remaining cell matter
6. Using a pestle or the plunger of a syringe, mash the cell matter through the strainer
7. Wash the strainer 2-3 times with 1 mL of DMEM + 10% FBS.

Note

If liquid is not moving through the strainer, carefully lift up the mesh strainer and using a P1000 with a clean tip, pull the media through the strainer and dispense into the conical tube

8. Discard the strainer and syringe
9. Centrifuge down the cell suspension at 1600 rpm for 5 minutes
10. Aspirate the supernatant
11. Resuspend the cell pellet in 2 mL of prewarmed RBC lysing solution

Note

I use 2 mL of RBC lysing solution per spleen

12. Incubate the cells at 37C for 2 minutes
13. Add 20-30 mL of PBS to the suspension and centrifuge at 1,600 rpm for 5 minutes
14. Aspirate off the supernatant and resuspend the cells in DMEM + 10% FBS

Note

The cells will be very sticky here and hard to resuspend, but try to break up some of the clumps

15. Prime another 70 um strainer and pass through the resuspended pellet. You will need to use a pestle here and wash the strainer at least 2-3 times.

Note

There will likely be a gooey mass that will not make it through the strainer and that is fine. This is likely most of the lysed RBCs

16. At this point you can either directly count the cells via a hemacytometer or centrifuge down the cells again and resuspend into a small volume to plate

Note

Depending on the downstream processing steps the number of cells can differ greatly. I usually plate 1 spleen per 10 cm dish and then after 3-4 days, seed cells for my experiments.

Note

For culturing techniques and dissociation methods, take a look at the *methods* (page **??**) under bone marrow isolation

Transgene expression control through ribozyme switch (96-well plate version)

Materials

- Cells
- Plates
- DMEM supplemented with 10% FBS
- KO DMEM
- PEI
- DNA containing the transgene-ribozyme switch cassette
  - Our lab most commonly uses two ribozyme switches: (1) the p2G6 ribozyme which is an ON-switch inducible with Guanine and (2) the Theo.CAUAA which is an ON-switch inducible with Theophylline.
- DNA with ribozyme controls (*Optional: These controls are useful comparisons for the dynamic range of inducible ribozyme switches*)
  - sTRSV ribozyme is the "maximum cleaving" control. This would be the lowest expression level you could expect with a Rz.
  - sTRSVctl ribozyme is the "minimum cleaving" control. This would be the highest expression level you could expect with a Rz.
- Small-molecule inducer such as theophylline or guanine (check the concentration that you will need)
- Vehicle for small molecule as a control (e.g. DMSO, NaOH)

Note

Aliquots are 25mM in NaOH (page **??**), and a final concentration of 100uM has been used by BAD/JCA.

Theophylline is used for the Theo.CAUAA ribozyme switch. Aliquots are 25mM in water, and a final concentration of 300uM is used by BAD/JCA.

To make Theophylline Stock:

1. Dissolve 4.50 mg Theophylline / 1 mL Elga Water. You may need to vortex and/or slightly warm the solution.
2. Filter sterilize with 0.22 uM filter before use.
3. Make sure the Theophylline is fully dissolved before use. It tends to crystallize out of solution when kept in the fridge.

Procedure

Day 1: Seed cells

1. Seed the cells at a density of 25-40k cells/well in a 96-well plate.
2. Incubate the cells at 37°C overnight.

Day 2: Transfection

1. Transfect 100μg of a plasmid expressing transgene-ribozyme switch cassette per well with PEI by following the *general transfection* (page **??**). If your small molecule or the solvent has cytotoxicity, using a lower PEI:DNA ratio of 3:1 is recommended.
2. Incubate the cells at 37°C for 4-6 hours.
3. Remove the media from the wells carefully to not lose the cells, and add 100μl of fresh media that contains the small-molecule inducer.
4. Incubate the cells overnight.

Note

If you are using ON-switch, where the ribozyme activity is inactivated by the small molecule (stabilizing the transcript), you don't need to treat the cells with the small molecules at 4-6 hours post transfection, you can do this next day.

If you are using OFF-switch, where the ribozyme activity is activated by small the molecule (destabilizing the transcript), you MUST do the small-molecule treatment at 4-6 hours post transfection so that transcribed mRNA binds with the molecule before being translated to protein.

Note

Be careful when changing the media for 293T cells—they easily detach, especially in a 96-well plate.

Day 3 or 4

- After 24 hours post small-molecule treatment, you should be ready for analysis via microscopy or flow cytometry.
- A 24-hour incubation with the small molecule should be generally enough to measure the output transgene expression, but longer incubations of 48 or 72 hours may improve detection.

3.5.3 Reprogramming

MEF-to-iPSC Reprogramming

Last updated: 3/31/2025 by MC

This protocol outlines the timeline of MEF-to-iPSC reprogramming and links to protocols as they are appropriate in the timeline. MC is currently optimizing this protocol to produce iPSCs using Oct4-GFP trasgenic MEFs.

Days 1 - 6: Production and transduction of viruses encoding reprogramming factors

See *here for the complete Plat-E virus production protocol* (page **??**).

At minimum, iPSC reprogramming requires the transduction of stem cell-specific transcription factors. Oncogenes such as p53DD are being evaluated for their ability to improve reprogramming efficiency. The following plasmids are frequently used as sources of stem cell TFs or oncogenes and are encoded on retroviral backbones compatible with production in Plat-E cells.

Single TF and polycistronic cassettes are being tested, with additional polycistronic cassettes in development.

pKG number	Plasmid Name
pKG03903	pMXs-Oct4
pKG03904	pMXs-Klf4
pKG00011	pMXs-Sox2
pKG01962	pMXs-MKOS
pKG03523	pMXs-CIDD-WPRE
pKG03525	pMXs-CISDD-WPRE
pKG03526	pMXs-SDDIC-WPRE
pKG03336	pMXs-SDDIR
pKG00060	pMXs-p53DD
pKG01292	pMXs-Snap-p53DD-IRES-hRasG12V

Day 1:

1. Seed **650,000** Plat-E cells per well of a *gelatin-coated* (page **??**) 6-well for each virus to be made.
    - Each well of a 6-well plate makes approximately 1.1 mL of virus. This is exactly enough to transduce 100 wells of 96-well scale (11 μL of virus per well of a 96-well plate). If more virus is needed, additional Plat-E wells should be seeded.

Tip

If starting from frozen, **start growing Plat-E cells 1 week prior** - they will be slow growing at first (don't change culture medium during the first 3 days). Split Plat-Es 4X-6X every 2-3 days when culture reaches 70-90% confluency.

2. Thaw MEFs on to gelatin-coated 10 cm dish or gelatin-coated T-75 flask. Expect 2-3 million MEFs to be recovered per frozen vial.

Day 2:

3. Transfect Plat-E cells using 1.8 μg of DNA per well of the 6-well plate. Typically, this is done late in the afternoon (~4:00 PM).
4. Check on MEFs to see if they need a media change.

Day 3:

5. Media change Plat-E cells. Remove media and add back 1.25 mL DMEM + 10% FBS + **25 mM HEPES** per well. Typically done in the morning around 10:00 AM to minimize PEI-related toxicity.
6. Seed MEFs
    i. Coat wells in 0.1% gelatin for approx. ~10 min.
    ii. Seed at **5k** cells/96-well in DMEM + 10% FBS.

Note

MC has been doing triplet plate assays to allow for 4 dpi flow, miscroscopy, and 21 dpi flow. Testing O+S+K and MKOS with oncogenes.

Day 4 (-1 day post infection):

7. Harvest and filter each Plat-E virus using a 0.45 μm syringe filter ~24 hours after previous media change. Replace media on Plat-E cells so that you may collect virus again the next day.
8. Mix Plat-E retroviruses in DMEM/FBS as outlined in your experimental design and according to the Plat-E production protocol, 11 μL of each Plat-E retrovirus per well of a 96-well plate.
9. Remove media on MEFs and replace with your completed virus mixtures.
10. Incubate cells overnight with virus.

Day 5 (0 days post infection):

11. Harvest and filter each Plat-E virus for the last time ~24 hours after previous virus collection.
12. Mix Plat-E retroviruses as outlined in your experimental design and according to their respective production protocol. Typically 11 μL of each Plat-E retrovirus and 2 μL of concentrated 293T-produced virus per well of a 96-well plate.
13. Remove media on MEFs and replace with your completed virus mixtures.
14. Incubate cells overnight with virus.

Day 6 (1 day post infection):

19. ~24 hours after previous transduction, remove virus-containing media and replace with fresh media.

> **Note**
>
> For R1-R3, MC has replaced media with DMEM+10% FBS, but will be trying *iPSC reprogramming media* (page **??**) on R4.

20. Perform *CellTrace Staining* (page **??**) to assess early proliferation.

Days 7 - 23: Media changes

Day 7 (2 days post infection):

No action required.

Day 8 (3 days post infection):

21. Media change plates to *iPSC media* (page **??**):
    i. Spike in 1,000X RepSox to media for desired experimental conditions.

> **Note**
>
> MC has been using RepSox in all experimental conditions except Puro controls. In R3 Puro controls did not recieve LIF.

Day 9 (4 days post-infection):

Assays for early indicators of reprogramming potential are performed on this day.

22. Perform *Snap-tag labeling* (page **??**).
23. Peform flow cytometry to quantify CellTrace dilution and Snap signal.

Days 10 (5 dpi), 12, ..,. 23 (19 dpi):

23. Change iPSC media every 2 days until done.

Note

MC has gone to 21 dpi in R1 and R2, 14 dpi in R3, and will do 21 dpi for R4.

Day 19 (21 dpi): Assay reprogramming rate

1. Fix one plate for *immunofluorescent staining* (page **??**) and microscopy.
2. Perform flow cytometry to measure Oct4-GFP expression.

Note

After 8 dpi, it is recommended to dissociate with (*DNAse/Papain* (page **??**)) instead of trypsin. MC has been using DNASe/Papain.

Human Fibroblast to iMN Reprogramming

This protocols outlines the reprogramming of human dermal fibroblasts (HDFs) to induced motor neurons (iMNs).

This protocol outlines the timeline of Human Fibroblast to iMN reprogramming and links to protocols as they are appropriate in the timeline.

Days 1 - 6: Production and transduction of viruses encoding reprogramming factors

See *here for the complete 293T virus production protocol* (page **??**).

At minimum, iMN reprogramming requires the transduction of motor neuron-specific transcription factors. Typically, the oncogenes (p53DD, hRasG12V, c-MYC, myrAKT, BCL2) are also introduced to enhance reprogramming efficiency. The following plasmids are frequently used as sources of motor neuron TFs or oncogenes and are encoded on retroviral backbones compatible with production 273Ts.

pKG number	Plasmid Name
pKG00121	pMXs-Ascl1
pKG00010	pMXs-Brn2
pKG00003	pMXs-Myt1L
pKG00006	pMXs-Ngn2
pKG00009	pMXs-Isl1
pKG00008	pMXs-Lhx2
pKG00002	pMXs-NeuroD1
pKG01169	pMXs-NIL
pKG01574	pMXs-LNI3xHA
pKG00060	pMXs-p53DD
pKG00121	pMXs-hRasG12V
pKG00747	pMXs-myrAkt-P2A-BCL2
pKG00751	pMXs-cMyc
pKG03523	pMXs-cMyc-IRES-p53DD
pKG03525	pMXs-cMyc-IRES-SNAP-p53DD

Day 1:

1. Seed 6 million 293Ts in each *gelatin-coated* (page **??**) 10 cm plate for each virus to be made.
    - Each 10 cm plate makes 200 uL of concentrated virus. If more virus is needed, additional 10 cm plates should be seeded.

Tip

If starting from frozen, **start growing 293Ts 1 week prior** - they will be slow growing at first (don't change culture medium during the first 3 days). Split 293Ts 4X-6X every 2-3 days when culture reaches 70-90% confluency.

You will likely need 2 to 3 T-182 flasks of 293Ts per human reprogramming experiment.

2. Thaw HDFs into a gelatin coated T-182 so they can recover over several days. When culturing HDFs, it is recommended to use DMEM + 15% FBS + non-essential amino acids.

Day 2:

1. Transfect transfer plasmids (containing your gene of interest), packaging, and envelope plasmids. Do this early in the day, as the media is changed 6-8 hours after transfection. *See here for the complete 293T virus protocol* (page **??**).
2. Media change 293Ts 6-8 hours after transfection. Remove media and add back 6.5 mL of DMEM + 10% FBS + **25 mM HEPES**.

Day 3:

Warning

Be sure to use *BSL2+ precautions* (page **??**) when working with the human-infectible retrovirus containing oncogenes.

1. Collect virus-containing media 22-24 hours after the previous media change in 50 mL conicals. Two plates can be combined into 1 tube. Replace with fresh media so that you can collect more virus the next day. Store collected virus in the 4°C refrigerator overnight.

Day 4:

1. Repeat the virus collection ~24 hours later, collecting virus in the same tubes.
2. Filter with a 0.45 um filter into a new 50 mL conical.
3. Add 1/3 volume of Lenti-X concentrator (e.g., for 36 mL virus, add 12 mL Lenti-X). Mix by inverting several times.

Tip

There is enough room in one 50 mL conical to collect 3 plates of virus for 2 days and add the appropriate volume of Lenti-X.

BAD prefers to add Lenti-X to the new 50 mL conicals and then filter the virus directly into the Lenti-X.

4. Store virus at 4°C overnight to precipitate the virus.
5. Seed HDFs at 3k/96-well (for 35dpi reprogramming) or 8k/48-well (for a 7dpi CTV experiment) onto gelatin coated plates.

Day 5:

1. Centrifuge precipitated virus at 1500 x g at 4°C for 45 minutes (use the lower centrifuge) to pellet the virus.
2. Remove supernatent and resuspend in 200uL per 10 cm plate with DMEM + 15% FBS + non-essential amino acids (HDF media).
3. Dilute virus according to calculations in fresh HDF media and polybrene. Use 1 uL of each concentrated virus per 96-well or 2.6 uL per 48-well.
4. To improve infection efficiency, spin the plate at 1500 x g for 90 minutes at 32°C *as described* (page **??**). Be sure to cover the centrifuge buckets with the plate spinner tray caps.

Day 6 (1 day post infection):

1. ~24 hours after transduction, remove virus-containing media and replace with fresh HDF media.
2. If you are interested in early proliferation during reprogramming, perform *CellTrace Staining* (page **??**) on ths day.

Days 7-11 (2-6 days post infection):

1. Change the media once around 4 or 5dpi with fresh HDF media. The cells are no longer BL2+ after this media change.

Day 12 (7 days post infection):

1. If assaying proliferation, complete flow cytometry quantification of CellTrace dilution on this day.
2. If assaying reprogramming rates, re-seed cells on this day.
    1. Gelatin coat a new 96-well plate as usual.
    2. Aspirate the gelatin and coat the plate with laminin (described *here* (page **??**)).
    3. Dissociate 7dpi cells with 3x diluted trypsin.
    4. Quench with 200 uL HDF media.
    5. Aspirate laminin and transfer dissociated cells to laminin coated plate.

Day 13 (8 days post infection):

1. 24 hours after replating the cells, media change to N3 media:
    i. N3 media = N3 base + BDNF/CNTF/GDNF (1,000X, 10 μg/mL) + FGF (10,000X, 100 μg/mL)
    ii. Spike in 1,000X RepSox to N3 media for all experimental conditions. This differs from MEF reprogramming.

Days 14-33 (9-28 days post-infection):

1. Do half N3 media changes every 3-4 days (2 times a week). Aspirate ~45 μL media, and add back ~50 μL fresh N3 media.

Day 33 (28 days post-infection): Assay Reprogramming

1. For flow staining quantification, dissociate cells with *DNAse/Papain* (page **??**), and combine 2 96-wells into 1 eppendorf tube.
2. *Optional*: Do a Live/Dead stain (e.g. Zombie).

1. Dilute ZombieNIR™[192] 1:1000x in PBS.
2. Wash dissociated and pelleted cells with PBS.
3. Resuspend cells in 100 uL diluted Zombie stain.
4. Incubate at room temperature for 15-30 minutes in the dark.
5. Wash with DMEM + 10% FBS.
6. Proceed to fixation step.

Note

Zombie NIR™ dye is excited by the red laser and has fluorescence emission maximum at 746 nm.

3. Proceed with plate or flow staining as *described here* (page **??**).

Note

BAD has used the following antibodies for adherent cell staining:

- 1:500 diluted mouse anti-Tuj1 (BioLegend Cat #801201) with 1:500 diluted anti-mouse AF-488.
- 1:500 diluted rabbit anti-MAP2 (Cell Signaling Technology Cat #4542S) with 1:500 diluted anti-rabbit AF-546 or AF-647.
- 1:10 diluted mouse anti-Hb9 (DSHB Cat #81.5C10) with 1:500 diluted anti-mouse secondary.

Laminin coating for human reprogramming

Important

This protocol is for **Corning mouse laminin**, which we use for human reprogramming. This is different from **human laminin-521**, which we use for iPSC culturing (that protocol is *here* (page **??**)).

Materials

- 100x mouse laminin[193] (Corning, 1 mg/mL; stored -80°C long-term and -20°C short-term)
- PBS

Protocol

[192] https://www.biolegend.com/en-gb/products/zombie-nir-fixable-viability-kit-8657
[193] https://ecatalog.corning.com/life-sciences/b2c/US/en/Surfaces/Extracellular-Matrices-ECMs/Corning%C2%AE-Laminin/p/354232

1. Add laminin to PBS, diluting 1:100.
2. Add 1 mL laminin-PBS to each well in a 6-well plate. For a 96-well plate, use 67 μL per well.
3. Allow wells to set for 1 hour at 37°C before removing excess liquid and plating cells.

Updates

9/30/25 - Nat aliquoted 60 μL and 7 μL of 0.9 mg/mL laminin

Specialized coatings for neurons

To improve adhesion of neurons, it is not uncommon to use 2 coatings. Usually coating options are:

- **Primary coating:** Positively charged polymers: PEI, PLO
- **Secondary coating:** ECM proteins: Gelatin, mLaminin, hLaminin

For positively charged polymers, they are often dissolved in borate buffer (pH approx 8.5) to improve adsoprtion onto cell culture surface.

1X Borate Buffer for TC

Materials

- Pierce™ Concentrated Borate Buffer Stock (20X)[194] (ThermoFisher, 20X; stored RT near TAE)

Protocol to make 1X sodium borate buffer (50 mM borate at pH 8.8)

1. Add 19 mL ELGA-water for every 1 mL 20X Borate
2. Sterile filter with 0.22μm filter
3. Store at 4°C

20X Borate (mL)	ELGA water (mL)	Total vol (mL)
1	19	20
25	475	500
50	950	1,0000

Note

NBW made a 1L sterile 1X Borate buffer stock in Oaken on 9/29/2025

PEI coating for neurons

Protocol adopted from Transcription-factor mediated differentiation of human iPSCs into neurons[195] and MaxWell (see Projects/neuron-electrophys/Protocols)

[194] https://www.thermofisher.com/order/catalog/product/28341
[195] https://pmc.ncbi.nlm.nih.gov/articles/PMC6993937

Materials

- Poly(ethyleneimine) solution in water[196] (Millipore Sigma, 50% (w/v); stored RT)
- 1X Borate buffer

Protocol for making stock 100X (7%) solution

Note

50% PEI in water is very viscous. MaxWell uses a 100X 7% PEI solution for a final 0.07% while Ward uses 10X 1% PEI solution for a final 0.1%.

1. Add 1 mL of 50% PEI solution into a 15 mL centrifuge tube and allow to settle
2. Add 6 mL of 1X borate buffer and mix well with serological pipet to make intermediate 100X 7% PEI/borate
3. Store in 500 μL aliquots (will make 50 mL coating solution, i.e. 8x6-well plates) and freeze at -20°C

Tip

Cut the tip of a 1,000 μL tip off to help pipet very viscous liquids

Protocol for working PEI solution (0.07%)

1. Add 500 μL of 100X PEI (7% in borate) to 49.5mL 1X borate buffer
2. **Sterile filter with 0.22μm filter**
3. Use 1 mL 1X PEI/Borate (0.07%) per 6-well
4. Place in 37°C, 5% CO2 incubator for 1 hr (can go overnight)
5. Aspirate PEI solution entirely
6. Wash x3 times with 1 mL sterile ELGA water
7. Aspirate water and let wells fully dry out in BSC for at least 1 hr (can tilt lid to help dry)
8. Coat wells with secondary (gelatin or mouse laminin)

Important

Ward lab reports PEI requires complete drying to prevent toxicity and can be washed 4 or more times but MaxWell is fine with 3 washes

[196] https://www.sigmaaldrich.com/US/en/product/sial/p3143

PLO coating for neurons

Protocol adopted from Transcription-factor mediated differentiation of human iPSCs into neurons[197] and MaxWell (see Projects/neuron-electrophys/Protocols)

Materials

- Poly-L-ornithine hydrobromide[198] (Millipore Sigma, 50 mg; stored 4°C)
- 1X Borate buffer

Protocol for making stock 10X (1 mg/mL) solution

1. Add 50 mL of 1X borate buffer to 50 mg bottle. Can rinse out bottle with buffer.
2. **Sterile filter with 0.22μm filter**
3. Store in 5 mL aliquots (will make 50 mL coating solution, i.e. 8x6-well plates) and freeze at -20°C

Protocol for working PLO solution (0.1 mg/mL)

1. Add 5 mL of 10X PLO (1 mg/mL in borate) to 45mL 1X borate buffer
2. Use 1 mL 1X PLO/Borate (0.1 mg/mL) per 6-well
3. Place in 37°C, 5% CO2 incubator for 1 hr (can go overnight)
4. Aspirate PLO solution entirely
5. Wash x3 times with 1 mL sterile ELGA water
6. Aspirate water and let wells fully dry out in BSC for at least 1 hr (can tilt lid to help dry)
7. Coat wells with secondary (gelatin or mouse laminin)

MEF-to-iMN Reprogramming

Our lab uses the reprogramming of mouse embryonic fibroblasts (MEFs) to induced motor neurons (iMNs) as a testbed system to study the process of cell fate transitions. To initiate and study reprogramming, a number of common lab protocols are combined, including virus production, CellTrace Labeling, and dissociation using DNAse and Papain.

This protocol outlines the timeline of MEF-to-iMN reprogramming and links to protocols as they are appropriate in the timeline.

Days 1 - 6: Production and transdcution of viruses encoding reprogramming factors

See *here for the complete Plat-E virus production protocol* (page **??**).

See *here for the complete 293T virus production protocol* (page **??**).

At minimum, iMN reprogramming requires the transduction of motor neuron-specific transcription factors. Typically, the oncogenes p53DD and hRasG12V are also introduced to enhance reprogram-

[197] https://pmc.ncbi.nlm.nih.gov/articles/PMC6993937
[198] https://www.sigmaaldrich.com/US/en/product/sigma/p3655

ming efficiency. The following plasmids are frequently used as sources of motor neuron TFs or oncogenes and are encoded on retroviral backbones compatible with production in Plat-E cells.

pKG number	Plasmid Name
pKG00121	pMXs-Ascl1
pKG00010	pMXs-Brn2
pKG00003	pMXs-Myt1L
pKG00006	pMXs-Ngn2
pKG00009	pMXs-Isl1
pKG00008	pMXs-Lhx2
pKG01169	pMXs-NIL
pKG01574	pMXs-LNI3xHA
pKG00060	pMXs-p53DD
pKG00197	pMXs-hRasG12V
pKG01112	pMXs-hRasG12V-IRES-p53DD
pKG01292	pMXs-Snap-p53DD-IRES-hRasG12V

Day 1:

1. Seed 700,000 - 850,000 Plat-E cells per well of a *gelatin-coated* (page **??**) 6-well for each virus to be made.
    - Each well of a 6-well plate makes approximately 1.1 mL of virus. This is exactly enough to transduce 100 wells of 96-well scale (11 μL of virus per well of a 96-well plate). If more virus is needed, additional Plat-E wells should be seeded.

Note

AMB uses 700,000 Plat-E cells, NBW uses 850,000 Plat-E cells to similar results.

Tip

If starting from frozen, **start growing Plat-E cells 1 week prior** - they will be slow growing at first (don't change culture medium during the first 3 days). Split Plat-Es 4X-6X every 2-3 days when culture reaches 70-90% confluency.

2. If using lentiviruses or other 293T-produced viruses that will be concentrated before infection, seed 293Ts on gelatin-coated or 10 cm dishes or in 6-well plates, depending on needed scale. See virus *production protocol* (page **??**) for more details.
3. Thaw MEFs on to gelatin-coated 10 cm dish or gelatin-coated T-75 flask. Expect 2-3 million MEFs to be recovered per frozen vial.

Day 2:

3. If using 293T-produced viruses, transfect transfer plasmids (containing your gene of interest), packaging, and envelope plasmids. Do this early in the day, as the media is changed 6-8 hours after transfection.
4. Transfect Plat-E cells using 1.8 μg of DNA per well of the 6-well plate. Typically, this is done late in the afternoon (~4:00 PM).
5. Media change 293Ts 6-8 hours after transfection. Remove media and add back 1.25 mL (for 6-well scale) or 6.5 mL (for 10 cm scale) of DMEM + 10% FBS + **25 mM HEPES**.

Day 3:

6. Media change Plat-E cells. Remove media and add back 1.25 mL DMEM + 10% FBS + **25 mM HEPES** per well. Typically done in the morning around 10:00 AM to minimize PEI-related toxicity.
7. If using 293T-produced viruses, harvest virus-containing media 18-24 hours after the previous media change. Replace with fresh media so that you can collect more virus the next day. Store collected virus in the 4°C refrigerator overnight.
8. Seed MEFs
    i. Coat wells in 0.1% gelatin for approx. ~10 min.
    ii. Seed at 10k cells/96-well. For larger sizes, scale accordingly according to surface area

Culture plate	Scale factor	# of 6-wells of Plat-E you'll need for a full plate
6-wells	30	2
12-wells	11	1.5
24-wells	6	1.5
48-wells	2.6	1.5
96-wells	1	1

Day 4 (-1 day post infection):

9. If using 293T-produced viruses, harvest (for the last time), filter, and begin concentrating each virus.
    i. Harvest virus-containing media 18-24 hours after the previous collection
    ii. Pool virus-containing media from with collection day 1
    iii. Filter media using a 0.45 μm syringe filter
    iv. Add Lenti-X concentrator.
    v. Store virus in the 4°C refrigerator overnight to precipitate the virus.
10. Harvest and filter each Plat-E virus using a 0.45 μm syringe filter ~24 hours after previous media change. Replace media on Plat-E cells so that you may collect virus again the next day.
    i. For conditions using the 6F transcription factor cocktail, the viruses encoding the 6 TFs (Brn2, Ascl1, Myt1L, Ngn2, Isl1, Lhx3) can be filtered through the same syringe together to simplify virus mixing.

Note

You can either 1. filter each virus then mix together (minimizes filtering) or 2. mix altogether then filter (standardizes mixing). Because filtering is the most annoying step, it is advised to minimized filtering.

Examples of mixing AFTER filtering

i. Example - 6F alone (96-well = 100 μl total/96-well):

   For 1 rxn, 96-well: 66 μL 6F (= 11 μL PER FACTOR*6) + 34 μL DMEM + 0.1 μL polybrene (1,000X) = 100 μL total/96-well

   For 3.5 rxn, 96-well: 231 μL 6F + 119 μL DMEM + 0.35 μL polybrene (1,000X) = 350 μL total for 3.5 96-wells

ii. Example - 6F + DD + RR (96-well = 100 μL total/96-well):

   For 1 rxn, 96-well: 66 μL 6F + 11 μL p53DD + 11 μL hRasG12V + 12 μL DMEM + 0.1 μL polybrene (1,000X) = 100 μL total/96-well

   For 3.5 rxn, 96-well: 231 μL 6F + 38.5 μL p53DD + 38.5 μL hRasG12V + 42 μL DMEM + 0.35 μL polybrene (1,000X) = 350 μL total for 3.5 96-wells

11. Mix Plat-E retroviruses as outlined in your experimental design and according to the Plat-E production protocol. Typically 11 μL of each Plat-E retrovirus per well of a 96-well plate. Only retroviruses are included on -1 dpi.
12. Remove media on MEFs and replace with your completed virus mixtures.
13. Incubate cells overnight with virus.

Day 5 (0 days post infection):

14. If using 293T-produced viruses, centrifuge precipitated virus at 4°C as described to concentrate. Remove supernatent and resuspend in desired volume with DMEM + 10% FBS. Typically 200 μL for each 10 cm dish of virus produced and 40 μL for each well of a 6-well used to produce the virus.
15. Harvest and filter each Plat-E virus for the last time ~24 hours after previous virus collection.
16. Mix Plat-E retroviruses and 293T-produced viruses as outlined in your experimental design and according to their respective production protocol. Typically 11 μL of each Plat-E retrovirus and 2 μL of concentrated 293T-produced virus per well of a 96-well plate.
17. Remove media on MEFs and replace with your completed virus mixtures.
18. Incubate cells overnight with virus.

Tip

Before returning cells to the incubator, cells can be centrifuged to improve transduction efficiency. The protocol for *spinfection is here* (page **??**).

Day 6 (1 day post infection):

19. ~24 hours after previous transduction, remove virus-containing media and replace with fresh media.
20. If you are interested in early proliferation during reprogramming, perform *CellTrace Staining* (page **??**) on ths day.

Days 7 - 18: Media changes

Day 7 (2 days post infection):

No action required.

Day 8 (3 days post infection):

21. Media change plates to N3 media:
    i. N3 media = N3 base + BDNF/CNTF/GDNF (1,000X, 10 μg/mL) + FGF (10,000X, 100 μg/mL)
    ii. Spike in 1,000X RepSox to N3 media for desired experimental conditions.

Day 9 (4 days post-infection):

Assays for early indicators of reprogramming potnetial are performed on this day.

22. If assaying early indicators of reprogramming potential, the following assays are typically performed on this date.

1. Flow cytometry quantification of CellTrace dilution.
2. Nascent transcription rate via *EU incorporation* (page **??**).
3. Protein expression via antibody staining *antibody staining* (page **??**)
4. And many others...

If assaying actual reprogramming rates, no action required on this day.

Days 10 (5 dpi), 12, ..,. 18 (13 dpi):

23. Change N3 media every 2 days (can do 3 days if after ~8 days and weekend but 2 is ideal) until done (usually 14 dpi).

Note

After 8 dpi, it is recommended to dissociate with (*DNAse/Papain* (page **??**)) instead of trypsin

Day 19 (14 dpi): Assay reprogramming rate

24. Assay reprogramming rate via microscopy or flow cytometry.

Hb9::GFP iMN sort

This protocol outlines how to obtain pure reprogrammed Hb9::GFP iMN cultures by FACS.

See here for the *complete reprogramming protocol* (page **??**).

Note

Plate coating and general practices for iMN culture could still be improved, pure iMN cultures are very prone to lift off so be extremely careful at all steps.

Prep plates (2 hours):

1. Coat plate with *gelatin* (page **??**)
2. Coat plate with *laminin-521* (page **??**) - takes 2 hours!!

Sort and replate cells:

3. Sort cells into N3 + pen/strep
4. Replate cells at 40k/96-well

Note

Increase sort purity (e.g. 4-way purity). If you're sorting Hb9::GFP negative cells don't do at the same time and there can be contamination.

Note on cell density - MEA recommended as high as 200-300k cells/cm^2 (62-96k/96-well) - O'Shea lab does 30-80k/96-well depending on cell proliferation rate

Culture replated iMNs:

5. Perform pre-warmed half media changes every other day and keep pen/strep in

Tip

- Only do half media changes
- Pre-warm media before doing media change
- Confluency seems like a big factor. If iMNs are not confluent, they will be extremely prone to washing off.

3.5.4 TC Basics

TC Best Practices

When handling mammalian cells (aka tissue culture) always work in the Biosafety cabinet (BSC). BSCs are located in 66-225B (the main TC room) and in 66-219 (for quarantined cells). Best practices are outlined below.

General Guidelines

- All TC room equipment (e.g., tube racks, timers, 70% ethanol bottles, gloves) should stay in the TC room. Also, outside equipment should not be brought into the TC room unless absolutely necessary — spray these with 70% Ethanol.
- Do not wear your TC coat into the bacteria area. The white coats should be primarily limited to use in the TC, the exception would be getting media from the fridge (Oaken).
- Discard gloves used for bacteria work before entering the TC room.

Getting started in the Tissue Culture (TC) Room

1. Wash your hands.
2. Don a fresh pair of gloves before touching things in the tissue culture room.
3. If you have long hair, it is recommended to pull it back before starting TC work.
4. Find your lab coat which should be singly hung on a hook (not stacked).
5. Spray your gloves with 70% ethanol before opening the incubators or hoods.

Getting into a biosafety cabinet (BSC)

1. Pull up the sash to the height indicated on the hood (the hood will beep if the height is incorrect). Spray down the surface with 70% ethanol to ensure a clean space.
2. Check the stocks of serological pipettes, pipette tips, and resupply anything that is low.
3. Check the waste bottle. It should be clear or slightly yellow. If it is pink, add bleach.

   If the bleach is above the taped line (~70% full):

   - Replace the old bottle with a new bottle (there are extras under the sink). Add some (~100 mL) bleach to the new bottle.
   - Add bleach to the old bottle if necessary, and wait at least 20 min for the waste to be decontaminated. Then pour the liquid down the sink and rinse out the bottle.
4. Optional: For ease of cleaning, place a clear trash bag or other disposable bag in the benchtop waste container. A trash bag is required for all BSL2+ work.

Note

- If you need to bring any extra materials (clean pipettes, pipette aids, markers, tube racks, etc) into the BSC, always spray down and clean with 70% ethanol.
- Ensure the blowers and vents are functional (laminar air flow running) by waiting for the BSC to say it is on. If the BSC alarm is going off the sash may be at the wrong height and must be adjusted.
- Only bring items you need into the BSC. Having too many items in the BSC interrupts the laminar air flow and can cause contaminants to be introduced.
- Do not place items on the BSC grill; this creates a gap in the air laminar flow.

5. Check the media and cells before using the BSC — turbid media usually means that the culture is contaminated. If contamination is suspected, follow *the guidelines below* (page **??**).
6. Bring any cells and reagents you are working with into the BSC. Spray down outside of all bottles and tissue culture plates with 70% ethanol, but be careful that no ethanol gets into the filter cap of T75 and T185 flasks.

> **Note**
>
> Plates coming directly from an incubator into a BSC do not need to be sprayed with ethanol.

While in the BSC

- The BSC is ordered from clean (left) to dirty (right) with clean tips and pipettes at far left and the dirty the waste container at the far right. Order things appropriately for the work you are doing.
- Use ethanol and Kim wipes to clean up small spills as soon as they happen. Wipe the bottom of plates before putting back into the incubators to limit liquid-mediated contamination.
- Always keep reagent bottles, tubes and culture dishes/flasks closed when not in use.
- Do not work above or over open bottles/tubes/cultures/etc. Open containers should be put far away from you such that no work is done above or over them.
- Be sure you are working INSIDE the BSC, a minimum 3 inches from the grill (not on or above the grill).
- Do not use your phone or computer while handling cells—this leads to contamination!
- Do not block the air in-take vents at the front and back of the BSC or the air flow will be disrupted. (For instance, do not make a wall of tip boxes at the back of the BSC.)
- Do not discard plates or tubes with media in them. Aspirate all liquids before putting plastics in the biohazard waste.

Finishing up work in the BSC

Important

These instructions are for normal BSL2 work only. After working with virus or BSL2+ materials, follow the *Virus BSC Cleanup* (page **??**) instructions.

1. Put away all reagents, cultures, and other materials you brought into the BSC.
2. Discard trash in the biohazard waste bin. If not using a trash bag, spray the benchtop biowaste container with 70% ethanol and wipe clean before placing it back in the BSC.
3. In order to minimize contamination and cross contamination, run dilute (10%) bleach through the aspirator tip after use.
4. Spray the surface inside the BSC with Pre-Empt, wait one minute for decontamination, then wipe down the surface with 70% ethanol. Pre-empt can be sticky so "rinsing" with ethanol can help remove residue that accumulates over time.

Important

We use Pre-Empt after all virus work, and should otherwise be used at least once daily by the last BSC user of the day.

1. Pull the sash down and allow the UV light to run for one cycle (minimum 15 min).
2. Check the liquid biowaste collection bottle. If contents looks pink, add more bleach.
3. Discard gloves in biohazard waste containers (NOT the regular trash can).
4. Remove lab coat.
5. Wash hands.

If your cell culture is contaminated

Media that appears cloudy/turbid or the clear presence of fungal growth (e.g. fuzzy/fluffy white ball) indicates the culture is likely contaminated. If contamination is suspected:

1. Immediately bleach the culture for at least 20 min **outside of a BSC** (we don't want to bring contamination into the BSC!). Pour the liquid down the sink.
2. Discard the flask/dish/plate in a biohazard waste bin (step can) and close and change the bin. Change your gloves immediately.
3. Decontaminate any surfaces that the contaminated culture came into contact with using bleach or 70% ethanol.
4. Clean any surfaces in the incubator where the contaminated culture was stored. (Do not directly spray into the incubator; instead wet a Kimwipe with 70% ethanol and clean the surface.)

5. Check the media bottle you used for the contaminated culture—if suspicious, bleach and discard the media.
6. Change gloves again and continue working with the rest of your cultures.

Adherent Cell Culture

General culture handling procedure

- Lift lid and tilt plate with one hand while aspirating/pipetting with the other.
- The lid should cover the plate as much as possible, providing just enough opening to perform the task
- The lid opening should face away from the hood opening (i.e., away from you and toward the back wall).
- Tilt the plate toward the back when aspirating.
- When aspirating, immerse the pipet tip in the media but don't touch the plate bottom until the very end to avoid aspirating away cells.
- Tilt media gently against the side wall of the dish to avoid dislodging cells.

Changing media

Unless you have very specific experimental conditions, it is good practice to check on your cultures every 2 or 3 days and change media if the cells are not ready to be split or expanded.

Recommended media volumes are listed below.

Culture dish/plate/flask	Approx. volume media
10-cm	10 mL
15-cm	15 mL
6-well	2 mL (1-3 mL)
12-well	1 mL (1-2 mL)
24-well	500 μL (500-1,000 μL)
48-well	250 μL (200-400 μL)
96-well	100 μL (100-200 μL)
T-75	10 mL (8-15 mL)
T-182	25 mL (20-25 mL)

Source: General culture volumes[199]

[199] https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html

Passaging cells

Passage number is an "indicator" of cell age. Every time the cells are detached from one dish and passaged into another dish, giving them room to divide/expand, the passage number increases by 1.

1. Aspirate away old media.
2. Wash with PBS (*optional*)
    i. Gently add PBS to the side of the dish/plate, not directly on the cells
    ii. Aspirate away PBS

Note

Washing with PBS may make it easier to detach cells during trypsin step

Note

Some types of cells do not require trypsin to detach (e.g. HEK293Ts, Plat-Es). In that case, just pipette up and down until cells detach. This looks like a thin film disappearing from the bottom of the dish/plate/flask.

3. Add 0.05% trypsin to detach cells from the plate.

    Note

    We buy 0.25% trypsin, so dilute it to the appropriate concentration in PBS. DO NOT DILUTE WITH ANYTHING WITH FBS! Using a higher concentration of trypsin will make the cells dislodge faster/easier, but can be more toxic.

    Trypsin should cover the entire surface area; this is typically half the media volume:

Culture dish/plate/flask	Approx. volume 0.05% Trypsin
10-cm	1 mL
15-cm	3 mL
6-well	500 μL/well
12-well	300 μL/well
24-well	150 μL/well
96-well	50 μL/well
T-75	5 mL
T-182	15 mL

4. Incubate at room temperature or in 37°C incubator for 3-4 min, checking cells to avoid over-digestion.

- Detached cells look rounded and refractile under the microscope, and by eye the media might look cloudy as it fills with cells.
- Different types of cells will require different incubation periods.
- The stronger the trypsin (higher %) the faster cells will detach.
- To help physically detach the cells from the plate, pipet trypsin along the sides to "wash" the cells or tap the side of the flask.

5. Neutralize with at least 2 volumes of media that contains FBS (e.g. neutralize 1 mL 0.05% trypsin with at least 2 mL FBS-containing media).
6. Mix well by pipetting and make sure that all cells are detached. Transfer to a 15 mL or 50 mL conical.
7. Centrifuge at 400g for 4 minutes. Cells should pellet at the bottom of the conical.
8. Aspirate all media/trypsin, carefully avoiding aspirating the cell pellet.
9. Resuspend pellet in 1-10 mL of media (volume is your choice).
10. If needed, *count the cells* (page **??**) to determine the proper dilution for passaging.
11. Seed new dish/flask with desired number or dilution of cells: mix the appropriate volume of resuspended cells with fresh media and add to a new flask/dish.

> **Tip**
>
> Make sure the cells are resuspended well before aliquoting to seed the new dish. Cells tend to settle quickly!

- General dilution guidelines will vary by cell type, but typical numbers for confluent HEK293T cells (doubling time ~24 hrs) are as follows:

Dilution	Confluent at day
1:2	1 (next day)
1:4	2
1:8	3

- For instance, cells from a T75 flask resuspended in 1 mL of media can be passaged 1:4 by adding 250 μL cells and 10 mL media to a new T75 flask.
- Passaging will generate several new flasks/dishes of cells; if only one flask/dish is desired, the extra cells can be *cryopreserved* (page **??**).

12. Cultures should be labeled with cell line name, date, passage number and initials of the owner.
    - Certain cell lines should also include the passage number of antibiotic selection, e.g. "Plat-E, Blast/Puro @ P12"

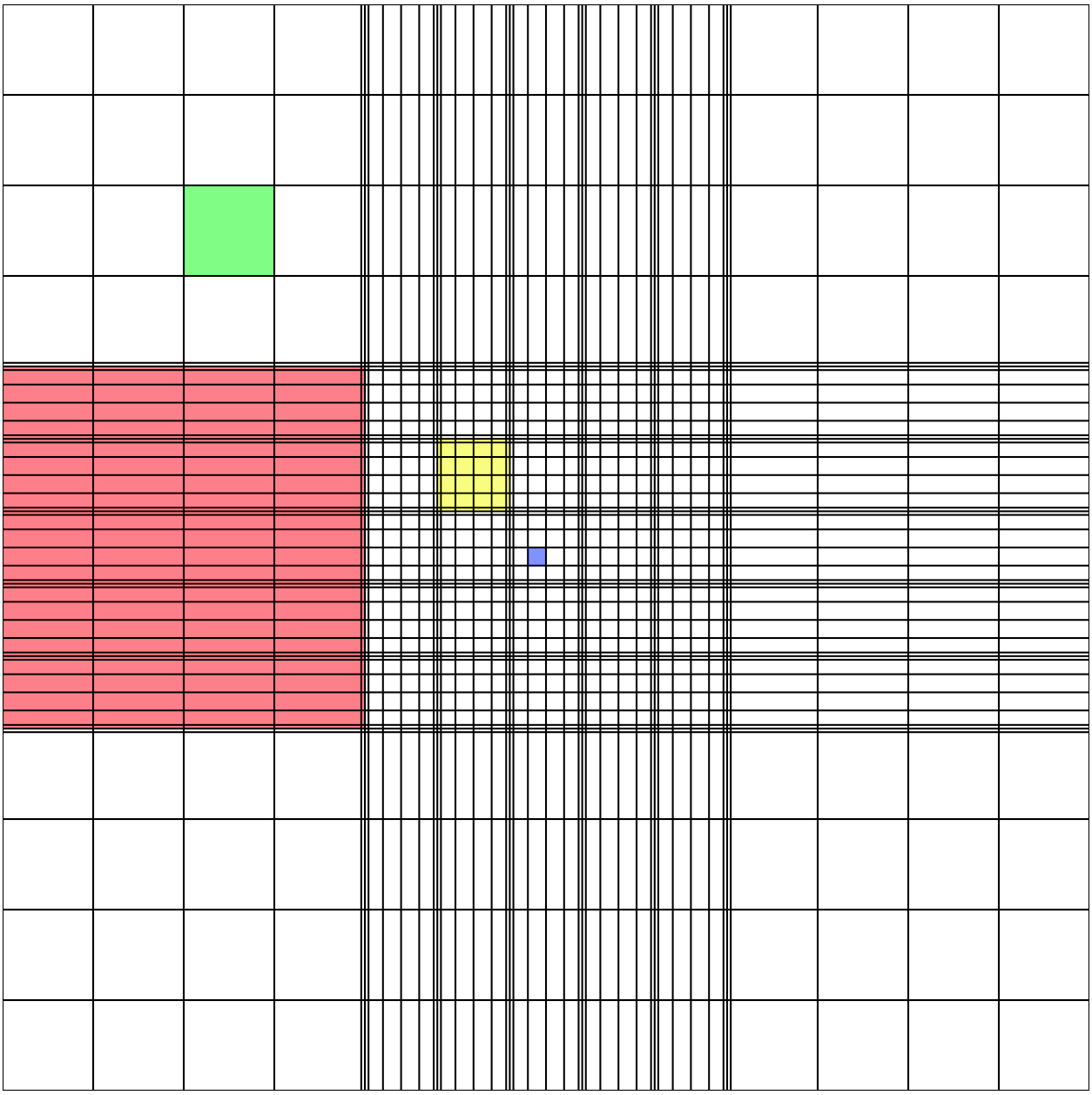
Note

Don't forget to increase the passage number by 1 on the new flask/dish!

Counting cells

1. Start with cells in suspension, i.e. dissociated from culture flask/dish and resuspended in a low volume (1-5 mL) of fresh media (see *Passaging cells* (page **??**) steps 1-9).
2. Dilute the cells as needed, based on a rough estimate of cell count.

   For instance, a typical dilution for a T75 flask of confluent HEK293T cells: resuspend in 2 mL media, then further dilute 1:10 by adding 10 μL cells to 90 μL media.
3. Prepare the hemocytometer by placing the glass coverslip on top of the chamber.
4. Pipet 10 μL diluted cells into the notch on the chamber, such that the liquid is held between the metal and the coverslip.
5. Under the microscope, count the number of cells in one of the marked regions on the hemocytometer, and calculate the cell concentration according to the volume of that region:



Dimensions	Area	Volume at 0.1 mm depth
1 x 1 mm	1 mm^2	100 nL
0.25 x 0.25 mm	0.0625 mm^2	6.25 nL
0.25 x 0.20 mm	0.05 mm^2	5 nL
0.20 x 0.20 mm	0.04 mm^2	4 nL
0.05 x 0.05 mm	0.0025 mm^2	0.25 nL

$$\text{concentration of cells in original mixture} = \left(\frac{\text{number of cells counted}}{(\text{proportion of chamber counted})(\text{volume of squares counted})}\right)\left(\frac{\text{volume of diluted sample}}{\text{volume of original mixture in sample}}\right)$$

$$\text{cells/mL} = \left(\frac{(\text{number of cells counted})(\text{dilution factor})}{(\text{number of large squares counted})}\right) \times 10000$$

Image source: https://en.wikipedia.org/wiki/Hemocytometer

For instance, for cells diluted 1:10, several regions the size of the red region above could be counted and averaged, then this number would be multiplied by 1e5 to obtain the concentration in cells/mL of the original suspension.

Avg # cells counted / 100 nL * 1e9 nL / 1e3 mL * 10x dilution = **Avg # cells counted** * 1e5 /mL

Note

Counting more cells—by diluting the cell sample less, counting a larger region of the hemocytometer, or averaging the counts of several regions of the same size—will increase the accuracy of the concentration calculation.

Seeding/Plating cells

Note

If applicable, *gelatin coat* (page **??**) new plates before beginning so the gelatin has time to sit. Plates should be gelatin-coated for culturing MEF cells and for protocols such as virus production.

1. Start with cells suspended in a low volume (e.g. 1-5 mL) of fresh media (see *Passaging cells* (page **??**) steps 1-9).

2. *Count the cells* (page **??**).
3. Mix cells with the appropriate volume of fresh media according to your experimental guidelines. The media volume should be the total media volume for all wells/dishes.

   Generally, for 80-90% confluent cells the next day:

Cell Type	Well Size	# Cells
MEF	6-well	100-160K
MEF	96-well	5-10K
HEK293T	10cm	750K
HEK293T	96-well	20-40K
Plat-E	6-well	800K

   For example, to seed a full 96-well plate of MEFs at 10k (1e4) cells/well: mix 1e6 cells (1e4 cells/well * 100 wells) with fresh media to a total volume of 10 mL (100 μL/well * 100 wells).
4. Pipet cells into new flask/dish or wells of the new plate. This should account for the entire flask/dish/well volume (e.g. 10 mL into a T75 flask, 100 μL into each well of a 96-well plate).
5. Any excess cells can be *passaged* (page **??**) to a new flask or *cryopreserved* (page **??**).

Gelatin Coating

Materials

- 0.1% Gelatin (sterile)

Protocol

1. Add enough gelatin to coat the bottom of the flask or well (approximately half of the media volume needed for the well).
2. Let sit for 10 min at room temperature.
3. Aspirate excess gelatin. Use for plating cells within 1-2 hrs maximum, otherwise gelatin may dry out.

Maintaining Integrated Cell Lines made with a selection marker in culture

To increase the stability of integrated cell lines (ie., Plat-Es, T-Rex 293 line, PiggyBac/CRISPR-edited lines), it is imperative to adhere to TC best practices, routinely passaging cell lines every 2-3 days. It is advisable to culture cells in a reduced (but non-zero) concentration of antibiotic used for the original selection *see Mirus guidelines on cell line creation <https://www.mirusbio.com/applications-stable-cell-line-generation/>_*.

For CRISPR/PiggyBac cell lines integrated with Puromycin, culturing the cells in 0.5 ug/mL Puromycin every 3 passages should increase the retention of the transgene on the genome and perhaps attenuate transgene silencing.

For maintenance of Plat-Es (Deon doesn't know what the lab typically does for Plat-Es)

For maintenance of the T-REx™ Cell Line, Thermo recommends culturing the cells in 100ug/mL zeocin (1:1000 dilution master stock) and 5ug/mL blasticidin (1:2000 dilution master stock). The T-REx Line stably expresses the tetracycline repressor protein. They save significant time and effort when using the T-REx™ System. The T-REx™ Cell Lines are functionally tested by transient transfection with the positive control vector pcDNA™4/TO/lacZ. T-REx™ Cell Lines exhibit extremely low basal expression levels in the repressed state and high expression upon induction with tetracycline (or doxycycline). For documentation of general (not Flp-in version) see *here <https://www.thermofisher.com/order/catalog/product/R71007#:~:text=T%2DREx%E2%84%A2%20Cell%20Line* For documentation of the Flp-in system see *here <https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fflpinsystem_man.pdf>*.

Freezing and thawing cells

Freezing cells

1. Make cryopreservation ("freezing") Media (should be fresh-ish, try not to use longer than 1 month)
    - Glycerol should be used if the cell type to be cryopreserved may be adversely or unwantedly affected by exposure to strong bi-polar compounds such as DMSO.
    - Important: The cryopreservatives, especially DMSO, must be used fresh each time. If you buy large volumes, transfer a small amount to another tube, wrap in aluminum foil (DMSO is light sensitive) and use the aliquot. Minimize opening and closing the reagent bottle. This is to avoid oxidization and/or absorbtion potentially toxic materials from the air.

Materials	Percent	Purpose
FBS	>20%	FBS binds toxic materials released if some cells are lysed during the freezing or thawing process.
DMSO or sterile glycerol	10%	Cryopreservative, prevents ice formation which destroys cells

1. Trypsinize, centrifuge, and resuspend cells in growth media with FBS
    - Typically resuspend in 1-3 mL for a T75 or T185.
    - *Optional*: count cells with a hemocytometer to know how many cells are frozen per cryovial.
2. Add cell suspension and freezing media to cryovial in a 1:1 ratio (typically, 0.5mL resuspended cells, 0.5 mL freezing media)

Tip

For sensitive cells like MEFs, resuspend in FBS so that final conc. is 90% FBS and 10% DMSO

4. Label cryovial with name, date, passage number (same as the trypsinized cells), and approximate number of cells (either from hemocytometer or fraction of confluent flask (i.e. 1/6th T185))
5. Freeze cryovial at -80°C inside a styrofoam container. This will allow slow freezing as to not incur cell damage.
6. After approximately 24 hours transfer cryovial to liquid nitrogen.

Plating Cells from Frozen Stock

See also *seeding and plating cells* (page **??**)

Materials

- Frozen vial of cells (i.e MEFs or HEK293Ts)
- Growth Media (ex. DMEM with 10% FBS)

1. Rususpend contents of frozen vial with 10-20 mL of media and spin down at 400G for 4 minutes.
2. Aspirate media to remove DMSO, careful to not aspirate the cell pellet.
3. Resuspend cell pellet in ~1 mL of growth media.
4. Use 10 μL to count with the hemocytometer.
    - Number of cells in one corner * 10,000 = # of cells / mL
5. Coat T75 with 0.1% gelatin (required for MEFs, optional for HEK293Ts)
    - Add 5-7 mL of sterilized 0.1% gelatin to T75.
    - Put T75 sideways and let sit at room temperature for minimum 10 minutes.
    - Right before use, aspirate gelatin liquid.
6. Plate onto a T75 with 10 mL of growth media. Label flask with your initials, the date, cell type, and passage number (+1 the passage number label on the cryovial)
7. Incubate at 37°C. These cells will take 1-2 days to recover.

Flow Cytometry

Preparing in a 96-well plate

Note

It is convenient to prep cells for flow cytometry in a 96-well plate because you can add media and do mixing with a multichannel pipette.

Important

Make sure you have at least 2 autoclaved tips per well (1 for triturating, 1 for transferring to U-bottom). For a full 96-well, that means at least 2x200 μL boxes. Plus more for PBS washes and adding in dissociation media!

1. Wash, dissociate, and triturate cells in plate (swap out tips every time for the trituration).
    - You don't need to swap tips if you hover above the plate while adding in wash and dissociation buffer but you **must swap tips for trituration** (i.e. everytime you put the tip into the well)
2. Take the plate out of the BSC (**the plate is no longer sterile at this point**).
3. *Optional* Transfer contents of (flat bottom) plate to a 96-well U-bottom or V-bottom plate
    - A U-bottom plate is not necessary, but cells pellet better on U- or V-bottom plates, and lose less volume running flow.
4. Spin the plate at 400 rcf (g) for 5 min using the plate bucket rotor.
    - Balance with another 96-well plate filled with roughly equivalent amount of water (there's a balance plate near the centrifuge) but balancing doesn't have to be exact at this low speed
5. Aspirate supernatant.
    - Tilting the plate and aspirating from corner helps to avoid disturbing the cell pellet. Do NOT aspirate straight from the middle of well.
6. If performing any staining (e.g. Live/Dead, surface markers, intracellular, etc.), skip step 7 and proceed to *Cell staining for flow* (page **??**).
7. Resuspend in ~200 μL PBS and pipet up and down to mix well (must be single-cell suspension—no clumps!).
    - Swap tips in between each transfer to avoid contamination between wells!
    - If using flat-bottoms, decrease the Attune uptake volume settings so you don't create bubbles (i.e. you will lose more volume with flat-bottom wells)
8. Load plate into the Attune CytKick autosampler and run.

Glass slide cell culture

Warning

NBW hasn't gotten this to work well yet, the DDRR makes MEF move off of them. I would use glass-bottom plates or try a diff coating than gelatin like laminin.

Cleaning Protocol *(optional)*

If cells are not attaching to glass coverslip try cleaning them. The commercially precleaned ones should not have to be treated like this.

1. Incubate coverslips overnight in 1N HCl on orbital shaker
2. Rinse in water at least 3 times
3. Wrap in foil and autoclave on dry cycle (30 min sterilization time)

Coating glass coverslip

1. Ethanol glass coverslip sleeve and tweezers, and bring into hood
2. Carefully place glass coverslip into suitable TC plate, being careful to not touch it with your glove and such that it lays flat
    - It will be difficult to make sure you're only grabbing a single coverslip
    - A 12 mm round cover glass coverslip should fit into a 24-well plate (16.2 mm ID)
3. Add enough *0.1% gelatin* (page **??**) to coat coverslip, incubate 10 min at RT
4. Aspirate excess gelatin and seed cells

Mounting glass coverslip

1. Carefully place a small drop (~10 μL) of mounting solution in the middle of the glass slide where the coverslip will be placed
    - How much mounting solution depends on glass coverslip size, you want enough to cover the whole thing
2. Carefully use tweezers to remove glass coverslip
    - It will be **difficult** to grab it without scraping cells off
    - Try to tilt plate one way and lift the coverslip with tweezer
    - Once the slidcoverslipe lifts a bit, **carefully** push the tweezer a little to shimmy it under the coverslip
    - Softly squeeze the tweezers to grip the coverslip
3. Place coverslip onto the glass slide such that the cells face down and will be submerged in the mounting solution

Important

Avoid bubbles in the mounting solution! It can help to set one edge of the slip into the mounting solution, then slowly set the rest of it down such that the act of putting the coverslip down will push the mounting solution towards the edges.

4. Let mounting solution set overnight in the dark before moving to a glass slide organizing case

Tip

When mounting, aspirate some of the media off but leave some liquid to help detach the glass slide from the bottom otherwise it will tend to stick to the bottom.

Culturing Jurkat Cells

Jurkat cells grow in suspension. Many of the techniques parallel *culturing adherent cells* (page **??**). The notable differents are described here.

Media

RPMI 1640, 10% FBS .. note:: Optional: 1X NEAA (not used in our lab.)

Culturing

Culture cells in suspension with media. Cells can be cultured in T75 or T182 in the 37C incubator. Rocking in not neccesary for cell growth.

Passaging & Media Changes

Spin down cells from suspension for 4 minutes at 400 G. Resuspend cells in RPMI 1640 (10% FBS). Replace cells into a culture flask at the desired passaging ratio.

Cryopreservation

Resuspended cells after centrifugation can be cryopreserved and thawed as described under *cryopreservation and thawing* (page **??**).

Transient HEK293T Transfection

Note

If performing transient transfection for virus production: please use the updated *Virus production in HEK293T cells* (page **??**) protocol for lentivirus production via HEK293Ts, and the *Plat-E Retroviral Production* (page **??**) protocol for Plat-E retrovirus production.

If performing transient transfection for an experiment, continue to the protocol below.

Calculations

For all calculations you can refer to the most updated transfection template in the shared projects folder[200] of the Galloway Lab sharepoint, example here.

To scale per well amounts according to plate size, divide by number of wells with the total amount per entire plate constant (e.g., 1 10cm-dish = entire 6-well plate = entire 96-well plate). For instance, each well in a 6-well plate would use $\frac{1}{6}$ the per-plate DNA and knockout DMEM amounts.

For conditions with multiple wells, make 110% of the calculated amount to account for pipetting loss. Additionally, make 120% KO DMEM + PEI MM to ensure there is enough extra.

Knockout (KO) DMEM + PEI Master Mix (MM) Calculations

Overall parameters:

Parameter	Value
Titrated PEI ratio	Specific for each PEI batch (e.g., 5 ug PEI : 1 ug DNA). The PEI concentration itself is 1mg/mL.
DNA/plasmid/well	10.8 ug/plate
KO DMEM/plasmid/wel	1.33 mL/plate

Example MM calculation (make 120% for extra):

Value	Example in a 96-well plate
Number plasmids/well	2 plasmids (*example*)
Total wells	50 wells (*example*)
Titrated PEI ratio	5 ug PEI : 1 ug DNA (*example*)
KO DMEM/well	(1.33 mL/plate/plasmid) / (96 wells/plate) * (2 plasmids) = **27.7 uL/well**
PEI/well	(10.8 ug DNA/plate) / (96 wells/plate) * (5ug PEI / 1ug DNA) / (1 ug/uL PEI) = **0.56 uL/well**
Total KO DMEM (120%)	(27.7 uL/well) * (50 wells) * 1.2 = **1.663 mL**
Total PEI (120%)	(0.56 uL PEI/well) * (50 wells) * 1.2 = **33.75 uL**

Example condition mix calculation (make 110% for extra):

[200] https://mitprod.sharepoint.com/sites/GallowayLab/Shared%20Documents/Forms/AllItems.aspx?id=%2Fsites%2FGallowayLab%2FShared%20Documents%2Fprojects%2Fshared&viewid=a7b740fe%2D9cf6%2D4f23%2D823f%2D41f507975686

Value	Example
Plasmid concentration	100 ng/uL (*example*)
Wells/condition	3 wells (*example*)
Plasmid volume/condition (110%)	(10.8 ug/plate) / (96 wells/plate) / (0.100 ug/uL) * (3 wells) * 1.1 = **3.71 uL**
Master mix/condition (110%)	(~ 27.7 uL/well KO DMEM) * (3 wells) * 1.1 = **91.41 uL**

Protocol

1. Seed healthy 293T cells 1 day prior to transfection. Cells must be ~80% confluent at time of transfection for highest efficiency. Recommended cell counts are:

Cell Type	Well Size	# Cells/Well
HEK293T	96-well	25-40K
HEK293T	10-cm	7.5M
Plat-E	6-well	800K

2. Make a mastermix (MM) of PEI and Knockout DMEM according to the calculations *above* (page **??**).

Important

Ensure that you add PEI to KO DMEM, *not the other way around!* Mix master mix well, then let sit for minimum 10 minutes.

3. For each condition, combine the DNA and the PEI+KO DMEM MM according to the calculations, then mix and wait 10-15 minutes. These are the "condition mixes."
4. Gently add the calculated amount of condition mix to each desired well on **TOP** of the existing growth media using the 100% NOT the 110% volumes. For larger well (anything above 24-well), add the condition mix *dropwise* and evenly around the plate, rocking the plate back and forth, side to side to mix.
5. *After 18-24 hours* (1 day post transfection, dpt), aspirate and replace with *fresh media* (page **??**) (e.g., DMEM + 10% FBS for HEK293T). Typically, any small-molecule inducers are added at this step (e.g., doxycycline).
6. It is standard to image and flow cells at 2 dpt (1 day after fresh media change). If small-molecule inducers were added at 1 dpt, it is common to flow at 3 dpt to allow the expression levels to reach steady state.

3.5.5 Virus

Virus safety

The highest hazard that we have in lab is likely human-infectible lentiviruses. The lentiviruses we make are *replication-incompetent*, i.e. they do not make more virus after they infect, but they can still be dangerous for two reasons:

1. Insertional mutagenesis: Even a benign GFP is bad for you if it randomly inserts into a key gene and causes misregulation leading to cancer.
2. Oncogene expression: Expressing known oncogenes from your constructs can cause cancer. Known or suspected oncogenes made in human-infectible lentiviruses falls under BSL2+. Only graduate students and postdocs that have done the special BSL2+ training are allowed to work with these.

Other virus types are also potentially hazardous if they express oncogenes, even if they are non-integrating like AAVs. The following still applies in those cases!

Control methods

Elimination/Mitigation

The easiest way to not be exposed to a human-infectible virus is to not make viruses! There are other integration techniques, such as PiggyBac/transposon-insertion which may be suitable for your process.

Substitution

If you need to make virus, we can reduce the hazard by using more modern viral vector systems or by reducing tropism.

We do this in two ways. First, we can use third-generation lentiviral vectors (which separate viral genes across multiple plasmids *and* have self-inactivating LTRs) which makes it less likely that inserted DNA can be re-recognized via the LTRs. Second, you can use a different envelope plasmid. The VSVG envelope has broad tropism; it infects human cells, mouse cells, and so on. If you are only targeting mouse cells, you can make virus in PlatE cells, which have a different envelope that does not infect human cells.

Engineering controls

The key way that we separate virus particles from ourselves is by working with our equipment safely. There are three main ways we do this:

1. Only do work in our BSCs, and work with the sash at the right height.
2. Only spin down cells in the virus centrifuge, and use the centrifuge caps when spinning.
3. Do not use sharps (or *glass aspirator pipettes*) when working with virus. If you think it is necessary to use sharps or glass, make sure you have a safety discussion.

PPE

When working with virus, we use disposable sleeves and double gloves. The proper order to put on your PPE is first, your lab coat, then your first pair of gloves, then sleeves, then the second pair of gloves.

For BSL2 virus, you can reuse your sleeves through several days of a whole virus production, but **only** if you check for any droplets. Swap sleeves if you got virus on your gloves, as the sleeves may be contaminated. For any BSL2+ virus, dispose of sleeves upon completion of BSL2+ work each day.

Virus protocols

We have specific virus procedures in place, which change the way that we work in TC to protect us. You should treat virus-infected cells or media using virus protocols until **both** three days and two media changes have passed.

BSL2+ work requires additional protocols that will be highlighted at each step. Work involving human-infectible viruses, even if the viruses are only BSL2, benefits from these additional safety protocols.

We have specific protocols for before, during, and after using viruses in BSCs.

Virus BSC setup

Important

The **most important** rule to follow when doing virus work is to use **double gloves and sleeves** and **never take your hands out of the BSC** until you have removed your second layer of gloves. If you forget something and need to exit the BSC to grab it, do a glove change.

1. When starting up your BSC hood to do virus work, make sure that you have enough serological pipettes, tips, and other plastics. You want to make sure that you don't have to take your hands out of the BSC. Add them now if needed.
2. Make sure that you have all of the plates, tubes, filters and other things that you need. You may want to do non-virus work (e.g., gelatin-coating plates, aliquoting media) before you add any virus to your hood.
3. Make sure you have enough media (and other reagents) in the hood. You may want to aliquot out of your larger 500mL bottle.
4. Add the following extra items to the BSC:
    a. A secondary plastic bag to the benchtop waste container, to catch any liquid on tips or pipettes.
    b. (**BSL2+**) Plastic jar to fill with 10% bleach, for rinsing tips and serological pipettes.
    c. (**BSL2+**) Squirt bottle of Pre-Empt and 2-3 paper towels, for wiping down materials before they leave the BSC.

Plastic rinsing during viral work (BSL2+)

1. Fill the plastic jar with 25-100 mL of 10% bleach.
2. Plastics that contact virus-contaminated media should be bleached before disposal.
    - **Pipette tips**: After dispensing and before ejecting the pipette tip, depress the plunger on the pipette to the second stop and pull up bleach from the bleach jar. Then, eject the pipette tip into the bleach. This ensures all surfaces that contacted virus also contact bleach.
    - **Serological pipettes**: After dispensing, pull up bleach from the bleach jar up to or beyond the level that contained virus previously. For example, if 9 mL of virus-containing liquid was previously measured and dispensed, pull up at least 9 mL of bleach. Bleach can be immediately dispensed and the serological pipette can be disposed.
    - **Empty conicals or Eppendorf tubes**: Fill with enough 10% bleach to ensure all surfaces that contacted virus are covered. Rotating the tube to ensure all surfaces are contacted by bleach is sufficient; tubes do not have to be completely filled with bleach.

Virus BSC cleanup

1. For any virus that is going to be removed from the BSC for use at a later time, transfer the virus to a screw-top tube if it is not already in one.
2. For any liquid waste you have not aspirated (e.g., 10cm dishes you are done with), use a serological pipette to add bleach to any waste plates/containers/tubes.
3. Spread the waste out slightly so all waste will get UV'd.
4. (**BSL2+**) Soak a paper towel with Pre-Empt and wipe down the exterior of any materials that are to be removed from the BSC and stored (e.g., media, concentrated virus, plates and dishes with cells).
5. (**BSL2+**) With a Pre-Empt soaked paper towel, wipe down any pipettes, tip boxes, aspirator, or any other equipment that you may have touched while working with virus-containing media.
6. Remove your second pair of gloves, putting them in the benchtop biowaste container.
7. Remove any non-waste cells, collected virus, small molecules, etc. from the hood (you may put on a fresh second pair of gloves, but the outside of these tubes should be decontaminated by Pre-Empt in Step 4).
8. Leaving all trash inside, close the sash on the BSC. Set a fifteen minute timer!
9. Fifteen minutes later, with full PPE (double gloves and sleeves, only required for **BSL2+**), open the hood again. Aspirate bleached liquid waste, finish with another bleach rinse of the aspirator, and collect all trash. Take the trash bag out and throw it in the biowaste containers.
10. Spray Pre-Empt on the surface of the BSC. Wait one minute or longer, and then wipe up the Pre-Empt by spraying 70% ethanol on the surface. Once the surface is looking nice, close the hood.
11. The hood is usable after the second UV cycle!

Exposure plan

Our PPE and processes mean that it should not be possible to get virus-containing droplets on your skin without needlesticking yourself, and it should not be possible to needlestick yourself if you do not use glass or sharps when working with virus.

However, it is still possible that an exposure happens, for instance if you have a pre-existing cut on your hand. Simply having virus-containing media on unbroken skin is not a large concern, though you can and should discuss this with a doctor if the virus was BSL2+ or if you are otherwise worried.

The main treatment is a 28-day course of HIV post-exposure prophylaxis drugs that interfere with lentivirus retrotranscription, among other things. This is a prescription drug, so you need to go to urgent care / somewhere else to get it prescribed, then get it from a pharmacy.

Lentiviruses are generally poor at infecting humans, even with direct puncture wounds (<20% of needlestick incidents directly with HIV lead to infections). The post-exposure drugs are *very effective* if started within 3 days (ideally within 3-6 hours), dropping this number to effectively 0%.

If you believe that you have been exposed, you should:

1. Make sure the BSC is in a safe state, e.g. nothing precariously perched on something else.
2. Wash your hands and/or the exposed area of skin for ten to fifteen minutes with soap and water.
3. If possible while washing your hands, inform someone else in lab to contact:
    a. the EHS rep, Katie, or department EHS Coordinator[201] (Brian Smith), to kick off the paperwork side of things. Do not worry about this paperwork now if you can't reach these people, it's not your job to deal with it.
    b. anyone, to contact MIT Medical urgent care (617-253-1311) if during business hours (8am-8pm M-F, 10am-4pm on weekends)
    c. if outside of business hours, any other urgent care that is open (or ER if everywhere is closed).
4. Tell the doctor that you were exposed to an HIV-derived lentiviral vector, and how you were exposed. If the virus was BSL2+, tell them that the vector encodes an oncogene. Any prescriptions should be covered under the base insurance that all students get.
5. Follow up with other people in lab to handle the paperwork filing, finishing cleaning out the BSC, and so on.

Lessons learned from incidents

Spilled virus incident

While a former member of the lab was filtering a human-infectible lentivirus encoding a fluorescent protein, too much pressure was applied on the syringe. Their hold on the syringe slipped, and knocked over the tube containing the filtered lentivirus. Most of the lentivirus-containing media

[201] https://cheme.mit.edu/people/staff/

stayed within the BSC, though some was knocked onto the person's lab coat, around the mid-chest area.

Response: The person carefully removed their PPE, washed their hands, and asked other people in lab for advice. Due to good PPE use and protocols, all virus-containing media ended up only on PPE, and the person decided that medical attention was not needed.

Katie filled out the incident paperwork. The lab coat was soaked in bleach and Pre-Empt and sent out for laundering after being decontaminated in lab.

Lessons learned: When filtering virus, if the filter gets too clogged with cells or other debris, it can be hard to apply enough pressure to filter the virus. If this happens, you can carefully transfer the remaining virus to filter back into a separate tube, and use a second syringe to complete the filtration. Do not apply more force than you can control.

Virus production in HEK293T cells

This protocol describes how to produce lentivirus and retrovirus in HEK293T cells, including an optional final concentration step.

Estimated time

4-6 days. Note that on the transfection day, you must change the media 6-8 hours after the transfection.

Important

We use precautions beyond those for normal BL2 tissue culture when producing human-infectible virus. Read over the *Virus safety* (page **??**) protocol before beginning.

Setup

The plasmids and exact amounts to use at each step vary based on the virus type (lentivirus or retrovirus) and scale. We mostly make lentivirus in HEK293T cells, though we can also produce retrovirus for transduction of human cells (e.g., for human cell reprogramming). The protocol below is written for a default 10cm-dish scale (i.e., one virus per dish), which is recommended. It seems that virus production is less efficient in a 6-well plate, so smaller production scales are not usually effective, especially if you'll be titering the virus.

Check out this spreadsheet as a template for the transfection calculations. Note that the "transfer plasmid" refers to the plasmid containing the lentiviral backbone with your cargo between viral LTRs. You'll also need to co-transfect separate packaging and envelope plasmids specific to the virus type.

Scale:

Scale	Area (cm^2)	Seeding amount (# cells)	Total DNA mass (µg)	Collection media volume (mL)	Concentrated volume (μL)
10cm	56.7	6.0 x 10^6 *	24	6.5	200
6-well	9.6	1.0 x 10^6	4.1	1.25	40

If using a different virus production scale, the total DNA mass should be scaled by surface area. The collection media volume should be roughly half of the standard amount of media used for that scale.

Note

* Lab members have successfully used seeding densities between 6 and 7.5 million cells per 10cm dish. We have not directly assessed how this variable impacts viral titer, though it is important to have an appropriate cell confluency for transfection the next day. For consistent titers across batches, use the same seeding density. Always ensure your cells are growing healthily before seeding, as they will be under a lot of stress during the production process.

Virus type:

Type	Packaging plasmid	Envelope plasmid
Lentivirus	psPAX2 (pKG0362, Addgene #12260)	pMD2.G (pKG0096, Addgene #12259)
Retrovirus	pIK-MLVgp (pKG0015)	pHDMG (pKG0022, Addgene #164440)

Typically, we prep common stocks of the packaging and envelope plasmids (TC aliquots box in Bruni 4ºC, additional aliquots in common box in Olaf -20ºC). These are maxipreps normalized to 500 ng/μL, but double-check the concentrations on the tubes to be sure. We sequence-confirm each prep of these plasmids to make sure they are high quality.

Virus Production

The following is given for a 10cm-dish production scale. For other scales, substitute the correct values from the scale table.

Day 0

1. Seed HEK293T cells onto gelatin-coated 10cm dishes. (For other scales, use the correct seeding amount above.)

Note

In theory, any HEK293T cells can be used to produce virus. In lab, we have the Lenti-X 293T line (Takara[202]) which is a particular HEK293T clone shown to produce high-titer lentivirus.

However, in our hands, there is not much difference between these Lenti-X and regular HEK293T cells—so either cell line can be used effectively.

Tip

If making many plates of virus, you'll need a lot of cells. A good rule of thumb is to culture one T182 flask of cells for every ~6-8 10cm dishes you need. A very confluent T182 can yield 60 million cells, which is enough for 8 x 10cm dishes (7.5 million cells per dish).

Day 1

Co-transfect your cells with transfer, packing, and envelope plasmids.

1. Prepare a mastermix of PEI and knockout DMEM. It is helpful to prepare a 110% master mix (include 10% extra mix) to account for pipetting losses. Ensure that you **add the PEI to the KO DMEM** and not in the other order.
    - As included in the transfection calculation spreadsheet, we use 1.33 mL of KO DMEM per 10cm dish (though the transfection seems relatively insensitive to this ratio).
    - The amount of PEI is set by the mass ratio of PEI:DNA experimentally determined for each batch, as for any PEI transfection. We use 24 μg total of DNA per dish.
2. Incubate the PEI-KO DMEM mastermix for 10-15 minutes at room temperature. While waiting, prepare the DNA mixes (next step).
3. Combine the transfer, packaging, and envelope plasmid DNA for each condition in the following ratios.

Scale	Transfer	Packaging	Envelope
Ratio	1	1	2
10cm	6 μg	6 μg	12 μg
6-well	1.02 μg	1.02 μg	2.05 μg

    - Use the calculation spreadsheet to determine appropriate volumes.
    - If you are transfecting multiple dishes per virus, consider mixing 10% extra of the final condition mix to account for pipetting loss when adding to the dishes.
    - When transfecting multiple virues, it can be helpful to create a mastermix of the packaging and envelope plasmids to reduce pipetting. Add these to the empty condition mix tubes first, then add the transfer plasmid DNA.
    - It it important to **mix all the DNA together** before adding the PEI-KO DMEM mastermix in the next step. That way, the transfection complexes contain equal ratios of all the

[202] https://www.takarabio.com/products/gene-function/viral-transduction/lentivirus/packaging-systems-and-cells/lenti-x-293t-cells

plasmids. (You don't have to pipet a ton, just don't add the packaging and envelope plasmids to the PEI-KO DMEM mastermix separately.)

4. Pipet the PEI-KO DMEM mastermix into the DNA mixes to form the final condition mixes. Incubate at room temperature for 10-15 minutes.
5. Add the mixture *dropwise* and *evenly* around the dish, then gently rock to mix. Place the cells back in the incubator for 6-8 hours.

Important

The six-to-eight hour timing is important for this protocol. We use a relatively large amount of transfected DNA, which means we are also adding a high concentration of PEI. Media changing after 6-8 hours can greatly reduce transfection-related cell death. Changing the media sooner can also work, but it may reduce transfection efficiency and thus viral titer. Being consistent with the timing of this media change can help reduce variability in titer across batches of virus.

6. Six-to-eight hours after transfection, replace the media on the plates with HEPES-buffered DMEM + 10% FBS. Use the collection volume of media, which is 6.5 mL for a 10cm dish.

   The recipes to prepare both the 1M stock solution of HEPES and the HEPES-buffered DMEM can be *found here* (page **??**). Typically, there is a common bottle of HEPES-buffered DMEM in Bruni (TC 4ºC).

Warning

Viral particles may be present after the first media change. Be sure to use proper PPE (i.e., lab coats, disposable sleeves) and wipe down the hood with Pre-Empt after use, from here on! **If the virus contains oncogenes or inhibitors of tumor suppressors (e.g., HRASG12V, p53DD), BL2+ precautions are required.** See the *Virus safety* (page **??**) protocol for details.

Day 2

1. 18-24 hours after the last media change, collect the media, replacing it with the same collection volume of fresh HEPES-buffered DMEM.
2. Store the collected media at 4°C. The collected media from separate days can be stored in the same conical tubes.

Note

Two collections (on Days 2 and 3) elicit sufficiently high titer virus. However, a third collection (on an extra day between Days 2 and 3 written here) may further increase titer.

Day 3

1. On the last day of collection (typically the second collection), combine all collected media for the same virus. If making multiple dishes of the same virus, the collected media can be combined into a single tube (two days of collection for 3 dishes can fit into a single 50 mL conical). Filter the media through a 0.45 μm filter using a syringe to remove cells/cell debris.

Note

It is okay to store unfiltered or filtered virus at 4ºC for several days before concentrating or transducing cells.

2. Then, either:
    a. Proceed to the *Virus Concentration* (page **??**) protocol (below). This is useful for long-term storage.
    b. Within a few days of collection, transduce cells (*protocol here* (page **??**)) directly with unconcentrated virus.

Note

Unconcentrated virus appears to lead to more cell death than does concentrated virus, possibly because a much larger volume of viral media is required. Therefore, concentrating the virus is advised.

Virus Concentration

Estimated time

1 hour in-TC time, 1 day overnight time

1. To the collected and filtered virus, add 1/3 volume of Lenti-X concentrator (e.g., for 30 mL of virus, add 10 mL of Lenti-X). Mix by inverting several times.
2. Store at 4°C overnight.
3. The next day, centrifuge at 1500 x g at 4°C for 45 minutes (use the lower centrifuge). Be sure to use the caps/lids on the centrifuge buckets.
4. Aspirate the supernatant. There will be a little liquid left; this is okay.

5. Resuspend the pellet gently in the remaining liquid. Add media to reach the desired final volume suggested in the "Concentrated volume" column of the scale table above.

Note

Measuring to the exact desired final volume can be tedious. If you are planning to titer the virus before use, it is okay to resuspend in a set volume without confirming the final volume, as titer is a concentration value (transducing units per μL). Of course, measured titers will be more consistent if you resuspend precisely.

6. Use or store the concentrated virus.
    a. To use: Transduce cells according to the *Transduction of concentrated virus* (page **??**) protocol.
    b. To store: Store the virus (or any remaining after transduction) in a cryovial at -80°C.

Note

Store virus only in cryovials with threaded lids. Normal Eppendof tubes can pop open when cooling, which is an exposure risk.

Note

Freeze-thaw cycles may affect transduction of concentrated virus. It is best practice to store an aliquot of concentrated virus for titering, separate from the main aliquot for later transduction. However, we have not rigorously quantified loss in effective titer over freeze-thaw cycles, so a limited number of freeze-thaw cycles may not have much of an impact.

Viral transduction

This protocol describes how to transduce cells with unconcentrated or concentrated virus (see *Virus Concentration* (page **??**) protocol). The method for each is the same, though the volume of viral solution to add to the cells will differ (much more for unconcentrated virus). Virus produced in HEK293T cells with the VSV-G envelope protein can be used to transduce either human or mouse cells.

Before beginning, calculate the amount of virus to use. The following calculations are for transduction in a 96-well plate; volumes can be scaled up accordingly for other plate sizes. Below are three common scenarios for virus amounts:

- As an initial rule-of-thumb, ~1% of the total virus produced in one 10cm dish (e.g., 1-2 μL if concentrated and resuspended in 200 μL total) is a good starting place for transducing one well in a 96-well plate.
- When transducing to calculate viral titer, follow the *Measuring viral titer* (page **??**) protocol, transducing several wells with a serial dilution of the virus.

- If the viral titer (transducing units, TU, per μL) is known, calculate the volume to use based on the number of cells and the desired multiplicity of infection (MOI):

  virus volume (μL) = # cells * MOI (TU/cell) / titer (TU/μL)

  Typically, an MOI of 0.3 or lower is used to ensure single-copy transduction. An MOI of 3 or higher is useful to ensure most of the cells are transduced with at least one copy.

Note

Viral titer is reported to decrease during freezing and thawing. Therefore, you may wish to increase the amount of virus used for every freeze-thaw cycle. However, we have not rigorously quantified the degree to which this matters, so it may be fine to ignore this for few freeze-thaw cycles. It is best practice to store an aliquot of concentrated virus for titering, separate from the main aliquot for later transduction.

Cells may be transduced in suspension (i.e., at the same time as seeding) or after plating. Transduction is more efficient in suspension, and efficiency can be increased for plated cells through spinfection. However, there may be a tradeoff between efficiency and cell viability. All three methods are described below.

Important

We use precautions beyond those for normal BL2 tissue culture when transducing any human-infectible virus. Read over the *Virus safety* (page **??**) protocol before beginning.

Transduction of plated cells

1. The day before (18-24 hours prior), seed cells on plates with the appropriate coating. Seeding densities vary by target cell type. Typical densities are 10k cells/well for mouse embryonic fibroblasts and 10-15k cells/well for hiPSCs.
2. Combine the calculated amount of virus with the appropriate amount of media and 1000X polybrene. For a 96-well plate, this is:

Component	Volume
Virus	calculated above
1000X polybrene	0.1 μL
Media	to 100 μL

Tip

Since the amount of polybrene for a single well is so small, if you are not infecting several wells with the same virus, you may wish to make a media-polybrene

master mix. For instance, make a virus-media mix (without polybrene) to 50 μL/well, then add 50 μL/well of a 2X polybrene + media solution.

Note

When transducing unconcentrated virus, the virus volumes may be large. Calculate amounts to add at least a half-well volume (e.g., 50 μL for a 96-well plate) of fresh media per well, even if this leads to a larger total well volume. This will make sure the cells have enough fresh media to stay healthy. Don't forget to adjust the volume of polybrene if the total well volume changes!

3. Aspirate the media from the cells.
4. Add the virus mix to each well.
5. The next day (1 day post-infection, dpi), aspirate and replace with fresh media. This is a good time to add any small molecule inducers, if applicable.
6. Continue culturing cells, observing proper virus precautions. Typically, a good end point for flow cytometry is 3 dpi.

Important

Viral particles are no longer present after 3 days AND 2 media changes. Use proper *virus safety* (page **??**) until then.

Transduction of cells in suspension

1. Coat plates. Do this first to provide sufficient time for coating (e.g., at least 10 min for 0.1% gelatin), as plates will be used at step 5.
2. Dissociate and count the cells that you will transduce.
3. Resuspend cells in fresh media at **double** the final concentration. For instance, if using 20k cells/well in a 96-well plate (a typical amount for HEK293T cells), resuspend at a concentration of 20k cells per 50 μL. To this mix, add 2X polybrene (i.e., 1:500 dilution of the 1000X stock).

Note

Polybrene can be added either to the cell solution or to the virus-media mix (next step). Typically, adding to the cells is easier because it reduces pipetting.

4. Combine the calculated amount of virus with the appropriate amount of media to a **half-well volume**. For a 96-well plate, this is 50 μL/well.

5. Aspirate the coating from the prepared plate and add a half-well volume of the cell solution to each well (e.g., 50 μL/well for a 96-well plate). Use the multichannel for easier pipetting.
6. Add a half-well volume of the virus-media mix to the appropriate wells and pipet to mix. Each well should now contain a full well volume of cells, polybrene, virus, and media.
7. The next day (1 day post-infection, dpi), aspirate and replace with fresh media. This is a good time to add any small molecule inducers, if applicable.
8. Continue culturing cells, observing proper virus precautions. Typically, a good end point for *flow cytometry* (page **??**) is 3 dpi.

Important

Viral particles are no longer present after 3 days AND 2 media changes. Use proper *virus safety* (page **??**) until then.

Spinfection

After following the transduction protocols above for either plated cell or cells in suspension, spinning the plate can increase transduction efficiency. This may help the viral particles settle on, and ultimately be taken up by, the cells. The combined protocol of transduction plus spinning is dubbed "spinfection."

1. Immediately after combining the virus with cells, polybrene, and fresh media as described above (with either *plated cells* (page **??**) or cells *in suspension* (page **??**)), move the plate into the bottom TC centrifuge rather than into the incubator.

Important

Cover the centrifuge buckets with the plate spinner tray caps.

2. Centrifuge the plate at 1500 x g for 30 min at 32ºC.

Note

The centrifuge won't heat to 32ºC before the spin, but it will warm up during.

1. After the spin is complete, transfer the plate to the 37ºC incubator. Continue as normal with the 1 dpi media change and subsequent treatments, readouts, etc.

Plat-E Retroviral Production

Plat-Es are a retrovirus packaging line sold by Cell Biolabs[203] that produce retroviruses that can only infect mouse or rat cells. Plat-E cells contain gag, pol and env genes, allowing retroviral packaging with a single plasmid transfection. Supposedly, Plat-E viruses can't be frozen but we have seen frozen Plat-E virus work fine.

[203] https://www.cellbiolabs.com/platinum-e-plat-e-retroviral-packaging-cell-line

Note

Do a **10 μg/mL blast and 1 μg/mL puro selection every ~3 passages**. This is to ensure cells are expresssing the retroviral structure proteins.

Plat-E Transfection

Day One (seed Plat-Es):

1. *Gelatin coat* (page **??**) a 6-well plate (1x6-well makes enough virus for 1x96-well plate)
2. Seed 2 mL of 425k cells/mL onto 6-well plates (850k Plat-E's/6-well total) onto the gelatin coated plates.
3. Rock the plate back and forth, side to side to mix, then place back in incubator. *Rocking side to side prevents cells from clustering at the well edge.*

Tip

If starting from frozen, **start growing Plat-E cells 1 week prior** - they will be slow growing at first (don't change culture medium during the first 3 days). Split Plat-Es 1:4 to 1:6 every 2-3 days when culture reaches 70-90% confluency.

Day Two (transfect Plat-Es):

1. Make a mastermix (MM) of PEI and Knockout DMEM as follows (MM for 1 well/each virus):
    a. For *just 1 well of a 6-well plate*:
        i. In a 1.5 mL tube, FIRST add 180 μL KO DMEM.
        ii. SECOND, add 7.2 μL PEI (1 mg/mL) for a 4:1 ratio (of μg PEI:μg DNA ratio).
        iii. Gently flick to mix. Let sit for 10-15 minutes.
    b. For *3.5 rxns or wells of a 6-well plate*, same as above but use 630 μL KO DMEM + 25.2 μL PEI (1 mg/ml) for a 4:1 ratio

Important

Ensure that you **add PEI to KO DMEM, NOT the other way around!!** Also make sure to use KO DMEM as KO DMEM facilitates lipid-mediated transfections (e.g. PEI) which might be disrupted by regular DMEM.

Note

PEI optimal concentration varies by batch and must be tested before transfection. Current batch is 4:1 as of 2022.03.17 (typical range 4:1 to 6:1).

2. Add DNA to this MM as follows, then mix and wait 15 minutes:
    a. 1.8 μg DNA/well, (or 6.3 μg DNA total for 3.5 rxns)
    b. For example:

Component	Volume/ 1 rxn	Volume / 3.5 rxn	**Total Vol/well**
pMXs-mGL (500 μg/μL)	3.6 μL	12.6 μL	**190.8 μL/well**

3. Add each KO DMEM + PEI + DNA mix to a single 6-well (1 VIRUS PER WELL) **DROPWISE** and evenly around the plate, rocking the plate back and forth, side to side to mix. Place back in incubator.

Day Three (Plat-E media change + seed mouse cells):

1. Change with 1.25 mLs fresh media (*DMEM/HEPES + 10% FBS* (page **??**)) after 24 hours. Note: NBW transfects ~4pm and media changess ~10am next day to minimize PEI cytotoxicity.

Note

Instead of infecting MEFs with unconcentrated retrovirus for two days, you can also media collect for 2 days and concentrate, same as the *HEK293T protocol* (page **??**). BAD and MC resuspend concentrated retrovirus in 100 uL media per well of a 6 well, and *spinfect* (page **??**) MEFs for 1 day with 2 uL concentrated virus.

2. Seed mouse cells.

i. Coat wells in 0.1% gelatin for approx. ~10 min.

ii. Seed at 10k cells/96-well. For larger sizes, scale accordingly according to surface area

Culture plate	**Scale factor**	**# of 6-wells of Plat-E you'll need for a full plate**
6-wells	30	2
12-wells	11	1.5
24-wells	6	1.5
48-wells	2.6	1.5
96-wells	1	1

Day Four (Plat-E media change + infect 1):

1. Harvest media after another 24 hours and add 1.25 mL fresh media (*DMEM/HEPES + 10% FBS* (page **??**)) to Plat-E plates for a second time.

2. Transduce mouse cells with retroviruses made from the Plat-E cells.

Note

Each virus will make ~1 mL/well from each 6-well of Plat-E (enough for 1x96-well plate). 11 μL of each virus will be added to each well of a 96-well plate ALONG WITH POLYBRENE (1,000X at 5 mg/mL)

a. Filter each virus through a 0.45 μm filter

b. Master mixes will be made for simpler "aliquoting" into wells. The following table is a guide for the final total volume for each well depending on the plate.

Culture plate	Total DMEM (i.e. MEF media) + virus per well
6-wells	2 mL
12-wells	1 mL
24-wells	500 μL
48-wells	250 μL
96-wells	100 μL

Note

You can either 1. filter each virus then mix together (minimizes filtering) or 2. mix altogether then filter (standardizes mixing). Because filtering is the most annoying step, it is advised to minimized filtering.

3. Add virus mixes to each well dropwise, rocking back and forth to mix.

Day Five (infect 2):

1. Collect media from Plat-Es again and reinfect/retransduce the cells for a second day.

Note

Centrifugation of target cells with virus (spinfection) can improve infection efficiency. Spinfection protocol is *here* (page **??**).

Day Six (1 dpi):

1. Change media on transduced mouse cells

Note

NBW has found you can freeze PlatE virus. I will concentrate a single 6-well collected over 2 days (~2.1 mL + 700 μL Lenti-X concentrator) and resuspend into 100 μL where I use 2 μL/96-well. **You will lose ~50% of your virus so I go with frozen virus from 2x6-wells will infect 1x96-well plate.**

VSV production

Materials

- Cells
- Plates
- DMEM supplemented with 10% FBS
- PEI
- Plasmids:
  - pCAG-VSVN (pKG00756)
  - pCAG-VSVP (pKG00755)
  - pCAG-VSVL (pKG00757)
  - pCAG-VSVG (pKG00758)
  - pCAG-T7 (pKG00759)
  - pVSV eGFP dG (pKG00760)

Procedure

Day 1. Seed cells

1. Seed HEK293T cells in a 6-well plate (2ml of 5.0 x 10 ^ 5 cells/mL) (**Plate_A**).
2. Incubate at 37°C.

Day 2. Transfection

1. Transfect the following plasmid mixture to the cells on **Plate_A** through the *general transfection protocol* (page **??**):

Plasmid	Amount of DNA
pVSV eGFP dG	5μg
pCAG-VSVN	1.5μg
pCAG-VSVP	2.5μg
pCAG-VSVL	0.5μg
pCAG-T7pol	5μg
pCAG-VSVM	4ug
pCAG-VSVG	4μg

2. Incubate the cells at 37°C for 5-6 hours.
3. Remove the media and add fresh complete media (2 mL/well of a 6-well).
4. Incubate at 37°C for about 48 hours.

5. After transfection, seed HEK293T cells in an another 6-well plate or 10cm dish for the following amplification process (**Plate_B**).

Day 3. VSVG transfection for viral amplification

1. Transfect pCAG-VSVG to the HEK293T cells on **Plate_B**, for viral particle amplification:
    - If using a 6-well plate, transfect 2μg of pCAG-VSVG
    - If using a 10cm dish, transfect 10μg of pCAG-VSVG
2. Incubate at 37°C.

Note

There is nothing that you have to do for the cells on **Plate_A** at Day 3.

Day 4. Collect the viral particles and amplify with HEK293T

1. Remove the media from the cells on **Plate_B** (HEK293T cells transfected with pCAG-VSVG at Day 3), and tansfer the supernatant of HEK293T cells on **Plate_A**.
2. Incubate at 37°C for about 48-72 hours to amplify the viral vector in the cells on **Plate_B**.

Day 6-14.

1. Monitor the cells for the development of virus-induced CPE or GFP expression if you are making GFP-expressing VSV. When 40-100% of the cells show signs, the supernatant can be harvested.
2. Harvest the supernatant and remove cells and cell debris by centrifugation for 10 min at 450xg or by filtering the supernatant with 0.45μm filter.

Note

Amplification period depends on the RNA structure of the genome. Usually, reporter gene expression should be able to be seen by day 7-8.

AAV production in HEK293T

This protocol describes how to produce AAV in HEK293T cells.

Estimated time

6 days. Note that on the **transfection day**, you must change the media 6-8 hours after the transfection.

Scale and Plasmids

Scale	Area (cm^2)	Seeding amount (cells)	Total DNA mass (ug)	Collection media volume (mL)
10cm	56.7	6.0 x 10^6	24	6.5
6-well	9.6	1.0 x 10^6	4	1.25

If using a different virus production scale, the total DNA mass should be scaled by surface area. The collection media volume should be roughly half of the standard amount of media used for that scale.

Type	Packaging plasmid	Helper plasmid
AAV	pAAV2/2 (pKG1378)	pAdDeltaF6 (pKG1548)

Note

It is highly recommended to full plasmid sequence the packaging and helper plasmids before use.

Note

Two other serotypes have been tested (AAV8 and AAV9n), but AAV2 was the consistently the best at infecting 293Ts, MEFs, and HDFs.

Self-complementary AAV (scAAV) also outperforms single-stranded AAV (ssAAV). With the current protocol ssAAV is only able to consistently infect 293Ts.

ssAAV has a total cloning capacity of 4.4kb between the ITRs (4.7kb including ITRs).

scAAV has a total cloning capacity of ~2.2kb between the ITRs.

Virus Production

The following is given for a 10cm-dish production scale. For other scales, substitute the correct seeding amounts/total DNA mass/other values from the scale table.

Day 0

1. Seed confluent 293T cells onto a gelatin-coated virus production plate/dish. For a 10cm dish, this should be 6-6.5 million cells (use the calculated **seeding amount** for other scales).

Day 1

1. Transfect your cells when they are 70-80% confluent. This should be roughly 18-24 hours after you seeded the cells.
2. Prepare a mastermix of PEI and knockout DMEM. It is helpful to prepare a 110% master mix (include 10% extra mix) to account for pipetting losses. Ensure that you **add the PEI to the KO-DMEM** and not in the other order.
    a. Use the total number of micrograms of DNA (24 for a 10cm dish, 4 for a 6-well) times the experimentally-determined PEI ratio to get the number of microliters of 1mg/mL PEI you need to add. In other words, the PEI ratio is a PEI:DNA mass ratio.
    b. We use 1.33 mL of KO-DMEM per 10cm dish (222uL for a 6-well).
3. After waiting for 10-15 minutes, mix the following ratios of DNA with the PEI-KO DMEM master mix. You can add the master mix to the DNA or add the DNA to aliquoted master mix.

Plasmid	Size	Molar Ratio	Approx. Mass Ratio	Approx. Mass 10cm (ug)	Approx. Mass 6-well (ug)
Helper	15420 bp	1	3	13.1	2.18
Packaging	7468 bp	1	1.5	6.54	1.09
Transfer	variable (usually 4-5kb for scAAV)	1	1	4.36	0.73

Note

BAD has also used a molar ratio of 2:1.5:1 (as recommended by Braatz Lab) and didn't notice any difference in 293T or MEF infection efficiency. It might be fine to use a set mass ratio like with Retro and Lenti virus, but this has not tested in lab yet.

4. After waiting an additional 10-15 minutes, add the mixture *dropwise* and *evenly* around the plate. After adding, be sure to gently rock the plate side-by-side to mix.

Important

The six-to-eight hour timing is important for this protocol. We use a relatively large amount of transfected DNA, which means we are also adding a high concentration of PEI. Media changing after six-to-eight hours can greatly reduce transfection-related cell death.

5. Six-to-eight hours after transfection, replace the media on the plates with HEPES-buffered DMEM. Use the collection volume of media, which is 6.5mL for a 10cm dish or 1.25mL for a 6-well.

    Use the following recipes to prepare both the 1M stock solution of HEPES and the HEPES-buffered DMEM.

1M HEPES stock solution (filter sterilize after pH-ing)

Component	Concentration	Amount/50 mL
HEPES-potassium salt	1M	13.82 g
DI H2O	main solvent	50 mL
Hydrochloric acid	to pH 7.0	

HEPES-buffered DMEM

Component	Concentration	Amount/50 mL
DMEM + 10% FBS	main Component	48.75 mL
Sterile 1M HEPES	25 mM	1.25 mL

Warning

Viral particles may be present after the first media change. Be sure to use proper PPE (i.e., lab coats, disposable sleeves) and wipe down the hood with Pre-Empt after use, from here on!

AAV is human-infecible, so be sure to use BSL2+ precautions if delivering oncogenes.

Day 4 or 5

1. 48-72 hours after the last media change, collect the media.
2. Filter the collected virus using a 0.45um filter. You can keep the unconcentrated virus at 4°C for up to a few days. The virus media can be used instead of normal DMEM in order to directly infect cells.

Tip

BAD finds the addition of polybrene does not affect AAV infection efficiency, unlike with Retro and Lenti virus.

Infecting multiple times or for a longer duration can increase infection efficiency.

BAD has not tested making AAV in a 10cm dish yet. Only 6-well plates have been used so far.

Measuring viral titer

Viral titer can be measured in several ways. We mainly use functional titer, in which we measure the fraction of transduced cells expressing the viral payload for several different virus volumes. This metric, in units of **transducing units (TU) per volume** accounts for the amount of particles, their ability to enter cells, and the vector's ability to integrate and express in the cell of interest—which are all necessary for downstream uses of the vector. The titer is specific to each batch of virus and,

because it relies on expression, transduced cell type. The viral titer can be used in future experiments to standardize the **multiplicity of infection (MOI, TU per cell)** in subsequent infections. This controls for differences in viral production efficiency across constructs or batches of virus.

Below, the protocol describes transduction with a serial dilution of virus and a flow cytometry readout, then how to use the flow cytometry data to compute viral titer. Note that expression can be detected easily if one or more of the cargoes is fluorescent, or via staining for non-fluorescent payloads.

Transduction with a serial dilution of virus

This protocol follows the steps to transduce *plated cells* (page **??**) or cells *in suspension* (page **??**) using a serial dilution of virus as your virus-media mix.

1. Begin with a plate containing cells, polybrene, and a fresh half-volume of media.

   If transducing plated cells:

   1. Seed cells at an appropriate, known density one day prior (e.g., 10k MEFs or iPSCs) on appropriately coated plates.
   2. On the day of transduction, replace media with a half-volume of fresh media containing 2X polybrene (i.e., 1:500 dilution of 1000X polybrene).
   3. *Optionally*, dissociate a few extra wells and count to determine the current cell count per well. Tip: Resuspend several wells in a very small volume for more accurate counts.

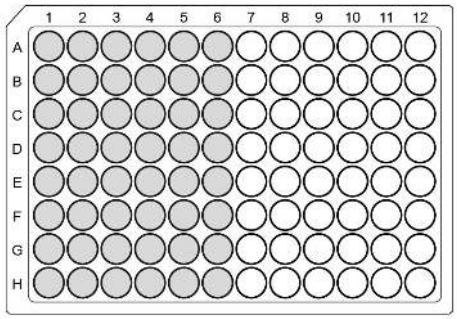
   If transducing cells in suspension:

   1. Prepare plates with the appropriate coating.
   2. Dissociate and count cells. Resuspend cells at double the final concentration (e.g., 20k cells in 50 μL fresh media per well for HEK293T cells) with 2X polybrene (i.e., 1:500 dilution of 1000X polybrene).
   3. Remove the coating and add a half-volume of cells to each well (e.g., 50 μL/well in a 96-well plate) using a multichannel pipette. This mix includes the correct total amount of cells and polybrene per well.

2. **Serially dilute each virus in fresh media**, enough for a half-volume per well. Do this in an auxiliary 96-well plate or fresh tubes. Start with a virus volume estimated to transduce most of the cells, then dilute by factors of 2-10 to generate 4-8 dilutions.

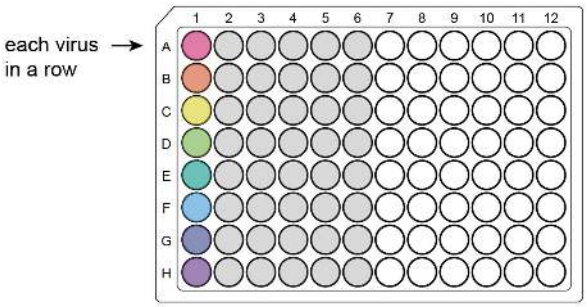
   To measure titer for several viruses, the easiest way to do this is in an **auxiliary 96-well plate** with a multichannel pipette. One way in particular is the following, using 6 two-fold dilutions per virus (half a row on the auxiliary plate), making enough virus-media mix to transduce 2 wells per condition of cells. Tip: Make 20% extra mix for each condition to account for pipetting loss. (The flat-bottom plates make pipetting the entire volume difficult.)

   i. Add 120 μL fresh media to all the wells you will use in the auxiliary plate, half a row per virus, with a multichannel. (Fill wells 1-6 in all columns before wells 7-12 in any column, as you will pipet by columns later.)



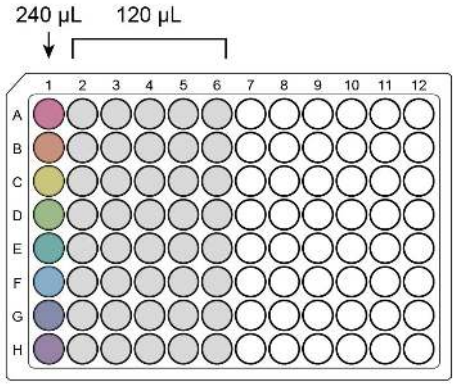
Add 120 µL of fresh media

ii. Add each virus to a new well in column 1, using enough for 4 wells plus 20% extra (e.g., for a final volume of 4 μL virus/well when added to the cells, add 19.2 μL virus to column 1).



Add virus to column 1

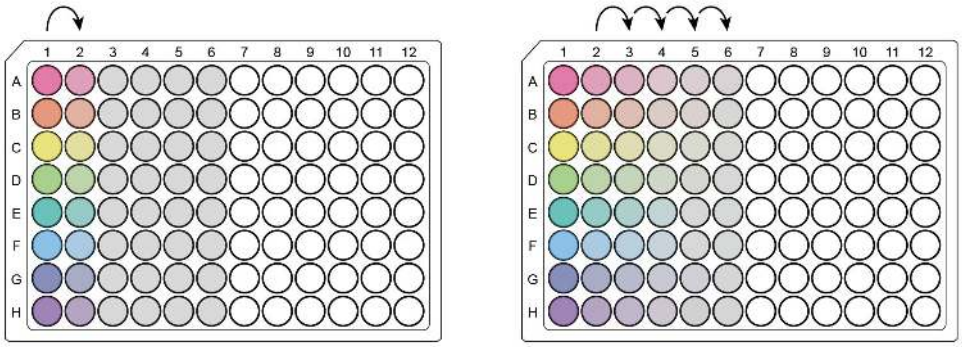
iii. Pipet additional media into column 1 to bring the total well volume to 240 μL. (If you are using the same volume of each virus, you may wish to do this with the multichannel after step 1.)



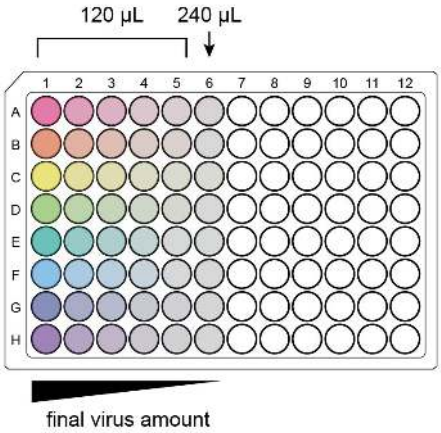
Add media to column 1,
final volume 240 µL

iv. Using the multichannel, pipet in column 1 to mix, then transfer 120 μL to column 2. Repeat for columns 3-6.

v. The final auxiliary plate now has 6 serial dilutions (columns) per virus (rows).



Columns 1-5 have 120 μL/well, and column 6 has 240 μL/well.



Note

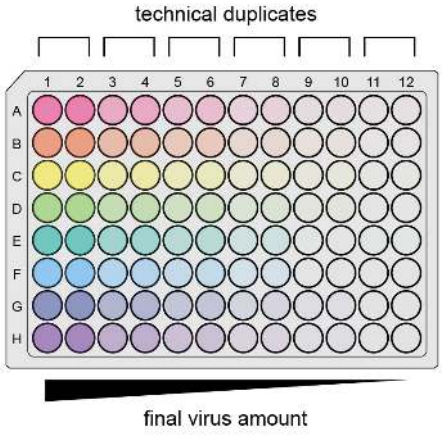
Alternatively, you can use one of these spreadsheets (1[204], 2) to compute dilution volumes / virus amounts to avoid making 2x volume of the final dilution. Note that this pipetting is less amenable to the multichannel.

3. **Add a half-volume per well of the virus-media mix to the plate from step 1** containing cells, polybrene, and fresh media. Pipet gently to mix.

   If using the auxiliary plate as described above, this can be done by pipetting 50 μL/well with the multichannel, columnwise. Tip: It is okay to use the same tips if you start with the most dilute column and mix carefully.

4. The plate now contains a full well volume (e.g., 100 μL/well for a 96-well plate) of virus, cells, polybrene, and fresh media. Return the plate to the incubator, or *spinfect* (page **??**), whichever you plan to do for subsequent transductions.

[204] https://mitprod.sharepoint.com/:x:/s/GallowayLab/EQEnStlTAd1NqKOPG0S18LIBFpaybN1_KckEwNsxueirOw?e=V5Gy4G



5. As in the standard transduction protocol, perform a media change at 1 day post-infection (dpi), making sure to add any inducers so the virus cargo maximally expresses.
6. At 3 dpi (or a similar endpoint you will use for subsequent transductions), collect the cells for *flow cytometry* (page **??**).

Important

Viral particles are no longer present after 3 days AND 2 media changes. Use proper *virus safety* (page **??**) until then.

Computing titer from transduction data

From the transduction experiment described above, we obtain fluorescence measurements for known numbers of cells transduced with known volumes of virus. Note that individual cells with detectable expression may be contain one or more copies of the viral payload, representing at least one transduction event.

First, let's define the **multiplicity of infection (MOI)**, λ, as the ratio of viral transducing units (TU) to number of cells, n:

$$\lambda = \frac{\text{number of TU}}{n}$$

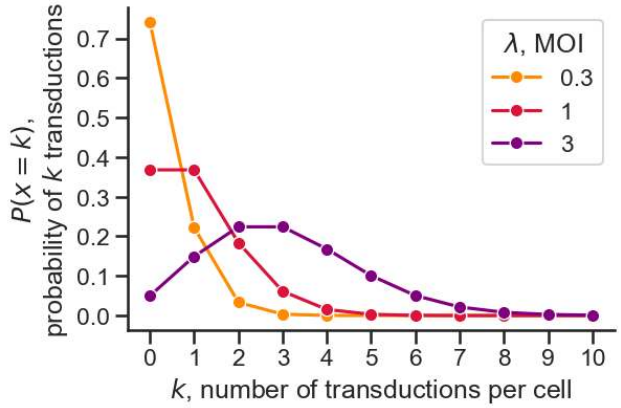
We can obtain a particular MOI in an experiment by using a calculated **volume of virus**, v, if we know the **viral titer**, t:

$$\lambda = \frac{t \times v}{n}$$

Assuming transductions follow a **Poisson process**, where discrete events occur in a fixed interval and events are independent, the expected rate of events (transductions) is the MOI, λ. Then, the probability of k events in the interval, or k transductions in a cell, is

$$P(x = k) = \frac{\lambda^k e^{-\lambda}}{k!}$$

We can see that the probability of transductions in a cell increases with λ (MOI), as expected. Notice that at an MOI of 0.3, the probability of transduction ($P(x > 0)$) is low, but the probability of multiple transductions ($P(x > 1)$) is negligible; this is useful for ensuring single integrations. On the other hand, at an MOI of 3, the probability that a cell will not be transduced ($P(x = 0)$) is low, useful for ensuring most cells are transduced.



Across a population of cells, we expect the fraction of cells with k transductions to reflect the probability of k transductions ($P(x = k)$). However, it is difficult to quantify the exact number of transductions in individual cells with flow cytometry. But we can easily compute the fraction of cells with at least one transduction:

$$\begin{aligned} P(x > 0) &= 1 - P(x = 0) \\ &= 1 - \frac{\lambda^0 e^{-\lambda}}{0!} \\ &= 1 - e^{-\lambda} \\ &= 1 - e^{v/n \times t} \end{aligned}$$

Thus, the flow cytometry data from the experiment above can give us a fraction of expressing cells, which we can use with known values of v and n (experimental parameters) to find t, the viral titer. To make this calculation more accurate, we can measure the fraction of expressing cells for several different volumes of virus—exactly the experiment described above—and curve fit to better estimate the viral titer when measurements are noisy. Note that viral titer has units TU/mL (or TU/μL, if you use virus volumes in μL).

We have written a function in the `rushd` Python package to simplify this calculation (coming soon!). See also the example Jupyter notebook in the `example_training` repo on the Galloway lab GitHub (also coming soon).

CHAPTER

FOUR

RECIPES

4.1 Bacterial recipes

4.1.1 LB selection plates

1. Dissolve 32 g of LB agar powder in 800 mL of sterile/ELGA water.
2. Add stir bar to the bottle, leave unscrewed, and cover with aluminum foil.
3. Autoclave on 20 min liquid cycle at 121C.
4. Wait for the solution to cool to about 45-50 C by putting on stir plate at room temperature.

Tip

If you can keep touching it with bare hands, that indicates the temperature of the LB is around 45-50C and it's time to add antibiotics.

4. Add 800 uL of 1000x antibiotics to the autoclaved LB agar-water solution.
5. Dissolve the antibiotics by stirring with a stir bar.
6. Before pouring, ensure no agar has solidified in the bottle (as a result of uneven cooling). If this happens, shake manually to re-melt the agar.
7. Pour ~12 mL of the antibiotic-containing LB to petri dishes using a pipette.
8. Gently move the plates around to evenly distribute the agar.

Tip

Use a 50 mL serological, fill to 60 mL, and pipette out 12 mL at a time onto sets of 5. Alternatively, you can pour LB by hand if you experience solidification when pipetting.

9. Incubate the poured plates at RT until the LB agar solidifies (~1 hr minimum-overnight).
10. After plates solidify, place plates back in the sleeve bag and store at 4C.

4.1.2 Antibiotic Stock Recipes

1000X Ampicillin (Amp)

1. Dissolve 100 mg of Ampicillin powder in 1 mL sterile/ELGA water (1 g of Ampicillin powder in 10 mL sterile/ELGA water).
2. Sterilize with 0.22 uM filter.
3. Make 1 mL aliquots and store at -20ºC.

1000X Kanamycin (Kan)

1. Dissolve 50 mg of Kanamycin powder in 1 mL sterile/ELGA water (500 mg of Kanamycin powder in 10 mL sterile/ELGA water).
2. Sterilize with 0.22 uM filter.
3. Make 1 mL aliquots and store at -20ºC.

1000X Chloramphenicol (Chlor)

1. Dissolve 34 mg of Chloramphenicol powder in 1 mL 70% EtOH (340 mg of Chloramphenicol powder in 10 mL 70% EtOH).
2. No filtration step is needed.
3. Make 1 mL aliquots and store at -20ºC.

4.1.3 Competent cells (NEB stable)

Estimated time

Overnight + 3 hours + 2 days

Day 1. Start an overnight culture

Start a culture of the relevant bacterial strain (i.e., NEB Stable, ccdB resistant) from the glycerol stock stored in the same box as the aliquots of competent cells. Use ~4 mL plain LB media, and grow overnight at 30°C.

Day 2. Prepare competent cells

Important

All pipetting should be done with filter tips, under a flame, and with fresh gloves to prevent contamination. Wipe down pipets with Obliterase prior to pipetting.

1. Combine 1 mL of the overnight culture with 99 mL of fresh LB (plain) in a 500 mL Erlenmeyer flask.
2. Shake at 37°C until the OD (600 nm) measures 0.4-0.6 (0.5-0.6 is ideal). Use the Nanodrop to measure OD with default settings.

Note

Usually, it takes 2-2.5 hours to reach 0.4-0.6 OD (600 nm). Check the OD after ~90 min to estimate how much longer it will take—consider that bacteria double approximately every 30 min. Be careful not to overshoot! If so, you'll need to restart the 100 mL culture.

3. Once the OD (600 nm) reaches 0.4-0.6, place the cells on ice for at least 10 min.
4. Using the Zymo Mix & Go! kit[205] (stored in green bin in Deli fridge), prepare 10 mL of 1X Wash Buffer and 10 mL of 1X Competent Buffer by diluting the 2X solutions with Dilution Buffer. At this point, set the centrifuge to 4°C to cool and label 100 Eppendorf tubes with the strain (e.g., "NS" for NEB Stable). Mark each labeled tube in the batch with a color different from those of any competent cells remaining in the freezer (the whole batch should be the same color).
5. After incubating the culture on ice, pellet the cells by centrifugation at 3000xg for 10 min at 4°C.
6. Remove the supernatant completely and suspend the pellet GENTLY with 10 mL of ice-cold 1X Wash Buffer. Bleach the collected supernatant for 20 min and dispose down the sink.
7. Re-pellet the cells by centrifugation at 3000xg for 10 min at 4°C.

[205] https://www.zymoresearch.com/products/mix-and-go-e-coli-transformation-kit

8. Remove the 1X Wash buffer and collect it in a separate tube.

Important

To dispose of 1X Wash: Add a few mL of bleach to the collected 1X Wash Buffer. A dark brown/black precipitate will form. This can be left to settle over several days or can be centrifuged after 20 min of decontamination. Then, discard the supernatant down the drain and dispose of the tube with precipitate in the biowaste.

9. GENTLY resuspend the cells in 10 mL of ice-cold 1X Competent Buffer.
10. Aliquot the cell suspension ON ICE into the labeled Eppendorf tubes, 100 μL per tube.
11. Store the cells in the relevant competent cell box at -80ºC (Elsa). Update the spreadsheet on the freezer door with the batch color and date.
12. To confirm there is no plasmid contamination, innoculate LB + ampicilin and LB + kanamycin cultures with competent cells. It is convenient to use any leftover cells insufficient for a final aliquot for this step. Grow the cultures at 37ºC for two days.

Day 4. Check for cell growth

After two days in culture, check whether cells grew in the LB + ampicilin and LB + kanamycin conditions. If there is no growth, the competent cells are good to use. Mark the batch as such on the spreadsheet on the freezer door.

Important

If cell growth is observed, **discard the entire batch** of competent cells. This indicates likely plasmid contamination. Note the contamination on the spreadsheet on the freezer door and message the lab if any aliquots may have been used. To discard the competent cells, combine the contents of all the aliquots and follow the protocol for disposing of the 1X Wash Buffer waste.

4.1.4 DNA wash buffer

This wash buffer can be used for the *DNA cleanup* (page **??**) washes or the second *miniprep* (page **??**) wash.

DNA wash stock solution (5x)

This is stored in a bottle on the shelf above the large thermocyclers.

Ingredient	Amount (per 80 mL)
80 mM NaCl	0.374 g
8 mM Tris-HCl	0.101 g
Water	80 mL
HCl to pH 7.5	

DNA wash buffer

Ingredient	Amount (per 100 mL)
5x stock solution	20 mL
Ethanol	80 mL

Other Qiagen recipes are available here.

4.1.5 Bacterial media recipes

TB (Terrific Broth)

The following recipe can be directly autoclaved in Erlenmeyer flasks if desired.

1. Combine and autoclave the following components. The final **10%** volume will be added afterwards.

Ingredient	Amount per 1L final volume
Tryptone	12 g
Yeast extract	24 g
Glycerol	4 mL
Deionized water	900 mL

2. After the mixture has cooled, add 100 mL / liter final volume of *10x TB salts* (page **??**) and desired antibiotics.

10x TB salts

The potassium phosphate salt solution acts as a pH buffer. It must be autoclaved separately to prevent high-temperature reactions between the salts and other components of TB.

1. Combine the following, filling to the desired final volume with deionized water.

Ingredient	Target concentration	Amount per 1L final volume
KH2PO4	0.17 M	23.1 g
K2HPO4	0.72 M	125.4 g

Note

Because we are making a fairly concentrated solution, you will need to add less than the total final volume of water (e.g. for a liter of TB salts, you may only have to add 900 mL water).

I tend to add 70% of the water, wait until all of the salt dissolves, then fill up to the relevant line on the flask. This doesn't have to be super precise.

2. Autoclave the salt solution.

SOC Media

Note

We use 2x concentrated SOC for better bacterial growth. This is reflected in the amounts below.

1. Combine 62 g of SOC powder with deionized water to a final volume of 1 L.

2. Autoclave and let cool.

LB Media

1. Resuspend 20 g of LB Broth powder with deionized water to a final volume of 800 mL.
2. Autoclave and let cool.

4.1.6 Other Solutions

Glycerol (60%)

The following protocol makes 200 mL which can be used for 50 stocks.

1. Into a 200 mL glass bottle, add 80 mL DI water.
2. Add 120 mL glycerol and pipet up and down in water to help mix and get rid of glycerol stuck in pipette.
3. Autoclave in liquid cycle.

1xTAE buffer

The following protocol makes 2.5 L of 1X TAE Buffer.

1. Add 50 mL of 50X TAE concentrate (Genesee catalog #20-194) to a 500 mL graduated cylinder.
    - 50X TAE concentrate is kept under the sink to the left of the gel runners.
2. Fill graduated cylinder to 500 mL with DI water and add to 1X TAE carboy.
3. Add an additional 2000 mL (2 L) of DI water to the 1x TAE carboy.
4. Close the carboy lid and shake thoroughly to mix.

4.2 Recipes for biochemical and analytical protocols

4.2.1 FISH buffers

Probe Hybridization Buffer

1. Combine the following components fresh before use

Ingredient	Amount
30% Formamide	3 mL
20X SSC	2.5 mL
1M Citric Acid, pH 6	90 uL
10% Tween 20	100 uL
50X Denhardt's Solution	200 uL
50% Dextran Sulfate	1.5 mL
20 mg/mL BSA	50 uL
H20	Bring volume up to 10 mL

Probe Wash Buffer

1. Combine the following components

Ingredient	Amount
30% Formamide	3 mL
20X SSC	2.5 mL
1M Citric Acid, pH 6	90 uL
10% Tween 20	100 uL
H20	Bring volume up to 10 mL

2. Store at -20 degrees C, expiration in 7 days

Probe Amplification Buffer

1. Combine the following components:

Ingredient	Amount
Na2HPO4	70.98 mg
NaCL	292.2 mg
H20	Bring volume up to 10 mL

2. Bring the pH to 6.8 (approximately 50 uL of 2.4 M HCl). Store at room temp.

5X SSCT

1. Combine the following components

Ingredient	Amount
20X SSC	10 mL
10% Tween 20	400 uL
DEPC H20	Bring volume up to 40 mL

1. Store at 4 degrees C, expiration in 1 month.

4.2.2 Shared genomics buffers

The following buffers are used widely across genomics protocols and have practically infinite shelf life (all salts). All of these should be **0.22μm filter-sterilized** (use syringe filters) and stored near the genomics hood in 66-219.

Note

We typically mark filtered buffers with a red Sharpie line and the word “filtered”.

- 50 mL **1M Tris (pH 7.5)**

Component	Concentration	g / L	Amount / 50 mL
Tris-HCl	1 M	157.64	7.88 g
Elga water	to 40 mL		
NaOH	to pH 7.5		~700 uL of 12 N NaOH
Elga water	to 50 mL		
Filter sterilize			

- 50 mL **2M Tris (pH 7.5)**

Component	Concentration	g / L	Amount / 50 mL
Tris-HCl	2 M	157.64	15.76 g
Elga water	to 40 mL		
NaOH	to pH 7.5		
Elga water	to 50 mL		
Filter sterilize			

- 50 mL **1M Tris (pH 8.0)**

Component	Concentration	g / L	Amount / 50 mL
Tris-HCl	1 M	157.64	7.88 g
Elga water	to 40 mL		
NaOH	to pH 8.0		~900 uL of 12 N NaOH
Elga water	to 50 mL		
Filter sterilize			

- 50 mL of **1M HEPES pH 7.5 stock solution**

Component	Concentration	g / L	Amount / 50 mL
HEPES	1 M	238.3	11.915 g
Elga water	to 40 mL		
NaOH	to pH 7.5		~1.2 mL of 12 N NaOH
Elga water	to 50 mL		
Filter sterilize			

- 50 mL of **500 mM EDTA (pH 8.0) stock solution**

Component	Concentration	g / L	Amount / 50 mL
EDTA	500 mM	186.1	9.305 g
Elga water	to ~30 mL		
NaOH	to pH 8		~3.75 mL of 12N NaOH
Elga water	to 50 mL		
Filter sterilize			

  The EDTA makes the solution very acidic. Adding the NaOH will help it dissolve. It will still take a lot of vortexing to get the final solution!

- 50 mL of **10x TE (pH 8.0)**:

Component	Concentration	Amount / 50 mL
2M Tris	100 mM	2.5 mL
500 mM EDTA	10 mM	1.0 mL
Elga water	to ~40 mL	36.5 mL
NaOH	to pH 8	
Elga water	to 50 mL	
Filter sterilize		

- 50 mL of **5M NaCl stock solution**:

Component	Concentration	g / L	Amount / 50 mL
NaCl	5 M	292.21	14.611 g
Elga water	to 50 mL		
Filter sterilize			

- 50 mL of **2.5M CaCl2**:

Component	Concentration	g / L	Amount / 50 mL
CaCl2 dihydrate	2.5 M	147.02	18.38 g
Elga water	to 50 mL		
Filter sterilize			

- 50 mL of **1.0M MgCl2**:

Component	Concentration	g / L	Amount / 50 mL
MgCl2	1.0M	95.21	4.76 g
Elga water	to 10 mL		
Filter sterilize			

- 50 mL of **10% SDS**:

Component	Concentration	Amount / 50 mL
SDS	10% (w/v)	5 g
Elga water	to 50 mL	

4.3 TC recipes

4.3.1 DNase (2X) and papain (2X)

DNase and papain from 2X

This is based off of Worthington Papain Dissociation System[206] to achieve a final ** 20 U papain/mL + 2,000 U DNase I/mL solution with 0.5mM EDTA and 1mM L-cysteine**.

Add equal volumes 2X DNase and papain from the -80°C. e.g. 1 mL DNase (2X) + 1 mL papain (2x).

Solution	Details
DNase (2X) (page **??**)	4,000 U/mL in DMEM/F12
Papain (2X) (page **??**)	40 U/mL with 1mM EDTA, 2mM L-cysteine in DMEM/F12

L-Cysteine (50 mM)

1. Measure 60.6 mg L-Cysteine (MW: 121.16 g/mol) and add to a 15 mL conical
2. Add 10 mL DMEM/F12
3. Take into TC and sterilize by filtering through a 0.22 μm filter
4. Aliquot ~1.1 mL into 1.6 mL eppendorf tubes and freeze with papain aliquots at -80°C

Papain (2X: 1mM EDTA, 2mM L-cysteine, 40 U papain/mL)

To make a 50 mL solution of 2X papain from Papain 100 mg[207]:

[206] https://www.worthington-biochem.com/products/papain-dissociation-system
[207] https://www.fishersci.com/shop/products/papain-i-carica-papaya-i-latex-2/AAJ61875MC

Component	Final conc	Volume
EDTA (0.5M, pH=8.0)	1 mM	100 μL
L-Cysteine (50mM)	2 mM	2 mL
Papain (100 mg bottle)	40 U/mL	100 μL
DMEM/F12		47.9 mL

Sterilize with 0.22 μM filter and store aliquots at -80°C. *EDTA (0.5M, pH=8.0)* (page **??**) should be in Bruni and *L-Cystein (50mM)* (page **??**) should be in -80°C with papain aliquots.

DNase (2X: 4,000 U/mL solution)

To make a 2X solution from DNase I (100 mg, 2000 units/mg).

Add 50 mL DMEM/F12 to a 100 mg bottle, sterilize with 0.22 μM filter and store aliquots at -80°C.

4.3.2 FBS heat inactivation

- **Heat Inactivation (HI)**: a process by which serum is maintained at a temperature of 56 ± 2°C for 30 ± 2 minutes.

Note

You should use a control bottles to measure temperature. The control bottle should be equivalent to the product bottle. Controls bottles are volumed to the same level as the product being heat inactivated and fitted with a thermometer suitable for monitoring 56°C. The thermometer should not touch the sides or bottom of the bottle. **The temperature of the control must be 56± 2°C for 30 ± 2 minutes during the heat inactivation process.** A 15 mL conical works well to heat-inactivate a 10 mL sample (good for 100 mL of DMEM/FBS)

1. Thaw serum. If serum was thawed in a refrigerator allow serum to come to room temperature prior to placing in water bath.
2. Fill the water-bath with sufficient water so that the product and control bottles are immersed near the serum level.
3. Set water-bath temperature to maintain the product at 56± 2°C.
4. Place a lead weight on the control bottle and place into the center of the water-bath.
5. Mix the contents of the product bottles using a gentle swirling motion until the product is uniform.
6. Place lead circular weights over the tops of the bottles to keep them upright.
7. Place the bottles in the water-bath.
8. Swirl bottle thoroughly every 10 minutes or if applicable turn on the oscillating shaker unit. Check the temperature of the control bottle frequently as the temperature approaches 56±2°C.
9. When the temperature of the control reaches 56± 2°C start the timer for 30 minutes. If a shaking water bath is not available, ensure bottles are swirled every 10 minutes during the entire process.
10. After 30 minutes turn off the oscillating shaker and remove the bottles from the water bath. Cool to room temperature and either aliquot or return to the freezer.

Note

Denatured protein resembles a jelly like substance, usually at the bottom of the bottle of serum. If this occurs, decant serum and use. Denatured protein should not be confused with Fibrin, a fine precipitate sometimes seen in thawed serum. Fibrin precipitate is a fine whitish powder flake, which has no ill effects on cell culture.

4.3.3 Common aliquot sizes for TC

Neurotrophic factor stocks (10kx and 1kx)

Final concentration should be 10 ng/mL of each neurotrophic growth factor

Important

Dissolve neurotrophics into 0.1% BSA/PBS only, using **BSA, Millipore Sigma, A2058**. For the FGF, it is possible to do sterile PBS but it is recommended that you use the same filtered 0.1% BSA/PBS

Neurotrophic	Cat	Master stock aliquot	Working stock aliquot
BDNF	R&D Systems 248-BDB-050	90 μL, 100 μg/ml (10kx)	50 μL, 10 μg/ml (1kx)
CNTF	R&D Systems 257-NT-050	90 μL, 100 μg/ml (10kx)	50 μL, 10 μg/ml (1kx)
GDNF	R&D Systems 212-GD-050	90 μL, 100 μg/ml (10kx)	50 μL, 10 μg/ml (1kx)
FGF	PeproTech 100-18B	**45 μL, 1,000 μg/ml (100kx)**	**25 μL, 100 μg/ml (10kx)**

For Neurotrophic factors, from the reagents in the glass tubes:

1. Make a 0.1% BSA/PBS **(ONLY use Millipore Sigma, A2058)** solution and syringe filter through a 0.2 μm filter
2. Add 500 μL filtered 0.1% BSA/PBS to a 50 μg bottle of neurotrophics to get a 100 μg/ml (10kx) master stock

For FGF only

1. Make 45 μL @100kx master stock aliquots for long term -80°C storage
    - For 1 mg, add 1 mL 0.1% BSA/PBS. You'll end up with ~21 of 10kx aliquots
2. *Directly* add 405 μL filtered 0.1% BSA/PBS to the 45 μL 100kx master stock to make 100 μg/ml (10kx) stock aliquots
    - For the last 100kx master stock aliquot, you'll have to manually measure it day of to know how much BSA/PBS to add. e.g. 35.8 μL will need 322.2 μL.
3. Make 25 μL @10kx aliquots for **long term -80°C storage** (don't keep at -20°C!)
    - You'll end up with ~18 of 10kx aliquots (2 rows of a cardboard box)
    - Don't keep at -20°C because it lasts so long

For BDNF, CNTF, GDNF only

1. Make 90 μL @10kx master stock aliquots using for long term -80°C storage

- You'll end up with ~5.5 of 10kx aliquots

2. For BDNF, CNTF, GDNF only, *directly* add 810 μL filtered 0.1% BSA/PBS to the 90 μL 10kx master stock to make 10 μg/ml (1,000x) stock aliquots
    - For the last half 10kx master stock aliquot, you'll have to manually measure it day of to know how much BSA/PBS to add. e.g. 35.8 μL will need 322.2 μL.
3. Make 50 μL @1kx aliquots for medium term -20°C storage
    - You'll end up with ~18 of 1,000x aliquots (2 rows of a cardboard box)
    - Leave the last aliquot for short term use in 4°C
    - You can use the full 50 μL -20°C aliquots to directly make 50 mL N3 aliquots for reprogramming experiments

RepSox (1kx)

Property	
Selleck Chem #	S7223
MW	287.32
1000x stock	7.5 mM
Final concentration	7.5 uM

1. To make working 1,000x stock (7.5 mM), resuspend 25 mg RepSox in 11.6 mL DMSO.
2. Aliquot 50 uL in 600 uL tubes and store at -20°C in a labeled box.

- Can make a 10kx stock also

Fetal Bovine Serum (FBS) Aliquots (50 mL)

- FBS contains many nutrients and growth factors to support cell growth. To preserve FBS, it is usually stored in the -20°C freezer until used. To avoid multiple freeze-thaw cycles of FBS and to prevent contaminations we make FBS aliquots.
- Store aliquots in -20°C freezer in the TC room.
- Take responsibility for aliquoting new stocks, when older stocks are almost used up. Make sure to use sterile technique in preparing aliquots.

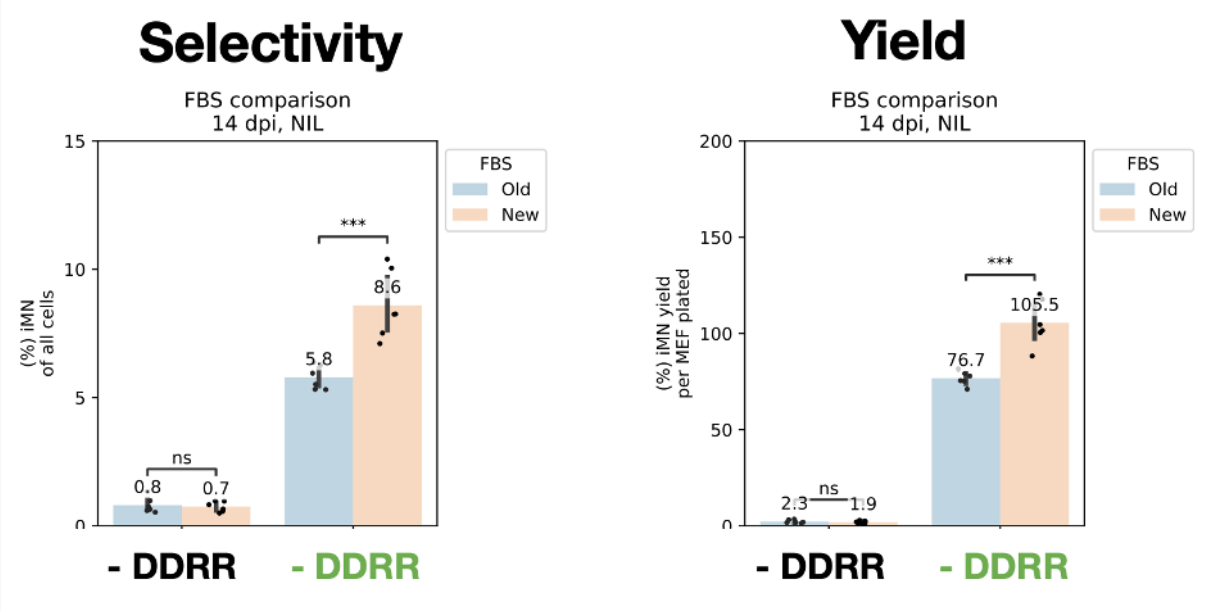
1. Thaw 500 mL FBS bottle in the fridge 2 days beforehand
2. Make ~10 aliquots of 50 mL each. Be sure to stop at the 50mL mark. Having larger volumes may cause the FBS to overflow (and be contaminated) when frozen again (FBS volume expands when frozen).
3. Make 12 mL aliquots with any leftover for *80% FBS/20% DMSO freezing media* (page **??**) (just add 3 mL DMSO)

Note

This is now a lab job. As of 5/6/2025, notify Sneha if more aliquots need to be made.

Important

As of 3/15/23, we will ask Brad from Genesee to set aside an FBS lot and send us a sample size. Bulk ordering saves us $170/bottle (~$6k over year) and helps reduce lot-to-lot variability. Test old and new FBS in *reprogramming* (page **??**). Use the sample FBS to defrost the MEFs, make the Plat-E virus, and the infection until 4 dpi. From 4 to 14 dpi, use N3 + supplements as normal. Assess 14 dpi reprogramming between the two lots. You should compare NIL (or LNI) +/- DDRR. You will have to *heat inactivate* (page **??**) the sample FBS as they do not heat inactivate small 50 mL FBS lot samples. **You should freeze it at -20°C to match number of freeze-thaw cycles as the currrent FBS.** An example of results



Pen-strep aliquots (100X, 5 mL)

- Penicillin-streptomycin is a mixture of penicillin and streptomycin and is added to the media to prevent bacterial contamination. However, it is always important to be very careful when handling cultures as contamination may still occur even when pen-strep is added.
- Thaw 50 mL pen-strep bottle in the fridge overnight
- Pen-strep is aliquoted into 5 mL aliquots and stored in the TC -20°C until used.
- Take responsibility for aliquoting new stocks, when older stocks are almost used up. Make sure to use sterile technique in preparing aliquots.

50X Geltrex aliquots

1. New orders of Geltrex (Thermo Fisher Scientific A1413302[208]) should be stored at -80ºC until aliquoting
2. Thaw the new vial overnight at 4ºC
3. The next day, prepare ice (use designated TC styrofoam) to chill reagents during aliquoting
4. Pre-chill DMEM/F12 and fresh, labeled 1.7 mL microcentrifuge tubes on ice
5. Dilute the Geltrex 1:1 with the cold DMEM/F12. Immediately aliquot 120 μL each into prepared tubes.
6. To use, further dilute a 120 μL 50X Geltrex aliquot in 6 mL cold DMEM/F12 (for a final 1:100 dilution). This is enough to coat one 6-well plate. See Geltrex coating plates for additional details.

Reference: https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/stem-cell-transfection-guide-app-note.pdf

[208] https://www.thermofisher.com/order/catalog/product/A1413301

4.3.4 TC media recipes

N3 media

Used for neuronal differentiation during direct conversion.

- Total volume: **500 mL**

Component	%	Volume
Glutamax (1%)	1%	5 mL
N2 (100x stock)	100x stock	5 mL
B27 (50x stock)	50x stock	10 mL
Pen/Strep (1%) *(optional)*	1%	5 mL
FBS *(optional)*	2%	10 mL
DMEM F12		500 mL

Add the following small molecules to small volumes immediatly before media addition, to a **final concentration of 10 ng/mL**:

- BDNF, from 1,000x stock (10,000 ng/mL)
- CNTF, from 1,000x stock (10,000 ng/mL)
- GDNF, from 1,000x stock (10,000 ng/mL)
- FGF, from **10,000x** stock (100,000 ng/mL)
- RepSox, **if RR**, from 1000x stock (7.5mM)

Note

1. 200mM glutamine is the same as Glutamax
2. We do not usually use FBS or Pen/Strep
3. Wrap N3 in foil after making

MEF media

Used for culturing MEFs and other fibroblasts.

Total volume: **500 mL**

Component	%	Volume
FBS	10%	50 mL
Pen/Strep *(optional)*	1%	5 mL
DMEM		add to 500mL

Breast cancer 1 media

Use for culturing the **LM2**, **BT-20**, and **MDA-MB-468** cell lines.

Component	Concentration	Volume / 50 mL
DMEM + 10% FBS	Base	Use MEF media
Anti-Anti	1%	500 uL

Breast cancer 2 media

Use for culturing the **HS 578T** and **BT-549** cell lines.

Component	Concentration	Volume / 50 mL
DMEM + 10% FBS	Base	Use MEF media
PSG	1%	500 uL
Insulin	10 ug/mL	125 uL of 4 mg/mL stock

Breast cancer 3 media

Use for culturing the **MDA-MD-231** cell line.

Component	Concentration	Volume / 50 mL
DMEM + 10% FBS	Base	Use MEF media
PSG	1%	500 uL

Breast cancer 4 media

Use for culturing the **SUM159** cell line.

Component	Concentration	Volume / 50 mL
F12	Base	47 mL
FBS	5%	2.5 mL
Anti-anti	1%	500 uL
Insulin	5 ug/mL	62.5 uL of 4 mg/mL stock
Hydrocortisone	1 ug/mL	
EGF	20 ng/mL	1 uL of 1 mg/mL stock

Epithelial cell 1 media

Use for culturing the **NCI-H1299** human lung cancer cell line.

Component	Concentration
RPMI + 10% FBS	Base

Epithelial cell 2 media

Use for culturing the **SAOS-2** human osteosarcoma cell line.

Component	Concentration
McCoy's + 15% FBS	Base

Glia media

Used for culturing glia cells

Total volume: **500 mL**

Component	%	Volume
Horse serum	10%	50 mL
Glucose	20%	100 mL
MEM	70%	350 mL

Sorting/Collection media

Used for cell sorting. Use DMEM/F12 for flow sorting and DMEM/F12 + 10% FBS for collection to help make cells happier. It is possible to use whatever though because you will have to spin-down and resuspend in the correct media (i.e. N3) anyways. You will want to include Pen/Strep as it will greatly reduce the amount of contamination.

Total volume: **50 mL**

Component	%	Volume
FBS *(for collection only)*	10%	5 mL
DMEM/F12	89%	44.5 mL
100X Pen/Strep	1%	500 μL

Motor neuron dissociation media

Used for dissociating iMNs or primary motor neurons (embMN) harvested from spinal cords for plating/sorting. Add 50 μL/96-well, let sit in 37°C for ~15 min. Lightly tap plae to see if cells are dissociating.

Total volume: **6 mL** (enough for 1x96-well)

Component	Volume
Papain	1 vial (>= 100 U/vial)
DNAse	1 vial (>= 1,000 U/vial)
DMEM/F12	6 mL

Freezing media

Component	Volume (1 mL)	Final Concentration
FBS (or DMEM/10% FBS)	900 μL	90%
DMSO	100 μL	10%

- It is easy to keep a 4°C stock of (*80% FBS/20% DMSO* (page **??**)) then use 500 μL 80%/20% FBS/DMSO + 500 μL DMEM/FBS cell solution

HEPES-buffered DMEM

For use during lentivirus or retrovirus production in HEK293T cells. We use HEPES Sigma-Aldrich (H3375).

1M HEPES stock solution (filter sterilize with 0.22 μm filter after pH-ing)

Component	Concentration	Amount/50 mL
HEPES	1M	13.82 g
DI H2O	main solvent	50 mL
NaOH	to pH 7.0	

Note

When NBW did this on 7/10/23, I did 41.46 g HEPES + 150 mL water. The HEPES takes up a lot of volume so I would dissolve in 100 mL ELGA-filtered water, then check volume and increase to 150 mL.

At the beginning, pH = 5.45 and I had to add 8.9 mL 4N NaOH to get to pH = 7.00.

HEPES-buffered DMEM

Component	Concentration	Amount/50 mL	Bottle
DMEM + 10% FBS	main Component	48.75 mL	550 mL
Sterile 1M HEPES	25 mM	1.25 mL	14.10 mL

iPSC Reprogramming Media

Used for reprogramming fibroblasts to iPSCs.

Last updated: 2025-04-01

From: Hoetker, Michael S., et al. “H3K36 methylation maintains cell identity by regulating opposing lineage programmes.” Nature cell biology 25.8 (2023): 1121-1134.

- Total Volume: **50 mL**

Component	%	Volume
KO-DMEM	1x stock	41.4 mL
de-activated FBS	15%	7.5 mL
Glutamax	1%	500 uL
MEM/NEAA	1%	500 uL
Leukemia inhibitory factor (LIF)	1000x stock	50 uL
β-mercaptoethanol	1000x stock	50 uL

Note

β-mercaptoethanol is not stable in dilute solution, so add fresh to an aliquot of media right before each media change. As of 2025-03-24, LIF is also aliquotted fresh from -20°C (do not refreeze).

50 mM β-mercaptoethanol stock solution

- Total volume: **1.2 mL** (enough for ~20 aliquots of 60 uL)

Component	Concentration	Volume
Starting stock	14.3 M	4.2 uL
PBS	1x	1195.8 uL

4.3.5 Other TC Solutions

Phosphate buffered saline (PBS)

1. Dissolve 10 tablets of PBS tablets into 2L of ELGA water in a large beaker (1 tablet/200mL) on a stir plate.
2. Cover the beaker with aluminum foil to prevent dust settling
3. After the solids are completely dissolved, adjust the pH of the solution to 7.40 by adding NaOH or HCl dropwise.
4. Keep stirring the PBS while adjusting the pH and keep measuring the pH until it completely equilibrates.
5. Transfer pH-adjusted PBS into 500mL or 1L bottles, label and mark with autoclave tape, and autoclave in liquid cycle.

0.1% Gelatin

1. Dissolve 0.5 g gelatin (Type A, from porcine skin) (Sigma G1890) into 500uL ELGA water in an autoclavable glass bottle. Scale volume as needed.
2. Label appropriately and mark with autoclave tape before autoclaving in liquid cycle. The gelatin will dissolve during the autoclave cycle.

Tip

- Use a regular Sharpie (not an ethanol-resistant lab marker) when labeling lab tape before autoclaving. Ethanol-resistant markers tend to blur and look fuzzy after an autoclave cycle, but Sharpie labels stay crisp and still won't wipe off with ethanol afterward.

EDTA (0.5M, pH=8.0)

To make 50 mL of EDTA using Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate[209] (MW: 372.24 g/mol). Note - EDTA disodium salt dihydrate MW is higher than just EDTA.

Solution is stable at 4°C for months.

1. Clean a stir bar and add to 100 mL glass beaker.
2. Add 9.306 g EDTA disodium salt dihydrate to 40 mL of ELGA water to beaker.
3. Adjust the pH of the solution to 8.0 by adding NaOH dropwise. Be aware you'll have to use quite a bit of NaOH and the EDTA will dissolve as the pH increases. You'll need roughly 1-2 mL 12N NaOH.
4. Keep stirring while adjusting the pH and keep measuring the pH until it completely equilibrates and dissolves. It will take >5 min for solution to become clear and >10 min for all sediment to dissolve.
5. Transfer pH-adjusted EDTA to a 50 mL conical and add ELGA water to 50 mL.

[209] https://www.sigmaaldrich.com/US/en/product/sigma/e5134

6. Take into TC and sterilize by filtering through a 0.22 μm filter.

4.3.6 PEI Stocks

Materials

- **Linear** PEI (located in "Chemicals" tray in Oaken 4ºC)
- Hydrochloric acid (HCl), to balance pH
- Elga water
- 0.22 μm filter (recommended 500 mL size)

Warning

Ensure you use the **linear** PEI, not the branched version!

Dissolve and Aliquot PEI

1. Before beginning, rinse all glassware and stir bar with Elga water.
2. In a beaker on a stir plate, combine 1 mg PEI powder per 1 mL Elga water.
3. While stirring, slowly add HCl to dissolve the PEI and achieve a final pH of 7.0.
    - PEI will not dissolve well in a basic solution, so slowly add acid to facilitate this process.
    - Add acid a few drops at a time to not overshoot. For a 500 mL batch of PEI (made 5/29/25), MC used ~800 uL of 4 N HCl.
    - After each addition, wait several minutes for the solution to stabilize. After pH 7.0 is reached, wait an extra 10 min to ensure the pH has not drifted.
4. In a BSC, filter sterilize the PEI solution through a 0.22 μm filter (the 500 mL ones are appropriate).
5. Make 1 mL aliquots of PEI in sterile 1.7 mL Eppendorf tubes.
6. Store the aliquots in Nokk (-80ºC, 2nd shelf from the top). Determine the optimal PEI ratio before use.

Test PEI Ratio

The optimal mass ratio of PEI to DNA for transfection can vary across batches. While more PEI typically improves transfection efficiency, it also increases toxicity. We perform a test transfection using varying amounts of PEI to determine the optimal μg PEI:μg DNA ratio. Previous tests have used ratios ranging from 1:1 to 6:1. Previous optimal ratios have been 4:1 or 5:1. The following protocol tests transfection efficiency in 6-well plates for ease of pipetting PEI volumes. Smaller well plates and volumes may be used if the number of cells and the amount of DNA are decreased in proportion to the decrease in culture surface area.

1. The day before transfection, seed 1 million 293T cells in 1 well of a gelatin-coated 6-well plate for each ratio to be tested.

2. Prepare a midiprep of a plasmid tht expresses a single fluorescent protein under the control of a strong constitutive promoter such as CMV or EF1α. Plasmids such as pKG1923 (pSHIP-EFS-mGreenLantern-bGH) or pKG2340 (pSHIP-CMV-mRuby2-bGH) are appropriate would work, along with any other simple plasmid. You will need 2 μg of DNA per ratio tested.
3. The day of transfection, prepare a knockout DMEM + PEI mix for each ratio to be tested. Add PEI to KO DMEM, invert to mix, and wait 10 minutes.
    - 1:1 - 200 μL KO DMEM + 2 μL PEI (1 μg / μL)
    - 2:1 - 200 μL KO DMEM + 4 μL PEI (1 μg / μL)
    - 3:1 - 200 μL KO DMEM + 6 μL PEI (1 μg / μL)
    - 4:1 - 200 μL KO DMEM + 8 μL PEI (1 μg / μL)
    - 5:1 - 200 μL KO DMEM + 10 μL PEI (1 μg / μL)
    - 6:1 - 200 μL KO DMEM + 12 μL PEI (1 μg / μL)
4. After 10 minutes, add 2 μg of plasmid DNA to each KO DMEM/PEI Mix. Invert to mix and wait 10 minutes.
5. After 10 minutes, add the entire KO DMEM/PEI/DNA mix dropwise to a single well of the 6-well plate. Repeat for all tested titrations.
6. Return cells to the incubator.
7. The next morning, replace the media with fresh DMEM + 10% FBS.
8. 2 days after transfection, assess transfection efficiency using the Keyence or the Attune.

Reference

https://www.addgene.org/protocols/transfection/

4.3.7 Small molecule stocks for TC

Antibiotic concentrations

Reference: Selection antibiotics[210] (ThermoFisher)

Name	Temperature	Stock conc	Common working conc.	293T conc. KL 2023.12.11
Blasticidin	-20°C	10 mg/mL (1kx)	1 - 20 μg/mL	10 μg/mL (1:1000) by 4-5 dpt
Geneticin (G-418)	-20°C	? mg/mL (1kx)	200 - 500 μg/mL	400 μg/mL (1:100) - no death observed
Hygromycin	-20°C	50 mg/mL (250x)	200 - 500 μg/mL	200 μg/mL (1:250) by 6-8 dpt
Puromycin	-20°C	1-10 mg/mL (1-10kx)	0.1 - 10 μg/mL	1 μg/mL (1:10,000) by 3-4 dpt
Zeocin	-20°C	100 mg/mL (100x)	50 - 400 μg/mL	1,000 μg/mL (1:100) >8 dpt

Other stock and working concentrations

Name	Temperature	Stock conc	Common working conc.
Doxycycline	-20°C	1 mg/mL (1kx)	1 ng/mL - 1 μg/mL
Grazoprevir (GZV)	-80°C	1 mM (1kx)	1 nM - 1 μM
Polybrene (Hexadimethrine bromide)	-20°C	5 mg/mL (1kx)	5 - 8 μg/mL

Indole-3-acetic acid (Auxin, IAA)

1. Resuspend IAA in ethanol at a ratio of approximately 11 uL ethanol to 2 mg IAA. Vortex until dissolved.
2. Add mixture to ELGA water to achieve a final IAA concentration of 1.75 mg/mL (10mM).
3. Sterilize with 0.22 μm filter.
4. Separate into 50 uL aliquots and store at -20°C.

Indole-3-acetamide (Auxin precursor, IAM)

1. Resuspend IAM in ethanol at a ratio of approximately 20 uL ethanol to 1 mg IAM.
2. Add mixture to ELGA water to achieve a final IAM concentration of 1.75 mg/mL (10mM).
3. Sterilize with 0.22 μm filter.

[210] https://www.thermofisher.com/us/en/home/life-science/cell-culture/transfection/transfection-reagents/selection-antibiotics.html

4. Separate into 50 uL aliquots and store at -20°C.

50mM O6-Benzylguanine (O6BG)

1. Dissolve 12 mg of O6BG in 1ml of DMSO.
2. Sterilize with 0.22 μm filter.
3. Separate into 50 uL aliquots and store at -20°C.

25mM Guanine

1. Dissolve 7.5 mg of guanine in 2 ml of 0.2N NaOH/Water. (Guanine can not be dissolved with DMSO/Water)
2. Sterilize with 0.22 μm filter.
3. Separate into 50 uL aliquots and store at -20°C.

50 μM AP1903/Rimiducid (1000x stock - old DSP)

1. Dissolve 1 mg of AP1903 in 14.2 mL of .1 % DMSO/water.
2. Sterilize with 0.22 μm filter.
3. Separate into 50 uL aliquots and store at -20°C.

1 mM or 100 µM AP1903/Rimiducid (100kx or 10kx stock - current NBW)

1. Dissolve 5 mg bottle of AP1903 in 3.542 mL DMSO.
2. Separate into roughly 7 x 500 μL 100kx aliquots and store at -20°C.
3. Further dilute each 500 μL 100kx aliquot into 1kx by adding 4.5 mL DMSO and separate into roughly 10 x 500 μL 10kx aliquots and store at -20°C.

Based on working conc. of 10 nM to activate iCasp9 (Mammalian Genomic Manipulation with Orthogonal Bxb1 DNA Recombinase Sites for the Functional Characterization of Protein Variants, 2023[211]).

Do not filter rimiducid with a PES filter! It will dissolve. You may use a PTFE filter.

Puromycin, 1 mg / mL (1,000x stock)

We use dissolved 10 mg/mL puromycin[212] (Invivogen, cat. ant-pr-1). It comes as 10 tubes x 1 ml @ 10 mg/mL dissolved in HEPES buffer (5 mM, pH 7.3).

To dissolve a single 1 mL @ 10 mg/mL tube:

1. Make 5 mM HEPES from the 1M HEPES stock: 45 μL of 1M HEPES + 9 mL sterile water
2. Add 1 mL of 10 mg/mL puro to the 9 mL of 5 mM HEPES
3. Sterilize with 0.22 μM filer

[211] http://pubs.acs.org/doi/10.1021/acssynbio.3c00355
[212] https://www.invivogen.com/puromycin

4. Split into 50 μL aliquots and store at -20°C

CHAPTER

FIVE

PLOT GALLERY

5.1 High-quality PDF figure export

a.k.a. how to not have journals munge your figures.

5.1.1 Intro

Exporting high-quality, vector figures is a real challenge. Despite PDF being a world standard, even programs like Illustrator can struggle with exporting PDFs that render properly everywhere, like in the printing pipeline that journals use.

Even when things render properly, you can run into color problems. Converting your nice RGB or CMYK colors to colors that render properly across computers and also *print* in the expected color is an extremely challenging problem. It is mostly solved nowadays with color profiles, but convincing all of the various software to use standard profiles is hard.

Luckily, Adobe Acrobat includes a tool called **Preflight** which will solve most of these issues.

The workflow for generating high-quality figures that stay high-quality when postprocessed by journals is generally:

1. Make sure you aren't using key drawing features that introduce problems.
2. Assemble your final figure in software that can export standard PDFs.
3. Export as a **non-standards-compliant** PDF. This is the normal default.
4. Use Preflight inside Acrobat to export a **PDF X-1a** PDF using the **SWOP** color palette.

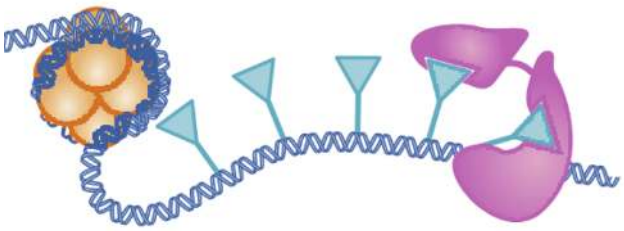
5.1.2 Problematic features

Transparency/opacity

While transparency/opacity works well if you are making a figure that will just appear online, transparency causes many problems when preparing files to print. Don't use it!

Transparency is not supported in the most standards-compliant PDF version. This is because the final post-processing steps need to properly assign colors, but there is not a good way to define what opacity fundamentally is when you have to represent both additive colors (e.g. your RGB monitor) and subtractive colors (e.g. printed inks on paper) in the same file.

Because of this, regions overlapping partial transparent regions will often get rasterized when sent to the journal. Sadly, here's an example from the final published version of our first review:



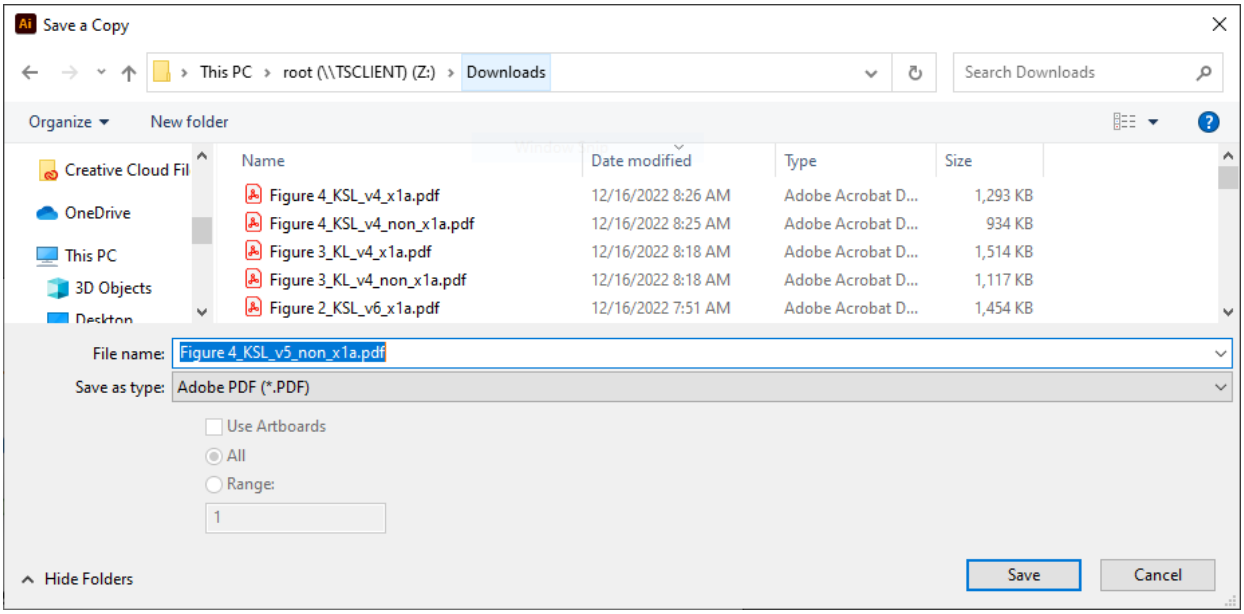
Gradients/blurs

Blurs are often used to give some 3D feeling to objects. Gradients are fine, but you should do these with the explicit gradient tools. Other Illustrator or Inkscape methods to make color gradients, such as a Gaussian Blur tool or similar, are raster operations and will cause your beautiful vector graphics to be rasterized.

5.1.3 Final figure export

Illustrator

When exporting from Illustrator, hit “Save a Copy”, and switch the output type to PDF.

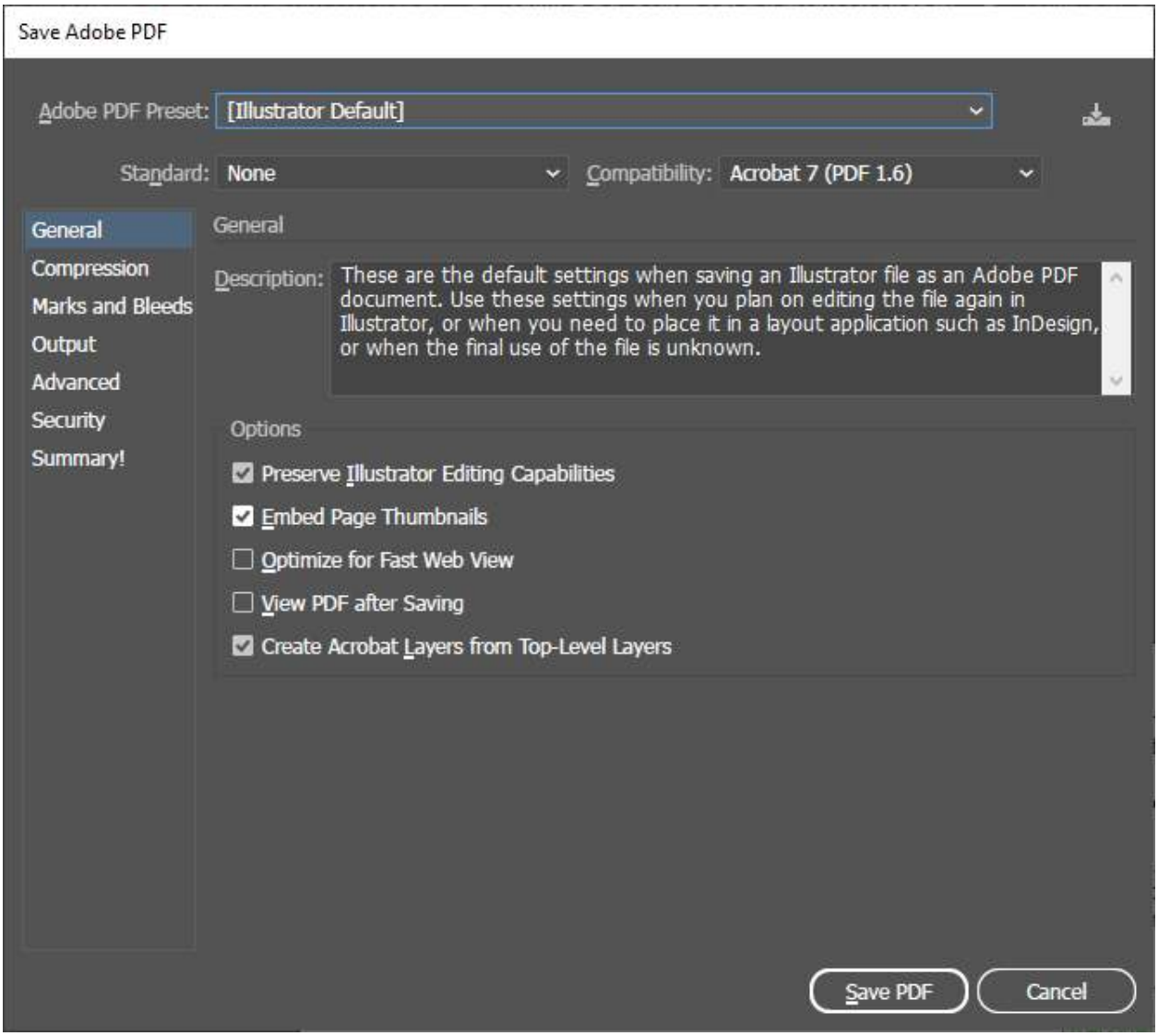


When you get to the export screen, make sure you select *[Illustrator Default]* as the output type:

Illustrator *does* have an option to directly export a X-1a PDF, but it sucks at doing so for some reason (it rasterizes things that should not be rasterized). Do not export in a mode other than the Illustrator default.

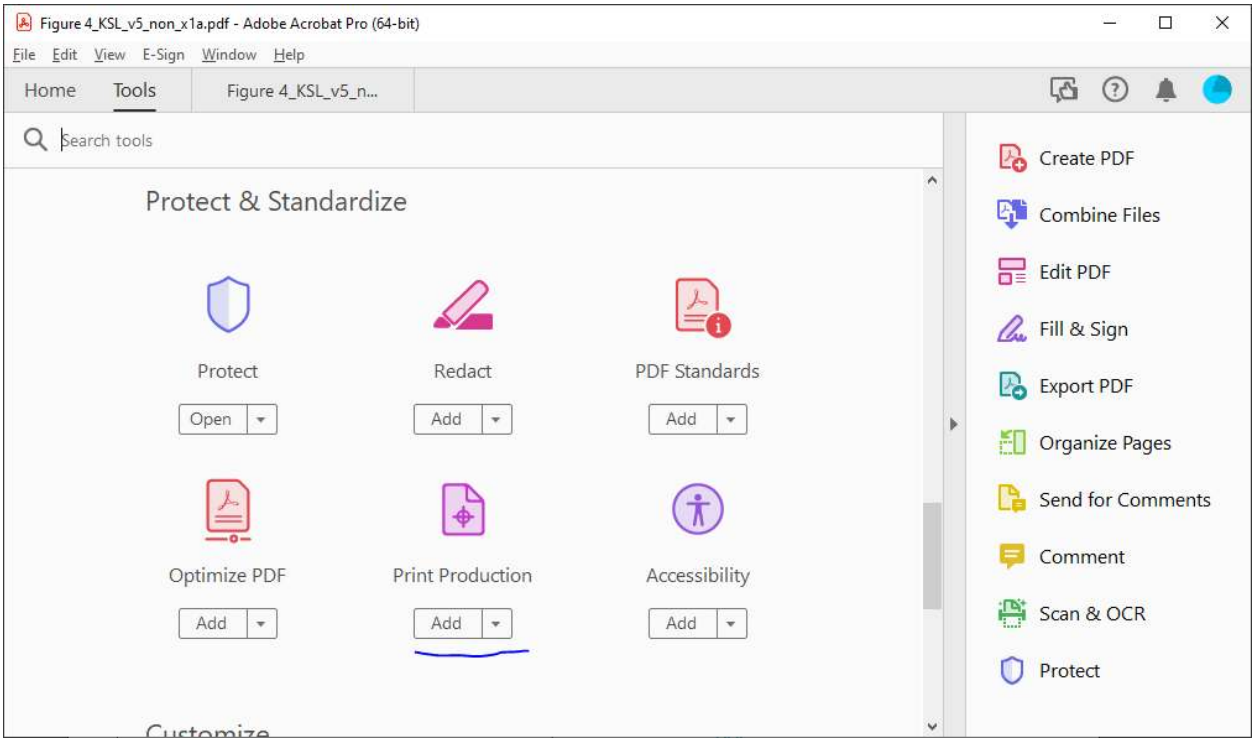
Matplotlib/Python

Doing *plt.savefig* with a *.pdf* filename is sufficient.


Save Adobe PDF
Adobe PDF Preset: [Illustrator Default]
Standard: None
Compatibility: Acrobat 7 (PDF 1.6)
General
Compression
Marks and Bleeds
Output
Advanced
Security
Summary!
General
Description: These are the default settings when saving an Illustrator file as an Adobe PDF document. Use these settings when you plan on editing the file again in Illustrator, or when you need to place it in a layout application such as InDesign, or when the final use of the file is unknown.
Options
Preserve Illustrator Editing Capabilities
Embed Page Thumbnails
Optimize for Fast Web View
View PDF after Saving
Create Acrobat Layers from Top-Level Layers
Save PDF
Cancel

5.1.4 Preflight

1. Open up the exported figure in Adobe Acrobat. Sanity check your figure to make sure that there wasn't errant rasterization or other problems.
2. Under your tools tab, hit "More Tools" and hit "Add" below the Print Production option to add this set of tools to the access bar.



3. Click Print Production to bring up the tools, and hit "Preflight":

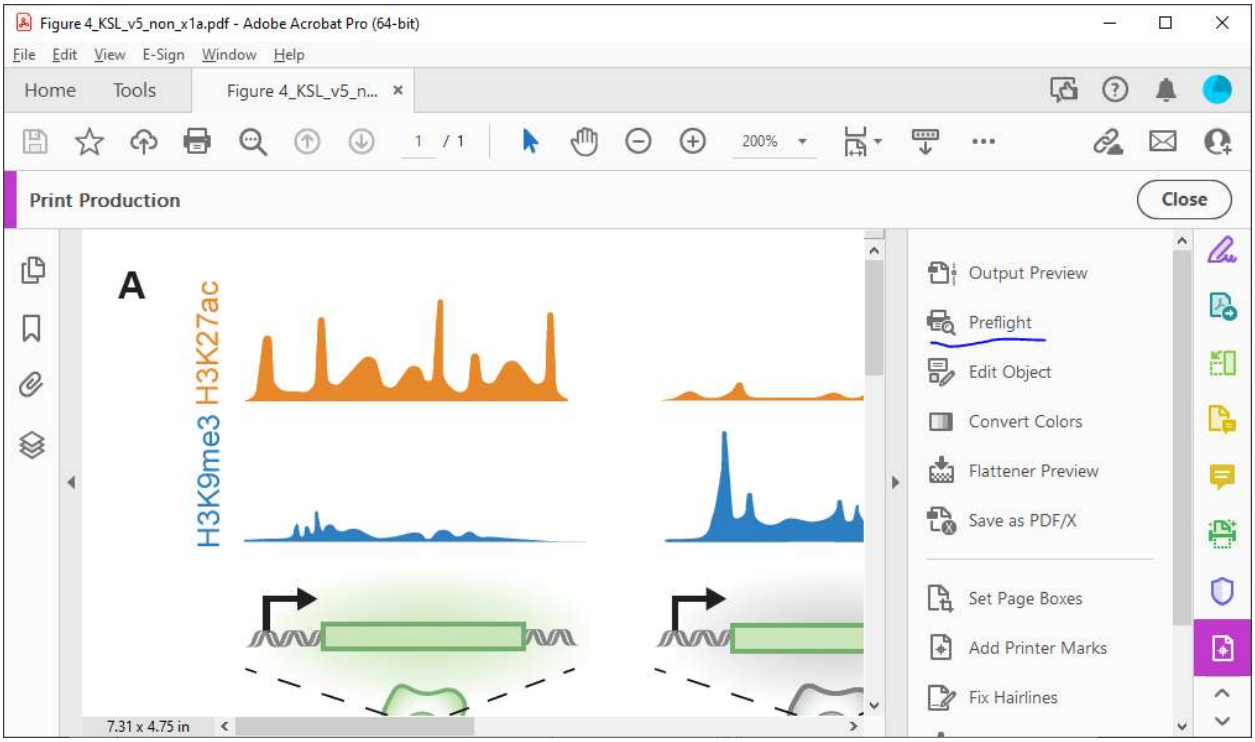
If this is the first time you have opened an advanced print production tool, Adobe may have to download some extra data before the window comes up.

4. Click the "Standards" tab at the top. Select "Save as PDF/X" and hit continue.
5. Select the first option, "Save as PDF/X-1a" and hit continue.
6. Check the checkbox, and scroll down and select the "Convert to PDF/X-1a (SWOP)" profile. Hit "Save as.." and select a new filename (we suggest *fig_name_x1a.pdf* so you can tell which files have been converted).

Note

SWOP originally was a US color profile. If publishing in international journals, carefully check the colors in your proofs. If they are off, you may want to resubmit using the FOGRA39 color profile (or see if you can ask someone which profile their production team uses).

In general, this shouldn't be a problem, as production staff should be able to use whatever color profile, as long as it is standardized.



7. Watch Preflight convert. You should see a successful run. If you don't, you should have some error messages telling you what to fix. By far the most common source of conversion errors are transparency and deeply nested clipping groups.

   By clicking on an error, you can hit "Show" to see where the problematic element is.

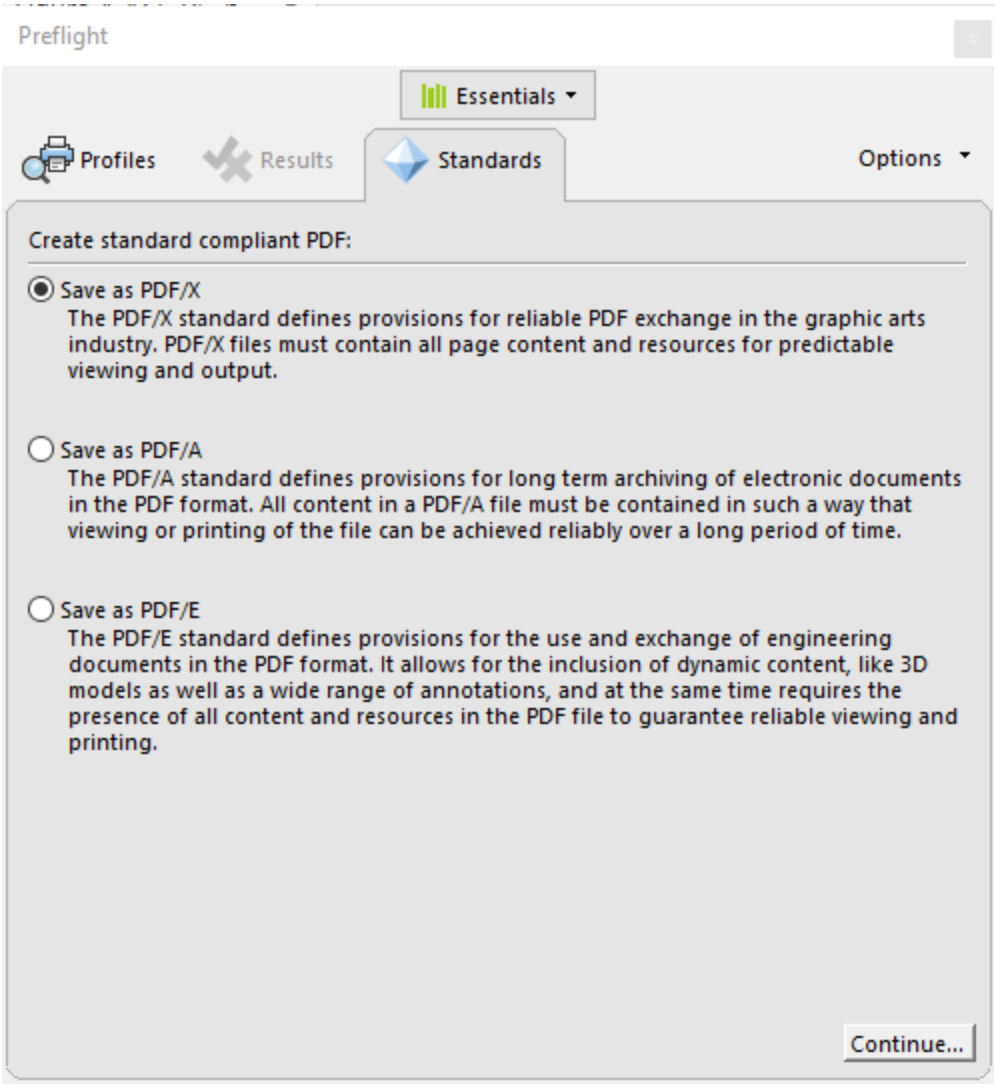
8. Sanity check the converted X-1a PDF, for rasterization and other problems. Edit your source file to remove transprency or other problems and repeat these steps until you are happy with the X-1a version.

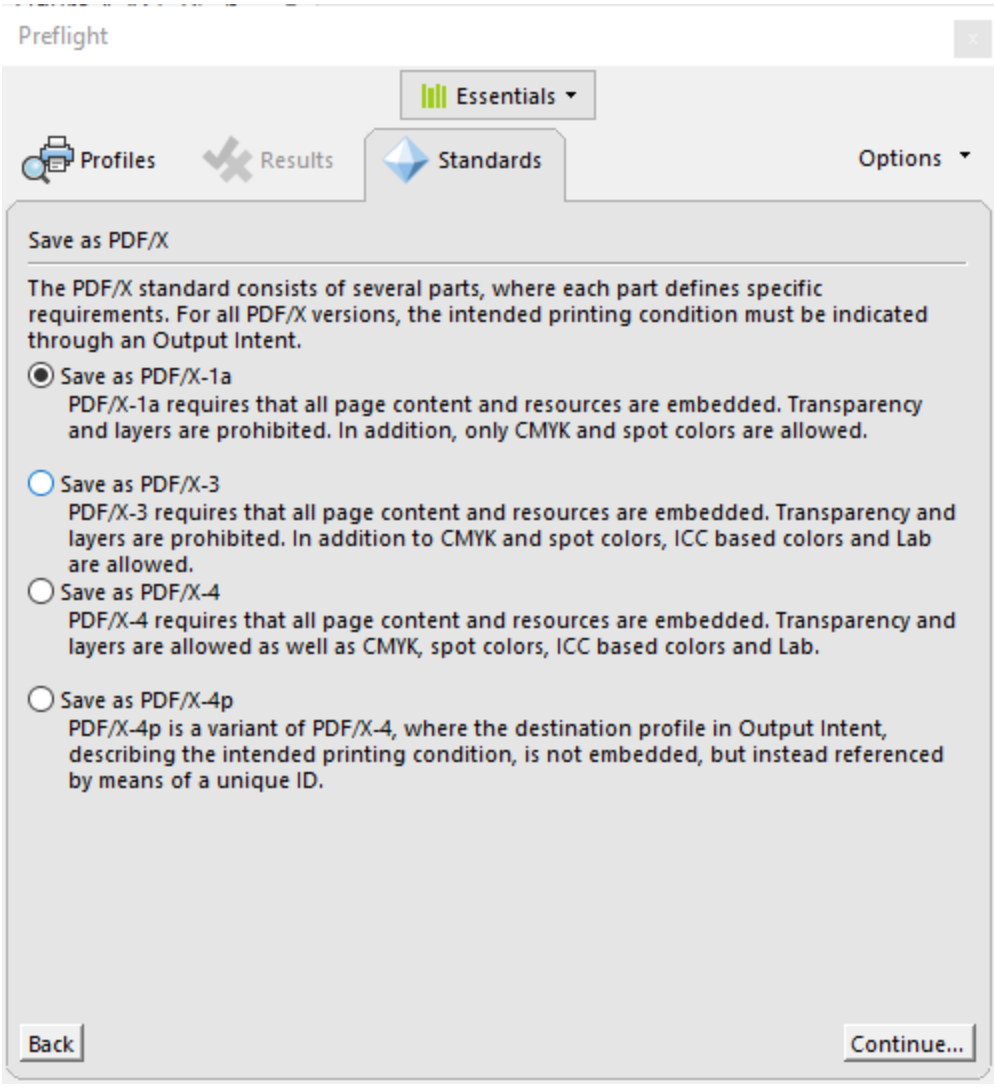
9. Submit the X-1a versions to the journal. A successful X-1a export practically guarantees that your figures will appear in the proofs as desired, with the correct colors.

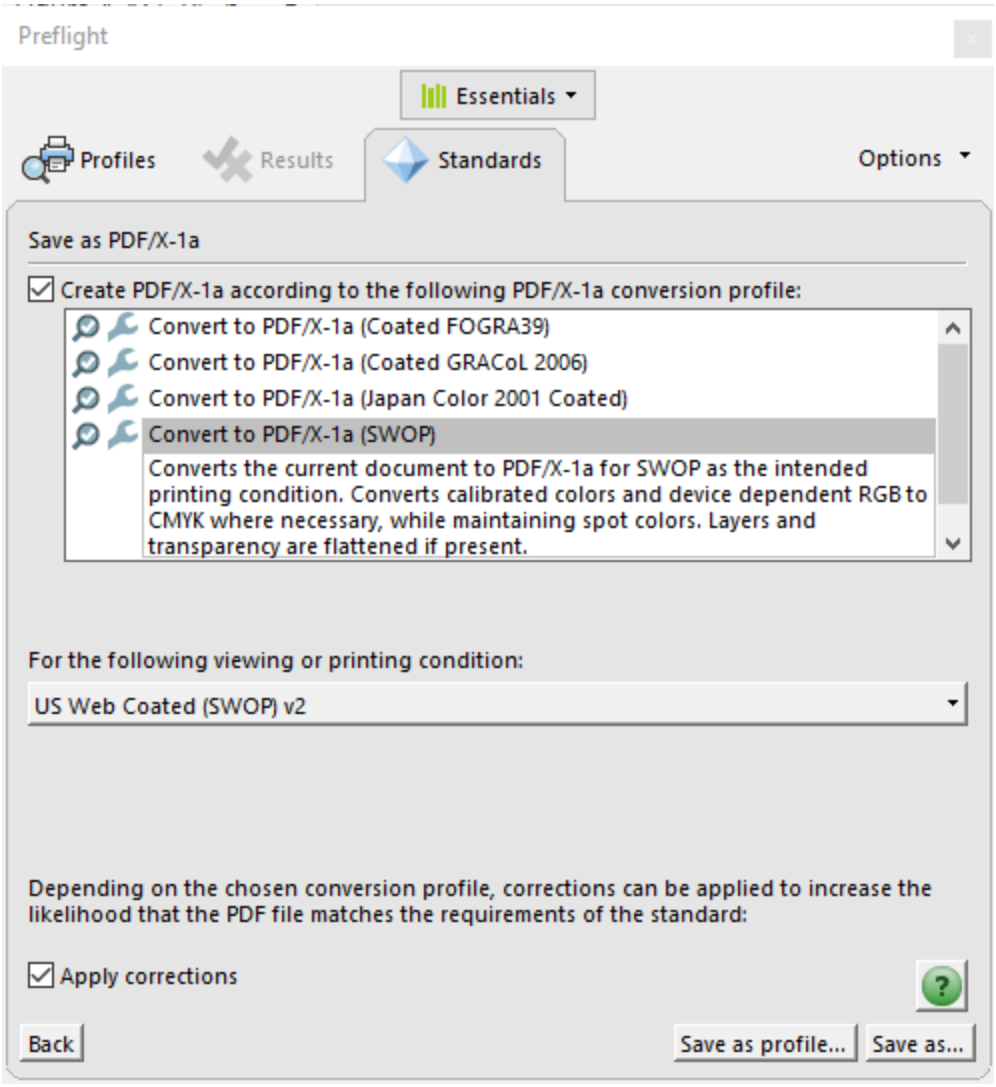
5.2 Flow plots with adjunct histograms

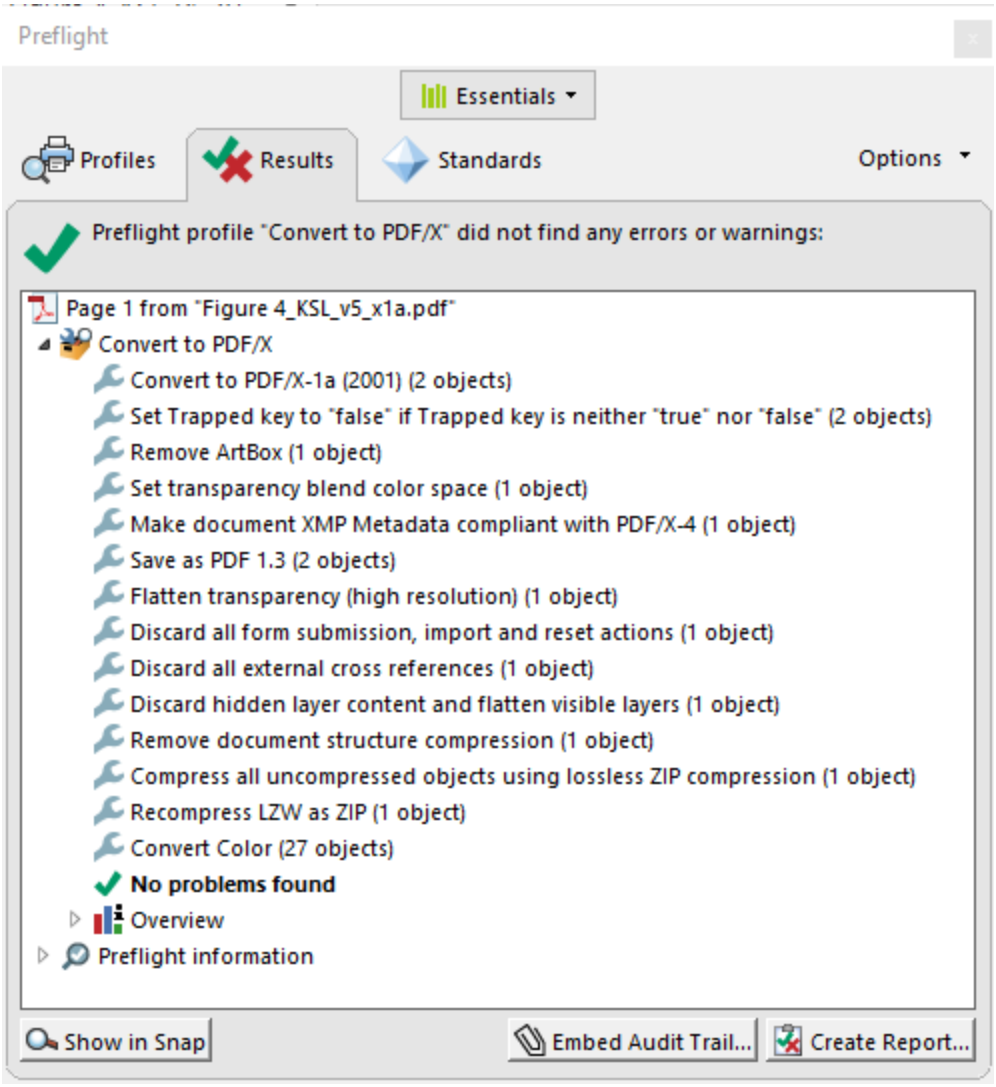
```
# Read in data
data = pd.read_csv('data/data_flow_with_adjunct_hist/data_flow_with_adjunct_hist.
↪csv')

# Randomly sample 10^4 samples from each condition to make representative flow␣
↪diagram
numSamples = 10**4
small_data = data.groupby(['cond']).sample(n=numSamples, random_state=1)

# General plotting params
x = 'CFSE-A'
```

(continues on next page)


Preflight
Essentials
Profiles
Results
Standards
Options
Create standard compliant PDF:
Save as PDF/X
The PDF/X standard defines provisions for reliable PDF exchange in the graphic arts industry. PDF/X files must contain all page content and resources for predictable viewing and output.
Save as PDF/A
The PDF/A standard defines provisions for long term archiving of electronic documents in the PDF format. All content in a PDF/A file must be contained in such a way that viewing or printing of the file can be achieved reliably over a long period of time.
Save as PDF/E
The PDF/E standard defines provisions for the use and exchange of engineering documents in the PDF format. It allows for the inclusion of dynamic content, like 3D models as well as a wide range of annotations, and at the same time requires the presence of all content and resources in the PDF file to guarantee reliable viewing and printing.
Continue...


Preflight
Essentials
Profiles
Results
Standards
Options
Save as PDF/X
The PDF/X standard consists of several parts, where each part defines specific requirements. For all PDF/X versions, the intended printing condition must be indicated through an Output Intent.
Save as PDF/X-1a
PDF/X-1a requires that all page content and resources are embedded. Transparency and layers are prohibited. In addition, only CMYK and spot colors are allowed.
Save as PDF/X-3
PDF/X-3 requires that all page content and resources are embedded. Transparency and layers are prohibited. In addition to CMYK and spot colors, ICC based colors and Lab are allowed.
Save as PDF/X-4
PDF/X-4 requires that all page content and resources are embedded. Transparency and layers are allowed as well as CMYK, spot colors, ICC based colors and Lab.
Save as PDF/X-4p
PDF/X-4p is a variant of PDF/X-4, where the destination profile in Output Intent, describing the intended printing condition, is not embedded, but instead referenced by means of a unique ID.
Back
Continue...


Preflight
Essentials
Profiles
Results
Standards
Options
Save as PDF/X-1a
Create PDF/X-1a according to the following PDF/X-1a conversion profile:
Convert to PDF/X-1a (Coated FOGRA39)
Convert to PDF/X-1a (Coated GRACoL 2006)
Convert to PDF/X-1a (Japan Color 2001 Coated)
Convert to PDF/X-1a (SWOP)
Converts the current document to PDF/X-1a for SWOP as the intended printing condition. Converts calibrated colors and device dependent RGB to CMYK where necessary, while maintaining spot colors. Layers and transparency are flattened if present.
For the following viewing or printing condition:
US Web Coated (SWOP) v2
Depending on the chosen conversion profile, corrections can be applied to increase the likelihood that the PDF file matches the requirements of the standard:
Apply corrections
Back
Save as profile...
Save as...


Preflight
Essentials
Profiles
Results
Standards
Options
Preflight profile "Convert to PDF/X" did not find any errors or warnings:
Page 1 from "Figure 4_KSL_v5_x1a.pdf"
Convert to PDF/X
Convert to PDF/X-1a (2001) (2 objects)
Set Trapped key to "false" if Trapped key is neither "true" nor "false" (2 objects)
Remove ArtBox (1 object)
Set transparency blend color space (1 object)
Make document XMP Metadata compliant with PDF/X-4 (1 object)
Save as PDF 1.3 (2 objects)
Flatten transparency (high resolution) (1 object)
Discard all form submission, import and reset actions (1 object)
Discard all external cross references (1 object)
Discard hidden layer content and flatten visible layers (1 object)
Remove document structure compression (1 object)
Compress all uncompressed objects using lossless ZIP compression (1 object)
Recompress LZW as ZIP (1 object)
Convert Color (27 objects)
No problems found
Overview
Preflight information
Show in Snap
Embed Audit Trail...
Create Report...

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```
y = 'sulfo-Cyanine5-A'
hue = 'cond'
cond_list = ['Ctrl', 'NIL', 'NILDD', 'NILDDRR']
colormap = {'Ctrl': 'grey', 'NIL': 'crimson',
            'NILDD': 'purple', 'NILDDRR': 'dodgerblue'}

# definitions for the axes
left, width = 0.1, 0.65
bottom, height = 0.1, 0.65
spacing = 0.005

rect_scatter = [left, bottom, width, height]
rect_histx = [left, bottom + height + spacing, width, 0.2]
rect_histy = [left + width + spacing, bottom, 0.2, height]

# Set up figure
fig = plt.figure(figsize=(6, 6))
ax_scatter = plt.axes(rect_scatter)
ax_scatter.tick_params(direction='in', top=True, right=True)
ax_histx = plt.axes(rect_histx)
ax_histx.set_axis_off()
ax_histy = plt.axes(rect_histy)
ax_histy.set_axis_off()

# Set limits
xlim = (10, 1*10**5)
ylim = (5*10**1, 1*10**5)
ax_scatter.set_xlim(xlim)
ax_scatter.set_ylim(ylim)
ax_histx.set_xlim(xlim)
ax_histy.set_ylim(ylim)

# Make density plots
sns.kdeplot(ax=ax_scatter, data=small_data, x=x, y=y, hue=hue, hue_order=cond_
↪list,
            log_scale=True, common_norm=False,
            palette=colormap, alpha=0.7, fill=False, legend=True)

# Plot histograms
sns.kdeplot(ax=ax_histx, data=small_data, x=x, hue=hue, hue_order=cond_list,
            log_scale=True, common_norm=False,
            palette=colormap, alpha=0.1, fill=True, legend=False)
sns.kdeplot(ax=ax_histy, data=small_data, y=y, hue=hue, hue_order=cond_list,
            log_scale=True, common_norm=False,
            palette=colormap, alpha=0.1, fill=True, legend=False)
```

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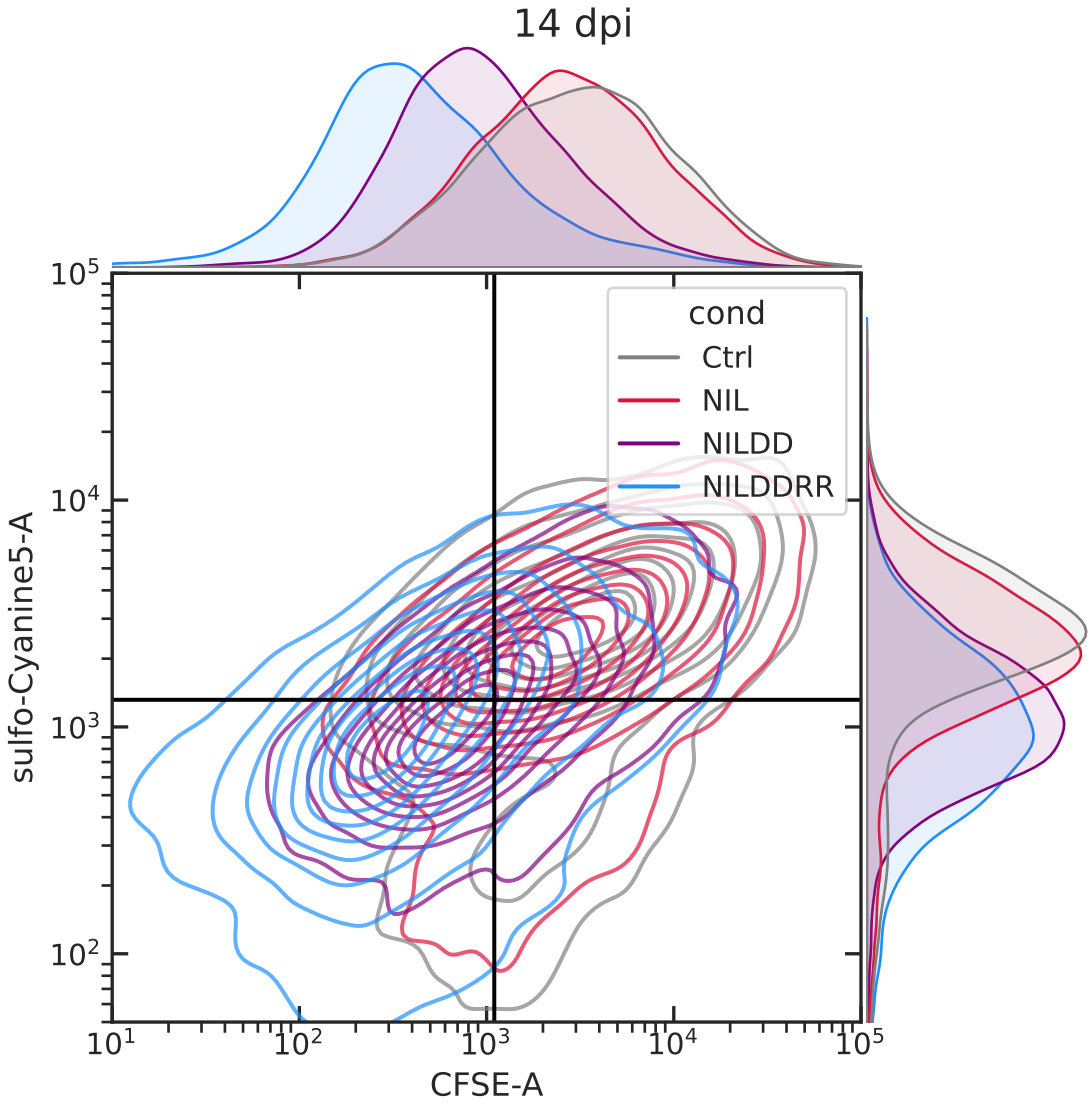
```
# Add in CFSE and EU lines
CFSE_low_thresh = np.percentile(data[(data['cond'] == 'Ctrl')]['CFSE-A'],  20)
EU_thresh = np.percentile(
    data[(data['cond'] == 'NIL') & (data['CFSE_cat'] == 'CFSE-low')]['sulfo-
↪Cyanine5-A'], 50)
ax_scatter.axvline(CFSE_low_thresh, 0, 1, color='black')
ax_scatter.axhline(EU_thresh, 0, 1, color='black')

# Title
fig.suptitle('14 dpi')
# Misc plotting stuff
fig.tight_layout()  # Helps improve white spacing
plt.show()
```



5.3 Histograms

5.3.1 Function to make subplots for several variables

```
# ----- PLOTTING FUNCTION -------

def plot_hists(df,              # DataFrame with data
               x,               # variable (str, column header of df) or list of␣
↪variables to plot
               sample_list,     # list of samples (values of column
↪'ConditionLabel') to plot in order
               name,            # short name (str) for saving the figure
               plot_title=None, # Super-title for plot (optional str)
               xlim=(1e1,1e6),  # tuple or list of tuples (optional) for x-axis␣
↪range on subplot(s)
               gates=None       # list (optional) of x-values at which to draw␣
↪vertical lines, i.e. gates
              ):

    # Specify additional parameters
    lin = set(['FSC-A','FSC-H','FSC-W','SSC-A','SSC-H','SSC-W']) # list of␣
↪variables to plot on linear scale (size-related params)
    condition_palette = pd.read_pickle('data/histogram/exp42_palette.pkl') #␣
↪custom color palette (dict mapping Condition values to colors)
    sns.set_context('talk',rc={'font.family': 'sans-serif', 'font.sans-serif':[
↪'Helvetica Neue']})

    sz = len(x) # number of subplots
    fig, axs = plt.subplots(1,sz,figsize=(sz*7,5))

    # Create each subplot
    for i in range(sz):
        g = sns.kdeplot(data=df, x=x[i], ax=axs[i],
                        hue='ConditionLabel', hue_order=sample_list,    #␣
↪samples are specified by the 'ConditionLabel' column in df
                        palette=condition_palette,
                        fill=True, alpha = 0.1,                         # shade␣
↪under the curve, but faintly
                        log_scale=(x[i] not in lin),                    #␣
↪default to log scale unless variable is in the linear list
                        legend=(i==sz-1),                               # add a␣
↪legend only to the last subplot
                        common_norm=True)                               #␣
↪supposed to normalize area under the curve (?)

        # Formatting
        sns.despine(ax=g)      # remove top and right plot border
```

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```
        g.minorticks_off()

        # Set the x-axis range based on provided list or value
        if isinstance(xlim,list): g.set_xlim(xlim[i])
        else: g.set_xlim(xlim)

        # Add a vertical line (gate) if specified
        if gates is not None: g.axvline(gates[i], c='gray', ls='--', alpha=0.5)

    # Adjust the legend to the right of the last subplot
    sns.move_legend(axs[-1], title='Condition', loc='upper left', bbox_to_
↪anchor=(1,1), frameon=False)

    # Add a super-title to the plot, if specified
    fig.suptitle(plot_title)
    fig.tight_layout()

    # [NOT APPLICABLE HERE] Save the figure as an image to the path specified by␣
↪output_path (not defined here)
    #   uses rushd outfile function to save metadata associated with the figure
    # fig.savefig(rd.outfile(output_path/('hist-'+name+'.svg')),bbox_inches='tight')

    return fig
# ----- END PLOTTING FUNCTION -------

# ----- Load data -----
labeler = pd.read_pickle('data/histogram/exp42_labeler.pkl') # dict mapping␣
↪short Condition name to long ConditionLabel
data = pd.read_csv('data/histogram/exp42_data-small.csv') # stored DataFrame of␣
↪data with metadata

# ----- Call the plotting function in a loop to generate plots with subsets of␣
↪samples -----

# Specify variables -> this will generate two subplots
x = ['mGL-A', 'mCherry-A']

# Filter data
df = data.loc[(data[x[0]]>0) & (data[x[1]]>0)] # remove cells with log-
↪unfriendly values
# Normalize number of cells in each sample (Condition) by downsampling to␣
↪smallest sample size
num_cells = df.groupby(['Condition','Replicate'])[x[0]].count().min()
df = df.groupby(['Condition']).sample(n=num_cells, random_state=1)

# Dictionary of plots (separate figures) to generate
```

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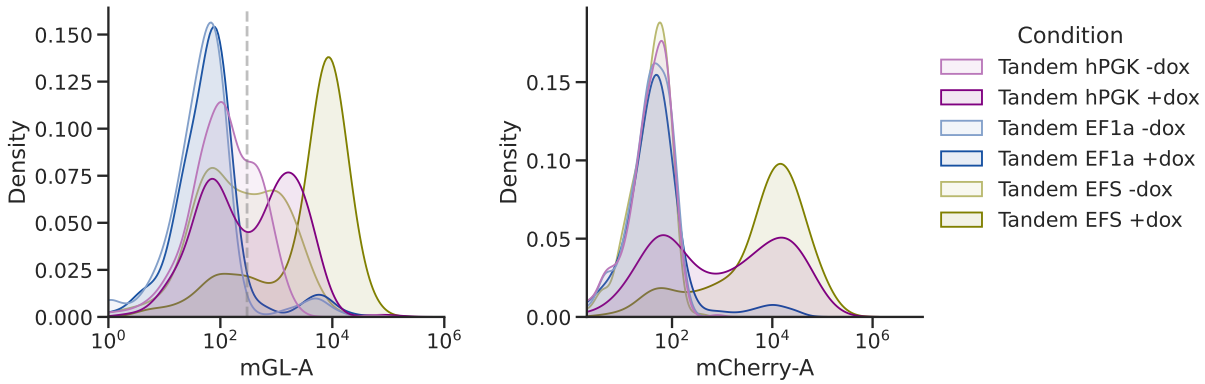
```
#  keys = short name that plots are saved under
#  values = list of samples (values in 'Condition' column) to include in the plot
plot_list = {
    'tandem': ['260_False','260_True','261_False','261_True','262_False','262_
↪True'],
    'divergent+dox': ['263_True','264_True','265_True'],
    'hPGK': ['260_False','260_True','263_False','263_True'],
}

# Loop to create each plot/figure
for name, sample_list in plot_list.items():

    # Convert short sample names above (Condition) to long names␣
↪(ConditionLabel) -> this is particular to my df organization
    sample_list = [labeler[s] for s in sample_list]
    # Identify the subset of the data to plot
    dd = df.loc[df['ConditionLabel'].isin(sample_list)]

    plot_hists(dd, x, sample_list, name,
               xlim=[(1e0,1e6),(2e0,1e7)],  # specify a different x-axis range␣
↪for each variable
               gates=[3e2,0])               # draw a gate on the first subplot␣
↪but not the second
```



5.4 Mixed distribution + mean plots

5.4.1 Gating plot

```
# Read in data
data = pd.read_csv('data/data_mGL_WPRE/data_mGL_WPRE.csv')
```

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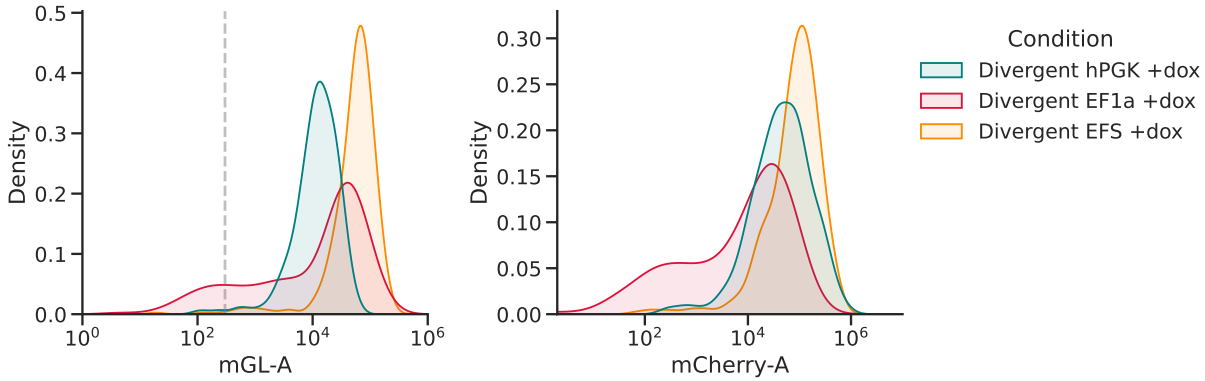
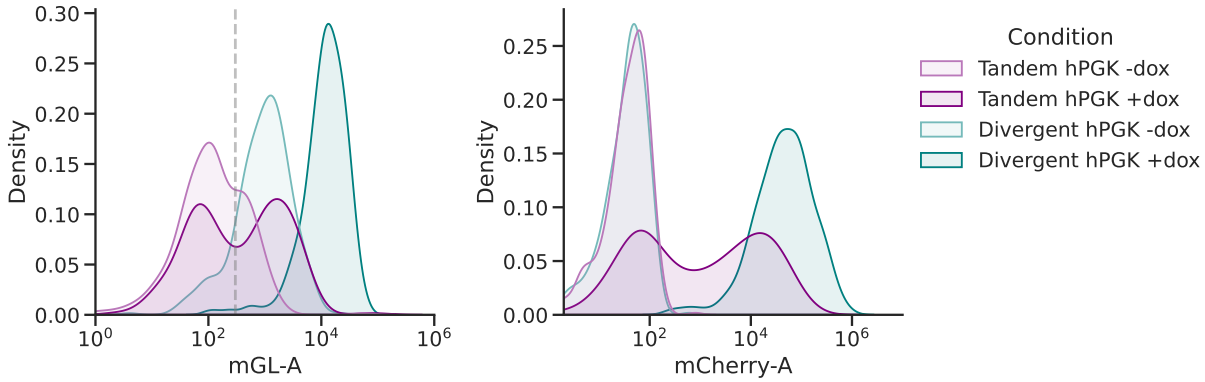

Density
mGL-A
mCherry-A
Condition
Divergent hPGK +dox
Divergent EF1a +dox
Divergent EFS +dox


Density
mGL-A
mCherry-A
Condition
Tandem hPGK -dox
Tandem hPGK +dox
Divergent hPGK -dox
Divergent hPGK +dox

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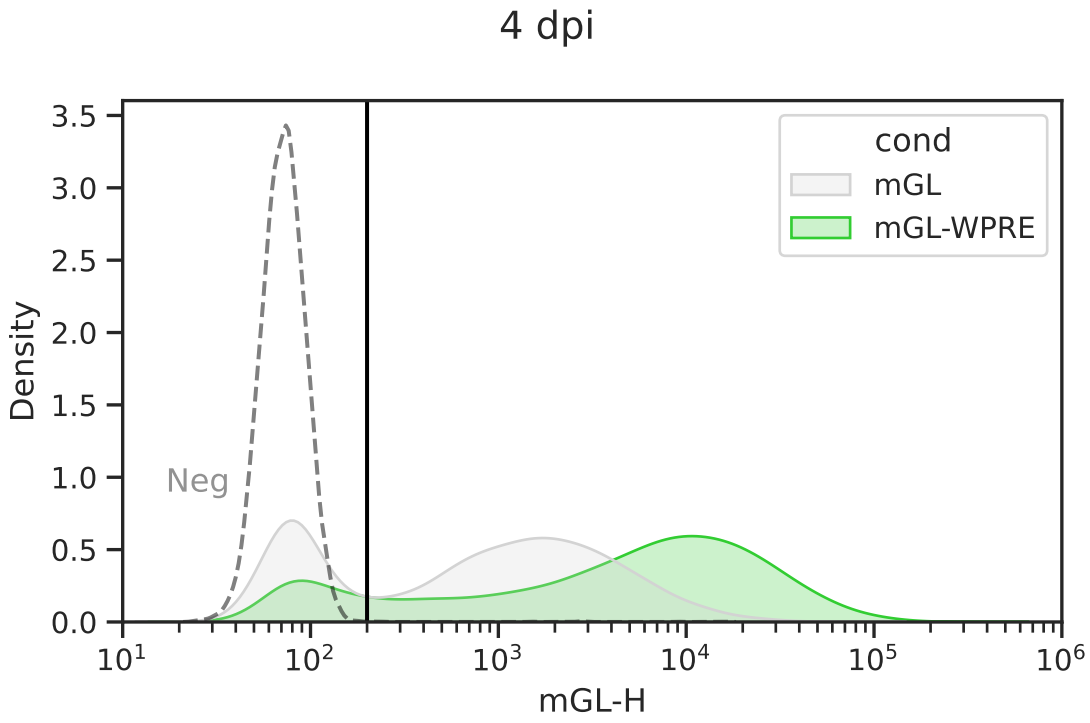
```
# Plot mGL-H
x = 'mGL-H'
hue = 'cond'
cond_list = ['mGL', 'mGL-WPRE']
colormap = {'mGL': 'lightgrey',
            'mGL-WPRE': 'limegreen'}

# Plot
fig, ax = plt.subplots(1, 1, figsize=(6, 4))
sns.kdeplot(ax=ax, data=data, x=x, hue=hue, hue_order=cond_list,
            log_scale=(True, False), common_norm=False,
            shade=True, palette=colormap)

# Plot neg ctrl
sns.kdeplot(ax=ax, data=data.loc[data['cond'] == 'Neg'], x=x,
            log_scale=(True, False), common_norm=False,
            shade=False, color='black', alpha=0.5, linestyle='--')
ax.annotate('Neg', (0.08, 0.25),
            xycoords='axes fraction', alpha=0.5, ha='center')

# Add threshold for mGL+ gating
mGL_H_thresh = 2*10**2
ax.axvline(mGL_H_thresh, 0, 1, color='black')

# Title
plt.suptitle('4 dpi')
# Adjust limits
mGL_lim = (10, 10**6)
ax.set_xlim(mGL_lim)

# Misc plotting stuff
fig.tight_layout()  # Helps improve white spacing
plt.show()
```


4 dpi
cond
mGL
mGL-WPRE
Density
Neg
mGL-H
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10^1
10^2
10^3
10^4
10^5
10^6

5.4.2 Box plot with well means

```
# Categorize if mGL+
mGL_cat = list()
for mGL_val in data['mGL-H']:
    if mGL_val > mGL_H_thresh:
        mGL_cat.append('mGL+')
    else:
        mGL_cat.append('mGL-')
data['mGL_cat'] = mGL_cat

# Get total counts and percent of mGL+ and mGL-
well_group = ['cond', 'replicate', 'sampleNum'] # specifies we're splitting by␣
↪cond >> bio rep >> tech rep >> etc...
count_df = data.groupby([*well_group, 'mGL_cat'])['mGL-H'   # Doesn't have to be␣
↪mGL-H, any column would work
    ].count().unstack(fill_value=0).stack().rename('count') # unstack()/stack()␣
↪puts 0 if no mGL-H+ rather than dropping row
percent_df = (count_df*100/count_df.groupby(well_group).transform('sum')
    ).reset_index(name='percent')

# Extract just the mGL+ cells
data_mGL = data.loc[data['mGL_cat'] == 'mGL+']
percent_df_mGL = percent_df.loc[(percent_df['mGL_cat'] == 'mGL+')]

# Calculate geom mean of mGL+ cells
well_mGL_gmean_df = data_mGL.groupby(well_group)[
    'mGL-H'].apply(scipy.stats.gmean).reset_index(name='mGL-H (gmean)')

# Plotting parameters
x = 'cond'
y = 'mGL-H'
order = ['mGL', 'mGL-WPRE']
pairs = [('mGL', 'mGL-WPRE')]
colormap = {'mGL': 'lightgrey',
            'mGL-WPRE': 'limegreen'}

# Plot
fig, ax = plt.subplots(1, 1, figsize=(3, 3))
sns.boxplot(
    ax=ax, data=data_mGL,
    x=x, y=y, order=order,
    boxprops={'facecolor': 'None'}, showfliers=False) # Gets rid of boxplot␣
↪colors and outliers
sns.stripplot(
    ax=ax, data=well_mGL_gmean_df,
    x=x, y=y+' (gmean)', order=order,
```

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```
    dodge=True, palette=colormap, size=5)

# Add in stats
annot = Annotator(ax=ax, data=well_mGL_gmean_df, x=x, y=y+' (gmean)',␣
↪pairs=pairs, order=order)
annot.configure(test='t-test_ind', text_format='star', loc='inside', verbose=2)
annot.apply_and_annotate()

# Adjust labels
plt.ticklabel_format(axis='y',style='sci',scilimits=(0,0))
plt.ylabel(y)
plt.title('4 dpi, HG')
fig.tight_layout()  # Helps improve white spacing
plt.show()
```

5.4.3 Violin plot with well means

```
# For violin plots, you must first log10 transform data
data_mGL['log({})'.format(y)] = np.log10(data_mGL[y])
well_mGL_gmean_df['log({})'.format(y+' (gmean)')] = np.log10(well_mGL_gmean_df[y+
↪' (gmean)'])

# Plot
fig, ax = plt.subplots(1, 1, figsize=(3, 3))
# Plot all points as violin
sns.violinplot(
    ax=ax, data=data_mGL,
    x=x, y='log({})'.format(y), order=order,
    palette=colormap, inner="quartile")
# Plot log10 transformed -> well geometric means of mGL-A as points
sns.stripplot(
    ax=ax, data=well_mGL_gmean_df,
    x=x, y='log({})'.format(y+' (gmean)'), order=order,
    dodge=True, color='white', size=5)

# Make log axis label:
ax.yaxis.set_major_formatter(
    mticker.StrMethodFormatter("$10^{{{x:.0f}}}$"))
ax.yaxis.set_ticks(
    [np.log10(x) for p in range(1, 7) for x in np.linspace(10**p, 10**(p+1),␣
↪10)],
    minor=True);

# Add in stats
annot = Annotator(ax=ax, data=well_mGL_gmean_df, x=x, y=y+' (gmean)',␣
↪pairs=pairs, order=order)
annot.configure(test='t-test_ind', text_format='star', loc='inside', verbose=2)
annot.apply_test().annotate(line_offset_to_group=0.3) # Offset helps account for␣
↪height of violin

# Adjust labels
plt.ylabel(y)
plt.title('4 dpi, HG')
fig.tight_layout()  # Helps improve white spacing
plt.show()
```

5.5 Multiple-axes plots

```
# -------- EXAMPLE DATA GENERATION --------------------
# Generate example data. Each condition will
# be a differently-sized and placed bivariate normal
```

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```
# distribution.
np_rand = np.random.RandomState(seed=2022)
example_data = np.vstack((
    np_rand.multivariate_normal( # A
        [5, 15],[[1,0.2], [0,3]], size=(1000,)),
    np_rand.multivariate_normal( # B
        [15, 5], [[3,0], [0.2,1]], size=(1000,)),
    np_rand.multivariate_normal( # C
        [5, 5], [[1,0], [0,1]], size=(1000,)),
    np_rand.multivariate_normal( # D
        [15, 15], [[1,1], [0.2,-0.2]], size=(1000,)),
))
df = pd.DataFrame({
   'condition': (
        (['A'] * 1000) + (['B'] * 1000) +
        (['C'] * 1000) + (['D'] * 1000)
    ),
   'x': example_data[:,0],
   'y': example_data[:,1]
})
# -------- END EXAMPLE DATA GENERATION ---------------

# Specify a color palette
beach_towel = {
'A': '#225A9B',
'B': '#19D2BF',
'C': '#FFB133',
'D': '#FE484E',
}

# Create a multi-plot subplot layout, where we specify
# a one-row, two-column set of plots, where the first
# axis takes five times the space as the right axis.
_, axes = plt.subplots(
                ncols=2,
                sharey=False,
                gridspec_kw={'width_ratios': [5,1]})
# Use Seaborn to plot the joint distributions.
sns.kdeplot(data=df, x='x', y='y',
            hue='condition', palette=beach_towel,
            levels=8,
            ax=axes[0], legend=None)
# For each condition, calculate intrinsic and extrinsic noise
for condition in df.condition.unique():
    # subset the dataframe by condition
    df_subset = df[df.condition == condition]
```

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```
    extrinsic_noise = np.var((df_subset.x + df_subset.y) / 2.0)
    intrinsic_noise = np.var((df_subset.x - df_subset.y) / 2.0)
    # use matplotlib to plot circles for each condition.
    # Jitter the x-axis locations so the dots don't overlap.
    axes[1].plot(
        np.random.uniform(-0.1,0.1),
        intrinsic_noise / extrinsic_noise,
        'o',
        color=beach_towel[condition])
# Modify axis titles
axes[0].set_title('Joint distribution')
axes[1].set_title('Noise\nratio', loc='left')
# Set uniform x and ytick labels
axes[0].set_xticks([5,10,15,20])
axes[0].set_yticks([5,10,15,20])
# Despine both axes, also removing the bottom spine
# on the right axis.
sns.despine(ax=axes[0])
sns.despine(ax=axes[1], bottom=True)
# Hide the right-most x axis.
axes[1].xaxis.set_visible(False)
# Scale the axis so our jittered points are visible.
axes[1].set_xlim([-0.3, 0.3])
```

5.6 Testing matplotlib directives

5.6.1 Subheading

```
df = pd.read_csv('data/test_data.csv')
sns.scatterplot(data=df, x='x', y='y')
sns.despine()
plt.show()
```

5.7 Notes on reproducing examples

All plots in the plot gallery have a set of implicit imports and setup code that runs before any given code examples:

```
import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
import scipy
import seaborn as sns
from matplotlib import ticker as mticker
```

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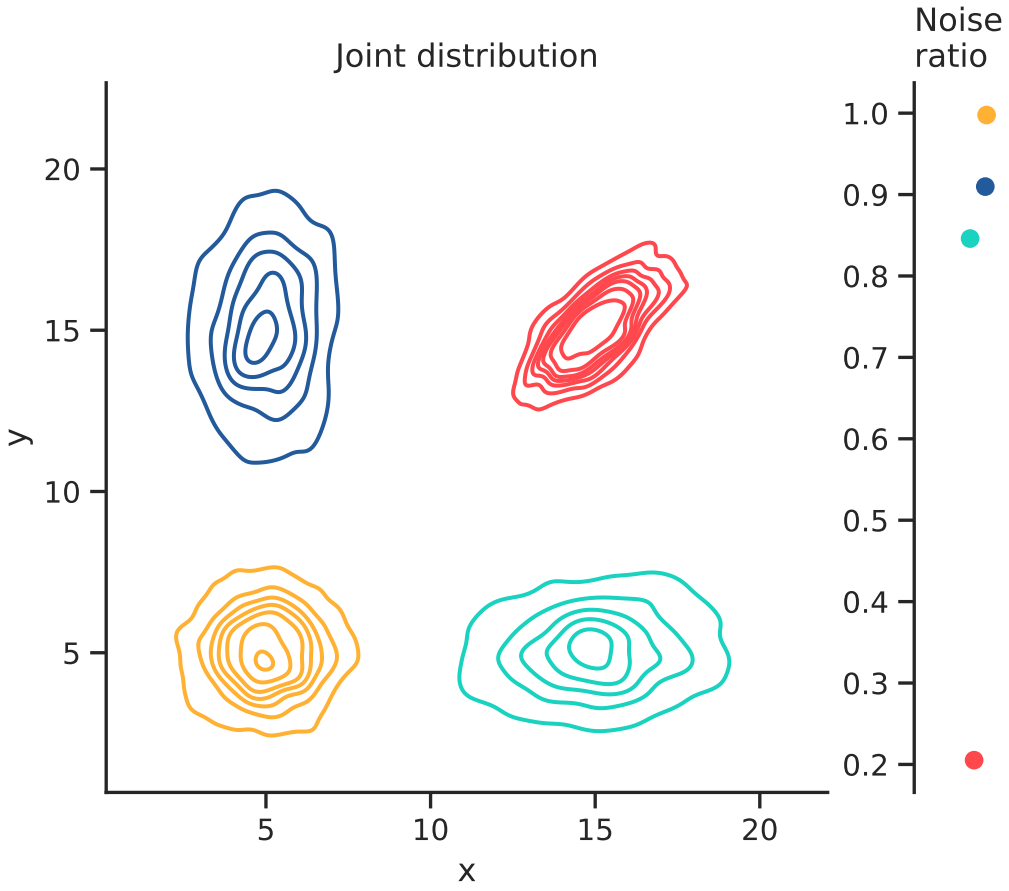
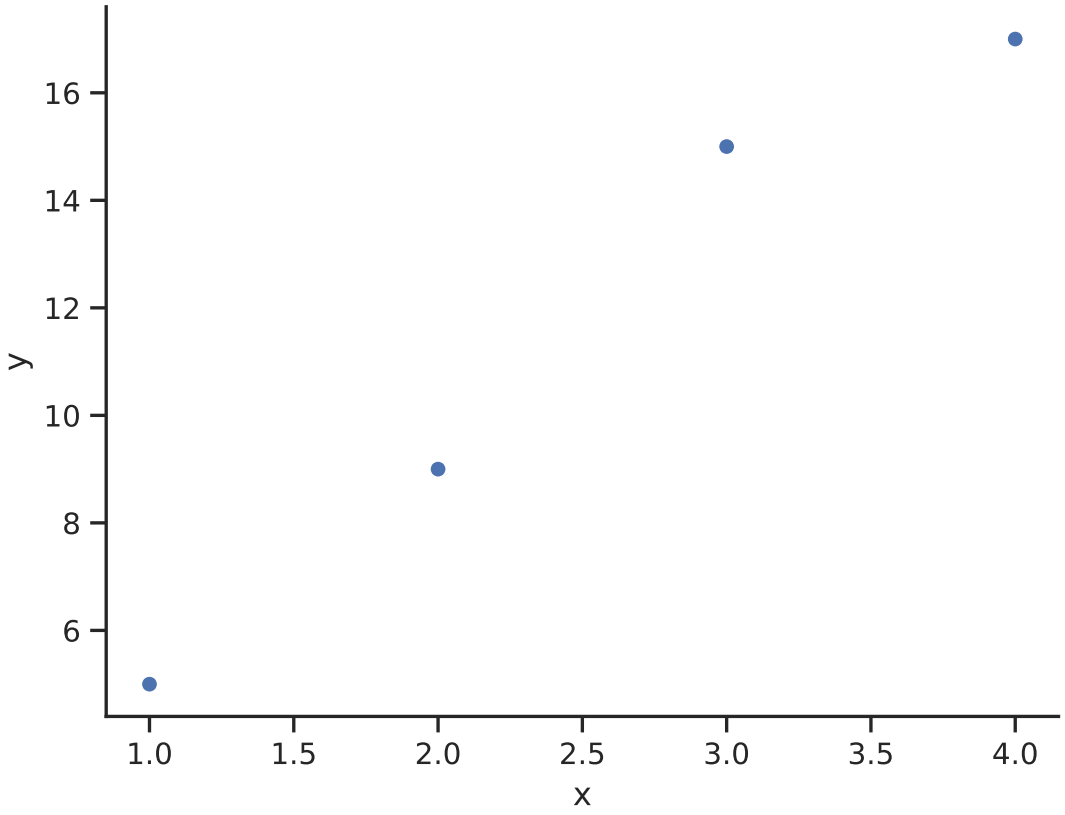

Joint distribution
Noise ratio
y
x
20
15
10
5
5
10
15
20
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2


16
14
12
10
8
6
y
1.0
1.5
2.0
2.5
3.0
3.5
4.0
x

(continued from previous page)

```
sns.set_theme(style="ticks")
```

If you are reproducing the code on your own, make sure that these lines have been run!

All example data needed is available as a zip file: plot_gallery_data.zip.

CHAPTER

SIX

WRITING GUIDE

The goal of this writing guide is to make writing more straightforward.

6.1 Additional resources

Resouces for writing inspiration and greater detail!

6.1.1 Big picture resources

Below are great resources on how to write papers. If you read through them, you will start to notice themes. Those themes are represented in the paragraph above. You might find more details and specific approaches in these documents useful.

- How to write a paper by George Whitesides[213]
- Suo group paper template by Zhigang Suo[214]
- How to write a paper by Dave Weitz[215]
- Ten simple rules for structuring papers by Konrad Kording[216]
- Thread on how to write a paper by Marcos Morgan[217]

[213] https://intra.ece.ucr.edu/~rlake/Whitesides_writing_res_paper.pdf
[214] https://docs.google.com/document/d/1a3ElMO7XzBgdkg2k6z7wsHm8zzbVYAycq8Tt5PzXtQk/edit
[215] https://projects.iq.harvard.edu/files/weitzlab/files/131.5_weitzlab_guide_to_good_paper_writing_10-2012.pdf
[216] https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005619
[217] https://threadreaderapp.com/thread/1581853226001125376.html

6.1.2 Getting into the finer details

Details matter! If you want to become more precise and persuasive you need to learn how to use language to build clear and compelling narratives.

- The science of science writing: how to structure sentences[218]
- Words to avoid from Arjun Raj[219]
- Figure scripting from Raj lab[220]

6.2 Points of style

6.2.1 Specific and nonspecific subjects

As much as possible, use specific subjects to replace nonspecific subjects such as "this." It makes the reader work harder when you use nonspecific subjects. Often when you revise, you should remove them. Including specific subjects will make the writing more powerful.

For example:

Original: We aim to build efficient delivery methods for genetic cargoes. This requires...

Revised: We aim to build efficient delivery methods for genetic cargoes. Efficient delivery of genetic cargoes requires...

If you remove nonspecific subjects, you may be able to eliminate or restructure some of the preceding text. Do we need to state our aim in the preceding sentence? Possibly or perhaps it is understood from the either the sentence prior or following text. Generally, we can assume if we are writing about the topic then it is an important and meaningful subject and the real task is simply explaining why and what we aim to do to tackle the challenges. Note: It is normal for first drafts to include nonspecific subjects. In first drafts, we are aiming to get ideas out. In revising, we are looking to make our ideas clear and concise.

Revised-compact: Efficient delivery of genetic cargoes requires...

[218] https://www.americanscientist.org/blog/the-long-view/the-science-of-scientific-writing
[219] https://docs.google.com/document/d/1r6nDcF43esu3xBjmk3ERAmaEHKEB75_HflSkk3zZhBk/edit
[220] http://rajlaboratory.blogspot.com/2017/08/figure-scripting-and-how-we-organize.html

6.2.2 Hyphenation

Compound nouns that function as adjectives should be hyphenated. For example: Cell-state promoter is hyphenated because it's an adjective... but when using cell state as a noun (as in "wow, look at that cell state!") it is not hyphenated

6.2.3 Numbers in text

Numerals should be used for numbers 10 and above, but numbers nine and below should be spelled out.

6.2.4 How to write gene and protein names?

TL;DR: Genes are italicized. Human genes are all caps, Mice are first cap only, Mammalian proteins are always all caps. Reference: https://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx

6.2.5 References

You can use different reference styles. If you're submitting a paper, you should check which format is preferred. In general, if you use the box format like Science does the box reference is inside the period as shown here [2-4]. If you use name format like the Cell journals, here is the style (Last et al., 2012). If you want the foot-note like style, it's outside the period like this.2-4

6.3 Guiding principles

1. Outline each section first. See below.
2. If you are writing a paper, the claims structure the outline. Outlines accelerate our ability to identify deficits in logical clarity are best. The experiments are the evidence that support the claims.
3. Consistency is key. Style choices must be made but the same ones should be made throughout a single document and ideally throughout a single lab. Clarity trumps style.
4. When in doubt, turn it into two simpler sentences. See editing/revising.
5. Find examples of writing that you like for the type of manuscript that you are writing and work from those. You can reverse engineer good writing.

6.3.1 Tips

6.3.2 Outline

Always, always, always outline your work first. If the logical flow sucks, the words do not matter. The structure must be solid. Do not waste your time getting a flimsy structure dressed up with fancy words, syntax, etc. Get the structure correct and then polish a strong framing.

6.3.3 Active Voice

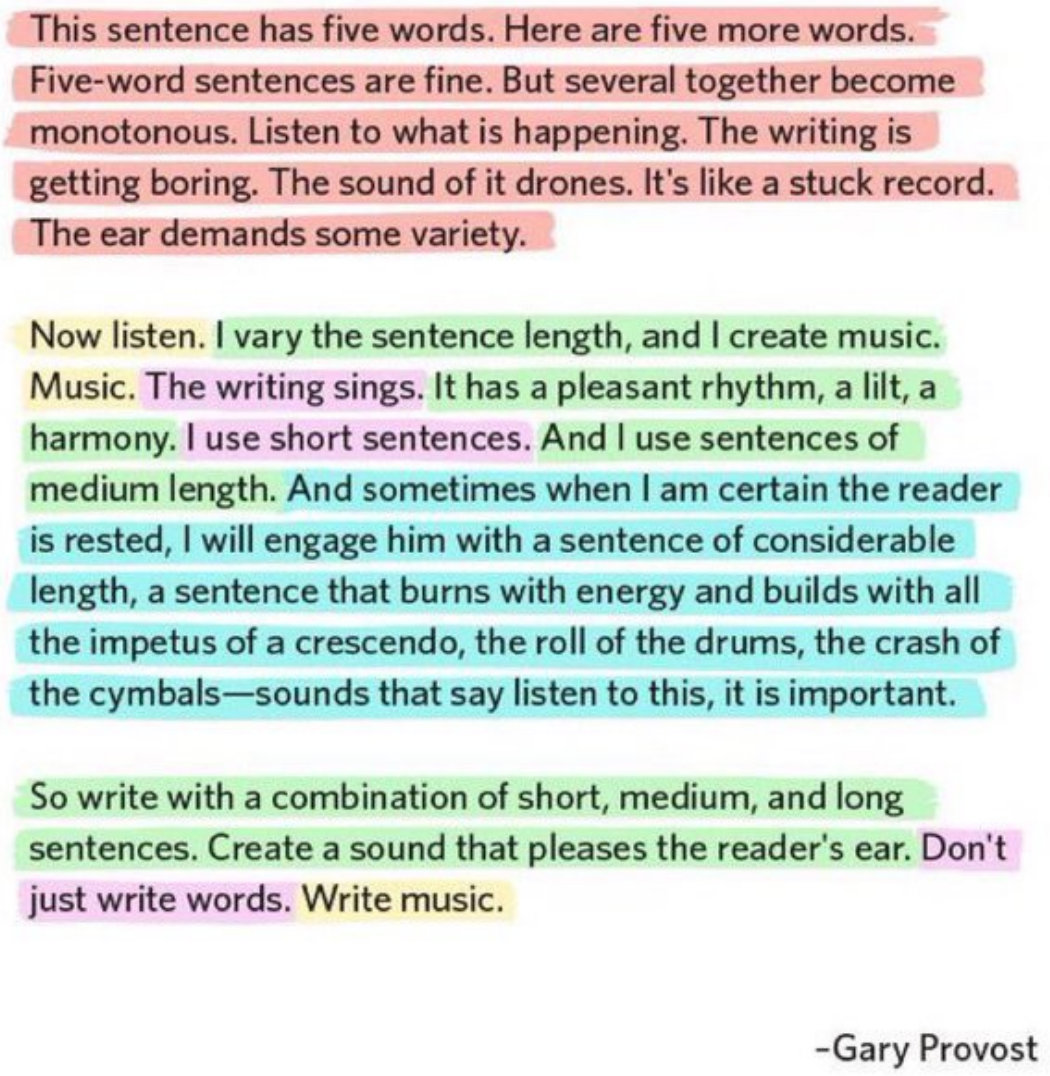
Write in active voice as much as possible! Your writing will be more comprehensible and often more compact. You can delete every instance of "has been shown to" to switch to active voice.

For example:

- **Passive**: This writing tip has been shown to work.
- **Active**: This writing tip works.

6.3.4 Editing and revising

When you start, the main goal should be to just make coherent thoughts. Through the process of revising, you can later reshape those coherent thoughts into beautiful writing which is more akin to "music."



6.4 What is a paper?

A paper is **NOT** a summary of your work. A paper is **a series of claims that support one big message or claim**. The subclaims should generally break down around figure. Each figure supports one subclaim figure to build a coherent narrative. Putatuively, your reviewers and readers are looking at the logical flow and support of your claims. Thus, to efficiently write an interesting and

engaging paper, you will not write linearly with respect to time or series of experiments. You write the paper to most clearly and robustly support your main message.

Determining how to most efficiently and effectively structure a paper requires iteration. You are unlikely to start with the optimal structure because you should NOT start when you have all the data. As you collect data, you will need to revise your paper to best integrate that new information into the bigger message. New data may also entirely redirect the main message. This is the normal correction that science allows. Writing is iterative, so if you don't start somewhere you will never get anyway. **Start early, revise often, iterate.**

Estimated time

hours, days, weeks, months, years...

CHAPTER

SEVEN

TRAINING

7.1 Onboarding

New to the Galloway lab? Start here!

Before your first day in lab, you can get started setting up important software. See *Day 0: Software and training setup* (page **??**) and *Software tips and tricks* (page **??**) in particular. Once you've installed everything, you may also wish to learn (or refresh your memory) on *Git* (page **??**) and *terminals* (page **??**), and test your knowledge by confirming that you can *set up a computational environment* (page **??**). If this is confusing, don't worry! We can help you do these steps when you arrive in person.

On your first day, follow the instructions at *Day 1: First day in lab* (page **??**). Ask a lab member (or your assigned mentor) to help you complete the onboarding form and show you around the lab. Connect with the current EHS rep to schedule your lab-specific safety training (required before you start lab work).

As you get settled in lab, you may want to peruse this protocol site and the lab SharePoint[221] (requires access). There's a lot of interesting info there! For instance, at *Day 2+: Content-specific trainings* (page **??**), you'll find written resources from past trainings on a variety of lab techniques. Elsewhere on this site, you'll find detailed protocols for performing them in lab. Tip: If you ever need help doing something, search the protocols page—there might be instructions already written up (e.g., for *ordering* (page **??**), *using the Keyence* (page **??**), *culturing iPSCs* (page **??**), *adding to this site* (page **??**), and more)! On the SharePoint, you'll find recent presentations in the `Group_Meeting` folder, and you can create a folder for your project (for plasmids, experiment calculations, graphics, etc.) in the `projects` folder.

Feel free to ask your new labmates questions—we're all happy to help you get started. :)

7.1.1 Day 0: Software and training setup

Our lab uses a various software and webservices for digital infrastructure and organization. Before beginning work in the lab, you must complete all required Environment, Health & Safety (EHS) trainings. New grad students and postdocs should also install or set up accounts for everything listed here; others should ask their mentor which are essential.

[221] https://mitprod.sharepoint.com/sites/GallowayLab/Shared%20Documents/Forms/AllItems.aspx?sortField=LinkFilename&isAscending=true

Check off each task as you complete it on the **lab Onboarding Form** (download the one for `grad students/postdocs` or `undergrads/visiting students`). The most recent forms are also located in this folder[222] in the lab SharePoint.

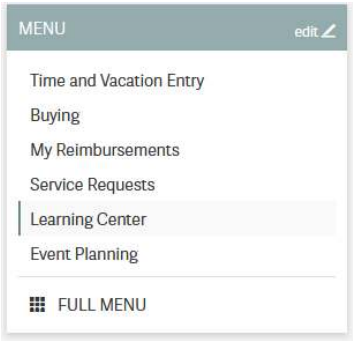
Important

Most of the software and webservices **require an MIT ID/email**. You should prioritize getting this set up. However, if you are waiting on paperwork, you can download most of the software (even if you won't be able to log in yet). After that, you may wish to read through the *Day 1* (page **??**), *Day 2* (page **??**), and linked pages on the protocols site.

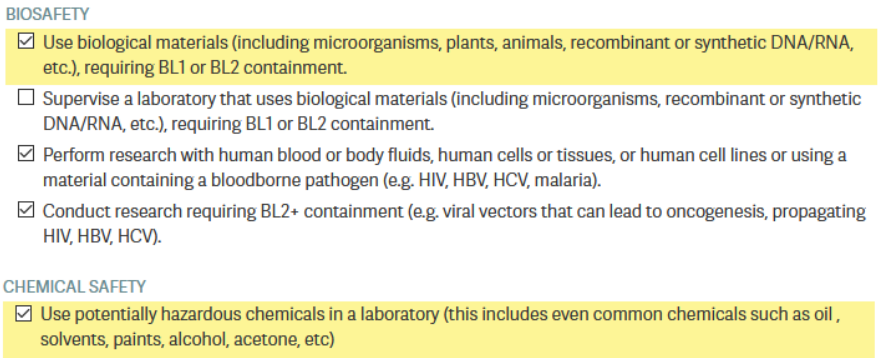
EHS setup and trainings

Adding yourself to the lab's training group will register you with the EHS system and add all necessary trainings to your profile.

1. Go to https://atlas.mit.edu and go to the learning center, through the tab on the left:



2. In the upper right, select "My Profile", then "Update PI/Activities".
3. Add Kate E. Galloway as your PI.
4. Select the following training types (5 total). If you are an undergrad, do not select the BL2+ training group.



[222] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgDFT2QaHvFyRK8T-mimny_OAWjTpmM18Te5DVKyJORWO10?e=KXd2iO

SPECIALIZED SAFETY

- ☐ Work in a confined space(s) such as tanks, manholes, boilers, or shafts
- ☐ Supervise those who work in a confined space such as tanks, manholes, boilers, or shafts
- ☐ Work or supervise those that work from scaffolding, staging, lifts, powered platforms, or from any work location that presents an unprotected fall hazard of four feet or more.
- ☐ Supervise those who weld, cut or solder with a torch, braze, or grind. Supervise a designated hot works area
- ☐ Operate cranes or hoists
- ☐ Operate a forklift, battery powered pallet jack, or other material handling equipment
- ☐ Involved in planning construction or renovation activities in you department, lab, or center
- ☐ Climb a tower
- ☐ Supervise those who climb towers
- ☑ Work with cryogenic liquids (recommended training unless your DLC requires it)

5. After submitting, many required trainings will be added to your Learning Center.
    - Complete the online trainings. This will likely take a few hours!
    - Some trainings have a required "classroom" component, such as the *Lab Specific Chemical Hygiene* training, which will be completed with an in-lab walkthrough.
    - To complete the *Signature: Read Dept. Chemical Hygiene Plan* training, please read the Chemical Hygiene Plan and then sign the attestation form, available at: https://web.mit.edu/cheme/resources/lab/ehs/ehs_cert.html

> **Note**
>
> One of the components of the bloodborne pathogen training is the opportunity to be vaccinated for Hepatitis B or to have an antibody titer test for free.
>
> Most of us were vaccinated for HepB as children, but that vaccine was only ~90% effective, so you may want to get the free antibody titer test. You can get a free booster or get doses of a new, more modern HepB vaccine if you no longer have HepB antibodies.

6. (*Grad students and postdocs only*) Also add and complete the following:

- Autoclave Safety Training[223], required for access to the autoclave/ice room.
- Shipping Training[224] (*new as of June 2026*), required for shipping any materials on behalf of MIT.
- If you are going to be helping with mouse work, in the "My Profile" tab under "Training Groups" click "Join Another Group" and add the **68N: Mouse** training group. Complete the additional trainings. Note that some of these require in-person trainings in the mouse facilities, which can be completed over the next few months.

[223] http://web.mit.edu/training/course.html?course=EHS00254w&sys=PS1
[224] https://us.list-manage.com/x50viiGiYi6?e=3aa52f1b11&c2id=e3a0eacb0edf1dd8510d3cfd3e0194a2

Software and webservices

Core webservices

- Create a Github account[225]. You can use either a personal or MIT email.
  - Activate your student benefits by going to https://education.github.com/discount_requests/student_application. You will be asked to connect your MIT email and send in a picture of your student ID. (Because MIT does not remove emails for alums, they need to confirm active student status.)
- Create a Zotero account[226]. Using a personal email is recommended for permanence reasons.
- Create an ORCID[227]. Adding all of your active emails is recommended.
- Request an MIT Google Workspace[228] account. This provides access to Google services like Drive, Docs, Calendar, etc. Note that it may take 24 hours to activate. Alternatively, you can use a personal Google Account for access to a lab calendar.
- (*New grad students and postdocs only*) Create a Quartzy account[229]. Using your MIT email is recommended.

After creating these accounts, request access to the lab's group on the relevant webservices. Most of these are managed by one or two lab members; see the onboarding form for who to contact. Message them with the following information: your **Kerberos ID (MIT email)**, your **Github username**, your **Zotero username**, the email you'd like added to the lab **Google Calendar**, and the email associated with your **Quartzy** account.

Then, you must accept the Github invitation to the GallowayLabMIT organization[230] and the Zotero invitation to the `gallowaylab` group, checking that it appears in your group list[231].

Shared storage

We use two file storage services in the lab: **OneDrive / SharePoint** for "small" files like documents, plasmids, primers, posters, and so on, and **Smithsonian / Nextcloud** for data files (microscopy images, flow data, NGS data). We used to use OneDrive for everything, but OneDrive has a 5-TB storage limit, which we reached after seven years.

Luckily, you login to **both** Smithsonian and OneDrive through Touchstone, so you don't need separate accounts.

[225] https://github.com/
[226] https://www.zotero.org/user/register
[227] https://orcid.org/register
[228] https://ist.mit.edu/g-suite/request
[229] https://www.quartzy.com/
[230] https://github.com/gallowaylabmit
[231] https://www.zotero.org/groups/

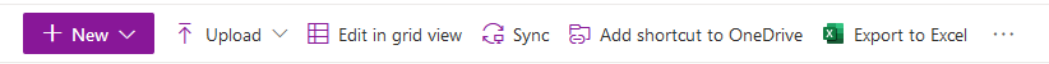
(1) OneDrive / SharePoint

OneDrive is Microsoft's file syncing service, and SharePoint is the version for teams (we use these terms interchangeably). The web interface of OneDrive is slightly clunky, and the official file syncing client is, honestly, not great. There are sync delays and sometimes things do not update. However, OneDrive has tight integration with Office products, allowing Google Drive-esque live, multi-person editing of Office documents saved within it. This is the largest reason why we still use OneDrive.

OneDrive uses "files-on-demand"/"online sync", where all files in the shared storage *appear* to be accessible, but do not actually take up local disk space until you open them/unless you manually trigger a download, at which point the software invisibly downloads files in the background. There's no cost to having the entire shared folder locally synced. Additionally, you can override this behavior and request that OneDrive download files before you access them (normally via a right-click menu)

After being given access:

1. If you are not on a recent version of Windows, download the OneDrive client[232]. Recent versions of Windows come with this preinstalled.
2. Bookmark the web version here: https://mitprod.sharepoint.com/sites/GallowayLab/Shared%20Documents
3. On the web version, select the **Sync** button in the top tab:



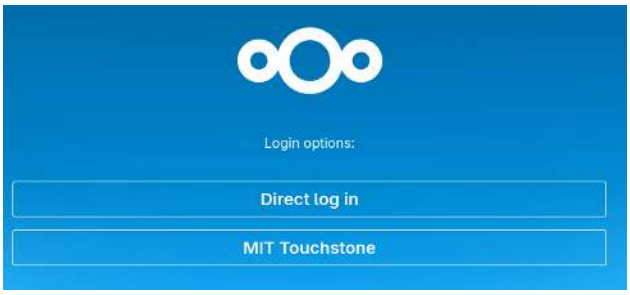
4. This will trigger the OneDrive software you installed. It will ask you for a local folder to sync into (the default location is usually fine). After several minutes, it will show "OneDrive is up to date", and all files should be accessible.

(2) Smithsonian

Smithsonian is the name of our data storage server that lives in the lab. We run this server ourselves, and it (currently) has a much larger capacity than OneDrive / Google Drive / MIT Dropbox: nearly 45 TB. Data stored on here is also backed up to an MIT-run backup system called Spectrum Protect / TSM. (If you're curious how this works, see *the tech documentation* (page **??**).) Our storage server is running software called Nextcloud Server. Unlike other cloud-syncing services, Nextcloud (the mostly open-source organization) does not run servers themselves, so our instance of Nextcloud is accessible at `smithsonian.mit.edu`.

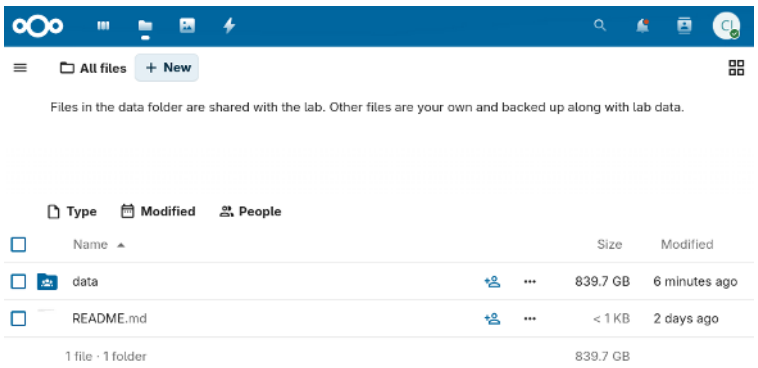
Like OneDrive, there is both a web interface to quickly browse files, and a local sync client that you can download that lets you access the files. The Nextcloud sync client also does the same virtual-file / "files-on-demand" that OneDrive does. You can access the **web interface** at https://smithsonian.mit.edu. You will see a login page that looks like this:

[232] https://www.microsoft.com/en-us/microsoft-365/onedrive/download



The "Direct login" option is only used for special accounts that are not attached to a person, namely, the administrator account and the account that the lab computers use. Both of these account details are in the password database and are accessed as described in *the tech documentation* (page **??**).

To login, use the MIT Touchstone option, which will redirect you through Touchstone and eventually land you on the files page:



Lab computers automatically save data into the `data` folder and are automatically shared with everyone. Other files and folders you create within your account are not shared with the lab by default (but are backed up and accessible with the administrator account).

To setup the **local sync client**, you need to download the Nextcloud client software and point it at Smithsonian.

1. Download the appropriate version of the Nextcloud Files app for your computer from the Nextcloud site[233].
2. Install the software.
3. Launch the software. It will ask you what server to connect to. Type in `smithsonian.mit.edu`
4. A web browser should open showing the Smithsonian login page. Login with Touchstone. You will reach a "grant access" page to allow sync access for this computer.
5. After granting access, return to the sync client. If it asks you to pick a location for the local sync folder, pick anything convenient. On macOS, it will appear in the default location: `/Users/[your-user]/Library/CloudStorage/`.

[233] https://nextcloud.com/install/#desktop-files

Coding and collaboration

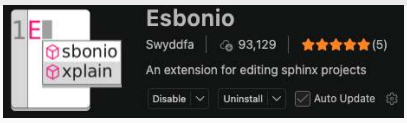
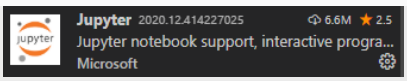
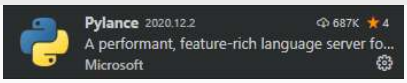
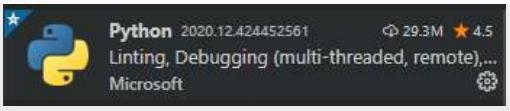
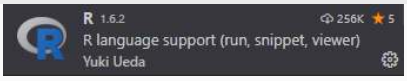
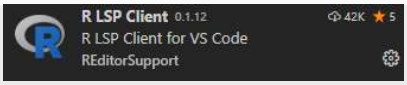
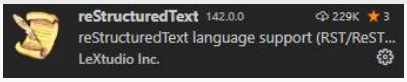
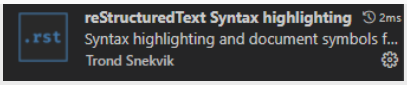
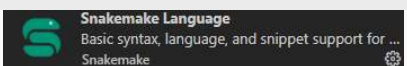
- **Slack** is how we communicate! After downloading it[234], sign into https://gallowaylab.slack.com. In addition to the default channels, you may want to join `#sequencing` to get your sequencing orders delivered right to you via Slack, and join `#memes` for obvious reasons. Ask your mentor or point of contact to add you to any other relevant private channels.
- **VS Code:** Having a good *plain-text editor* (not Word) is important for coding, and is ultimately up to personal taste. We recommend Visual Studio Code (VS Code), downloadable here[235]. However, if you have a different favorite editor, you may use that. If you are used to language-specific IDEs like MATLAB, IDLE, or RStudio, VS Code allows you to do editing, debugging, previewing, source control, etc in a mostly language-agnostic manner; once you customize it to your preferences, you can use it for all of your coding.

  After installing, you should click the extensions button: 

  and search and install the following extensions (type in the name, click the install button).

[234] https://slack.com/downloads
[235] https://code.visualstudio.com/

Table 1: Recommended VS Code extensions

Name	Image	Description
Code Spell Checker		Inline spell checker that is intelligent enough to not flag specific language-specific words, but still can spell check comments and variable names.
Esbonio		Support for editing Sphinx projects, e.g., this protocols site. The live preview function is super helpful!
Jupyter		Inline Jupyter notebook support. No more need to launch Jupyter in a web browser, just do it inside VS Code!
Pylance		Faster 'language server' for Python, which means the IntelliSense is faster and more accurate.
Python		Enables Python debugging, running, and IntelliSense (in-line help while typing).
R (*optional*)		Base language support for R.
R LSP Client (*optional*)		The VS Code side of the R language server. Before installing this, run `install.packages("languageserver")` inside an R prompt.
reStructuredText		Enables reStructuredText support, the language used to write this documentation, among others.
reStructuredText Syntax highlighting		Enables syntax highlighting for reStructuredText.
Snakemake		Snakemake syntax highlighting for

- **Git:** For any code/code-like files (LaTeX, other plain-text files), Git is the standard way to share and collaborate with others and to track version history.

  You must install the base command-line tools from here[236]. Select your operating system and not the "Download source code" button. For macOS, the easiest way is probably the "Xcode Command Line Tools" option.

  > **Tip**
  >
  > When installing Git, you may want to change Git's default editor to something other than Vim, such as VS Code.
  >
  > When asked about adjusting the PATH environment, choose the **Git from the command line and also from 3rd-party software** option; this makes sure all the other software also has Git access. All other defaults are fine, but can be changed if you want.

  After installation, you should set your global identity on that computer, i.e., the name and email that gets stored alongside the work you do. To do so, open a terminal (Terminal on macOS, Powershell on Windows) and type the following lines (without the beginning `$`, which identifies here that we are typing this into a terminal), substituting your name and email (giving an email you associated with your Github account). If you're not familiar with the terminal, check out our intro *here* (page **??**).

  ```
  $ git config --global user.name "Full Name"
  $ git config --global user.email email_address@example.com
  ```

  For a comprehensive introduction to Git, check out our intro *here* (page **??**) or this tutorial[237] from Git.

- *(Optional)* **Github Desktop:** This program is a good basic GUI Git tool, in case the command line interface or built-in editor interfaces aren't for you. Download it here[238].

- **Python:** Python is an excellent "Jack of all trades" language; we use it extensively. If you are on macOS, you may have Python3 pre-installed; you can check by typing `python3` at a terminal. If you do not have Python preinstalled, you should download it here[239]. Click the latest version download from the top, then scroll down and select the 64-bit installer for your OS.

  When installing, select **Add Python to PATH**; this ensures that when you type `python` at a terminal, you get this version you just installed. Other software can also access this "default" installation. After installing, restart VS Code.

[236] https://git-scm.com/downloads
[237] https://git-scm.com/book/en/v2
[238] https://desktop.github.com/
[239] https://www.python.org/downloads/

What is PATH?

`PATH` is a "environment variable", i.e., something that any program running in the "environment" of your computer can access. It is a list of folders where software can be found. In a command line, when you type a program name (like `ls`, or `python`, or `git`) without specifying where the program is, your computer iterates through every folder in `PATH` to see if it can find the program there.

Bonus fact: virtual environments work by temporarily messing with `PATH`, redirecting calls to programs like Python to the virtual environment install.

On snakes and Anaconda

If you have Anaconda installed and don't have an explicit reason to need it (e.g., conda-only packages), it is recommended to uninstall Anaconda and install Python directly this way.

With modern Python, the benefits that Anaconda initially brought to the field (virtual environments and pre-compiled packages) are now integrated into the normal Python ecosystem, making Anaconda unnecessary. We also don't want multiple Python versions competing.

To make sure the install worked, open a new terminal and type `python` (or `python3` on macOS), checking that the output looks similar to the following. Then exit the Python prompt by typing `exit()`.

```
$ python
Python 3.9.1 (tags/v3.9.1) [MSC v.1916 64 bit (AMD64)] on win32
Type "help", "copyright", "credits" or "license" for more information.
```

Fixing Python "command not found" (Windows & macOS)

If you see errors like

- `'python' is not recognized as an internal or external command`
- `command not found: python`

then you likely forgot do the above step (clicking Add Python to PATH), or you didn't restart VS Code. The easiest way to fix this is to simply **uninstall Python and reinstall it**, while clicking the box. If you don't want to do that for some reason, you can manually add Python to PATH.

Windows

You need to find where Python is installed. This will vary! The easiest way to do this is just search for `python.exe` to locate where that folder is. This might look something like

```
C:\Users\<USERNAME>\AppData\Local\Python\python-3.14\
python.exe
```

Then:

1. Press the Windows key on your keyboard to bring up the search.
2. Search for *Edit environment variables*
3. In the box that shows up, click *Edit the system environment variables*.
4. Click *Environment Variables*.
5. In the **User variables for <USERNAME>** box, find the **Path** variable and click *Edit*.
6. In the list of directories that shows up, click *New* and add the folder containing Python identified earlier.
7. Click OK and **restart any open terminals and VS Code to pick up the change**. (If you're unsure, log out and log back in to your computer.)

macOS / Linux

On macOS and Linux, you handle the path by editing your shell configuration file, normally either at `~/.zshrc` for zsh or `~/.bashrc` for Bash.

In these lines, you should add an export call to add the Python location to the end of path, like:

```
export PATH="$PATH:/path/to/python/that/you/found
```

- *(Optional)* **R:** Many bioinformatics tools are written in R, but there are also many good Python versions. You can install this now, or wait to see if you need it later. From here[240], download the main package (macOS) or both the `base` entry and the `Rtools` entry (Windows).
- *(Optional)* **RStudio:** If you don't feel like using VS Code for your R work, the excellent, well-polished standard IDE is RStudio Desktop, downloadable here[241].

Experimental software

- **SnapGene:** We use SnapGene for molecular cloning and plasmid design. Download it through MIT IST here[242], and access the registration code here[243] (MIT login required for both links).
- **FlowJo:** We have a single license on lab computers for analyzing flow cytometry data; we can show you how it works in-lab.
- *(Optional)* **FIJI:** For simple image analysis, Fiji (ImageJ) gives a nice GUI interface. Download it from https://fiji.sc

[240] http://lib.stat.cmu.edu/R/CRAN/
[241] https://rstudio.com/products/rstudio/download/#download
[242] https://downloads.mit.edu/released/snapgene/vendor-registration.html
[243] http://downloads.mit.edu/released/snapgene/group-name_registration-code.txt

- *(Optional)* **CellProfiler:** CellProfiler is an excellent tool for doing image cytometry (analyzing cell-by-cell in image data). In contrast to the GUI-only tools built into the Keyence software, CellProfiler enables repeatable, pipelinable analyses. Download it from https://cellprofiler.org/

Other

- **Zotero:** Zotero is an excellent free, open-source citation manager. After downloading Zotero from https://www.zotero.org/, it should prompt you to install the Zotero Connector, a browser plugin that lets you download paper citations with one click. If it doesn't prompt you, download the connector here[244]. We also have a shared Zotero group, which you should have requested access to above, to accumulate citations when writing manuscripts.

  Several helpful plugins can be downloaded; the recommended ones are:

Table 2: Recommended Zotero plugins

Name	Description
ZotFile[245]	Enables useful file operations, such as extracting annotations from a marked-up PDF, transferring new papers to a tablet for annotation, and auto-file renaming.
Zutilo[246]	Enables helpful tagging operations, such as the ability to copy/paste tags or easily add paper relationships.
Better Bibtex[247]	If you plan to use LaTeX, install this plugin before exporting to BibTeX. This addon makes nice-looking, stable citation keys that do not change on export.

Downloading Zotero plugins through Firefox

Since Zotero is built on modified Firefox, Zotero plugins appear similar to Firefox plugins. If downloading these plugins through Firefox, you will need to explicitly right click -> "download target"; left-clicking on download links will attempt to install the Zotero plugin as a Firefox plugin, which will fail.

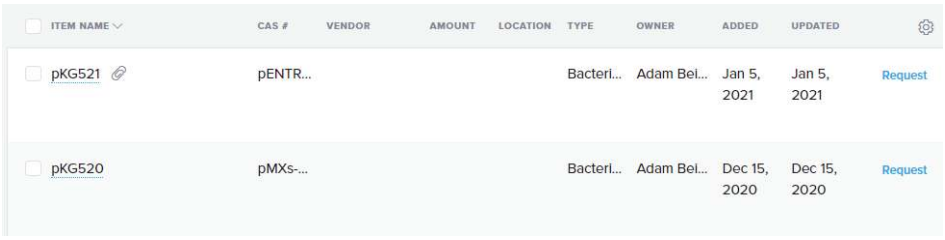
- **Better Quartzy:**

[244] https://www.zotero.org/download/connectors
[245] http://zotfile.com/
[246] https://github.com/wshanks/Zutilo
[247] https://retorque.re/zotero-better-bibtex/

TODO

Unfortunately, the Quartzy interface updated, so our userscript to customize the appearance is broken. :(

While Quartzy is great for inventory purposes and the interface for the plasmid database isn't too bad, the web interface leaves a lot to be desired. By default, you can't really read the plasmid names even after you move the "CAS #"" field to the second position:



To fix this, there is a Quartzy enhancer to make the plasmid field larger and to directly list the antibiotic resistance (Amp/Kan/Chlor) below the plasmid.

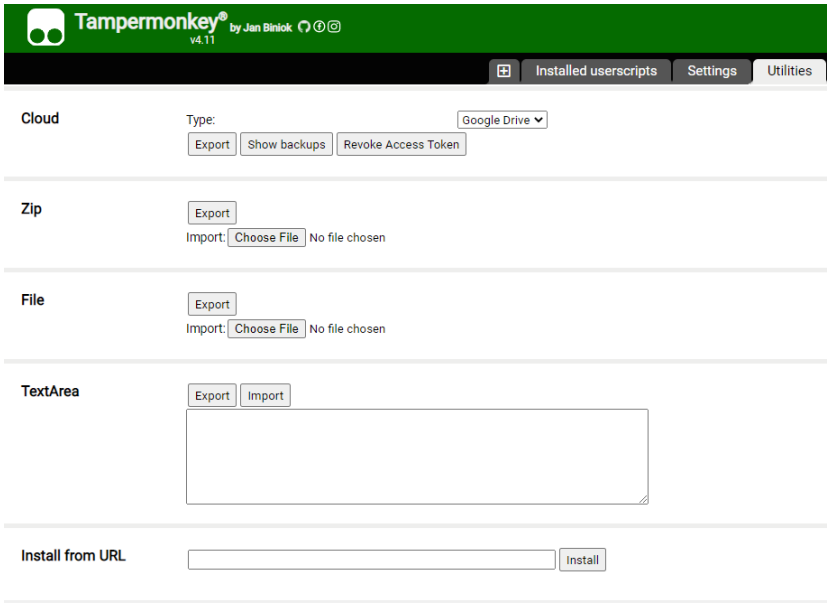
This feature is implemented using something called **userscripts**; these are small Javascript scripts that get injected into webpages; effectively they are mini browser extensions.

To set this up, install a userscript manager like Tampermonkey[248].

Then, click on this link to add the userscript: https://gist.github.com/meson800/f28e64d532da9b0fe2a1d22480ea5cda/raw/quartzy_enhancer.user.js

Or, in the Tampermonkey Utilities tab, you can use the **install from URL** option:

[248] https://www.tampermonkey.net/



- **Adobe Creative Cloud:** MIT has a site license for students and staff (but unfortunately, not for affiliates). After installing the Creative Cloud application[249], select "Work/School account" and login with your MIT credentials. You may have to wait 24 hours for activation after your first login. You should install **Acrobat** (for viewing PDFs) and **Illustrator** (for drawing graphics).

> **Note**
>
> As a free and open-source alternative to Adobe Creative Cloud, you can also check out **Inkscape** (download here[250]). Inkscape and Illustrator have many similar but not completely overlapping features. Inkscape's PDF importer (Cairo) may be superior for importing vector images from manuscript PDFs. Inkscape may be useful to know if you don't want to pay for Creative Cloud later; however, we use Adobe software for creating graphics and figures in lab.

- **Color palettes:** Having nice color-blind friendly, distinct colors is helpful when you begin creating graphics. Palettes help unify figures and convey consistent information via color.

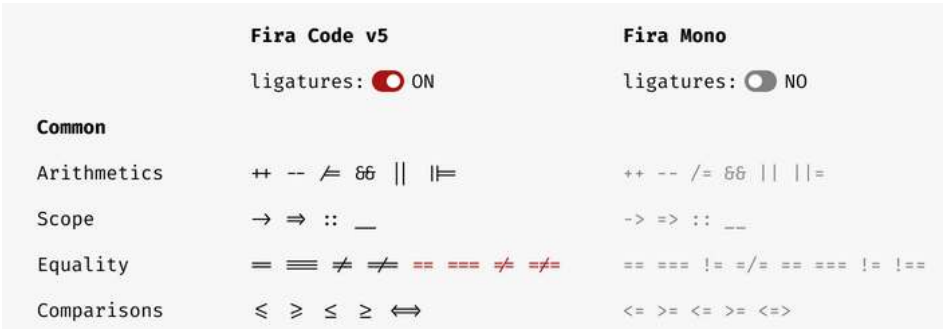
  You can download pre-created palettes for both `Illustrator` and `Inkscape` for the well-known Category20/20b color set, which is color-blind friendly (and becoming the default in more and more software packages):

[249] https://www.adobe.com/creativecloud/desktop-app.html
[250] https://inkscape.org/release/inkscape-1.0.1/

To use these palette files, see the Illustrator documentation[251] ("Create and open swatch libraries") or the Inkscape documentation[252].

- **Fonts:** *Helvetica Neue* is a good sans-serif font that is based on everyone's favorite font, Helvetica. While not required, many people in lab use this font, so their files (e.g., PowerPoint, Illustrator) won't render well if you don't have it installed. First, download it `here` (macOS, Linux) or `here` (Windows). Then, unzip the folder, select all the `.tff` files, and double click to open, which should prompt installation. Alternatively, right click and select "Install font".

  For a good monospaced/code/terminal font, *Fira Code* is excellent (download here[253]). Besides looking nice, Fira Code has something called **font ligatures**. These are originally defined for special letter combinations, like æ for adjacent ae. In Fira Code, common programming combinations are given special ligature symbols that appear as you type normally. You often have to enable ligatures in the editor you are using.



[251] https://helpx.adobe.com/illustrator/using/using-creating-swatches.html#share_swatches_between_applications
[252] https://inkscape-manuals.readthedocs.io/en/latest/palette.html
[253] https://github.com/tonsky/FiraCode/releases

7.1.2 Day 1: First day in lab

Before your first day in lab, be sure to complete required trainings and download lab software, as described in *Day 0: Software training and setup* (page **??**). Once you have lab access, complete the list below to become oriented to lab processes and procedures.

Onboarding form

Complete the tasks on the **lab Onboarding Form** (download the one for `grad students/postdocs` or `undergrads/visiting students`). The most recent forms are also located in this folder[254] in the lab SharePoint. Check off each task as you complete it. This list includes:

1. Setting up webservices (you should've done this in *Day 0* (page **??**))
2. Obtaining access to webservices (you should've requested this in *Day 0* (page **??**))
3. Getting the appropriate physical lab setup (lab notebook, bench, etc.)
4. Performing the required EHS trainings (online trainings in *Day 0* (page **??**), in-person described below)
5. Performing a walkthrough of the lab to familiarize yourself with its organization (see also the Lab Citizenship section below)
6. Adding a photo of yourself for the lab website to this folder[255] in the SharePoint

Lab-specific safety training

After completing all other safety trainings online, meet with the lab EHS rep to learn the relevant safety features of our lab (e.g., location of eyewash stations, processes for chemical waste disposal). To confirm that you understand these procedures, you must submit a Google form to be checked off by the lab EHS rep. This safety information is *documented here* (page **??**), and key points are highlighted in the lab-specific training slides (IAP 2025[256]).

For grad students and postdocs, an in-person training is also required for access to the room containing the autoclave, dishwasher, and ice machine (room 56-415). Once you have completed the Autoclave Safety Training[257] module in Atlas, a lab member will demonstrate how to operate the autoclave and describe relevant safety features. Then, email the Building 66 Facilities Manager and ChemE EHS Coordinator (Chris Monaco and Brian Smith, directory[258]) to request card access.

[254] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgDFT2QaHvFyRK8T-mimny_OAWjTpmM18Te5DVKyJORWO10?e=KXd2iO
[255] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgCB3AedU6JORrIMlIWW9UdQAVcKnwOU69Rr4hlMbVRB5TI?e=KmmG2E
[256] https://mitprod.sharepoint.com/:p:/s/GallowayLab/EffS_fJNCA1KgtfpJG7O6DgBQFc9nWfK2uXpOLrbIo9-lg?e=HzM0Cq
[257] http://web.mit.edu/training/course.html?course=EHS00254w&sys=PS1
[258] https://cheme.mit.edu/people/staff/

Lab citizenship

It is essential for lab members to display common courtesy and follow established procedures to ensure the lab runs smoothly. Professor Galloway expects all lab members to contribute to the lab mission and uphold lab values, as described on the lab website[259] and internally in a lab meeting (IAP 2026[260]). On your first day in lab, a current lab member will walk you through the lab space and describe our digital organization. This presentation (IAP 2026[261]) contains a good list of things to know.

Maps for lab organization:

- Building map[262] of lab rooms
- Lab equipment[263]
- Freezer organization[264]
- Incubator shelf assignment[265]

Selected lab protocols to be familiar with:

- *Lab safety* (page **??**)
- *Ordering materials and reagents* (page **??**)
- *Receiving orders* (page **??**)
- *Shipping* (page **??**)
- *Recycling* (page **??**)
- *Cleaning and autoclaving* (page **??**)
- *Plasmid database* (page **??**)
- *Basic TC procedures* (page **??**)
- *Virus safety* (page **??**)

[259] https://gallowaylab.mit.edu/bts/
[260] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQD9zyPd_-dBS5MBPrYZXVD7AabiBQJH33UpdkmKmh_5X9s?e=4uMhI5
[261] https://mitprod.sharepoint.com/:p:/r/sites/GallowayLab/_layouts/15/Doc.aspx?sourcedoc=%7B34FBAE9F-CA9A-40E5-B0BA-5EADE917E203%7D
[262] https://mitprod.sharepoint.com/:i:/s/GallowayLab/EbbxNb8qgvhFmFE0GKADaAEBCjVjy9nSm-bfoXdxJbo3kw?e=ay4Z9f
[263] https://mitprod.sharepoint.com/:b:/s/GallowayLab/EbjBy6vhAyZKqWVgSfxkRXABdfoGp_YYGrRKiLTGay74fg?e=zjkM5C
[264] https://mitprod.sharepoint.com/:x:/s/GallowayLab/ERVgIOi4w31JohZf3xSQIDQBK7t2Pm4gxTZNxHdolh4EOw?e=kSnjs1
[265] https://mitprod.sharepoint.com/:w:/s/GallowayLab/EediYQ9VgLFMpMWxMOpqOu8Bqo_zbC4BykKfnjKReiZqQ?e=4zv17t

Core facility access

If you anticipate using a core facility (e.g., flow sorter, confocal microscope, plate reader) check out the *relevant protocol* (page **??**) to obtain access. It's best to start this process early, since obtaining access may take several weeks — training can be difficult to schedule during busy times, and you may be required to book supervised time on equipment before independent access is permitted.

7.1.3 Day 2+: Content-specific trainings

In addition to the required basic orientations to lab safety and organization (see *Day 0* (page **??**) and *Day 1* (page **??**)), the lab has put together trainings on topics directly related to our research. Many of these trainings are presented yearly during IAP/Summer, and resources from recent presentations are posted here for reference.

Core lab techniques

The following are core topics/techniques used in our lab. Many are linked to the slides for related training sessions.

- **Molecular cloning:** general introduction (IAP 2026[266]), modular Golden Gate assembly (IAP 2026[267])
- **Mammalian tissue culture:** best practices (IAP 2026[268]), cellular reprogramming (IAP 2025[269])
- **Analytical methods:** flow cytometry (IAP 2026[270]), staining and microscopy (IAP 2026[271]), genomics (*overview page* (page **??**))

Computational skills

General

- Install *required software* (page **??**) and extensions
- *General software tips and tricks* (page **??**)
- Basics of the *command line* (page **??**)

Github

All lab code is stored on Github for version control and sharing. Check out the following protocols to learn the basics and refer back to as needed:

- *Introduction to Git* (page **??**)
- *Startup checklist* (page **??**) for working with Git repositories
- *Computational environment check* (page **??**) to confirm that you can edit Git repos
- *Contributor guide* (page **??**) for editing this protocol site

[266] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQA3vVP3JP5qRbN9GfGuHvU5AQucMAOWycOwvut-UR7X58Y?e=wP9pvO
[267] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQBd38tXyVq9Rbq7-FEZiAfFAejpTyzna_HolRxvQikgSnM?e=Oz6IFk
[268] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQAgKhXRx0uiRbabjPYw7oGoAbbs8CY8hp8k0VUlzglbLoY?e=ibijp5
[269] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQBaCEYrFtGLQ6yJTPeuPLW7Ac96vWqtTrysJUB5ZVYJj7s?e=isklZ4
[270] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQDJKQU0ACzMRJkckaxWEIYqAU5IKlwLuSKH4XubMdEIawY?e=mFVYsc
[271] https://mitprod.sharepoint.com/:p:/s/GallowayLab/IQCEeRxo0KawS41umxU04aSwAZSE4VzFF9gE_rbtAbGVuWE?e=0EGHPg

Data Analysis & Modeling

- **Data analysis tips & common approaches:** see the *Data Analysis* (page **??**) protocols pages, example Jupyter notebooks in the `example-training` repo (link[272]), and the user guide for the `rushd` Python package (link[273])
- **Introduction to gene expression modeling:** set of undergrad trainings (*Summer 2021* (page **??**))
- **High-performance computing:** tutorial for the MIT Engaging cluster (MIT ORCD[274])

Research reading and writing

Reading literature, communicating your findings, and composing compelling visuals are key research skills to master. The lab has put together several trainings on these **science communication** topics. A *"How to" training series* (page **??**) was presented in Summer 2021 (as well as Summer 2023[275] and Summer 2026[276]), to undergraduates and other interested lab members. Additionally, Katie has put together a *Writing Guide* (page **??**) relevant for academic writing in diverse forms (papers, proposals, etc.).

To produce high-quality, informative **graphics**, the lab mainly uses Adobe Illustrator. The best way to learn how to use Illustrator is by making graphics of your own! To get started, check out the following:

- *Section on Illustrator* (page **??**) in the Onboarding Day 0 page for instructions on setting up the software
- Illustrator keyboard shortcuts list[277] from Graphics & Illustrator training (IAP 2026[278])
- A lab Illustrator tutorial, including slides and video demo (Summer 2026[279])
- YouTube video[280] for a basic tutorial on many features relevant for scientific graphics

Journal clubs are an important component of reading and evaluating primary literature. Past lab journal club presentations are located in this folder[281]. This includes several journal clubs on some of the lab's papers and review articles (IAP 2024). For a complete list of papers from the lab, see the lab website[282] or Katie's Google Scholar profile[283].

[272] https://github.com/GallowayLabMIT/example-training
[273] https://gallowaylabmit.github.io/rushd/en/main/index.html
[274] https://orcd-docs.mit.edu/getting-started/
[275] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgCabz3F-8ZcRYFgF9DnyesTAV1B3twaBrkUFYim1lpEET4?e=aBCbSF
[276] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgAyMVRnhvKLRoctjrUNiy3YAQfkMzEhhvXRH1zB3gEcWes?e=DAtb9g
[277] https://mitprod.sharepoint.com/:w:/s/GallowayLab/IQAtam1dK9LiQqfk-UDpyb2bAS8J5FQOKeUqJ94R-dkRs9g?e=VHPmpM
[278] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgBqnHBMeeqlTpF_21nT0mweAc7Il02msz5xrXOR2L_5pYA?e=dMaudu
[279] https://mitprod.sharepoint.com/:f:/s/GallowayLab/IgACQyVA1uUKTrsu-DDLHkpfAa-AE8Xvjp6YwBt-zAGldEw?e=cMPzDC
[280] https://www.youtube.com/watch?v=3IuoK07YDds
[281] https://mitprod.sharepoint.com/:f:/s/GallowayLab/Eugaj4o4lw5Egeyo9_lQmjIB8un7AnIkFoRDG5BQ0Btwaw?e=OQuJTU
[282] https://gallowaylab.mit.edu/publications/
[283] https://scholar.google.com/citations?user=boemvUgAAAAJ&hl=en

7.1.4 Computational environment check

Most of the data analysis we do in lab depends on Python and uses Git for version control and collaboration. Additionally, lab infrastructure like this protocols site and our plasmid website rely on these tools. It's important to get these set up early so your labmates can help troubleshoot any issues that arise. That way, you can begin contributing and start off with best practices.

Following the instructions below serves as a minimal test of basic functionality. It's normal to run into issues as you work to complete it—your labmates are happy to help you troubleshoot and understand what's going on!

Before you begin

1. Double check that you've completed everything in *Day 0: Software and training setup* (page **??**). Specifically, you'll want to have Python, Git, and VS Code installed, and OneDrive and Nextcloud synced locally to your computer.
2. If you aren't very familiar with the terminal, check out *On terminals and shells* (page **??**) for explanations of some basic commands. You might want to try completing the *exercises* (page **??**) at the end to double-check your understanding.
3. It's *highly recommended* that you read through *An introduction to Git* (page **??**) or a similar tutorial before beginning. Understanding why and how we use Git will help you complete the steps below. Don't worry about being an expert yet, but a refresher before you begin is always a great idea.

Tip

General tip: If things really aren't working (especially during the very initial setup) try quitting and restarting the application, restarting your computer, or uninstalling and reinstalling the software. Sometimes things need a reboot, and it doesn't hurt to try if you're really stuck.

Initial environment check

1. Clone the `environment-check` repository at https://github.com/GallowayLabMIT/environment-check.

   To do so, follow the instructions in the "Existing repository (Python)" section of "Startup checklist when working with repositories" (*here* (page **??**)).

   Note

   This is intentionally a private repository, which means that you will have to be in the GallowayLabMIT Github organization and have your local Github credentials working in order to clone it.

Tip

If you get an error message about not having permission to run scripts, you may need to change your execution policy.

On Windows:

1. Open PowerShell on administrator mode
2. Run `set-ExecutionPolicy RemoteSigned`
3. Select "Y" to confirm

Now you should be able to run scripts. For more explanation, see here[284].

2. In the `environment-check` repo, create and switch to another branch. Name the branch with something like your name (for example, `cjohnsto`). See *this section* (page **??**) of the Git intro for instructions.
3. Install `rushd`, a package for sane data management, using `pip install rushd`. Because you added new packages, update the requirements file by using `pip freeze > requirements.txt` so someone else could use the same package versions in the future.
4. For this training, modify the `datadir.txt` file (or create one if you haven't yet) to contain the path to your locally synced lab OneDrive/SharePoint (*not the Nextcloud for this repo only*). You don't need any quotes or other characters around the path.

   For example, the path might look like this (on macOS):

```
/Users/username/Library/CloudStorage/OneDrive-SharedLibraries-
↪MassachusettsInstituteofTechnology/GallowayLab - Documents
```

[284] https://learn.microsoft.com/en-us/powershell/module/microsoft.powershell.core/about/about_execution_policies?view=powershell-7.5&viewFallbackFrom=powershell-7.2

> **Note**
>
> The environment-check script expects that you point the data directory at the *root* of the OneDrive, i.e., the folder with subdirectories like `projects`, `instruments`, etc.

5. Commit your changed files and push your new branch to Github. See *this step* (page **??**) in the "New repository (Python)" section of "Startup checklist when working with repositories".
6. From a terminal inside the repo, run `python check.py` or `python3 check.py` (macOS) to see if you get all green checks! Fix any errors until you do.

Protocols check

Getting all green checks above means that you can now clone repos and run Python! The next step is to make sure you can contribute to our lab protocols (this website). At base, this site is a collection of formatted text files that is built into a website and pdf using Python. Specifically, protocols are written in reStructuredText[285], a lightweight markup language that enables useful formatting in a relatively straightforward manner. These `.rst` files are converted into a nice-looking website via the Python package Sphinx[286]. (For more details on how we use this for the protocols site, check out the *Contributor guide* (page **??**). But don't worry about knowing the ins and outs of all this now.)

Instead, to start, it's good to check that you can edit one of the files.

1. Confirm that you have installed the "reStructuredText" and "reStructuredText Syntax highlighting" extensions in VS Code, as directed in *Day 0: Software and training setup* (page **??**).
2. Clone the protocols repo at https://github.com/GallowayLabMIT/protocols, using your new knowledge. (Hint: Check out *Startup checklist when working with repositories* (page **??**).)
3. Edit this file (`docs/training/onboarding/environment_check.rst`), adding your name to the completion list.
4. Save, commit, and push those changes, and you are done!

> **Tip**
>
> For tips when working with the protocols site, read the *Contributor guide* (page **??**). For instance:
>
> - Use *Local previewing* (page **??**) to see how the reStructuredText is rendered on the website as you edit.
> - Check out the *Basics of reStructuredText* (page **??**) for a primer on formatting in reStructuredText.
> - Follow the *Standard workflow* (page **??**) when editing protocols.

[285] https://docutils.sourceforge.io/rst.html
[286] https://www.sphinx-doc.org/en/master/

Completion date

- Christopher Johnstone (2022-06-01)
- Katie Galloway (2022-06-13)
- Emma Peterman (2022-06-13)
- Kasey Love (2022-06-17)
- Nathan Wang (2022-06-17)
- Christian Otero (2022-06-17)
- Brittany Lende (2022-06-17)
- Conrad Oakes (2022-06-20)
- Kei Takahashi (2022-06-20)
- Adam Beitz (2022-06-21)
- Patrick Han (2022-06-21)
- Sneha Kabaria (2022-06-21)
- Joji Teves (2023-02-24)
- Deon Ploessl (2023-06-27)
- Mary Ehmann (2024-01-15)
- Diya Godavarti (2024-06-03)
- Eliska Liang (2024-06-24)
- Yunbeen Bae (2024-08-08)
- Zahmiria Johnson (2025-01-07)
- Maria Castellanos (2025-01-07)
- Derin Gumustop (2025-01-09)
- Rachel Lee (2026-01-22)
- Paulina Naydenkov (2026-01-29)
- Adrian Shenvi (2026-07-28)
- Rohan Thakur (2026-08-18)
- Joey Huang (2026-08-19)

The next frontier

Now that your computational environment is set up, you're ready to move on to *the next frontier*: analyzing your data. This belongs separately in its own training, but there are a few things you can do to get started with the knowledge you have from this one.

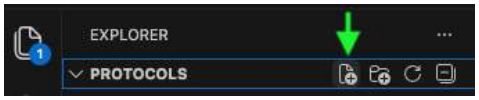
1. Create a new repository in the GallowayLabMIT organization for your project, which will use Python. (Hint: See *Startup checklist when working with repositories* (page **??**).)

   Call it something descriptive related to the project (but *probably not your name/initials*, as collaborators may contribute to it). You can always change this later on Github under the "Settings" tab in the repo.

   The repo should be private for now, but you'll make (a version of) the final code public when you publish!

2. Test that you can run a Jupyter notebook by creating a new file anywhere in the repo called `test.ipynb`.

   The easiest way to do this is probably with the "New file" icon on the left-side "Explorer" panel in VS Code.



3. Use your imagination to make a plot in the notebook!

   - The first time you run a code cell in the Jupyter notebook, VS Code will prompt you to select a kernel. Choose the Python instance from your virtual environment.
   - Don't worry about loading data for now; generate some random values or use one of the sample datasets from seaborn[287].
   - Consider checking out the seaborn gallery[288] or tutorials[289] to get inspired about the cool plots you can make!

Once you can successfully run a Jupyter notebook in a Git repo, you're ready to analyze data. If you're eager for a head start, check out the `example_training` repo here[290], which describes common analysis workflows for flow cytometry, qPCR, ddPCR, etc. Barring any bugs, you should be able to run these notebooks (and begin playing around with the data) if you have Smithsonian locally synced.

[287] https://seaborn.pydata.org/tutorial/introduction.html
[288] https://seaborn.pydata.org/examples/index.html
[289] https://seaborn.pydata.org/tutorial.html#
[290] https://github.com/GallowayLabMIT/example-training.git

7.1.5 An introduction to Git

Acknowledgements

This bootcamp session is heavily inspired by the Software Carpentry Git lesson[291] (CC-BY-4.0).

The initial motivation section is inspired by The Git Parable[292].

Motivation

Even if you haven't used version control software before, you have probably encountered two types of version control:

1. Linear time-based snapshots. This can either be manually done (e.g. you append a date to documents and copy files when you want to make a new version), or automatically done (e.g. you are storing documents in Dropbox, and it stores time snapshots for the last 30 days automatically).
2. Online, live multi-person editing, like in Overleaf and Google Drive.

These techniques are useful, but have limitations. Time-based snapshots often fail if you want to have multiple people editing simultaneously without massive headaches, and online live editing only works if you have permanent internet access, intermediate states are meaningful (e.g. saving mid-sentence is fine for manuscripts, but would throw a syntax error for code), and you don't actually care about saving a detailed history.

For us, having robust version control without these limitations makes our coding work easier to share, easier to reproduce (how do you regenerate a plot made with a previous code version you didn't know you needed?), and easier to do shared work.

In the CS community, this is a solved problem; the field has coalesced around using the version control system called `git`. There are competing version control systems, including Subversion, Mercurial, and Fossil, but `git` has emerged as the clear winner with the emergence of supporting webservices Github, Sourceforge, GitLab, and others.

Unfortunately for us, `git` is also by far the most powerful and complicated version control system, so before jumping into `git` commands, let's consider an example:

> Git is a simple, but extremely powerful system. Most people try to teach Git by demonstrating a few dozen commands and then yelling "tadaaaaa." I believe this method is flawed. Such a treatment may leave you with the ability to use Git to perform simple tasks, but the Git commands will still feel like magical incantations. Doing anything out of the ordinary will be terrifying. Until you understand the concepts upon which Git is built, you'll feel like a stranger in a foreign land.
>
> —Tom Preston-Werner, The Git Parable[293]

Say you are trying to do version control for files related to a paper manuscript. You have some figures, and a main `.docx`

[291] https://swcarpentry.github.io/git-novice
[292] https://tom.preston-werner.com/2009/05/19/the-git-parable.html
[293] https://tom.preston-werner.com/2009/05/19/the-git-parable.html

```
.
├─ figure1.ai
├─ figure2.ai
└─ manuscript.docx
```

How should you track the version history of this? First create a new directory/folder, which is called `working` here, as it is the directory you would be actively working on/editing.

```
.
└─ working
   ├─ figure1.ai
   ├─ figure2.ai
   └─ manuscript.docx
```

After some hacking in Illustrator, you're happy with the way the figures look. To save this version, you just copy the entire working folder to a `snapshot-01` folder and promise yourself that you won't further edit what is inside the snapshot folders.

```
.
├─ snapshot-01
│  ├─ figure1.ai
│  ├─ figure2.ai
│  └─ manuscript.docx
└─ working
   ├─ figure1.ai
   ├─ figure2.ai
   └─ manuscript.docx
```

After you do some more work, (some directories are shown collapsed, without showing content), such as adding new figures, you might have lots of snapshots:

```
.
├─ snapshot-01
├─ snapshot-02
├─ snapshot-03
├─ snapshot-04
│  ├─ figure1.ai
│  ├─ figure2.ai
│  ├─ figure3.ai
│  └─ manuscript.docx
└─ working
   ├─ figure1.ai
   ├─ figure2.ai
   ├─ figure3.ai
   └─ manuscript.docx
```

How are you supposed to remember what we changed in each of these? To help yourself, you decide to add a new file, `message.txt` to each snapshot created. Inside the message, you decide to

add the date, our name, and some free-form message that describes what happened in that version snapshot. A `message.txt` might look like:

```
Author: Christopher Johnstone <cjohnsto@mit.edu>
Date:   Jan 11 2021

    Made Figure 3 300% more aesthetic!
```

The snapshots now look like the following. Note that we add the `message.txt` after we copy the working folder to a new snapshot folder.

```
.
├─ snapshot-01
├─ snapshot-02
├─ snapshot-03
├─ snapshot-04
├─ snapshot-05
│  ├─ message.txt
│  ├─ figure1.ai
│  ├─ figure2.ai
│  ├─ figure3.ai
│  └─ manuscript.docx
└─ working
   ├─ figure1.ai
   ├─ figure2.ai
   ├─ figure3.ai
   └─ manuscript.docx
```

After some more work, you end up revising the manuscript while also trying out a new revision of `figure1.ai`. You want to save your updated manuscript version, but the figure isn't quite ready yet. How do we save the manuscript edits into a snapshot while leaving the in-progress figure edits out of the snapshot? There isn't an obvious way to do this with this folder setup, so you decide to make another special directory called `stage` (as in a stage to set and later snapshot).

With the edits marked with a *, we initially have:

```
.
├─ snapshot-01
├─ snapshot-02
├─ snapshot-03
├─ snapshot-04
├─ snapshot-05
│  ├─ message.txt
│  ├─ figure1.ai
│  ├─ figure2.ai
│  ├─ figure3.ai
│  └─ manuscript.docx
├─ stage
```

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```
   ├─ figure1.ai
   ├─ figure2.ai
   ├─ figure3.ai
   └─ manuscript.docx
└─ working
   ├─ *figure1.ai
   ├─ figure2.ai
   ├─ figure3.ai
   └─ *manuscript.docx
```

Now instead of directly copying the `working` folder into a snapshot, we first copy the changes we want into `stage`, while leaving files we are still actively editing in `working`. Copying the modified manuscript to `stage`:

```
.
├─ snapshot-01
├─ snapshot-02
├─ snapshot-03
├─ snapshot-04
├─ snapshot-05
│  ├─ message.txt
│  ├─ figure1.ai
│  ├─ figure2.ai
│  ├─ figure3.ai
│  └─ manuscript.docx
├─ stage
│  ├─ figure1.ai
│  ├─ figure2.ai
│  ├─ figure3.ai
│  └─ *manuscript.docx
└─ working
   ├─ *figure1.ai
   ├─ figure2.ai
   ├─ figure3.ai
   └─ *manuscript.docx
```

then we make a new snapshot from `stage`:

```
.
├─ snapshot-01
├─ snapshot-02
├─ snapshot-03
├─ snapshot-04
├─ snapshot-05
│  ├─ message.txt
│  ├─ figure1.ai
```

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```
│   ├── figure2.ai
│   ├── figure3.ai
│   └── manuscript.docx
├── snapshot-06
│   ├── message.txt
│   ├── figure1.ai
│   ├── figure2.ai
│   ├── figure3.ai
│   └── *manuscript.docx
├── stage
│   ├── figure1.ai
│   ├── figure2.ai
│   ├── figure3.ai
│   └── *manuscript.docx
└── working
    ├── *figure1.ai
    ├── figure2.ai
    ├── figure3.ai
    └── *manuscript.docx
```

Finally, this whole setup seems to be working well, and you want to go share your work with fellow lab-mates. While you could just copy the entire folder and share created snapshots, this would be inherently linear; only one person could work on this at a certain time! To solve these issues, among others, you decide to assign arbitrary, unique names to snapshots, such as `2a8aba`. With these arbitrary names, it's hard to tell what order you made the snapshots in, so you also add a `parent` field.

```
.
├── snapshots
│   ├── c3612a
│   ├── 86ac2d
│   ├── acd748
│   ├── fb9742
│   ├── 12d276
│   │   ├── message.txt
│   │   ├── figure1.ai
│   │   ├── figure2.ai
│   │   ├── figure3.ai
│   │   └── manuscript.docx
│   └── 2a8aba
│       ├── message.txt
│       ├── figure1.ai
│       ├── figure2.ai
│       ├── figure3.ai
│       └── *manuscript.docx
├── stage
```

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```
│   ├── figure1.ai
│   ├── figure2.ai
│   ├── figure3.ai
│   └── *manuscript.docx
└── working
    ├── *figure1.ai
    ├── figure2.ai
    ├── figure3.ai
    └── *manuscript.docx
```

After this renaming, the most recent `message.txt` (in `snapshot-06`, renamed to `2a8aba`) could read:

```
Snapshot: 2a8aba
Parent: 12d276
Author: Christopher Johnstone <cjohnsto@mit.edu>
Date:   Jan 12 2021

    Updated the manuscript with method details.
```

Now this gets confusing now; what was the last snapshot we took? To fix that, let's start a file, conveniently called `branches.txt`. Its contents could be:

```
last: 2a8aba
```

That way, when we are ready to make a snapshot, we use the `last` pointer and set that as the parent. Once we have the snapshot name, we write over the entry in `last`.

What happens if someone else happens to also be working on these files? This is fine; it just means that each person will have their own `last` pointer.

We can extend our pointer file such that everyone can **simultaneously edit** and keep track of where they are in history by having a different 'pointer' in the branches file!

Summary

This example has hopefully indicated a couple useful properties of a version control system:

- Taking **snapshots** instead of file-level versions ensures that every file is tracked in itscorrect context.
- Having multiple people work on the same project, or working on different parts of a project naturally leads to a system where we have a **non-linear, branched** history.
- It is useful to have a separation between the files we are ready to snapshot (e.g. the **staged changes**) and files we are not ready to snapshot (**unstaged** or **untracked**).

Keeping this in mind, we're ready to jump into Git; our little toy system is actually pretty representative of how Git keeps track of things.

Git by example

We will start you off learning Git by editing the very repository we are working on!

Prereqs

You should have a graphical Git tool installed, such as GitHub Desktop. You can also use the built-in Git tools in something like VS Code. You also should have **command line git** installed; there are certain functions that are really only possible through the command line interface, even though much of it can be done through the graphical tools.

Then, you should check that your Git global settings are set properly. Start off by seeing what the editor, name, and email settings you currently have. An example of the output is as follows:

```
$ git config core.editor
vim
$ git config user.name
Christopher Johnstone
$ git config user.email
meson800@gmail.com
```

If your name and email are not set properly, set them with the following commands. Set your email equal to one that you have connected to your GitHub, so your commits are properly attributed:

```
$ git config --global user.name "Your Name"
$ git config --global user.email "your_email@invalid.com"
```

Also, for the purposes of this bootcamp session, you should set your editor to a command line editor. Unless you know how to use `vim`, this should probably be set to `nano`:

```
$ git config --global core.editor "nano -w"
```

Finally, we'll be editing this documentation! To do so, make sure that you have Python installed and the following packages installed:

```
$ pip install sphinx sphinx-rtd-theme sphinx-last-updated-by-git
```

Basic Git terms

Thinking back to our example earlier, we had the concept of wanting to track everything within a folder, and we took various snapshots of this folder.

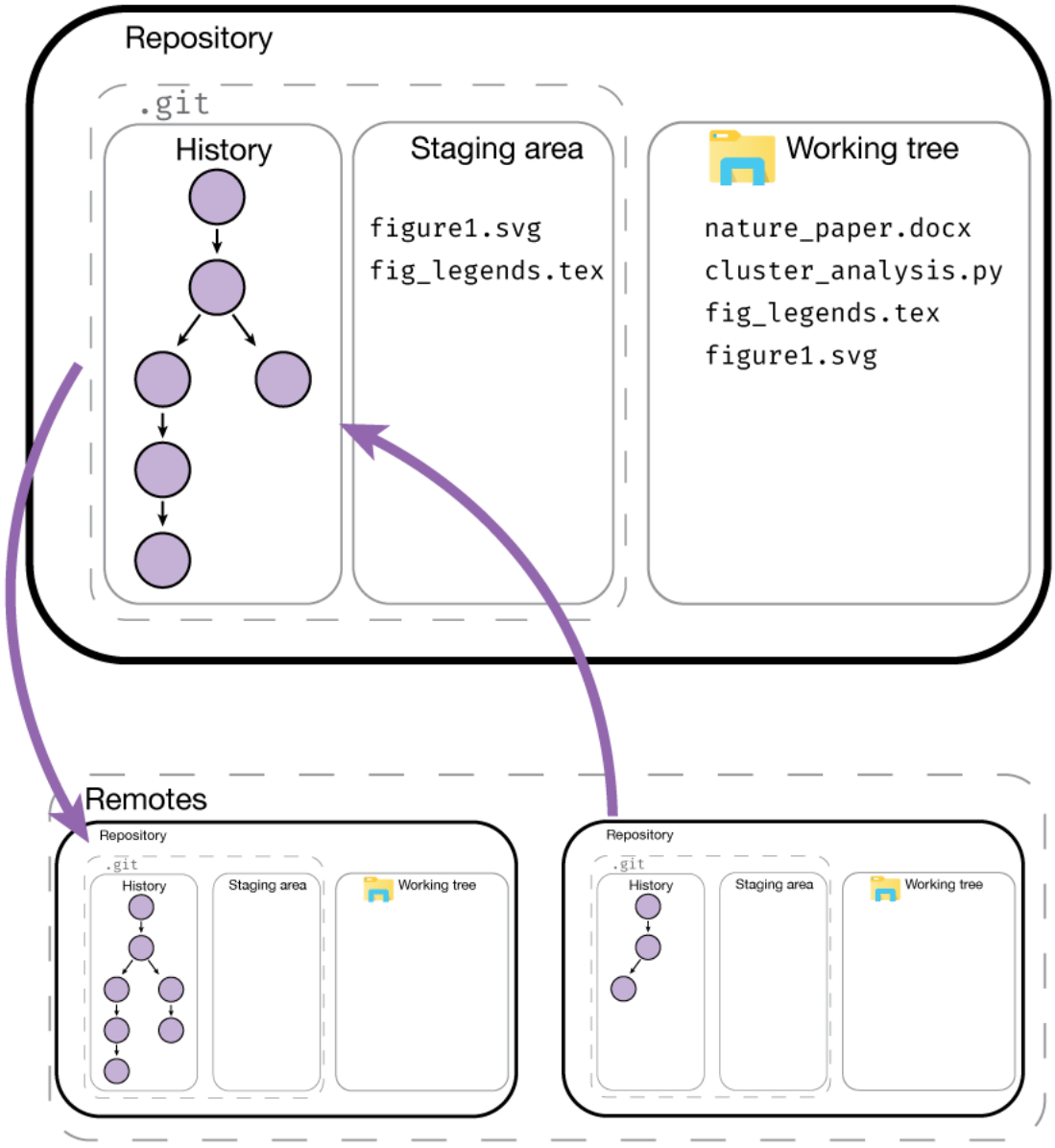
In Git, we call the entire set of files and folders that we are interested in a **repository**, or **repo** for short. Typically, repositories are going to include everything related to a single project. This could mean a set of analysis scripts for the same project, an entire R package that is being developed, or more exotic things like paper and figure drafts.

On a brief note, Git is a version control system for **plain text files**, which is a broad category of files that are readable by humans as well as machines. While Git does still work on other so-called **binary files** (like pictures, PDFs, docx's, and so on), Git is inefficent at versioning these types of files and you lose a lot of the power of Git, like seamless merging of simultaneous changes.

That being said, if it is plain text, such as all code, `.txt` files, LaTeX files, it could be a good fit for Git.

In our analogy, we had the **working tree**, e.g. the folder we are actively editing next to folders for the **staging area** and the various snapshots we took.

In Git, the files that make up the staging area and history are stored within the subdirectory `.git`. By default, this folder is hidden, though you can show it if you want. The rest of the files in the repository folder form the **working tree**.



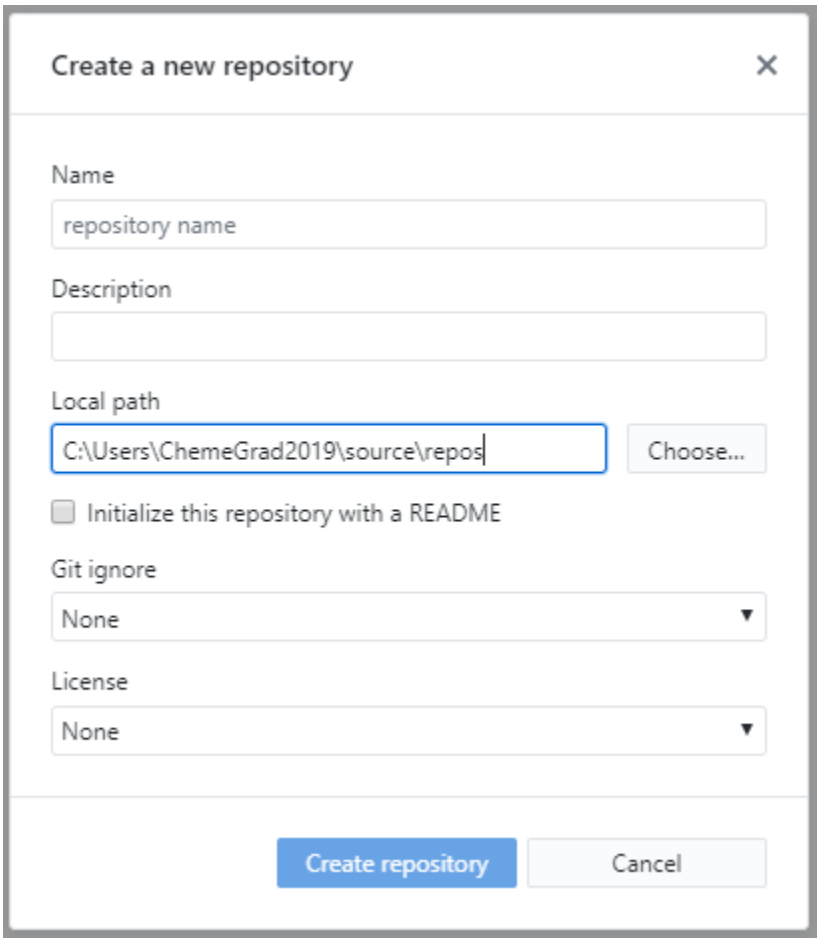
Downloading or starting a repository

If you wanted to start a new fresh repository, you can start a new repository by calling `git init`:

```
$ mkdir a_new_repo
$ cd a_new_repo
$ git init
Initialized empty Git repository in ~/a_new_repo/.git
```

If you start a fresh repository, you must add and commit some content before trying to push to a remote like Github; otherwise there is nothing to push!

You can also do this via a graphical interface.

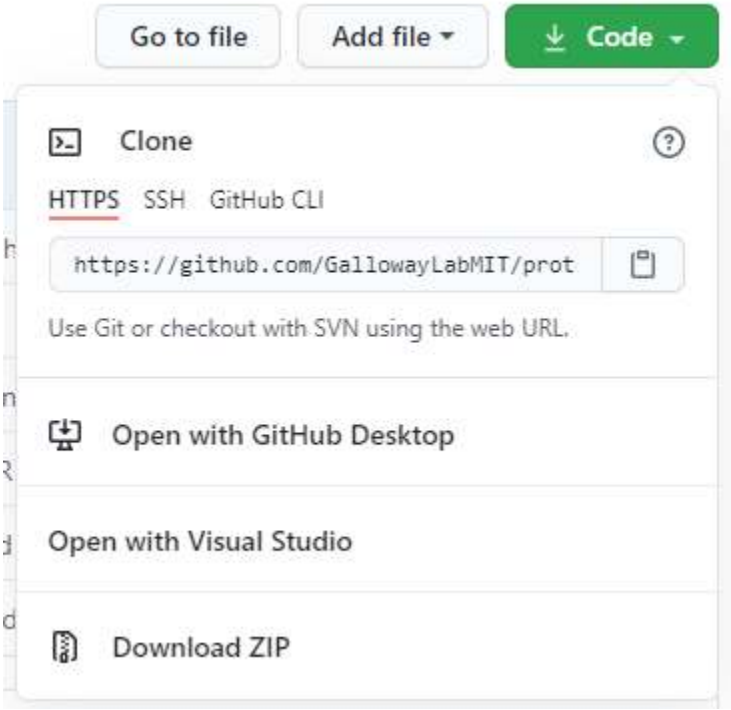


The graphical interface gives the additional option to start with some content pre-generated, in particular a **copyright license** and a **.gitignore** file. We will cover the ignore file later. If you choose one of these options, you can immediately push to a remote, because there is content to push!

For the purposes of this demo, we will be modifying the protocols website! If a repository already exists somewhere else, you can **clone** the repository to make a local version of it.

To clone a repository, we have to know where it is located. There's many possible ways to access another remote repository; it could be on a shared drive location, or accessible on a server. By far

the most common location for remotes is on websites like Github. To find the URL, we can look on the website and click the green code button:



If you have added an SSH key to your Github account, you can use the `ssh` URL:

`git@github.com:GallowayLabMIT/protocols.git`.

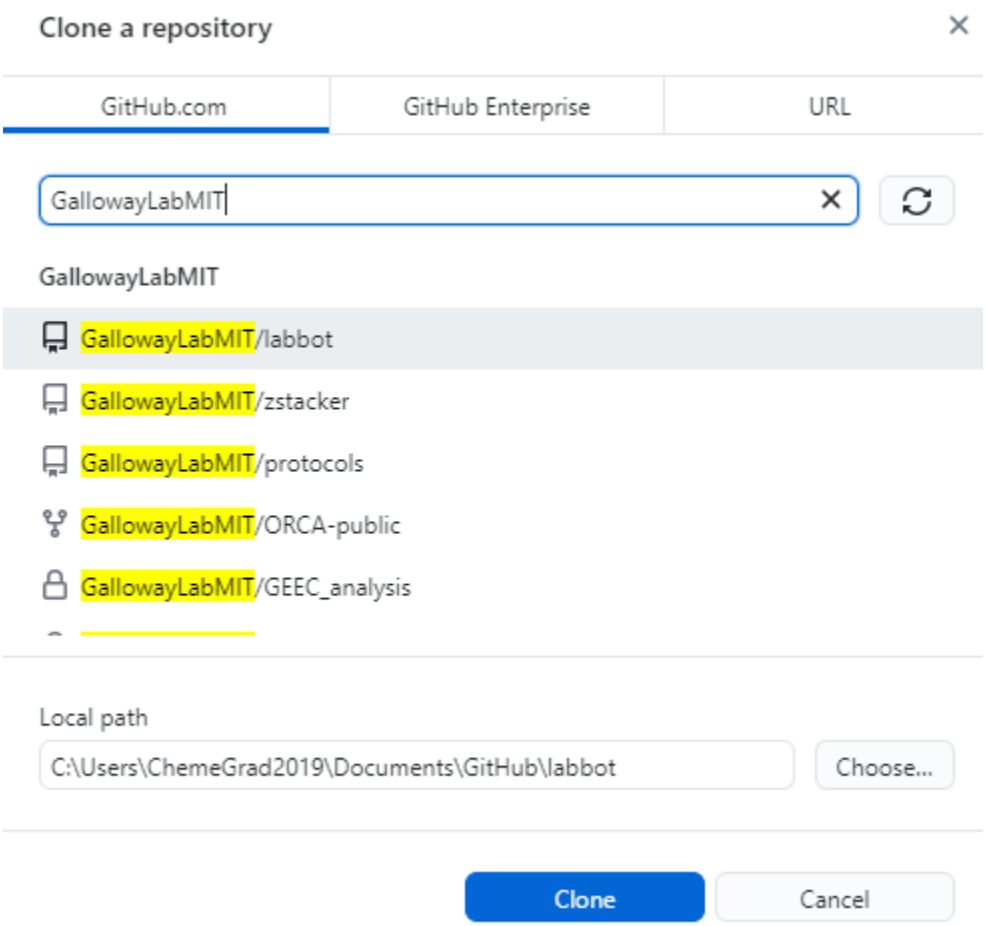
If you haven't added an SSH key, you can use the https URL:

`https://github.com/GallowayLabMIT/protocols.git`

To clone a repository, you use `git clone`. By default, it will create a new folder equal to the repository name:

```
$ ls
$ git clone https://example.com/example/example_repo.git
Cloning into 'example_repo'...
remote: Enumerating objects: 14, done.
remote: Counting objects: 100% (14/14), done.
remote: Compressing objects: 100% (11/11), done.
Receie: Total 1144 (delta 3), reused 12 (delta 3), pack-reused 1130 eceiving␣
↪objects: 100% (1144/1144)
ving objects: 100% (1144/1144), 6.53 MiB | 16.39 MiB/s, done.
Resolving deltas: 100% (562/562), done.
$ ls
example_repo
```

If the remote repository is stored on Github, you can also use Github Desktop to clone a repository:



Exercise

1. Create a new repository using the command line. What is the contents of the hidden `.git` folder?
2. Create a new repository using a graphical tool like Github Desktop
3. Delete both of these repositories.
4. Clone this protocols repo, using either the terminal or a graphical tool.

1. The `.git` folder contains several text files encoding the current branch among other information, with several folders that store the history.

```
$ ls .git
branches  config  description  HEAD  hooks  info  objects  refs
```

2. Completed with Github Desktop.
3. Completed with delition through the file manager or with `rm` in the terminal.
4. Unless you have SSH, the following is the correct command:

```
$ git clone https://github.com/GallowayLabMIT/protocols.git
Cloning into 'protocols'...
remote: Enumerating objects: 14, done.
remote: Counting objects: 100% (14/14), done.
remote: Compressing objects: 100% (11/11), done.
Receie: Total 1144 (delta 3), reused 12 (delta 3), pack-reused 1130 eceiving␣
↪objects: 100% (1144/1144)
ving objects: 100% (1144/1144), 6.53 MiB | 16.39 MiB/s, done.
Resolving deltas: 100% (562/562), done.
```

Basic history terms and git status: What's happening?

Now that we've cloned the protocols repository, we can start poking around.

Whenever you are feeling lost, you should use the `git status` command. The output is generally more concise than the output of many of the graphical tools. In this case, if we run the status command we will get:

```
$ git status
On branch latest
Your branch is up to date with 'origin/latest'.

nothing to commit, working tree clean
```

What does this mean? Let's take a look at an example history graph:

This is a pretty picture; how do we actually understand what is happening in the repository we just checked out? For this, we'd like to actually view the history.

We view the history using either graphical tools or using the `git log` command. Let's play around with the command line first! When you type `git log`, you will be in an interactive browser, use the arrow keys to scroll up and down, and press `q` to exit.

By default, `git log` prints out the entire author and commit message of the previous commits:

```
$ git log
commit 51313751defcf0798fce66c517abd0a20dd1feb1 (HEAD -> latest, origin/latest)
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Mon Feb 15 23:38:29 2021 -0500

    Removed assumptions about working directory from build script

commit 5031d35ad03e53a25ab748c57dea4cbf31b8b33b
Author: katiegal <katiegal@mit.edu>
Date:   Mon Feb 15 23:10:30 2021 -0500

    N3 table alignment
```

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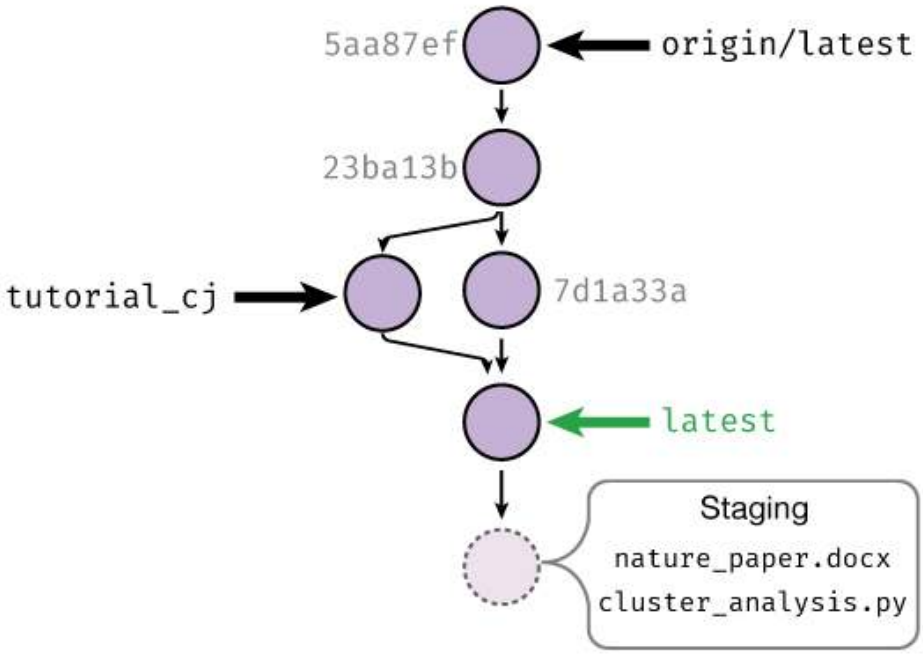

Fig. 1: An example history is shown. Each commit has a random name (in gray) that is assigned by Git. Shown with arrows are the various **branches**, which can be thought of as movable commit names. Shown in green is the currently checked out branch; staged changes will be committed on top of this branch.

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```
commit 756662c5c9f16a7adca2849863d07123a0a2815d
Author: katiegal <katiegal@mit.edu>
Date:   Mon Feb 15 23:08:01 2021 -0500

    N3 table formatting
```

If we want a more concise view, we can tell Git this with the `--oneline` option:

```
$ git log --oneline
5131375 (HEAD -> latest, origin/latest) Removed assumptions about working␣
↪directory from build script
5031d35 N3 table alignment
756662c N3 table formatting
5aa87ef formating of N3
74434ec Formatting repsox table
76541e4 typos fixed in repsox protocol
fa70197 Formatted RepSox info
77ac72b added RepSox concentrations
e0a749e Merge branch 'master' into latest
a8b29e4 Updating awesome RNA vs DNA image!
70075b9 Added BSA requirement for neurotrophics
0e96845 Removed settings.json from tracking and .vscode
```

Here, we see that we have a short name for each commit (the first 7 characters of the full name), and just the commit message, with no author attribution.

To see the whole branching layout, we can view the log with the `--graph` option. If we combine these two options, we can get a short graph view!

```
$ git log --graph --oneline
* 5131375 (HEAD -> latest, origin/latest) Removed assumptions about working␣
↪directory from build script
* 5031d35 N3 table alignment
* 756662c N3 table formatting
* 5aa87ef formating of N3
* 74434ec Formatting repsox table
* 76541e4 typos fixed in repsox protocol
* fa70197 Formatted RepSox info
* 77ac72b added RepSox concentrations
*   e0a749e Merge branch 'master' into latest
|\
| * a8b29e4 Updating awesome RNA vs DNA image!
| * 70075b9 Added BSA requirement for neurotrophics
* | 0e96845 Removed settings.json from tracking and .vscode
* | e9ca614 Fixed formatting
* | 14a7bad Use currently running python interpreter to run sphinx
* | 667a652 (origin/tech_wip) Added details on no-spaces rationale
```

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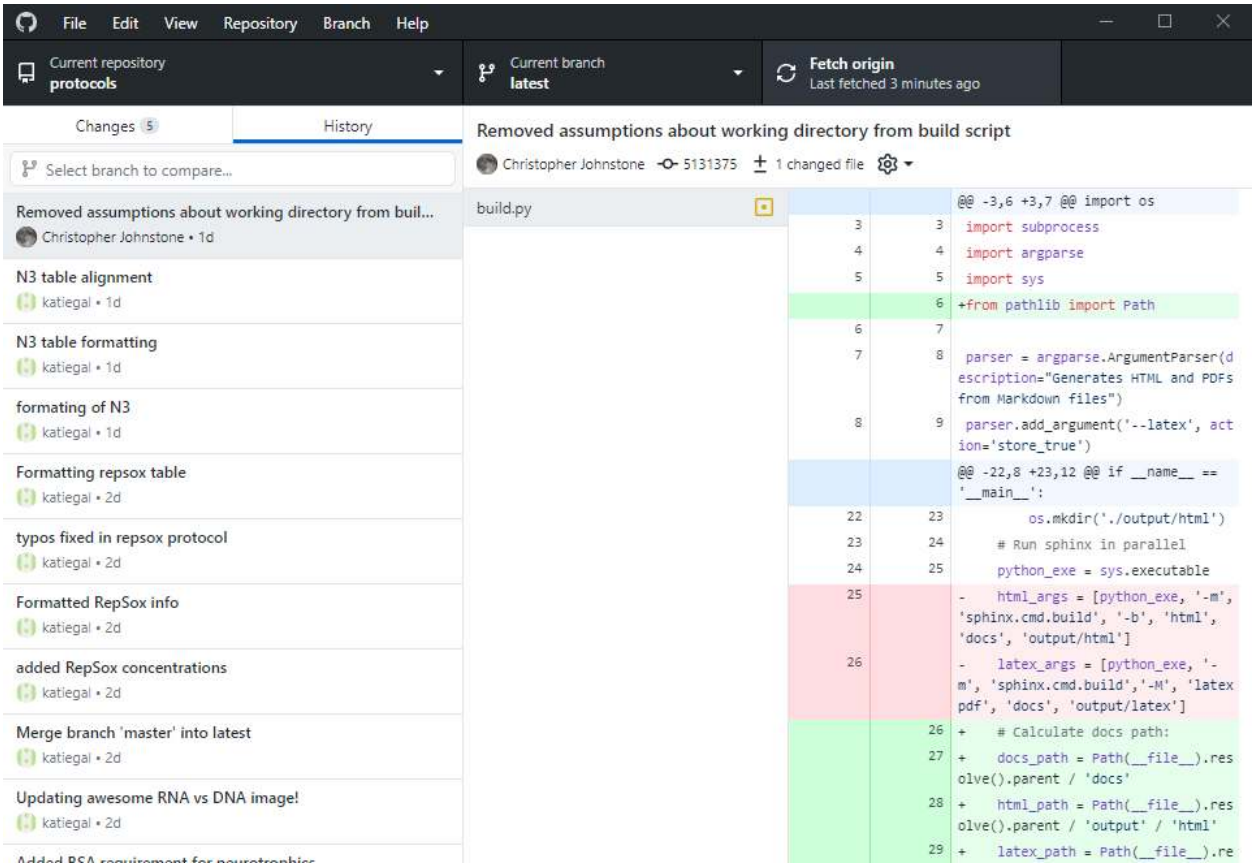
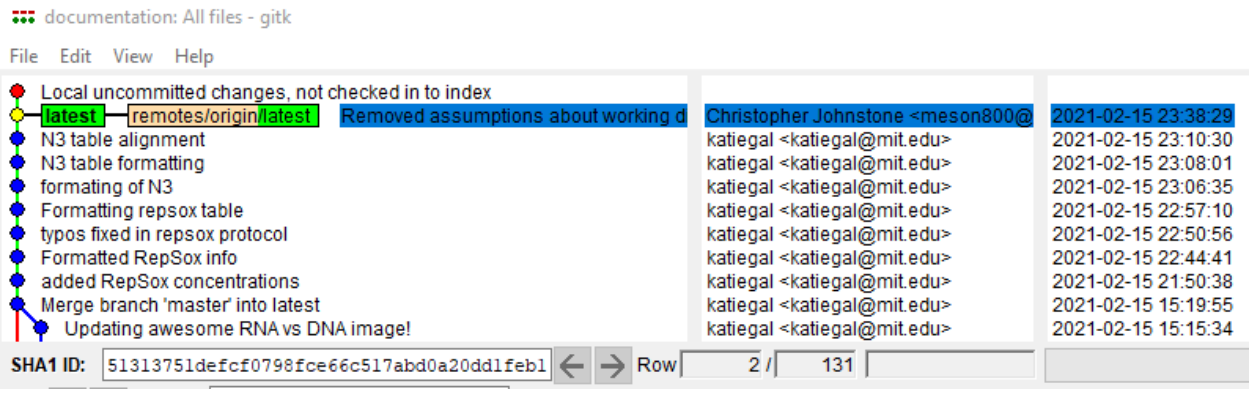
```
* | 9a3dda0 Switched ISO date to explicitly link to xkcd
* | 55bb3eb Updated shell and software tips and tricks with shell extras
* | f65c1e5 Added helper script run info
```

In this example graph view, we see that two branches came together at commit `e0a749e`.

We can use various GUI programs to get the same result! In Github Desktop, you can switch to the history tab to see a linearized history (e.g. like the default `git log`):



You can also run `gitk` in a terminal to bring up a different graphical view which shows the entire tree:



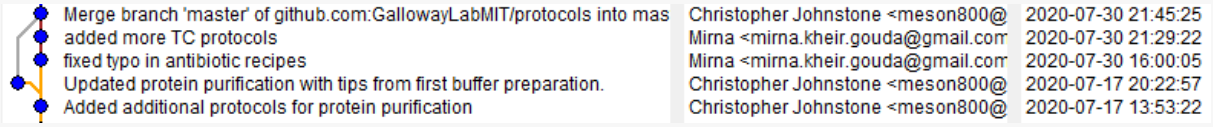
In summary, `git log` and `git status` and their GUI equivalents should be your go-to tool to find out what is happening in history. If you are ever feeling confused, take a look at the log!

Exercise

Do the following with the `git log` command or one of the graphical tools.

1. Find a "fork" in the history graph, where two different commit branches came together, other than the example given. Which commit did the merge occur at?
2. What was the second commit in the protocols repository?
3. Find the embedded emails of two contributors to the protocols repo.

1. One example is this one, shown in graph form with Gitk:



2. The second commit in the repo is the following:

```
commit 5a9a7f55fb53333ef9c642d019d9af67aa64a2a4
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Fri Feb 14 10:43:13 2020 -0500

    Updated navigation system to use a protocols dropdown
```

3. Answers will vary. You should use the full git log view to find these.

Branching out, adding, and checking out files

Now that we know how to view history, let's start making some history! We'll start by adding a convenient name to the commit we are on, so we don't have to refer to these snapshots/commits by their full name.

We view branches and create new branches with the `git branch` command. Typing `git branch` by itself returns all of the current branches, with a star next to the currently active branch:

```
$ git branch
* latest
tech_wip
gh_pages
```

Since we haven't done anything to this repository yet, we start off in the **default branch**. For the protocols repository, the default branch is `latest`: this naming derives from a relatively common naming scheme for documentation where the most recent branch is called `latest`.

Other default branches you may see include `master` and `main`; the default moving forward on our repos is `main`.

Sidenote

Historically, the default branch name has been called `master`. In the summer of 2020, the Software Freedom Conservancy recommended[294] that the branch name `master` be replaced with `main` because of its possible link to master-slave. Strictly speaking, the etymology is likely referring to **master recording** or **mastering**, both audiovisual production terms, but ultimately changing branch names is a minor change that can be made with major benefits for inclusivity in our community.

> I wanted to conclude that, at the end of the day, it doesn't matter where the name comes from (something that was touched upon a number of times in the thread). The fact that it has bad connotations, or inspires dread for individuals and whole communities, is reason enough to change it.
>
> —Bastien Nocera, https://mail.gnome.org/archives/desktop-devel-list/2020-June/msg00023.html

If you want to create a new branch, you do so by adding a name after the branch command:

```
$ git branch
* latest
$ git branch tutorial_cj
$ git branch
* latest
tutorial_cj
```

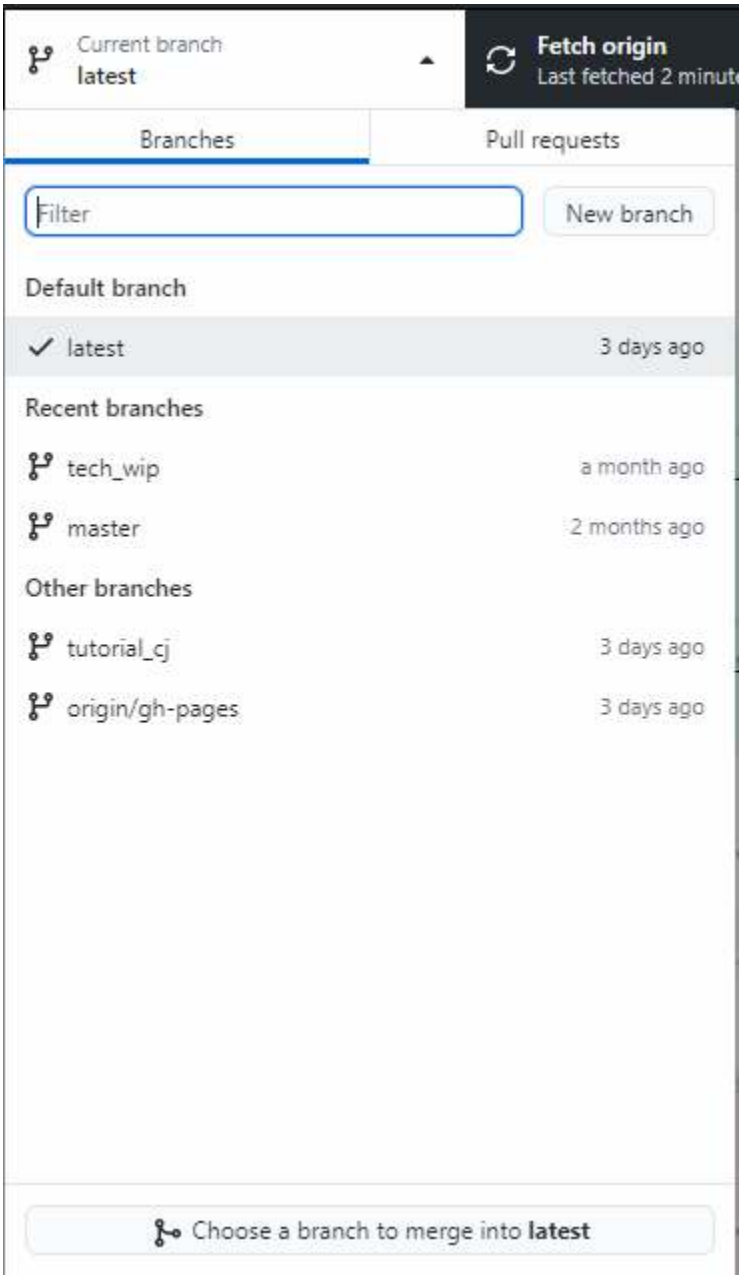
Note that creating a new branch **does not switch to it**. To actually switch branches, we need to

[294] https://sfconservancy.org/news/2020/jun/23/gitbranchname/

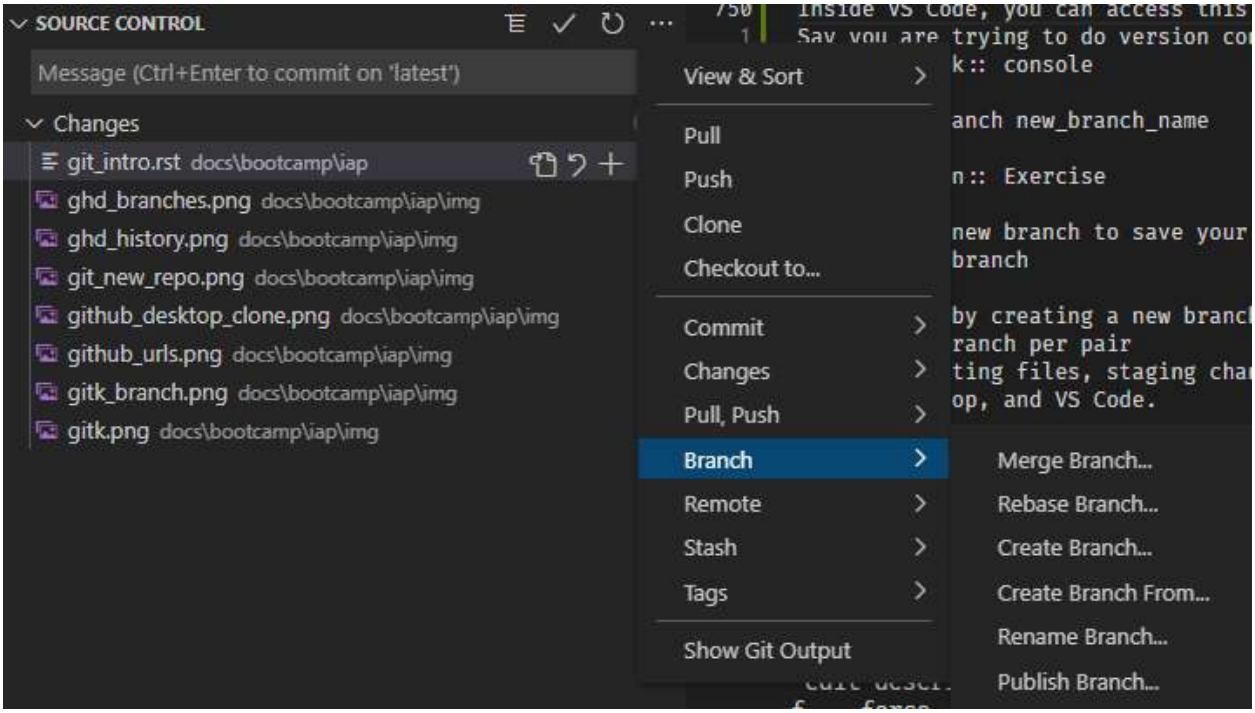
tell Git to update the directory we actually see (the **working tree**). We do this with the **checkout** command:

```
$ git branch
* latest
tutorial_cj
$ git checkout tutorial_cj
$ git branch
latest
* tutorial_cj
```

There are GUI equivalents of these commands. In Github Desktop, you get here by pushing on the branch button:



Inside VS Code, you can access this functionality with the drop down menu inside the source control tab:



Exercise

To do some of the later merging steps, it helps to find a partner for this step! If you don't have a partner, you can clone the repository into a separate folder to simulate multiple peoplele.

1. Have one of the partners create a new branch, deciding on a specific name between the two (suggestion: `tutorial_xx_yy` for initials `xx` and `yy`).

2. For future setup, have the partner that created the branch run:

```
$ git push --set-upstream origin tutorial_xx_yy
Enumerating objects: 28, done.
Counting objects: 100% (28/28), done.
Delta compression using up to 8 threads
Compressing objects: 100% (19/19), done.
Writing objects: 100% (22/22), 2.03 KiB | 346.00 KiB/s, done.
Total 22 (delta 13), reused 1 (delta 0), pack-reused 0
remote: Resolving deltas: 100% (13/13), completed with 5 local objects.
remote:
remote: Create a pull request for 'tutorial_xx_yy' on GitHub by visiting:
remote:      https://github.com/GallowayLabMIT/protocols/pull/new/tutorial_xx_
↪yy
remote:
To https://github.com/GallowayLabMIT/protocols.git
* [new branch]      tutorial_xx_yy -> tutorial_xx_yy
Branch 'tutorial_xx_yy' set up to track remote branch 'tutorial_xx_yy' from
↪'origin'.
```

3. Again, for future setup, have the other partner pull this update and checkout the same branch:

```
$ git pull
$ git checkout tutorial_xx_yy
```

3. With both a terminal and another GUI tool, repeatedly checkout/switch between `latest` and the new branch you created.

Now how do we start making changes? Remember that when we make changes in the (working) directory, we have to explicitly tell Git which changes we would like to snapshot. We inform Git of which changes we want(e.g. we *stage* the changes) using the `git add` command.

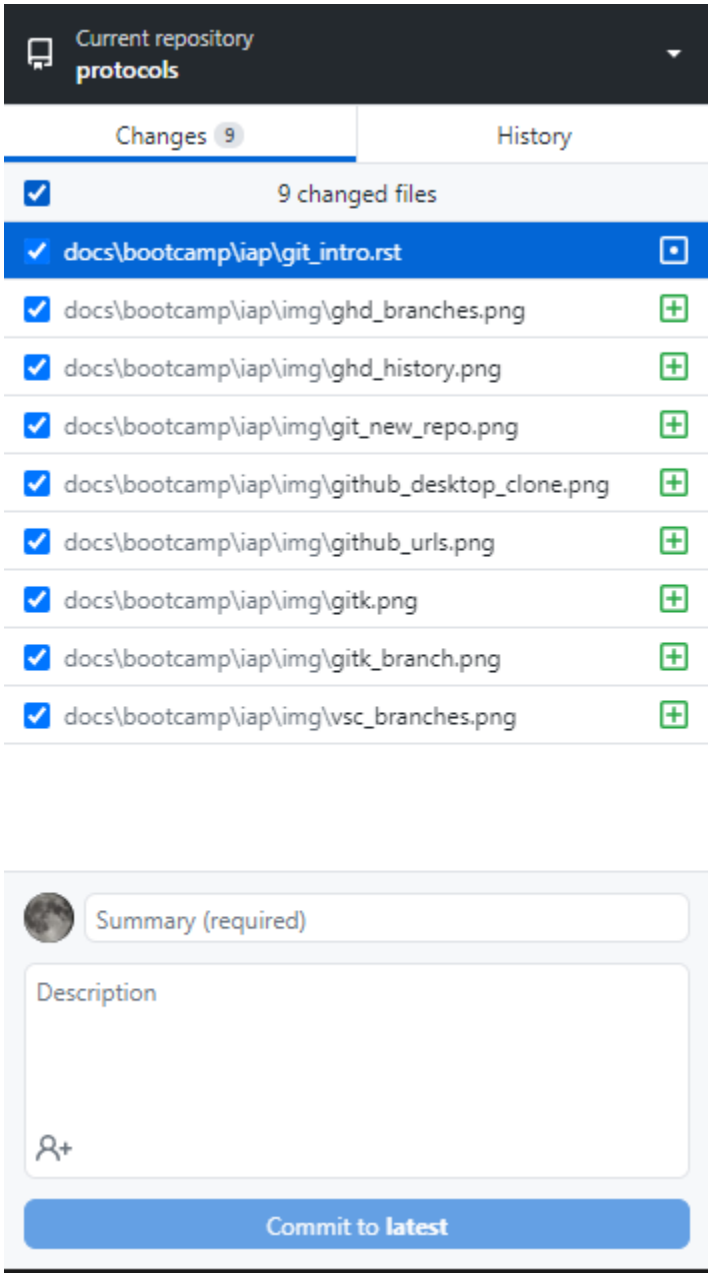
Note

Git reports changes in files in two ways. If a file has already been included in a previous snapshot, then changes to that file are marked as changes. If a file has *not* been included in a previous snapshot, then it appears as an **untracked file**. Even though these are two different types of changes, we use the same command, `git add` to add both of them.

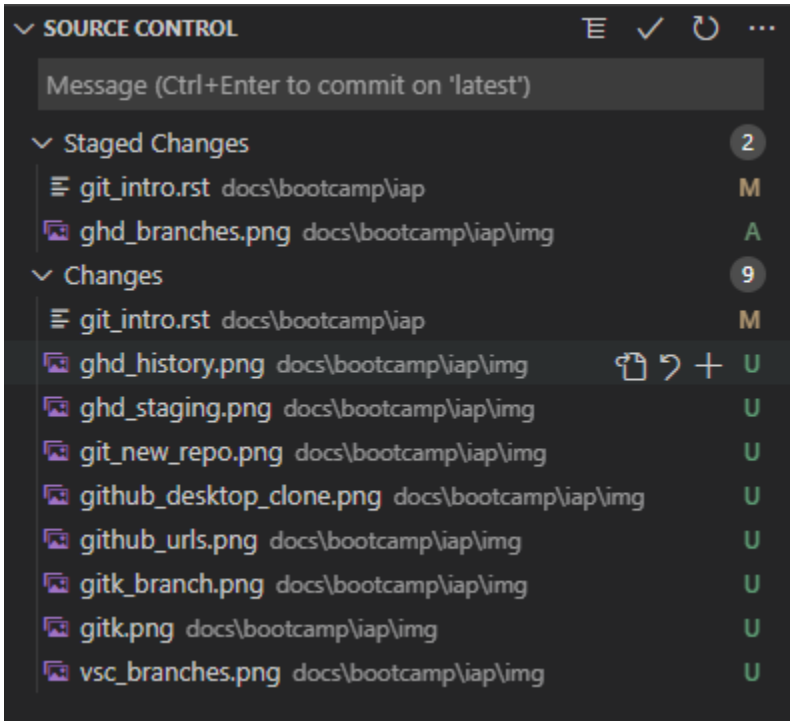
Assume that we are tracking the file `test.txt` (e.g. we have `git add`'ed it in the past, but not `new.txt`. Then the status command will look like:

```
$ git status
On branch latest
Your branch is up to date with 'origin/latest'.

Changes not staged for commit:
(use "git add <file>..." to update what will be committed)
(use "git restore <file>..." to discard changes in working directory)
        modified:   test.txt

Untracked files:
(use "git add <file>..." to include in what will be committed)
        new.txt
$ git add test.txt new.txt
$ git status
On branch latest
Your branch is up to date with 'origin/latest'.

Changes to be committed:
(use "git restore --staged <file>..." to unstage)
        modified:   test.txt
        new file:   new.txt
```

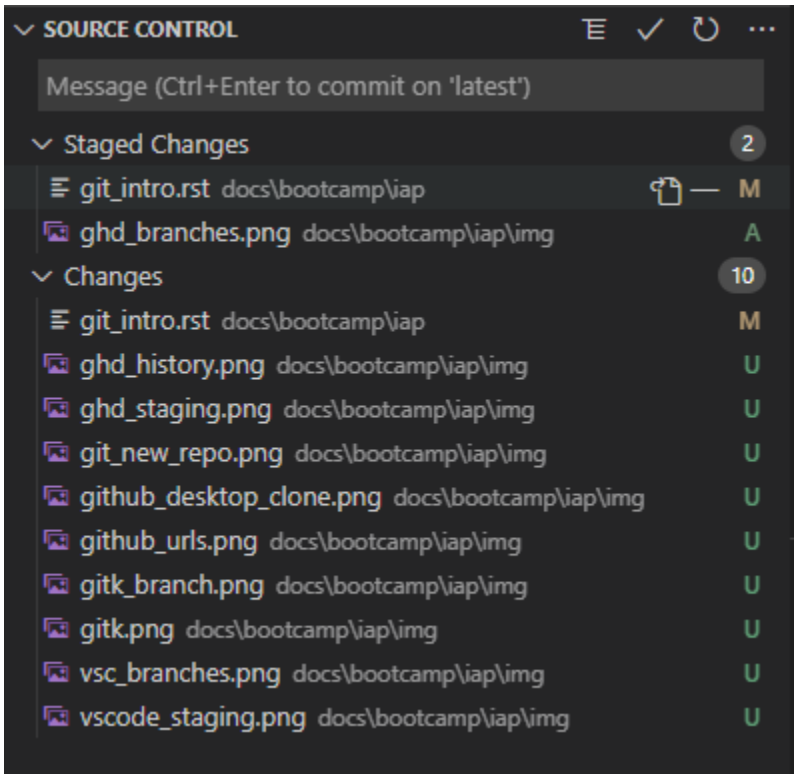
We can do the same thing in the GUI tools. In Github Desktop, changes are staged automatically:



VS Code has a clearer interface. In the source control tab, the new changes appear under the "Changes" column. By hovering over files, you can use the plus icon to add/stage the changes.



How do we remove our changes from being staged? In Github desktop, you can uncheck changes to unstage them. In VS Code, hovering over a staged change will give a minus button that unstages:



If we want to do this from the terminal, we can follow the suggestion given by `git status`:

```
$ git restore --staged test.txt
```

Note that unstaging doesn't actually remove the changes, they are still in your working tree! It just removes it from the set of changes you want to snapshot.

Exercise

1. Edit this file (`docs/training/onboarding/git_intro.rst`), adding your name below:

   ```
   Christopher Johnstone, Another name?
   ```

2. Add a new protocols file. It could be something random, or something the lab needs.
3. Using the terminal, **stage** these changes, e.g. adding them to the list of changes to snapshot.
4. Using a graphical tool, unstage and then re-stage these changes.

Snapshotting your work

Now that we've staged changes to be snapshotted, we need to actually perform the snapshot. We do this with the `git commit` command, or through one of the tools. Let's start with the terminal!

To make a new commit, you can just type `git commit`:

```
$ git commit
<editor opens. After you save and close the editor>
[latest 2cdfda4] Edited an in-progress version of git_intro, up to commiting
2 files changed, 491 insertions(+), 9 deletions(-)
```

When you run `commit` in this way, it will open up a text editor (which should be Nano, following the prereqs) and ask you to type in a message. After you are done, you can save and exit from the editor, and the commit completes. You will see a short summary, including the branch name, the short version of the commit name (the random string), the first line of your commit message, and a summary of how many files and line-based changes were made.

If you have a short commit message already in mind, you can skip the editor step by directly adding a message after the `-m` or `--message` flag:

```
$ git commit -m "Here's a short commit message!"
[latest 73ca1a9] Here's a short commit message!
1 file changed, 100 insertions(+)
```

In the various GUI tools, there is often a box to type in your commit message. For example, in VS Code, you can type in a message in the message box, adding newlines as required with enter, submitting the commit by pushing control-enter:

In Github Desktop, if you made a change to a single file, it will auto-suggest a default commit message. **Don't use this default message!** The suggested commit message is not descriptive:

Think of your commit messages as comments to either future you or other collaborators for your overall changes. We wouldn't write code comments like:

```
pi = 3.1415 # Sets the pi variable equal to the 5-digit truncated value of pi
```

so don't write commit comments like "Updated such and such file"; instead try to add the **why** you made this commit.

Exercise

1. Commit your changes, adding a relevant commit message using your interface of choice. What is the name of your newly created commit?
2. After commiting all of your changes, try committing again. What happens?
3. Make some more changes in a file, such as this one. Add and commit these changes with another interface.

1. This could be done with:

```
$ git commit -m "Your commit message here"
```

The output of this command will include the commit name!

2. When committing with no new changes, you will get a (nice) error message:

```
$ git commit -m "Nothing here?"
On branch latest

nothing to commit, working tree clean
```

3. Answers will vary.

Ignoring files

What happens to files we **don't** want to track version history on? Why would we not want to track history?

A good example of files that you don't want to track is temporary files. On MacOS, browsing to a folder using Finder creates hidden `.DS_Store` files, which store information like the x-y position of files within the Finder window. Programming languages also generate temporary files that do not need to be tracked by Git. For example, Python will sometimes compile `.pyc` or `__pycache__` files that speed un script execution. These files, however, do not need to be tracked as they are derived from the "real" source code. Similarly, we do **not** want to track the website or PDF output of this repository in source control, given that we build these outputs from the source code anyway.

Git will not track these until you add them, but they will always annoyingly sit in the `git status` output unless you tell Git to ignore these files. We do this by adding files we want to ignore to a special hidden file, `.gitignore`. Each line in this file is matched against a file. If it appears on the ignore list, Git pretends that it doesn't exist, making for nice clean history!

For example, the current `.gitignore` for this repository is as follows:

```
.DS_Store

# vscode ignores
.vscode

# Specific build gitignore
output/

# Python gitignore
# Byte-compiled / optimized / DLL files
__pycache__/
*.py[cod]
*$py.class

# C extensions
*.so

# Distribution / packaging
.Python
build/
```

Because we use Sphinx/Python to build the website, we have several Python-specific temporary files to ignore, as well as the output folder.

When creating a new repository on Github, you are given the option to start off with a language-specific `.gitignore`. These are generally a pretty good starting place; you can always add more ignore patterns as you go along!

Recovering history: reverting commits and file-level checkouts

Git is a wonderful tool for storing history, so you may be wondering how we go back to certain states.

In a typical Git workflow, we do not typically reset the entire branch state back to some previous commit. It is possible to do this, but it is a **destructive operation** that removes history from the tree (e.g. removes some commit nodes), so Git only lets you do it after you override several warnings.

Instead, Git provides utilities to **revert entire commits** and restore specific files from previous versions. In either case, we simply add to the history (e.g. add more commits) that simply reverse previous changes.

To reverse the changes introduced by a commit, you can give a commit name after `git revert`. As an example, consider the following terminal example introducing, and then reverting a commit:

```
$ git add test.txt
$ git commit -m "Just testing"
[tutorial_cj 57e1f4e] Just testing
$ git revert 57e1f4e
[tutorial_cj a0afdee] Revert "Just testing"
```

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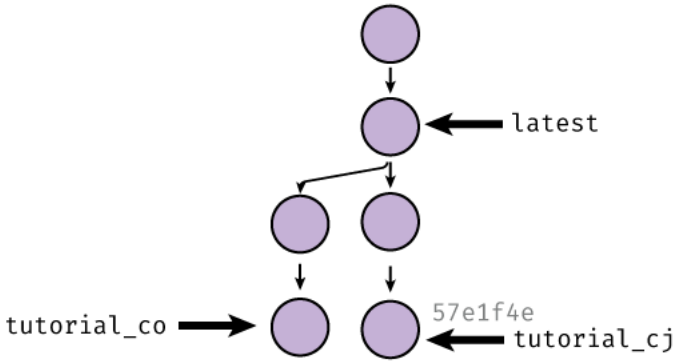
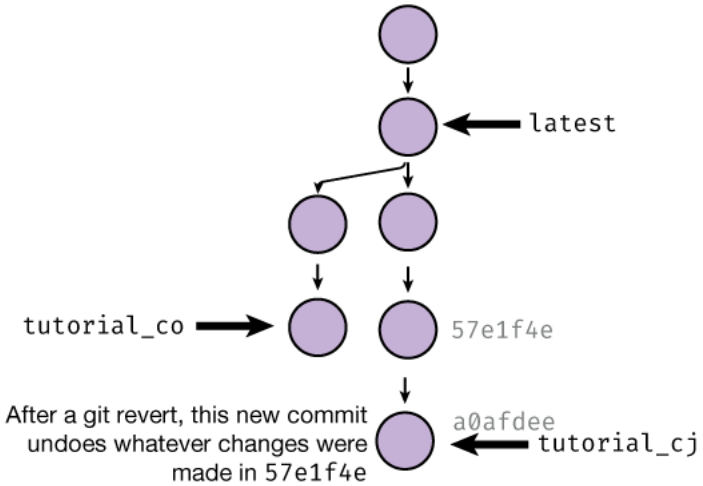
```
 1 file changed, 1 addition(+)
$ git log
commit a0afdeee60ac8acb9be8066abb2a95fde70a2df8 (HEAD -> tutorial_cj)
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:24:27 2021 -0500

    Revert "Just testing"

    This reverts commit 57e1f4ea06f25a7c05c6d03fcc297efa458bc8e4.

commit 57e1f4ea06f25a7c05c6d03fcc297efa458bc8e4
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:24:21 2021 -0500

    Just testing
```

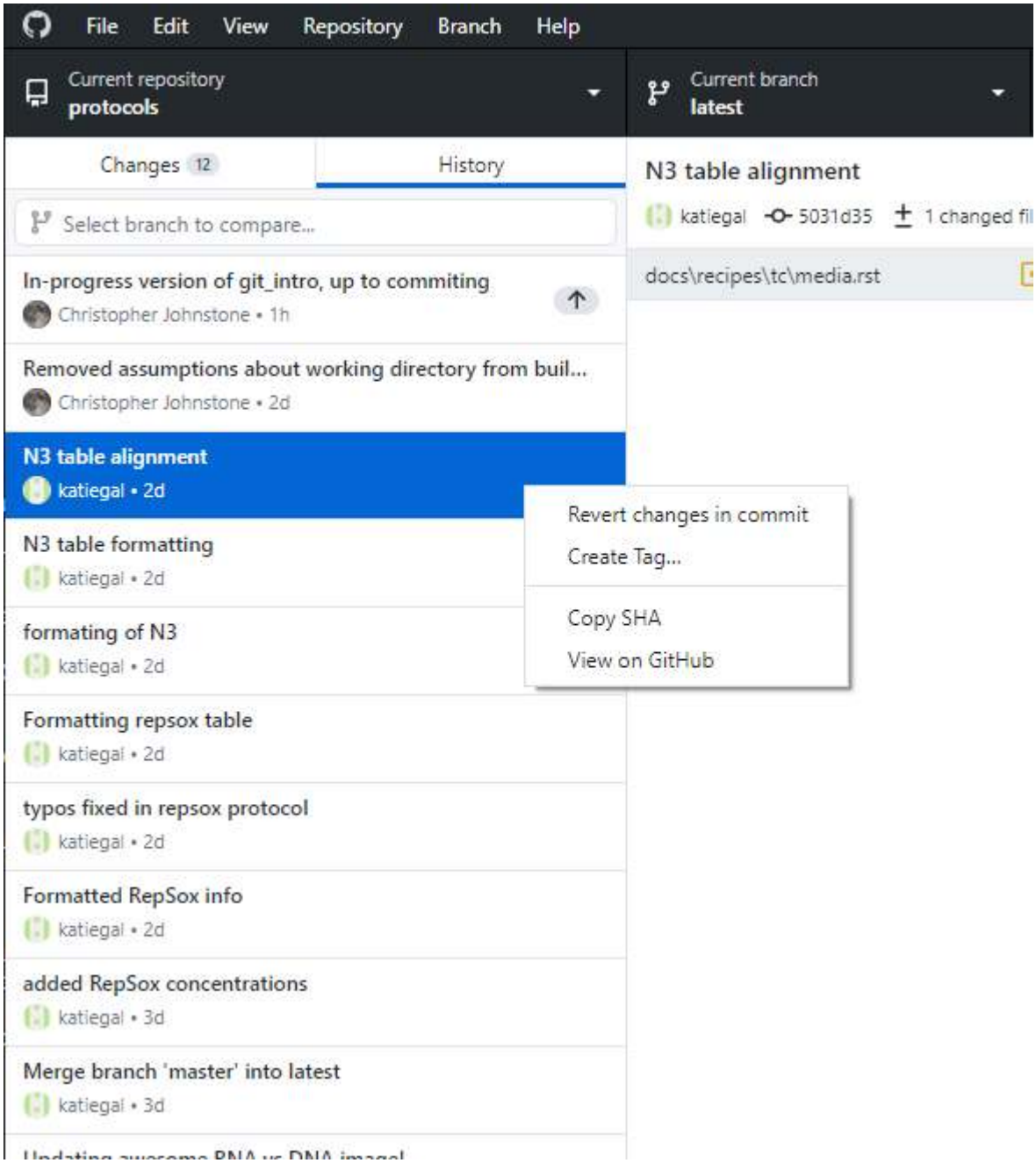
Visually, what has happened is that we have gone from this tree:



to this one:



If you'd like to use a GUI tool to revert changes, you can do so! Going to the History tab in Github Desktop and right-clicking on a commit gives you the ability to revert it:



How do we revert single file changes? We can use the same **checkout** command that we used to switch between branches, but this time limit it to specific files. If we specify a commit name to checkout from and a list of files, Git will fetch the state of those files from the previous commit. In the previous example, if we wanted to

```
$ git checkout 57e1f4 test.txt
Updated 1 path from 57e1f4e
```

Most GUI tools do not let you do specific-file checkouts; you have to use the terminal for this.

Exercise

1. Create some change to a file in this repository; just really mash the keyboard, add this change, and commit it (New commit #1)

2. Make a second change to **another location** in the same file, add, and commit it. (New commit #2).

3. Using `git log` or any other tool, find name of New commit #1, and revert it.

4. Use a selective `git checkout` to checkout the version of the file prior to New commit #1 (e.g. before you edited it for this exercise).

5. Commit this change.

6. You've now made four commits. What does your `git log` look like?

1. Say you edit the `test.txt` file. Then:

```
$ git add test.txt
$ git commit -m  "Some keyboard mashing"
[latest e033746] Keyboard mashing
1 file changed, 1 insertion(+)
```

2. After making a second change:

```
$ git add test.txt
$ git commit -m "A second change"
[latest 2b2e177] A second change
1 file changed, 1 insertion(+)
```

3. We could look at our history, but we also see the commit name (`e033746`) after our first commit. We revert this with:

```
$ git revert e033746
[latest ecd301c] Revert "Keyboard mashing"
1 file changed, 1 deletion(-)
```

4. Looking in our `git log` output, we see that the commit prior to "Keyboard mashing" is commit `65c5e3f`. We checkout `test.txt` from there and commit:

```
$ git checkout 65c5e3f test.txt
Updated 1 path from 171189d
$ git commit -m "Restored file back to its original version"
[latest 2da20a3] Restored file back to its original version
1 file changed, 1 deletion(-)
```

5. The `git log` looks like:

```
$ git log
commit 2da20a30521b41bbdf973e560c7549e1be3a0a4b (HEAD -> latest)
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:55:45 2021 -0500

    Restored file back to its original version

commit ecd301c798d162fc547661f897de277789b09d4f
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:52:53 2021 -0500

    Revert "Keyboard mashing"

    This reverts commit e03374683b76f9f2a3d70bafdb6ff257af90b0e4.

commit 2b2e1778e4ce0df4c31a351b54c9e11e52c15678
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:51:00 2021 -0500

    A second change

commit e03374683b76f9f2a3d70bafdb6ff257af90b0e4
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:49:12 2021 -0500

    Keyboard mashing

The ``.git`` folder contains several text files encoding the current branch
among other information, with several folders that store the history.
```

Working with others: remotes and inevitable merge conflicts

At this point, how do we share our wonderful work with others? This normally involves transferring our history with some central server.

Because Git has immutable history and we tend to only add new commits to the history tree, integrating our changes with others is easy; the hard part is **merging** multiple people's changes together.

Taking a quick "big-picture" break, in Git, you can connect to other versions of your repo through something called **remotes**. Any repository you want to interact with that is not your local version is called a **remote**.

If you initalize a new repository, it starts off with no remotes; it doesn't know about anything else! In this case, we **cloned** the protocols repo, so we do have a remote; the location on Github! By default, the first remote is called `origin`, as it is the origin which you cloned from.

You can see details about your remotes using `git remote`. Here, we'll add a `-v` flag to give more

verbose output:

```
$ git remote -v
origin  https://github.com/GallowayLabMIT/protocols.git (fetch)
origin  https://github.com/GallowayLabMIT/protocols.git (push)
```

We see that Git has two (identical) URLs, one for pushing changes (e.g. local to remote) and one URL for receiving changes (remote to local). In certain weird circumstances you might want these to be different URLs, but generally it's ok that they are the same thing.

To share your work with others, you **push** your branch *to the remote*. To get work from others, you **pull** the remote branch to yourself.

What happens if two people have both added their own local commits to a branch? Let's find out!
.. admonition:: Exercise

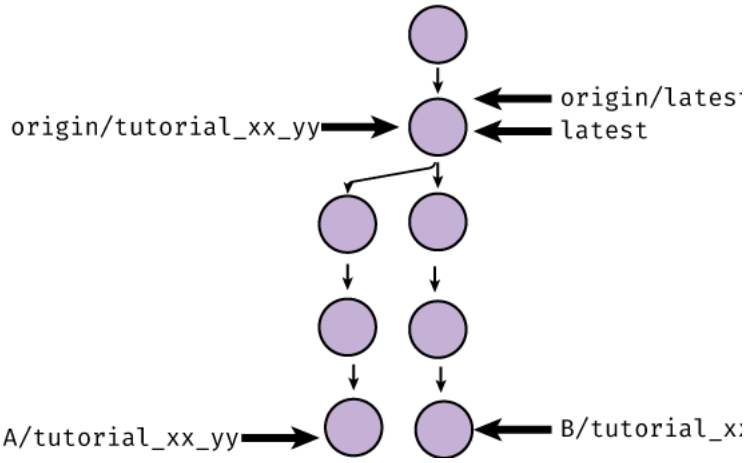
One partner should push the shared branch first:

```
$ git push
Enumerating objects: 28, done.
Counting objects: 100% (28/28), done.
Delta compression using up to 8 threads
Compressing objects: 100% (19/19), done.
Writing objects: 100% (22/22), 2.03 KiB | 346.00 KiB/s, done.
Total 22 (delta 13), reused 1 (delta 0), pack-reused 0
remote: Resolving deltas: 100% (13/13), completed with 5 local objects.
* [update]      tutorial_xx_yy -> tutorial_xx_yy
```

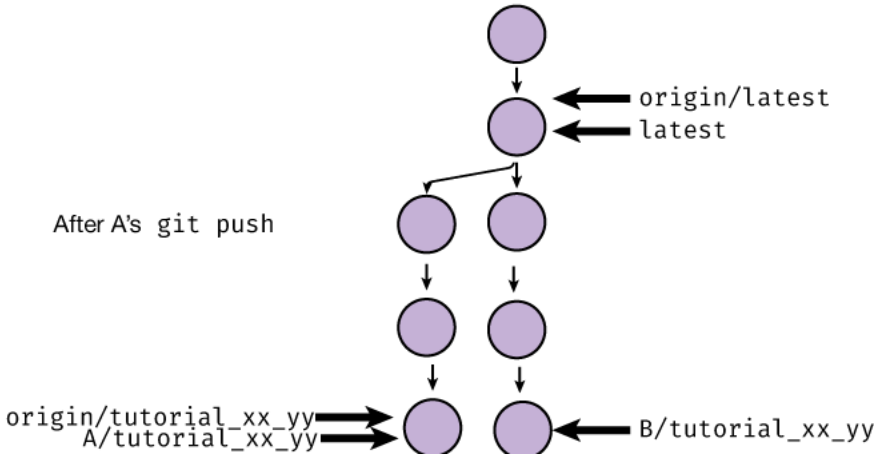
What happens when the other person now tries to push?

```
$ git push
To https://github.com/GallowayLabMIT/protocols.git
! [rejected]        tutorial_xx_yy -> tutorial_xx_yy (fetch first)
error: failed to push some refs to 'https://github.com/GallowayLabMIT/
↪protocols.git'
hint: Updates were rejected because the remote contains work that you do
hint: not have locally. This is usually caused by another repository␣
↪pushing
hint: to the same ref. You may want to first integrate the remote␣
↪changes
hint: (e.g., 'git pull ...') before pushing again.
hint: See the 'Note about fast-forwards' in 'git push --help' for␣
↪details.
```

To explain this message, let's look at how the history changed. Initially, the branch on Github was where it was initalized at the beginning, with each person having local commits:

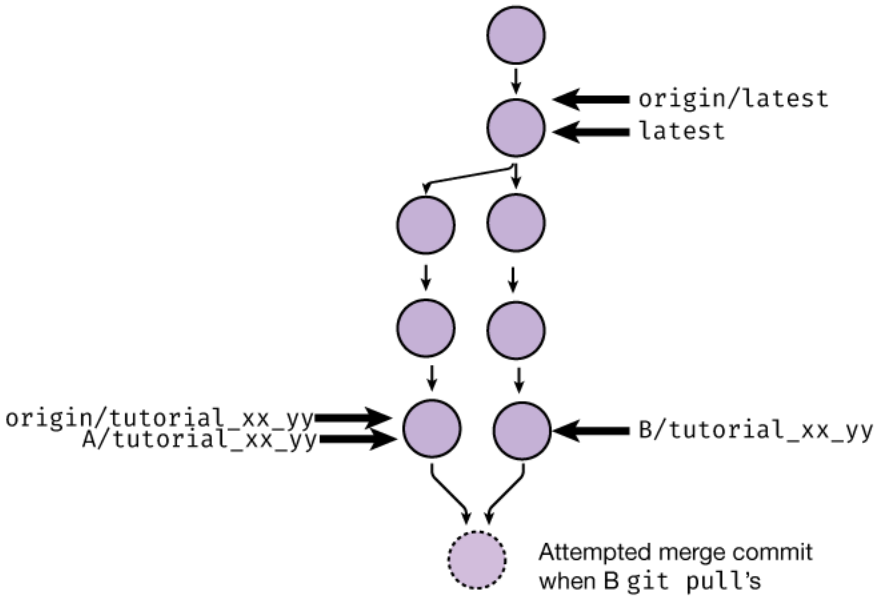


The first person's push (A) set the remote branch `origin/tutorial_xx_yy` equal to **their** version of `tutorial_xx_yy`:



Then, when the second person (B) went to push, Git rejected the push because setting `origin/tutorial_xx_yy` to the location of `B/tutorial_xx_yy` would erase A's changes! Git only allows so-called "fast-forward" pushes, where the branch pointer moves down the tree without backtracking.

We solve this by having person B **pull** the changes. Starting a `git pull` will attempt to create a **merge commit** that has two parents: the old `B/tutorial_xx_yy` and `origin/tutorial_xx_yy`:



Git is very smart about merging changes; it is capable of automatically merging relatively complicated scenarios, such as one person moving a function within a file and another person editing within the same function. If this was implemented like "Track Changes" in Word, everything would explode; the move would be marked as a deletion/insertion, which would conflict with the other edits. Luckily, Git tracks this in a sane way.

However, sometimes Git cannot automatically merge changes. This most often happens when two commits edit the exact same lines within a file. When this is a case, you will get a scary-looking message when you pull:

```
$ git pull
Auto-merging docs/training/onboarding/git_intro.rst
CONFLICT (content): Merge conflict in docs/training/onboarding/git_intro.rst
Automatic merge failed; fix conflicts and then commit the result.
```

This long error message is just Git informing you that it could not automatically merge.

If we run `git status`, we will get more information on what to do:

```
$ git status
$ git status
On branch tutorial_xx_yy
You have unmerged paths.
(fix conflicts and run "git commit")
(use "git merge --abort" to abort the merge)

Unmerged paths:
(use "git add <file>..." to mark resolution)
        both modified:   docs/training/onboarding/git_intro.rst
```

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```
no changes added to commit (use "git add" and/or "git commit -a")
```

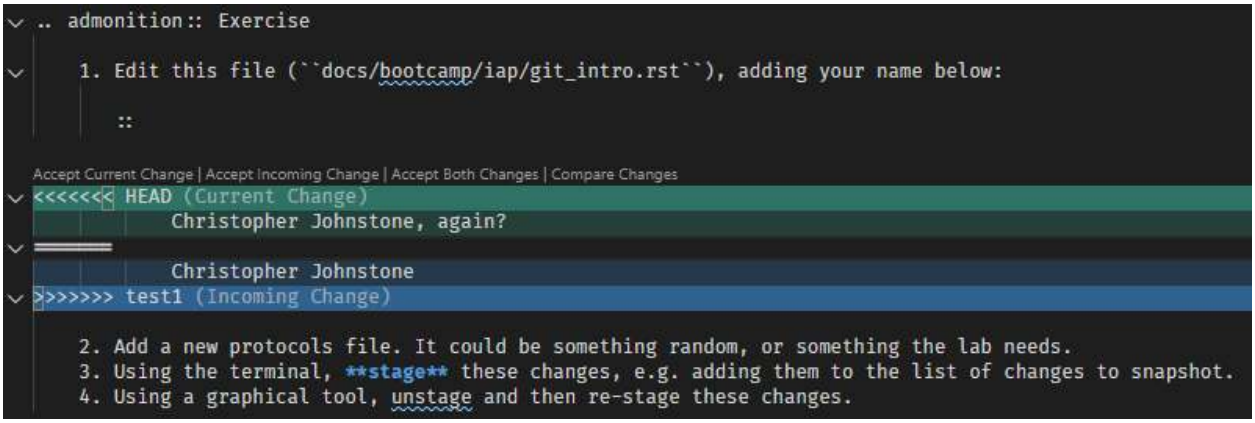
It tells us that we have to **1)** fix the merge conflicts, **2)**, use `git add` to tell Git that we fixed the conflicts, and **3)**, run `git commit` to finish the merge.

How do we know where the conflicts are? In each file listed as conflicted, Git added **conflict markers**, which are <<<<<<<, =======, and >>>>>>>. For example, a likely conflict you will get is in the name editing portion. This means that the file now looks like:

```
    1. Edit this file (``docs/training/onboarding/git_intro.rst``), adding your␣
↪name below:

    ::

<<<<<< HEAD
            Christopher Johnstone, again?
======
            Christopher Johnstone
>>>>>> test1

    2. Add a new protocols file. It could be something random, or something the␣
↪lab needs.
    3. Using the terminal, **stage** these changes, e.g. adding them to the list␣
↪of changes to snapshot.
    4. Using a graphical tool, unstage and then re-stage these changes.
```

Between the markers, you can see the two different versions of the file that conflicted. It is up to you, the human, to decide what to do.

If you are using VS Code, these markers trigger special syntax highlighting:

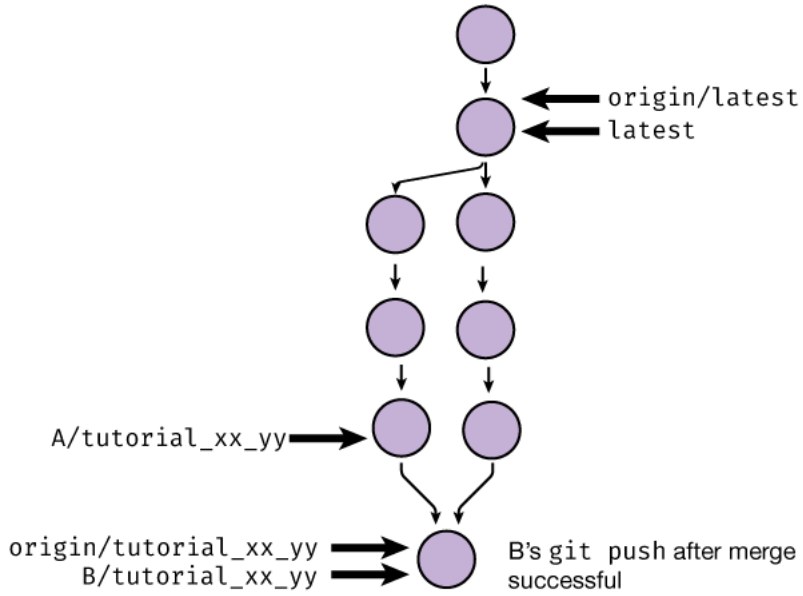


If it is indeed the case that you simply want to take one change, deleting the other, you can use the options at the top to do so. **However, this is often not what you want to do**. After doing whatever you think the correct merge is, you should delete the marker lines.

After we have resolved the conflict, we can finish the commit:

```
$ git add docs/training/onboarding/git_intro.rst
$ git commit
```

Finally, after we have a successful merge commit, the other partner can push their branch; it is now a fast-forward and is accepted:



Exercise

```
$ git push
Enumerating objects: 28, done.
Counting objects: 100% (28/28), done.
Delta compression using up to 8 threads
Compressing objects: 100% (19/19), done.
Writing objects: 100% (22/22), 2.03 KiB | 346.00 KiB/s, done.
Total 22 (delta 13), reused 1 (delta 0), pack-reused 0
remote: Resolving deltas: 100% (13/13), completed with 5 local objects.
* [update]      tutorial_xx_yy -> tutorial_xx_yy
```

Exercise

1. The other partner should run `git pull`, resolve any merge conflicts, then push.
2. After the other partner has pushed, the first partner should pull in the freshly-merged changes. You're done!

Conclusion

Git is much more powerful, but these form the basics. Go and celebrate; you can now collaborate with others with code, while keeping the entire history safe :)

7.1.6 Software tips and tricks

Naming, and one date format to rule them all

Fig. 2: Courtesy of xkcd[?]

In file naming schemes, we typically use the `YYYY-MM-DD` format. The separators don't typically matter, you could use dashes or underscores or periods. (Most of the time, we use periods.)

This date format is both unambiguous and also sorts well (i.e., a lexicographic/alphabetical sort sorts in the correct time order).

[295] https://imgs.xkcd.com/comics/iso_8601.png
[296] https://xkcd.com/1179/

Finally, **avoid using spaces** in file and directory names! It's not a problem for most code, but it's a best practice that makes typing these paths in easier in a terminal.

Paper RSS feeds

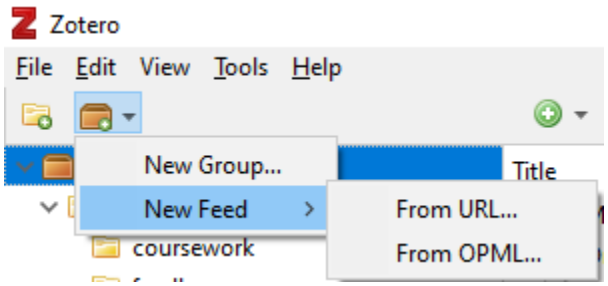
Staying on top of the firehose of papers can be difficult. Using a RSS (Really Simple Syndication) aggregator can be helpful. RSS is a relatively old technology that allows for websites to push lists of content over time; the first podcasts were actually syndicated/released using RSS.

Most journals have RSS feeds; you can find it by searching for a journal name + `rss`, or by looking around with the RSS icon:

For example, the main Nature feed lives at: http://feeds.nature.com/nature/rss/current

After finding some feeds you are interested in, you likely want to use a **feed aggregator**, something that combines all of the new pushed papers into a single feed. A very popular feed aggregator is Feedly[297]. It has a website and mobile apps. The free version is more than sufficient for most purposes; it lets you combine up to 100 RSS feeds into 3 separate feeds.

You can also view feeds directly in Zotero! If you export your feed list from Feedly as a OPML file, you can also direct-import it into Zotero.



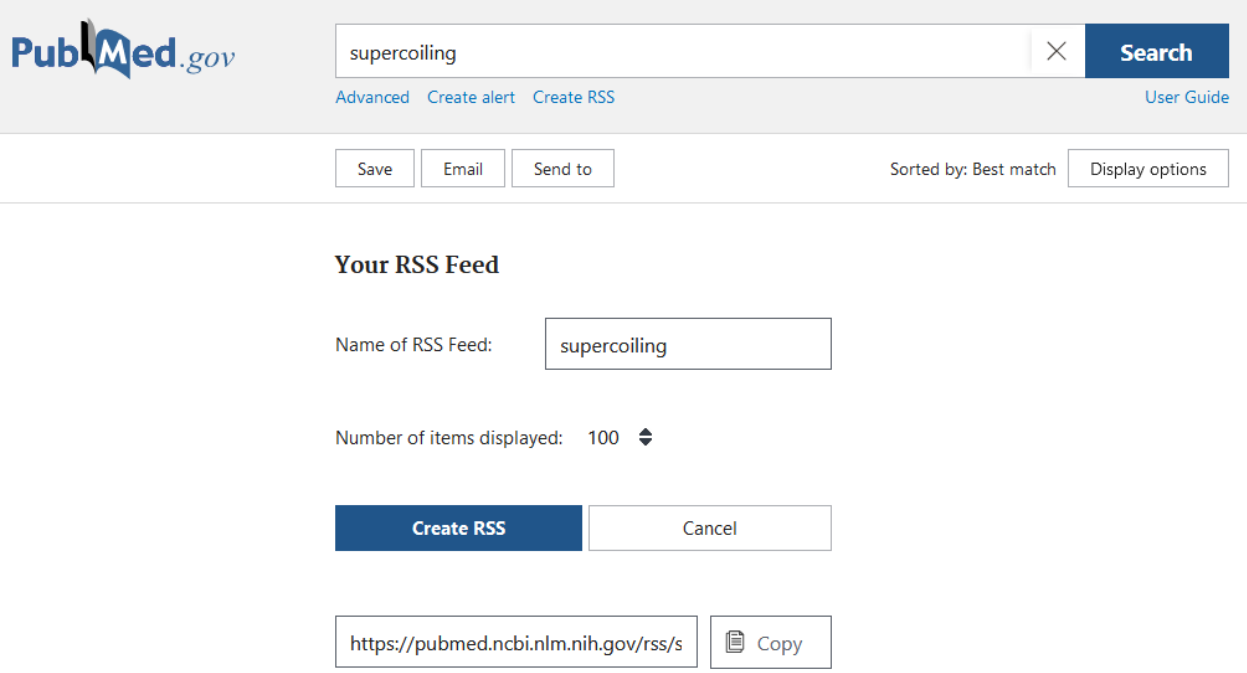
Creating feeds from searches

Sometimes you want to have a feed of papers matching some search terms. You can easily do this with Pubmed and ProQuest; whenever a new paper that matches your search terms gets added, it will get added to your RSS feed aggregator. Unfortunately, Google Scholar does not allow you to create RSS feeds (this would easily enable competition with their services).

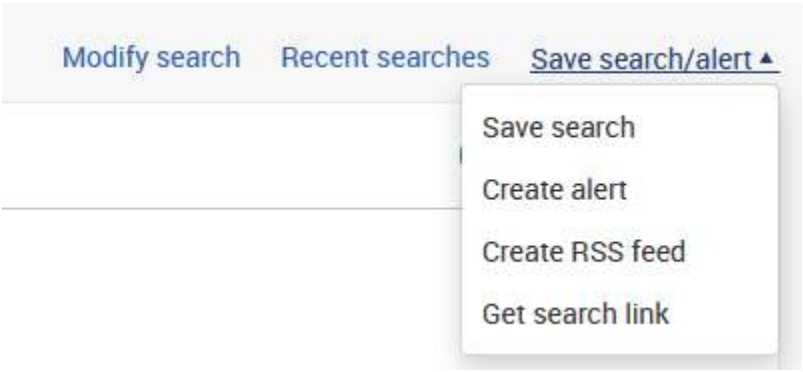
For Pubmed searches, start at https://pubmed.gov, and design your search. You may want to be pickier; a search for just `p53` is going to return a lot of junk!

After you have a search of your liking, click the `Create RSS` button, and bump up the returned number of items to 100. Then, you can directly use that feed URL in Feedly or Zotero!

[297] https://www.feedly.com/



To add a Proquest feed, you can go to https://search.proquest.com, make a search, then click the "Save search/alert" button to create a RSS feed:

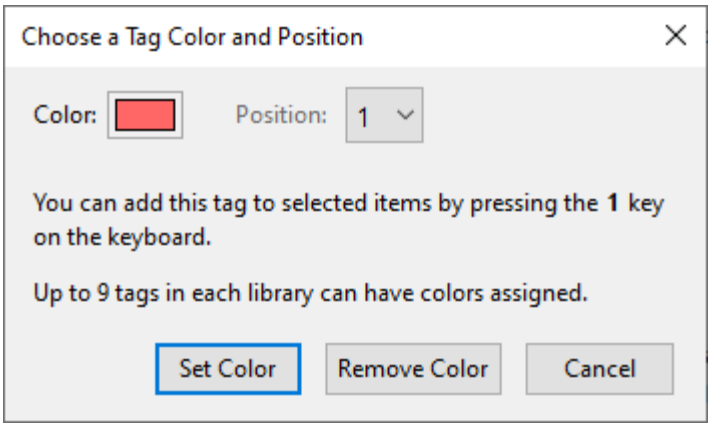


However, because Proquest is a MIT-sponsored database, you may have trouble accessing feed items outside of the MIT network.

Zotero

There are several built-in features of Zotero that make paper reading more efficient.

1. **Color tags**: Zotero sometimes adds automatic tags based on article metadata, but you can also add tags of your own under the details menu. In addition, you can select up to 9 tags at any one time as quick/color tags. In the tag menu in the bottom left, right clicking on a tag lets you assign a number and a color; tagged entries will have small colored squares viewable at a glance.

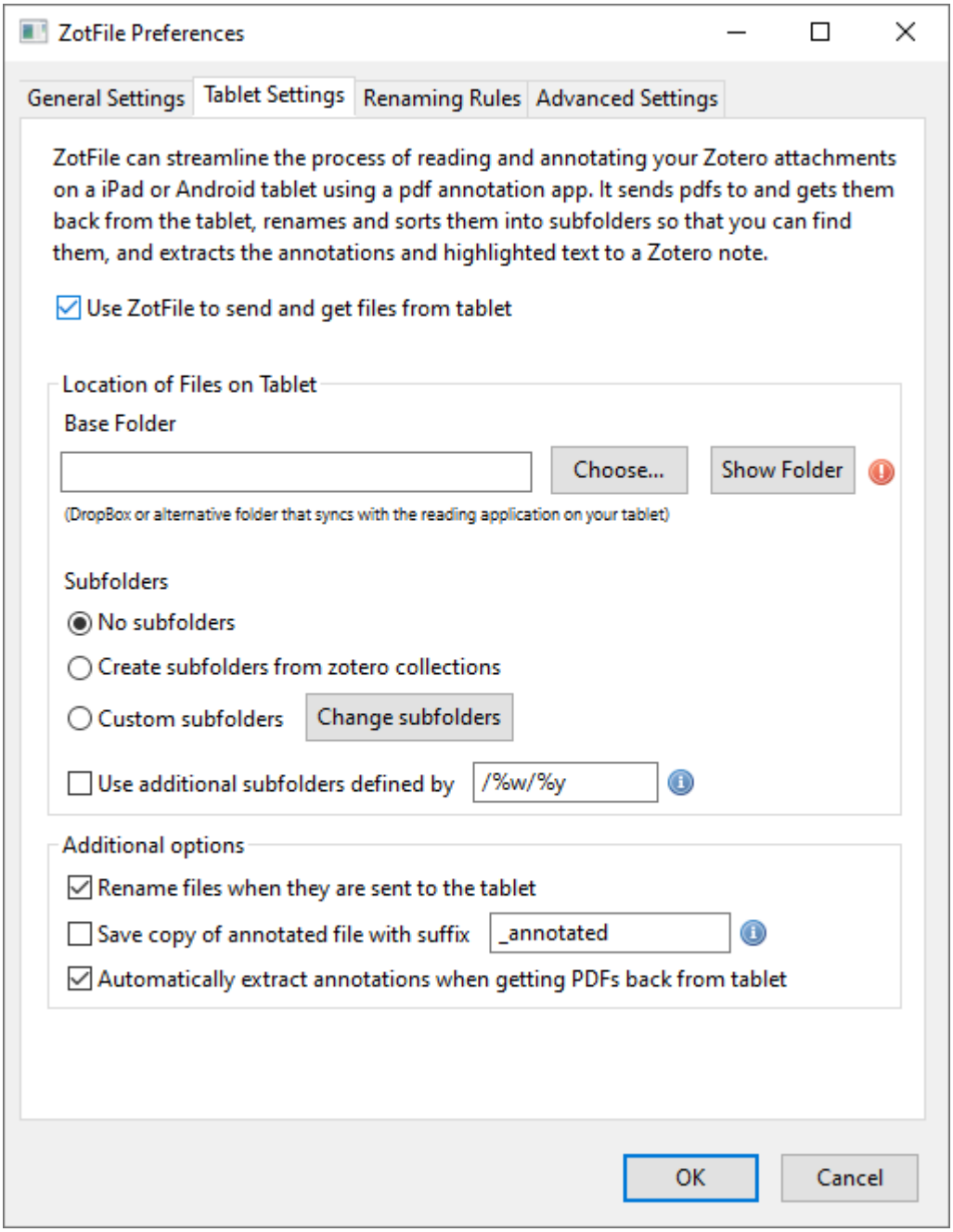


2. **Notes**: When you add notes through the sidebar, they become fully searchable! You can also embed images and other content within notes. This is particularly helpful for summarizing papers. A template for notes might look like the following:

```
One-sentence summary:


Highlights/claims:

Relevance:

Limitations:

Extensions:
```

3. **Full-text searching**: The search bar has several available options through the dropdown. Switching to "everything" includes full-text searching of linked PDFs (e.g. it will look for phrases inside every linked PDF)!

4. **Tablet syncing**: If you install the Zotfile extension, you can setup automatic transfer and syncing with a tablet for annotation purposes. You will need some cloud syncing application (Dropbox, OneDrive, Google Drive) that is accessible both on your computer through your annotation app on your tablet. Point ZotFile at this folder, and it will automatically check for updates and pull the annotated PDFs back into Zotero.



Regex

Regular expressions, or "regex"s are used for pattern-matching strings (words). This is useful for searching for files with names that follow a particular structure. For instance, the Python package `rushd` developed by the lab uses a regex to extract metadata from filenames when loading flow cytometry data in Python. Here's one tutorial[298] with examples to learn more about regular expressions.

If you ever need help designing or debugging a regular expression, try using https://regex101.com/

[298] https://librarycarpentry.github.io/lc-data-intro/01-regular-expressions.html

Be sure to change the "Flavor" on the left to Python, or whichever language you're using.

YAML files

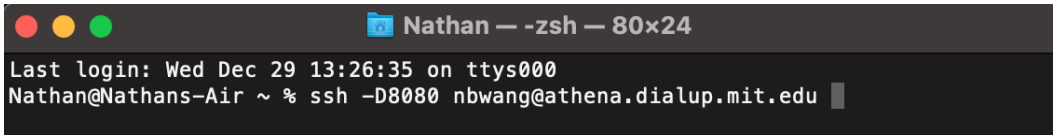
Several lab workflows use YAML files to add metadata to images, flow data, etc. YAML is a language/syntax commonly used for metadata or configuration files, similar to the JSON format.

A quick tutorial can be found here: https://learnxinyminutes.com/docs/yaml/ and the official introduction is here: https://www.yaml.info/learn/index.html

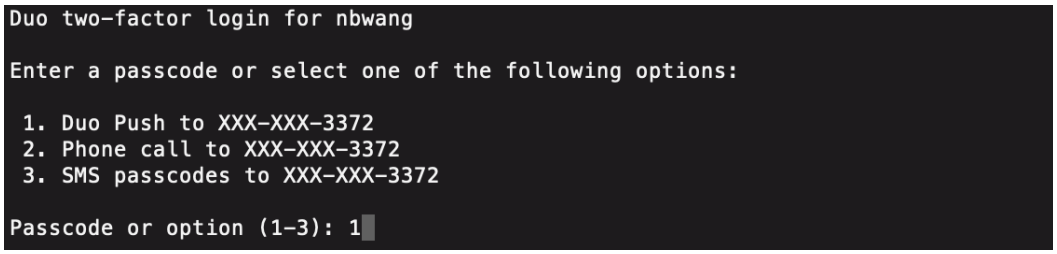
Activate SnapGene remotely

MIT uses a network license that can only be activated from an MIT IP address. To activate remotely, you can tunnel traffic through the Athena cluster via the following:

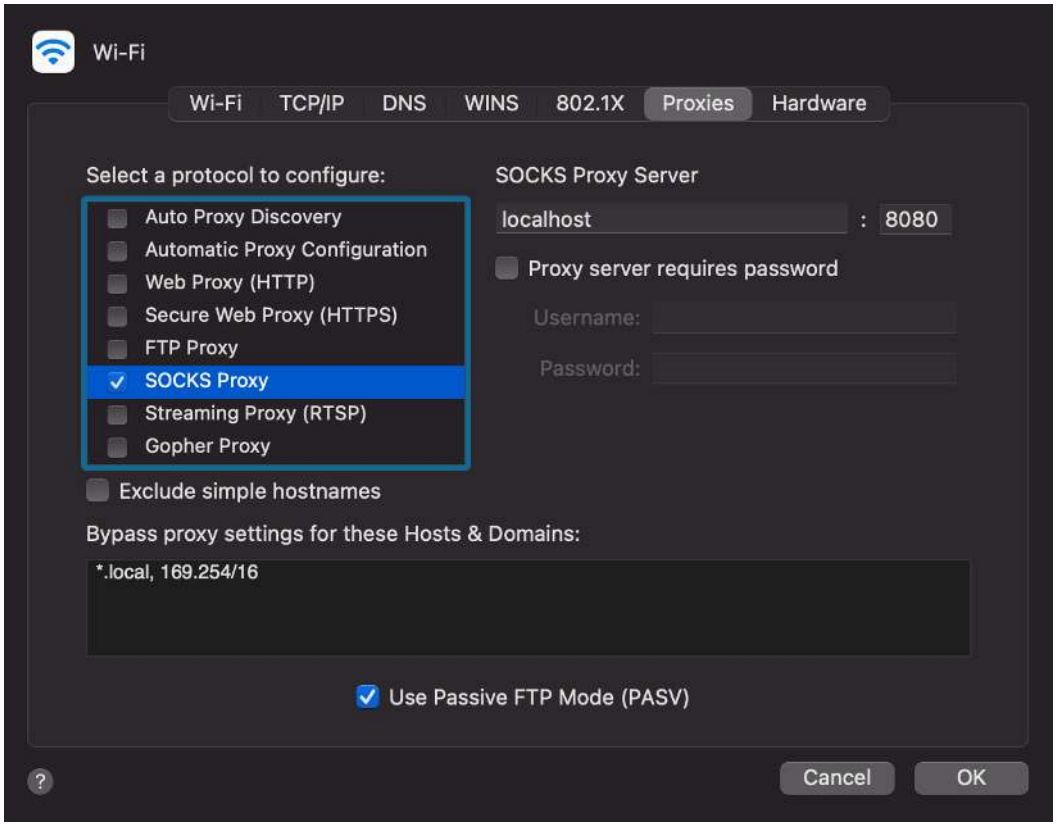
1. Run `ssh -D8080 KERBEROS@athena.dialup.mit.edu`



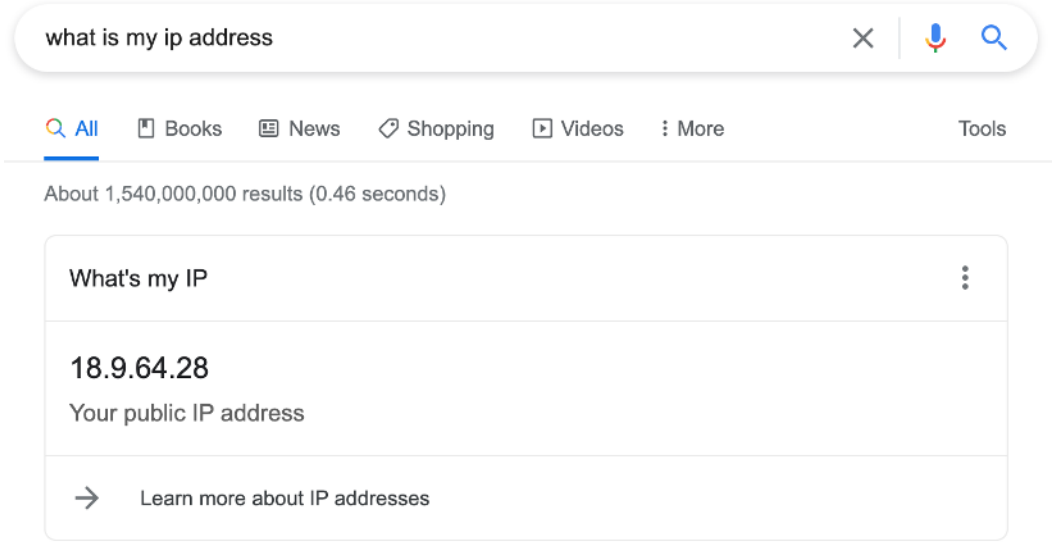
2. Login with your MIT password and do a Duo push in bash/powershell/etc. **Leave this window open.**



3. Set your system proxy settings to use a SOCKS v5 proxy to localhost , port 8080



4. Check that the system proxy works by typing “what is my ip address” into Google. You should get an IP starting with 18

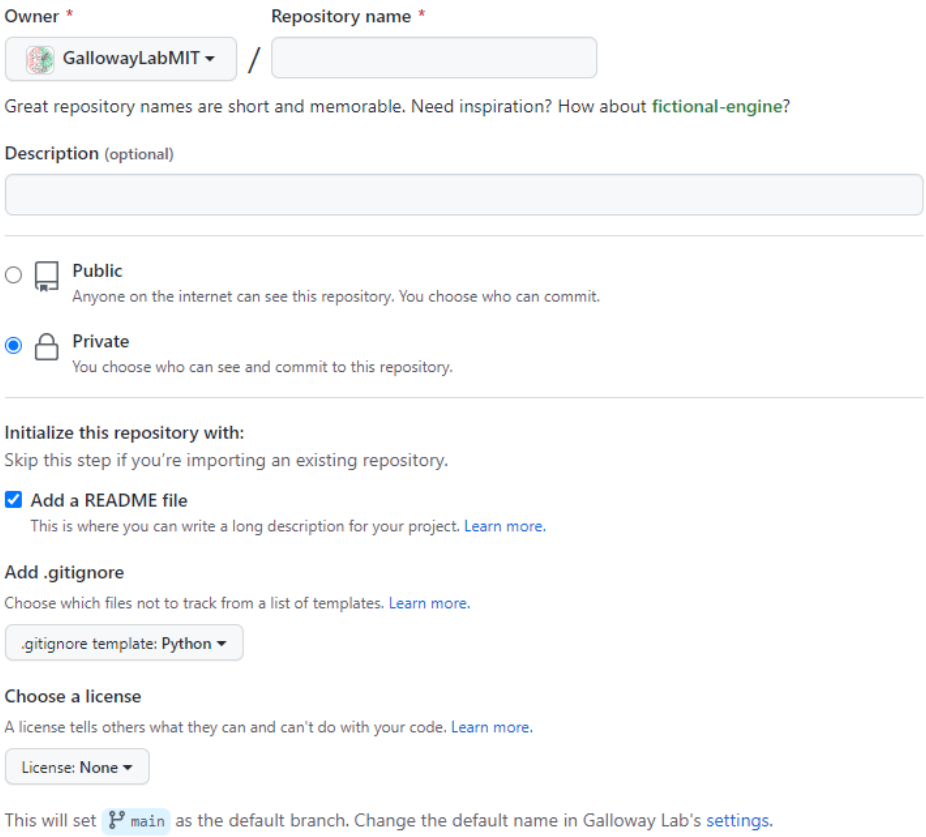


7.1.7 Startup checklist when working with repositories

When both creating a new repository or cloning (creating a local copy) of an existing repository, certain "startup tasks" need to be completed. These typically only have to be performed once, when you create the local copy, not every time you work with the repository.

New repository (Python)

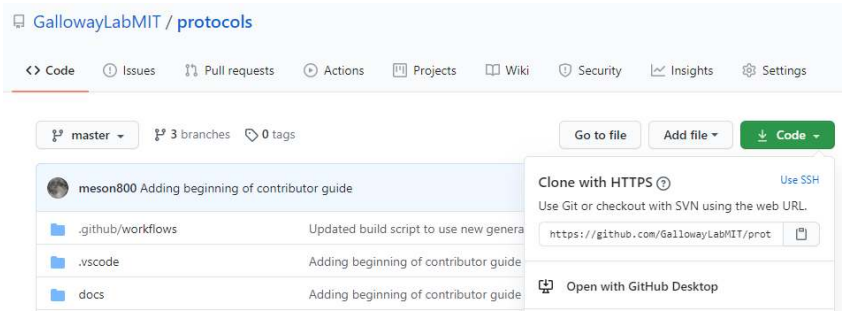
1. Create a new repository (aka repo) on Github, likely inside the GallowayLabMIT organization, by going to: https://github.com/organizations/GallowayLabMIT/repositories/new
    - When creating the repository, you likely want to check **Add a README file**. You should update this later with a description of the repository contents, as well as any non-standard setup instructions.
    - You should select **Python** as the `.gitignore` template. Setting the `.gitignore` means that Git will start off by ignoring all Python-related temporary files. You can update and modify the ignore list later.
    - Unless you know what you are doing, you can leave the License field set to None initially.



2. Clone the repository to some local folder.

> **Note**
>
> A common pattern is to put all of your git repositories in a `repo` folder in your home directory. Importantly, don't put git repositories inside OneDrive or another cloud-synced folder; in addition to duplicated version control, `git` tracks lots of small files internally which means a lot of syncing effort.

First, find the URL to the repository. You can get the link at the repository online under the green "Code" button. It probably looks something like `https://github.com/GallowayLabMIT/YourRepoName.git`



Then, *clone* (make a local copy of) the repo. There are several ways to do this, including the following:

- In a terminal:
  1. Navigate (`cd`) to the local folder you want to put the repo in.
  2. Run `git clone URL`, replacing `URL` with the one you found above.
- In VS Code:
  1. Open the Command Palette (`ctrl-shift-p` or `command-shift-p`) and select "Git: Clone".
  2. Paste the URL you found above, or search within your repos.
  3. In the pop-up file explorer, select the local folder you want to put the repo in.

3. Open a terminal in the repository folder (i.e., `cd` into the folder). It's easiest to do this and the following steps inside VS Code.
4. Create a **virtual environment** for this project.

   Virtual environments enable standardization by creating local copies of packages. That way, the correct package versions are associated with your code, allowing for reproducibility by running in the same "container" each time. This only needs to be performed when you first clone the repo. If weird package errors happen later, you can always delete the environment folder and recreate it.

   From the root of the repository (i.e., the folder containing `README.md`) create the environment using the `venv` Python module, passing the name of the virtual environment as an argument. In the command below, we give it the customary name `env`, but you can choose anything.

```
$ python -m venv env  # On Windows, most Linuxes
$ python3 -m venv env # On modern macOS
```

5. **Activate** the virtual environment. This typically has to be done every time you open a new terminal or when you switch between projects with different virtual environments. Once the environment has been activated, any Python changes you do (installing packages, etc.) will only affect this environment.

```
$ source env/bin/activate # On macOS, Linux
```

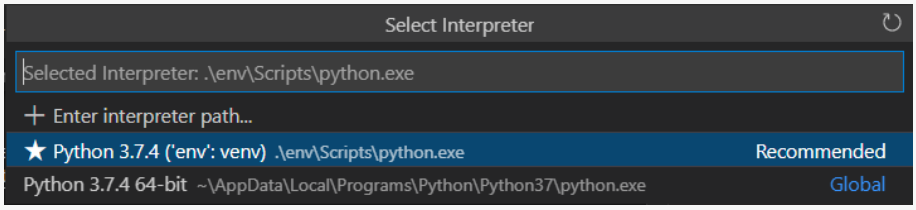
```
> .\env\Scripts\activate # On Windows
```

Now, the prompt in terminal should begin with `(env)`, indicating your environment is active.

Note

If you are working inside VS Code, right after you create the virtual environment, you may get a popup that says something akin to "New virtual environment detected. Do you want to set this environment as your project environment?" Answering yes means that all launched Python instances will use that environment by default. (Convenient!)

If you don't see the popup, you can also set the Python environment through the Command Palette. Press `ctrl-shift-p` or `command-shift-p`, search for "Python: Select Interpreter", and click the Python installation in your newly created virtual environment.



6. Install the packages you need. For data analysis projects, this is likely `pip install ipykernel matplotlib nb-clean numpy pandas rushd scipy seaborn`

   These packages are useful for computing with arrays and statistics (`numpy`, `pandas`, `scipy`); plotting (`matplotlib`, `seaborn`); and running Jupyter notebooks (`ipykernel`, `nb-clean`), an interactive computing workspace that combines sections of code with text. Finally, `rushd` is a package developed by the lab for common tasks related to data loading, analysis, and plotting.

   If you are using `nb-clean`, in the terminal run `nb-clean add-filter`. From then on, this package will automatically run alongside Git as a filter to remove extraneous notebook metadata.

7. Save your environment into a `requirements.txt` file using `pip freeze > requirements.txt`. This means other people can reproduce exactly the set of packages you just installed. If you install or update packages later, remember to update the requirements file by repeating `pip freeze > requirements.txt`.

8. If you will eventually load data from Smithsonian, create a `datadir.txt` file in the top-level folder of the repository. This file should contain one line with the full, absolute path to where Smithsonian syncs locally on your computer. You don't need any quotes or other characters around the path.

   For instance, a path on macOS might look something like:

   ```
   /Users/username/Library/CloudStorage/Nextcloud-kerberos@mit.edu@smithsonian.
   ↪mit.edu/data
   ```

9. Mark items that Git should not track by adding them to your `.gitignore` file. This means adding a line in `.gitignore` (usually at the top) for each file or directory.

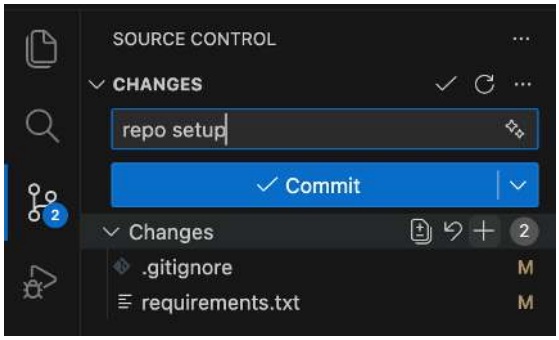
   Typically, this includes your virtual environment `env` and any output files you save in a directory like `output`, as you can always re-create these later. Other files like `datadir.txt`, `.DS_store` (on macOS), and `.vscode` save information relevant only to your local computer, so these should not be tracked by Git.

   Here's an example:

   ```
   # custom
   env/
   datadir.txt
   output/
   .vscode
   .DS_store

   # defaults continue below
   ...
   ```

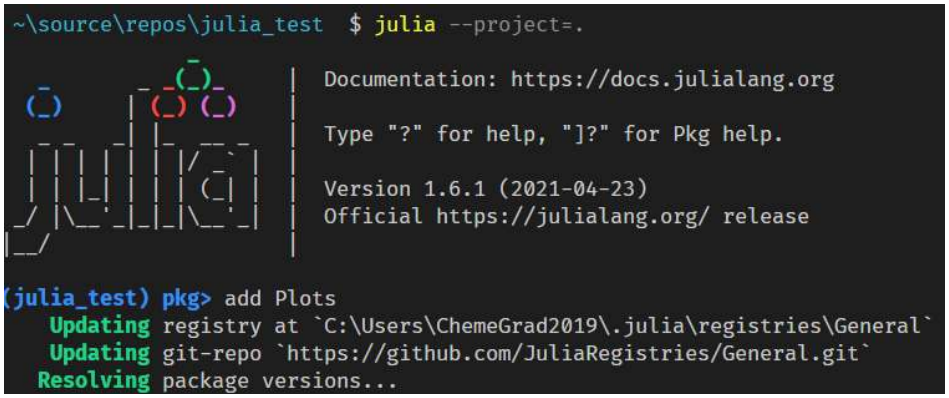
10. This is a good time to commit your changes, probably with a commit message like "repo setup".

    You can do this in the terminal with `git commit -m "Your message"`, or in VS Code in the "Source Control" pane. For the latter, stage changes first by clicking the plus icon next to each change, type your commit message in the box, and click the blue "Commit" button.



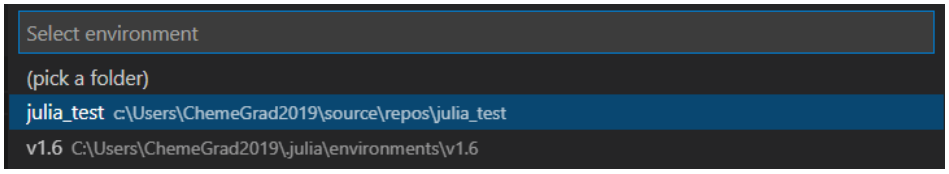
New repository (Julia)

1. Follow steps 1-3 above for creating a new repository. Except, select **Julia** as the `.gitignore` template.
2. Start a Julia instance inside a local virtual environment by typing `julia --project=.` into a terminal. Unlike Python, you do not have to pre-create a virtual environment, and you specify a virtual environment at launch using the `--project` syntax.
3. Inside the Julia prompt, press `]`. The prompt should change to `(folder_name) pkg>`. Type `add pkg1 pkg2` to install packages (replacing `pkg1`, etc. with package names) into the virtual environment.



> **Note**
>
> When you add or update packages later, be sure to commit the `Manifest.toml` and `Project.toml` files! These describe how others can reproduce your set of packages.

4. Once you have `.jl` files, VS Code should auto-select your local virtual environment. If it doesn't, you can open the command palette (`ctrl-shift-p` or `command-shift-p`) and search for "Julia: Change Current Environment" and select your newly created environment.



New repository (R)

> **Warning**
>
> TBD. R does not have a virtual environment system built-in, and this system is also bad compared to others. It is really hard to decouple system state in a reproducible way in R compared to Julia and Python.
>
> A possible best practice environment (Dockerized containers) is currently under beta testing.

Existing repository (Python)

1. Follow steps 2-5 for "New repository (Python)" *above* (page **??**) to clone the repo, create a virtual environment, and activate it.
2. Install the current package versions for this project using `pip install -r requirements.txt`.
3. If using Jupyter notebooks, run `nb-clean add-filter` to register the cleaning filter with Git.
4. If using `rushd`, add a `datadir.txt` file to the root folder of the repository. This file should contain one line with the full, absolute path to where Smithsonian syncs locally on your computer. You don't need any quotes or other characters around the path.

   For instance, a path on macOS might look something like:

   ```
   /Users/username/Library/CloudStorage/Nextcloud-kerberos@mit.edu@smithsonian.
   ↪mit.edu/data
   ```

5. Any additional setup should be described in the `README.md` file of the repository.

Existing repository (Julia)

1. Clone the repository, following steps 2-3 for "New repository (Python)" *above* (page **??**).
2. Start Julia within the virtual environment using `julia --project=.`
3. Enter package mode by pressing `]`
4. Run `instantiate` to automatically install the reproducible list of packages in the Manifest and Project files.
5. Any additional setup should be described in the `README.md` file of the repository.

7.1.8 On terminals and shells

Motivation

Knowing the basics of using a command line interface is occasionally helpful. While a lot of software has nice GUI interfaces, making these interfaces takes a large amount of work. Software written by a small number of people (like research groups!) often are only accessible via a CLI. In many cases, a command line interface can also be a faster way to do certain operations.

Even if you avoid using a terminal in daily computing, a terminal is often the only way that you can access high performance computing clusters.

The basics

Lots of words get thrown around: CLI, terminals, bash, shells, command prompts. For these purposes:

- A **terminal** is the program that runs on your computer and handles all of the low-level input output details. It is responsible for drawing to the screen, getting keyboard input, handling the clipboard, selecting fonts, and so on.

  Common terminals might be the programs *Terminal* or *iTerm2* on MacOS or *Windows Terminal*, *Powershell*, and *Command prompt* on Windows (more on this later).

- A **shell** is a command-line program (e.g. instead of interacting with us through a graphical interface, you type stuff in line by line) that lets the user take actions like modify files, view program output, run other command-line programs, and so on.

  Inside a terminal, you run a shell. Somewhat confusingly, the default terminals on both MacOS and Windows run a default shell, so that the shell and terminal appear identical.

  Common Unix shells (runnable on MacOS and Linux) are the original shell, `sh`, along with `bash` (common default on Linux), `zsh` (recent default on MacOS), and others like `csh` and `dash`. The two major Windows shells are `cmd` (old-school, going back to DOS) and `powershell` (Windows' modern shell).

When a shell starts it, it displays a **prompt**, showing that it is ready for input. Once you are done typing in your command, you hit enter to run that command.

When you run a command line program, the shell will show the prompt again when it's ready for more input. In these notes, we will use `$` to represent generic prompts (and > for the default Powershell prompt); lines without the `$` are example output of what you will see when you enter the command. When typing in commands, you should *not* type in the dollar sign! Your specific shell may display a more complicated prompt than a dollar sign, such as showing the current directory that you are currently in.

An example is:

```
$ echo "test"
test
```

[300] MacOS image from https://ohmyz.sh/

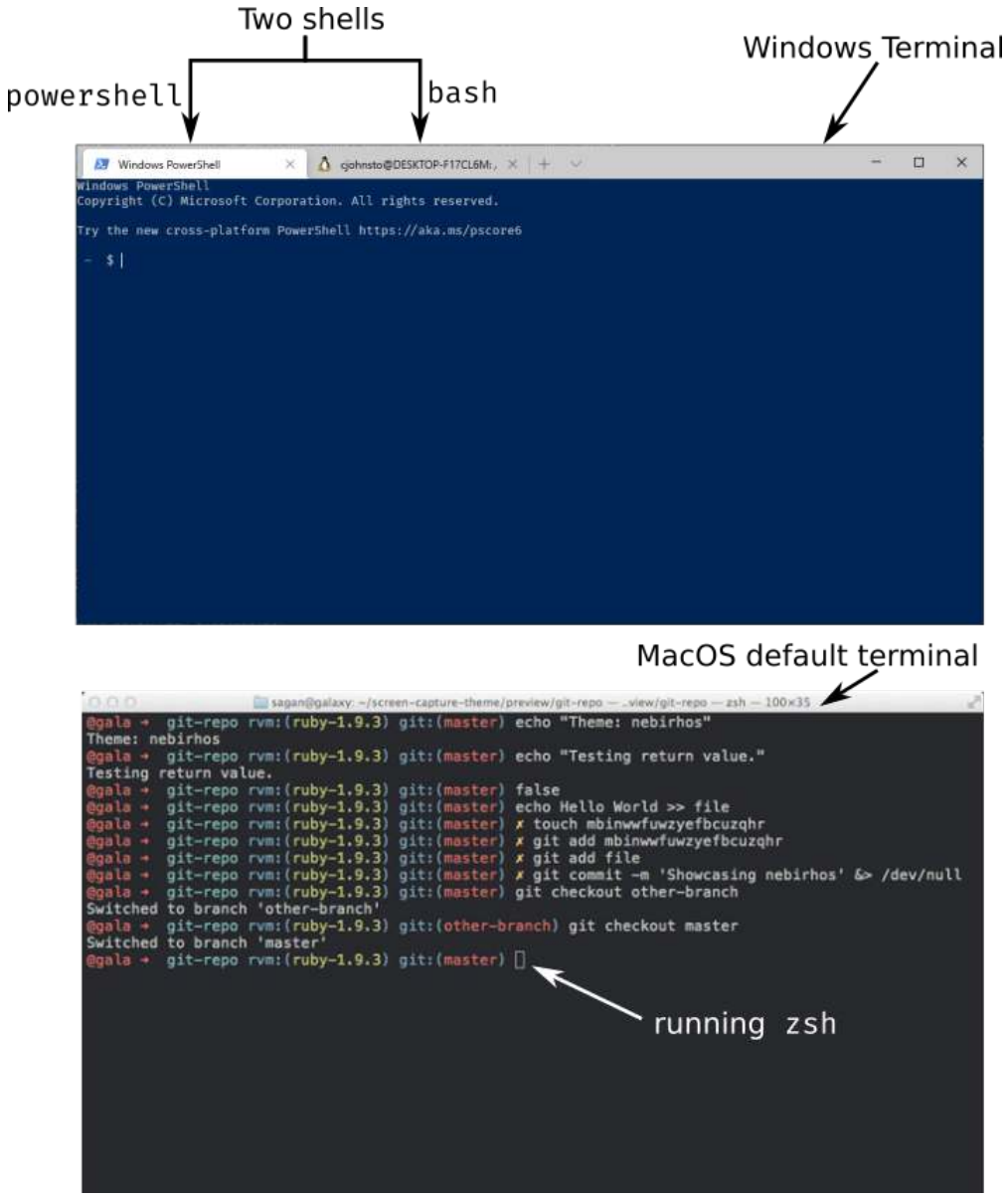


Fig. 3: An example of the difference between terminals and shells[7]

If we have an operating-system specific command to show, we'll show them in specific boxes, and we'll use the Powershell prompt symbol > for the Powershell examples.

```
$ echo "test"
test
```

```
> echo "test"
test
```

Finally, throughout this we will talk about **files** and **directories**. Directories are more commonly known as **folders**, but **directory** is the common shell terminology, so will be used here for consistency. However, saying folder is totally fine unless someone is being *really* pedantic.

Commands

In a shell, you enter commands to run them. When processing a line, the line you entered is first separated by the spaces present. Consecutive spaces are treated as a single space, with text in quotes being represented as a single "word".

Then, the first word is taken as the command name, and looked up in the list of installed command-line programs (e.g. see if it is present in `PATH`). The rest of the line is given to that program as it's **command line arguments**.

By convention, command line arguments that start with dashes are normally **options**. By convention, these options typically either have long names and start with two dashes, or have a "shorthand" form with a single dash and a single letter. Arguments that don't start with dashes are typically user-specified.

For example, in the following commands:

```
$ git commit --message "Testing out a commit"
$ git commit -m "Testing out a commit"
```

the command `git` is called in both cases, and is given the argument list:

(`commit`, `--message`, `Testing out a commit`) in the first case or (`commit`, `-m`, `Testing out a commit`) in the second. These two calls are identical in this case, because `-m` is shorthand for `--message`.

Demo

For the command line commands:

```
$ python -m venv env
$ git add testing.py module.py "explanation revised.docx"
```

what command is called in each case? What arguments are given to that command?

In the first example, the program `python` is ran with arguments (`-m`, `venv`, `env`).

In the second example, the program `git` is ran with arguments (`add`, `testing.py`, `module.py`, `explanation revised.docx`).

Interface basics

While the shell is minimalistic, there are three features that make our life easier:

1. **Job control**: Pressing Control and C (notated as `Control-C`, `Ctrl-C`, or `^C`) does **not** copy inside a terminal. Instead, `^C` is the command to quit the actively running program. The program is given a chance to clean up after itself (e.g. this is like hitting the exit button in a program, not force-closing it).

   On Unix-derived systems (Linux and MacOS), you can additionally pause a running program by pressing `Ctrl-Z`. When you pause the program, you will see `[1]+ Stopped` and you will be back at the shell prompt. To resume the program, type `fg` (to bring the program back to the **f**ore**g**round.

2. **Clipboard**: Because control-C does not copy, we need some other way of using the clipboard. On Mac, this is easy; copy and paste are typically bound to Command-C and Command-V. On Windows and Linux, it depends on what terminal you are using. A typical copy/paste solution binds the keyboard to right-click. On standard Powershell, you can select a region with your mouse and press enter to copy that to the clipboard. To paste, right click inside the terminal region.

3. **Tab completion**: Typing out full file names gets tiring, especially when you have long file names or deeply nested directory structures. Tab completion saves us: if you have a filename partially written, hitting `Tab` attempts to auto-fill the rest of the name. If there is a single unique file that matches what you have typed so far, that name is filled. If there are multiple files that might match, then the exact behavior differs per shell. Bash and similar shells will typically complete as much of the name as possible, but then stop. If you double-tap `Tab` in Bash, it will print a list of all possible matching files. In Powershell, pressing `Tab` cycles between files that match.

4. **History**: Retyping common commands also gets tiresome. You can access your command line history (e.g. the previous lines you have typed) by pressing the up and down arrows.

Starting off at home

When you first open a terminal, your shell will likely start off in your **home directory**, also known in shorthand as `~`. Each user has its own home directory. All of the user directories that you are used to accessing through Windows Explorer or Finder, such as `Desktop`, `Downloads`, or `Documents` are subdirectories of your home directory.

The actual location of your home directory differs, but is typically something like `C:\Users\Username` on Windows, `/users/Username` on MacOS, and `/home/Username` on most Linuxes.

So that we don't have to type that large thing every time, `~` is short-hand notation for whatever your home directory is. That is, the location of your downloads folder could be written either as `C:\Users\Username\Downloads` or more simply as `~\Downloads`

Note

You may have noticed earlier that these directory paths have been written differently between the two operating systems. In short, due to backwards compatibility, Windows uses the backslash \ as the path separator (written between directory names), whereas all Unix-derived operating systems including MacOS and Android use the forward slash / as the path separator.

Most of the time you can just use the forward slash without worry; `powershell` on Windows will auto-convert from forward slashes to backslashes if you use forward slashes, but when programming you should keep this in mind and not manually use slashes when constructing paths to filenames. It still may work, but you should ideally use filesystem-aware techniques, like using `os.path` or `pathlib` in Python.

The shell has a current location; imagine it as having a Finder/Explorer window open to some directory on your computer. This current location is called the (current) **working directory**. This is How do we know what directory we are in while using the shell? Our first command we will learn is `pwd`:

Command: `pwd`

`pwd` stands for **print working directory**, and does just that; it tells you what your current location is, in full detail (e.g. the entire path, not in shorthand). If you ever get lost, just type `pwd`!

This output is similar across operating systems; it is a little more verbose in Powershell.

Example output right after launch, so that you are starting in your home directory:

```
$ pwd
/users/username
```

```
> pwd

Path
----
C:\Users\Username
```

If we want to know what is inside the current directory, we can use `ls`:

Command: `ls`

`ls` stands for **list**, and lists every file and directory inside the current working directory.

If you were to run it in your home directory, you might get something like:

```
$ ls
Desktop    Downloads  Pictures
Documents  Music      Videos
```

If you want to see what is inside one of these directories, `ls` takes command line arguments specifying which directory you'd like the view:

```
$ ls Documents
10-50     10-40    10-34
research
```

To view **hidden files** (on MacOS/Linux, these are files/directories that start with a period; on Windows, these are files/directories with a hidden attribute set), we need to pass `ls` a command line option. This differs between shells, but on bash/zsh/etc, you use `--all` or `-a` to show hidden files as well:

```
$ ls -a
.         Desktop    Music     Videos
..        Documents  Pictures
.bashrc   Downloads  .profile
```

In Powershell, we pass the option `-Force`:

```
> ls -Force
Directory: C:\Users\username

Mode                 LastWriteTime         Length Name
----                 -------------         ------ ----
d--h--         1/11/2021   7:19 PM                .git
d-----         1/11/2021   7:19 PM                Desktop
d-----         1/11/2021   7:19 PM                Documents
d-----         1/11/2021   7:19 PM                Downloads
d-----         1/11/2021   7:19 PM                Music
d-----         1/11/2021   7:19 PM                Pictures
d-----         1/11/2021   7:19 PM                Videos
```

Moving away from home

To move what directory we are in, we can use `cd`:

Command: `cd`

`cd` stands for **change directory**, and switches the current working directory to whatever directory you give it. This is the major way that you move around the various directories to find files.

```
$ pwd            # Start off in your home directory
/Users/username/
$ cd Downloads   # move into the Downloads directory
$ pwd
/Users/username/Downloads
$ cd ~           # return the the home directory
$ pwd
/Users/username
```

Relative and absolute paths

The earlier examples have hinted at the existence of two types of paths/ways to reference files.

The first is using an **absolute path**; this is what we call specifying the entire path from the filesystem "root" to the file of interest. On Windows, this means paths like `C:\Users\username\Downloads`, where we specify the drive followed by every path component.

On MacOS and Linux, absolute paths start at the root, which is the special name given to the path `/`, so absolute paths look like `/Users/username/Downloads`.

In contrast, **relative paths** allow you to more concisely reference files and directories, as the paths are calculated relative to the current working directory.

It is fairly intuitive how this works for going into subdirectories; just specify the subdirectory name. To be able to reference directories "above" yourself in the tree, we need some way to reference these parent directories.

Luckily this is standardized; there are two special pseudo-directories accessible everywhere on the filesystem; the 'current directory' `.` and the 'parent directory' `..`. The current directory is always a sort of empty operation, but is useful if you want to run scripts in the same directory as yourself.

When these are passed to a command, they are evaluated starting at the current working directory.

Say that we start off in our downloads directory, `/Users/username/Downloads`. Then changing directory to relative directory `..` means going one step "up", to `/Users/username`

```
$ pwd
/Users/username/Downloads
$ cd ..
$ pwd
/Users/username
```

We can go up multiple layers at a time by combining these pseudo-directories together. For example, to go up two directories to `/Users` from `/users/username/Downloads`, you could just write `cd ../..`.

You can actually combine absolute and relative paths; the parent directory `..` will always go "up" a directory, effectively removing what comes to the left if combined in this way. For example, the paths `/users/username` and `/users/username/Desktop/..` both point to the same thing.

Demo

If your shell starts in the Downloads directory `/Users/amanda/Downloads`, which of the following will navigate to the directory `/Users/amanda/data`? `/Users/amanda` is your home directory.[301]

1. `cd .`
2. `cd /`

3. `cd /Users/amanda/data`
4. `cd ../../`
5. `cd home/data`
6. `cd ../data`
7. `cd ~/data`

The 3rd, 6th, and 7th examples will navigate to the proper directory.

1. `.` will stay in the same directory, `/Users/amanda/Downloads`
2. `/` is an absolute path to the filesystem root, not the correct directory.
3. `/Users/amanda/data` is the full absolute path to the desired directory, so this works.
4. `../../` evaluates to `/Users`, the wrong directory.
5. `home/data` will give an error, as it tries to navigate to `/Users/amanda/Downloads/home/data`
6. `../data` evaluates to `/Users/amanda/Downloads`, the correct path.
7. `~/data` also works, as `~` expands to `/Users/amanda`

File operations

Now that we can navigate around, we can learn file operations. The first is conceptually the simplest, as it creates a new directory:

Command: `mkdir`

`mkdir` stands for **make directory**. It creates a directory name equal to that of the argument it gets passed.

```
$ pwd            # Start off in the home directory
/Users/username/
$ cd test   # try moving into the test directory; it fails!
cd: test: No such file or directory
$ mkdir test     # Create the test directory
$ cd test        # Now the cd succeeds
```

Importantly, `mkdir` on Linux/MacOS can only make a single directory by default, so an error will occur if you try to create nested directories in one command (e.g. if we want to create the directories `test/inner_test` without first creating `test`, we'll get an error). Powershell does not have this limitation.

If we do want to create multiple nested directories, we can use the `-p` or `--parent` flag to tell `mkdir` that it is allowed to create parent directories if they don't exist.

[301] Inspired by the Software Carpentry examples[?], license CC-BY-4.0
[302] https://swcarpentry.github.io/shell-novice/02-filedir/index.html

Now that we can create directories/folders, how do we actually move files around? Using *mv*!

Command: `mv`

`mv` stands for **move**, and takes at least two arguments. We use `mv` to both move and rename files (renaming is just moving!).

The input arguments are `mv <source> <destination>`. Source can be a single file/directory, or it can be multiple! However, if multiple source files are given, then the given destination **must be a directory**! Put another way, even thouhg there can be multiple source files/directories, there can only be one destination.

If we want to rename a file we can do so with move:

```
$ ls
test.txt
$ mv test.txt old_test.txt
$ ls
old_test.txt
```

We can also rename directories in the same way:

```
$ mkdir test_dir
$ ls
test_dir
$ mv test_dir new_dir
$ ls
new_dir
```

If we want to move files, we can move them by naming them one by one. Consider the case where we have several `.txt` files in a subdirectory. We can use a wildcard to move everything matching a certain wildcard pattern:

```
$ ls
inner_dir
$ cd inner_dir
$ ls
test1.txt  test2.txt  test3.txt
$ mv test*.txt ../ # Move files to the parent directory (where we started)
$ ls       # now there are no more files in inner_dir
$ cd ..
$ ls       # but they are in the parent directory!
inner_dir  test1.txt  test2.txt  test3.txt
```

Copying files is very similar to moving files. In fact, it has almost all of the same semantics: the one difference is in how you have to copy directories.

Command: cp

cp stands for **copy** and has the same `cp <source> <destination>` semantics as move, **except in the case of copying directories**. The reason for this is even though it is "free" (e.g. doesn't take up extra disk space) to move directories, copying directories may involve a very large amount of storage space, so you must confirm this action.

To copy directories, you need to use the `-r` flag, which stands for a **recursive copy**.

If you try to copy a directory in bash (Linux/MacOS) without `-r`, you'll get an error:

```
$ ls
test_dir
$ ls test_dir
test1.txt
$ cp test_dir another_dir
cp: -r not specified; omitting directory 'test_dir'
```

If you try to copy a directory in Powershell (Windows), you won't get an error, but the newly "copied" directory will be empty.

In both cases, add the `-r` flag to copy directories:

```
$ ls
test_dir
$ ls test_dir
test1.txt
$ cp -r test_dir another_dir
$ ls
test_dir  another_dir
```

Our final command is what we use to delete files:

Command: `rm`

`rm` stands for **remove**, and it **permanently deletes files!** There is no recycle bin; things get immediately deleted. If you truly delete something important, then you'll need to try various data recovery techniques to get it back.

Much like `cp`, when deleting directories you need to add the `-r` (recursive) flag. If you don't, you'll either get an error message (Linux, MacOS) or a confirmation dialog (Windows).

```
$ ls
test_dir  file1.txt  file2.txt
$ rm file*.txt
$ ls
test_dir
$ rm -r test_dir
```

Editing and viewing files

The last part of this covers basic file editing. You'll often be editing using some other GUI tool, but it's helpful to know how to use a basic command-line editor, especially when viewing files on computing clusters.

To print out the contents of a file to the terminal, you can use the `cat` command:

Command: `cat`

`cat` stands for con**cat**enate. While it can still be used to combine multiple files together, it is more often used to print the contents of a file to the screen.

```
$ ls
text_file.txt
$ cat text_file.txt
This is the file's contents!
```

How do we actually edit files? There are many different command-line editors, but the most commonly installed ones are `vi` and `nano`, which typically both come preinstalled on Linux and MacOS. `nano` is much more user-friendly; `vi` and its predecessor `vim` are often much faster at day-to-day typing, but come with a steep (vertical?) learning curve.

Note

Windows comes without a command line text editor installed. If you have installed Git though, we can adjust your Powershell profile to have access to `nano`. Assuming Git has been installed to the default location you can do the following in Powershell:

```
> Set-Alias nano 'C:\Program Files\Git\usr\bin\nano.exe'
> nano $profile
    C:\Users\Username\Documents\WindowsPowerShell\Microsoft.PowerShell_profile.
```

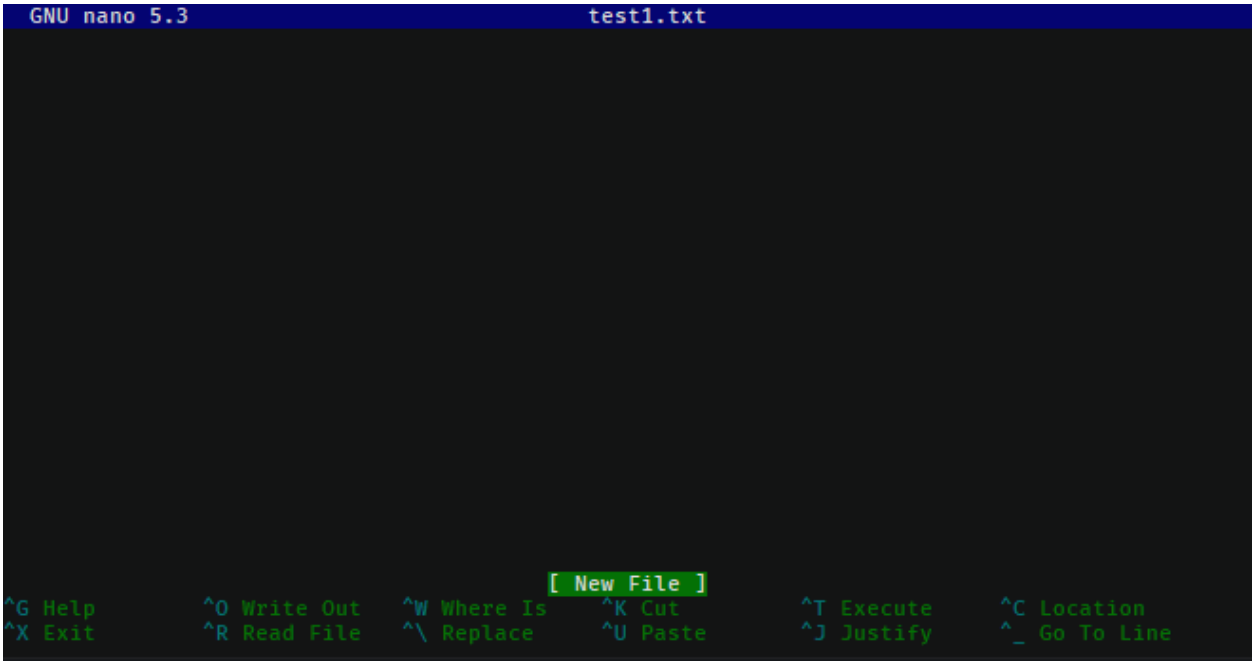
```
↪ps1

                        [ Read 1 lines (Converted from DOS format) ]
^G Help         ^O Write Out    ^W Where Is     ^K Cut          ^T Execute      ^C␣
↪Location
^X Exit         ^R Read File    ^\ Replace      ^U Paste        ^J Justify      ^_␣
↪Go To Line
```

Once in the editor, type the line `Set-Alias nano 'C:\Program Files\Git\usr\bin\nano.exe`, then press `Ctrl-O` (denoted `^O` in the bottom menu), press enter, accepting the filename, then press `Ctrl-X` to exit.

What this did was define what happens when you type `nano`; adding it to your profile script means that every Powershell session you start will have access to `nano`.

When opening Nano, you'll see the name of the file at the top. In the middle is your editing area; you can type and navigate around with arrow keys as you would expect.

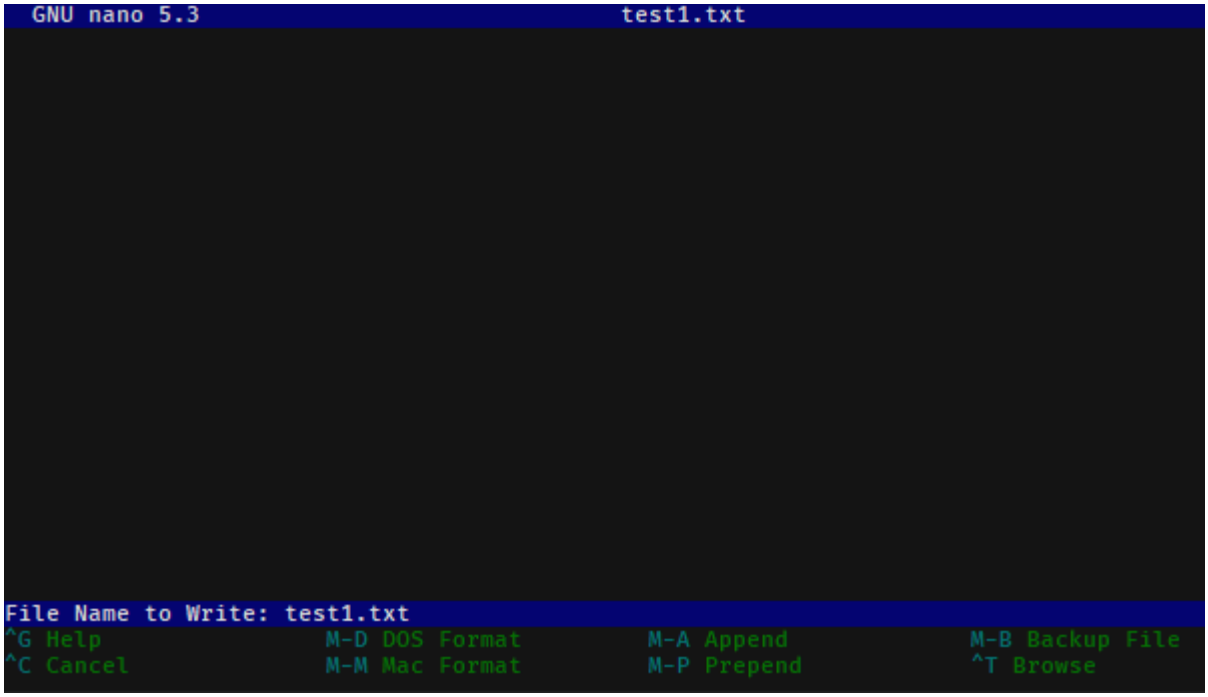
If you want to just open a blank editor, you can just type `nano`. If you want to edit a specific pre-existing file, then you can type `nano filename`.



At the bottom, you have a status bar that shows the available actions you can take. Here, control is represented with `^`, so `^X Exit` means you can press `Ctrl-X` to close Nano.

The important features are `Ctrl-X` to exit, `Ctrl-O` to save ("write out"), `Ctrl-W` for searching/find, and `Ctrl-\` for find-and-replace.

When you go to save a file after editing, you will bring up a bottom bar prompting you to give a filename. If you want to save with the same name as you opened, you can just hit enter; it auto-fills the current name. Edit the name if you want to save under a different filename.



Finding things in documents

`grep` is the tool you should use when searching through files. It lets you do basic searches and also regular-expression searches on one or multiple files.

Note

Windows also doesn't come with `grep` preinstalled. Add an alias to the Git-installed version as you did for `nano`:

```
> Set-Alias grep 'C:\Program Files\Git\usr\bin\grep.exe'
> nano $profile  # Add the above line to your profile and save.
```

Command: `grep`

`grep`'s name comes from commands inside `ed`, one of the first text editors ever created, where it means '**g**lobal/**r**egular **e**xpression/**p**rint'.

In short, it searches through entire files (global), using regular expressions as needed, and prints the search results it finds.

It's syntax is:

`grep <flags> <match_pattern> <files>`

When run without arguments, `grep` attempts to find `match_pattern` anywhere in the given files. Limited regualr expression syntax is supported in this mode.

Following the example in the Software Carpentries example[299], consider searching through a file `haikus.txt` for the word `not`:

```
$ cat haikus.txt
The Tao that is seen
Is not the true Tao, until
You bring fresh toner.

With searching comes loss
and the presence of absence:
"My Thesis" not found.

Yesterday it worked
Today it is not working
Software is like that.
$ grep not haikus.txt
Is not the true Tao, until
"My Thesis" not found
Today it is not working
```

By default, `grep` returns any line that contains the given pattern.

If you want to use regex matching, you should pass the `-E` flag, but that is beyond the scope here.

Other useful options are `-w` for "word" matching and `-n` for line numbers.

Let's consider what happens if we search for the word `is`:

```
$ grep is haikus.txt
The Tao that is seen
"My Thesis" not found.
Today it is not working
Software is like that.
```

It returned the second line because 'is' occurs inside the word Thesis! Adding the word flag forces `grep` to only match whole words:

```
$ grep -w is haikus.txt
The Tao that is seen
Today it is not working
Software is like that.
```

If we want to know more about where these lines were matched from, adding `-n` will give line numbers corresponding to lines returned:

```
$ grep -w  -n is haikus.txt
1:The Tao that is seen
10:Today it is not working
11:Software is like that.
```

Finally, by adding the `-r`, the **recursive flag**, you can tell `grep` to search entire folders!

Exercise

Time for an exercise to test all of this out! Partner up with someone if desired.

After downloading this zip file, unzip it **without looking inside the ``content`` directory!** Then, open up a terminal and get started! For some of the file manipulation tasks, there is a helper Python script that can check if you have correctly completed parts of the exercise. Run it with `python check.py`.

Exercise

Do all of the following just in your terminal, without using GUI tools.

1. What directory did your terminal start in?
2. Where did you unzip the exercise directory to?

Use `cd` and `ls` to navigate into the `content` directory.

3. What is the directory layout of the `content` directory? You may want to draw out the directory tree.
4. Are there any hidden folders?
5. In the `data` directory, the `imaging` subdirectory was accidentally placed inside the `sequencing` directory; move it out so it is next to the `sequencing` directory.
6. Add your name to the `README.txt` file.
7. In the `data/mixed` directory, there is a mixture of `.fastq` and `.tif`, and `.fcs` files. Create a new directory, `flow`, and **move** these files into the `sequencing`, `imaging`, and `flow` directories, respectively. Delete the `data/mixed` directory after everything has been moved out.
8. In the `raw` directory, there are mixed data files from several years. Create a `raw` directory inside `data`, then create `YYYY` directories for each year (e.g. 2020), sorting the mixed data files by **copying** them into the year directories.
9. In the `primers` directory, there are (hint: use `grep`!). Which primers contain the repetitive sequence AAAA?

1. Will change per user.
2. Will also depend per user, but probably in their Download folder.
3. The directory layout is as follows:

```
content
├─ data
│  ├─ mixed
│  └─ sequencing
│     └─ imaging
├─ primers
├─ raw
│  ├─ 2019.08.06_SlowFT_reprogram
│  └─ 2020-02-19-PEI-titration
└─ scripts
   ├─ calc.py
   └─ env
```

[299] https://swcarpentry.github.io/shell-novice/07-find/index.html

4. There are hidden folders, the `scripts/.git` folder.
5. From inside `content`, this can be done with `mv data/sequencing/imaging data/`, or other ways.
6. This can be done with `nano README.txt`.
7. This can be done by using `mv` with a wildcard file specification. One solution is:

```
$ cd data
$ mkdir flow
$ mv mixed/*.fcs flow
$ mv mixed/*.tif imaging
$ mv mixed/*.fastq sequencing
$ rm mixed
```

8. This can again be done with a combination of `mkdir` and `mv`, with file specifications like `2020*`.

   Starting from the `content` folder:

```
$ cd raw
$ mkdir ../data/raw
$ mkdir ../data/raw/2019
$ mkdir ../data/raw/2020
$ mkdir ../data/raw/2021
$ cp -r 2019* ../data/raw/2019
$ cp -r 2020* ../data/raw/2020
$ cp -r 2021* ../data/raw/2021
```

9. The following primers have quadruple A's in a row:

```
geec_primers.txt:oGE024 Rsites-UbC_fwd  agtccagtgtCATCAACAAGTTTGTACAAAAAAG
nbw_primers.txt:GG_lenti_bb_fwd ACAGCGTCTCAtcctTAAAAGAAAAGGGGGGAC
nbw_primers.txt:GG_WPRE_fwd     ACAGCGTCTCAttgcCGATAATCAACCTCTGGATTACAAAATT
nbw_primers.txt:link-NLS-VPR_rev       ␣
↪gaaagctgggtctagatatcTCAAAACAGAGATGTGTCGAAGATGG
nbw_primers.txt:"mCherry-MCP-VP16_rev   "      ␣
↪gaaagctgggtctagatatcCTACAGCATATCCAGATCAAAATCGTC
nbw_primers.txt:Rsites_UbC_MCS_fwd     ␣
↪tccagtttgggcatgcgctagcctcgagGCATCAACAAGTTTGTACAAAAAAG
nbw_primers.txt:VPR_rev gaaagctgggtctagatatcTCAAAACAGAGATGTGTC
sequencing_primers.txt:oSEQ046 phage-dest-upstream-seq  CGACGTACTCCAAAAGCTCGAG
```

What directory did you unzip into?

Extras

If you'd like to customize your shell prompt so it is more useful, you can use something called Oh my Posh 3[303].

For example, my terminal looks like this when logged into a remote server:

First, it shows a lock symbol because I'm connected over a secure connection, followed by my username and the server name. In blue, the current working directory is shown. Finally, it shows git status directly on the prompt line (here, we are on branch `master` with no changes).

Before installing this, make sure you have a powerline-enabled font (like Fira Code). Then, follow the installation instructions here[304]. If you are on Windows, this is simple, just type:

```
> Install-Module oh-my-posh -Scope CurrentUser -AllowPrerelease
```

Then, create a JSON file with your desired prompt; you can do this with `nano ~/.omp_prompt.json`.

After this, there is typically a final setup step[305] that modifies your profile file and points it at your prompt file. On Powershell, this is `Set-PoshPrompt ~/.omp_prompt.json`, added via `nano $profile`. On Linux and MacOS, this is typically adding

`eval "$(oh-my-posh --init --shell zsh --config ~/.poshthemes/jandedobbeleer.omp.json)"` to either your .zshrc (`nano ~/.zshrc`) or .bashrc (`nano ~/.bashrc`), depending on which shell you use.

If you'd like to copy my prompt, mine is:

```
{
"final_space": false,
"blocks": [
    {
    "type": "prompt",
    "alignment": "left",
    "segments": [
        {
        "type": "session",
        "style": "plain",
        "foreground": "#ffffff",
        "properties": {
            "postfix": ":",
            "user_color": "#ffd93d",
            "ssh_icon": "\uE0A2 "
        }
        },
```

(continues on next page)

[303] https://ohmyposh.dev/
[304] https://ohmyposh.dev/docs/installation
[305] https://ohmyposh.dev/docs/installation/#4-replace-your-existing-prompt

(continued from previous page)

```
        {
        "type": "path",
        "style": "plain",
        "foreground": "#44B4CC",
        "properties": {
            "style": "agnoster_full"
        }
        },
        {
        "type": "git",
        "style": "powerline",
        "powerline_symbol": "\uE0B0",
        "foreground": "#193549",
        "background": "#a1c60b",
        "properties": {
            "local_working_icon": " W",
            "local_staged_icon": " S"
        }
        },
        {
        "type": "python",
        "style": "powerline",
        "background": "#00897b",
        "foreground": "#193549",
        "powerline_symbol": "\uE0B0",
        "properties": {
            "display_version": false,
            "display_virtual_env": true,
            "display_mode": "context"
        }
        },
        {
        "type": "text",
        "style": "plain",
        "properties": {
            "text": "$"
        }
        }
    ]
    }
]
}
```

7.2 Data analysis tutorials

7.2.1 Gating cells in FlowJo

Flow cytometers save data in `.fcs` files, one per sample. While it is possible to load these files in Python, it is (currently) easier to do an initial gating step in FlowJo, a paid flow cytometry analysis software from Waters Biosciences (formerly BD Biosciences). Follow the instructions below to gate single cells and save these as `.csv` files, one file per sample. The `.csv` files can then be loaded into Python as described in *Loading flow cytometry data* (page **??**).

We use FlowJo to gate single cells, i.e., to select events on the cytometer that represent measurements from individual cells and to exclude debris and cell doublets or clumps. This is commonly done by first gating **cells**: in an FSC-A vs SSC-A plot, select the main cluster of events to remove debris (smaller) and obvious clumps (larger). Then, gate **single cells**: in an FSC-A vs FSC-H (or SSC-A vs SSC-H) plot of the gated cell population, exclude the population with similar height but larger area to remove doublets.

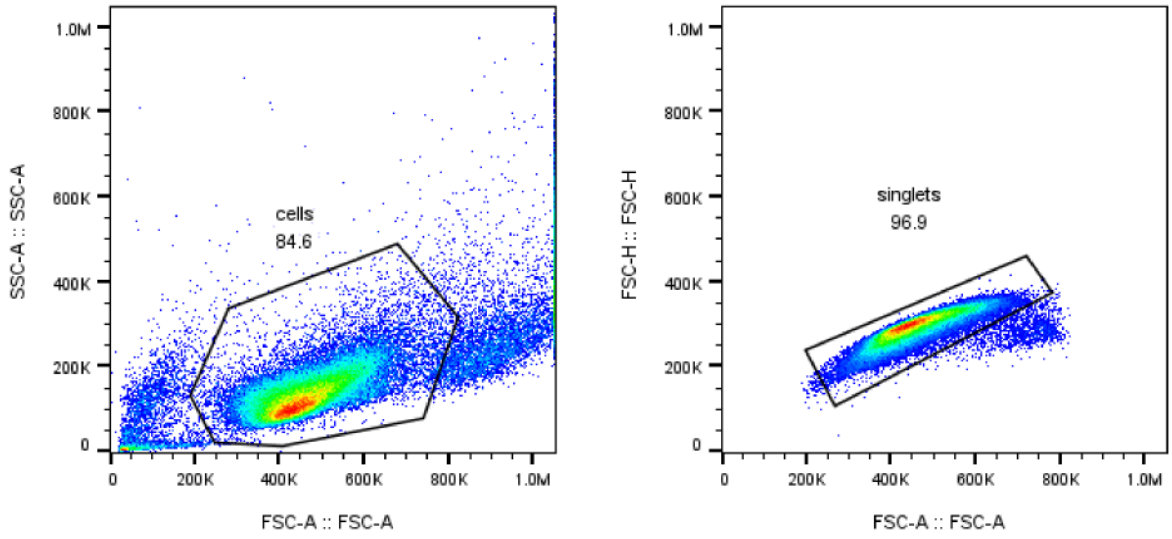


Fig. 4: **Left:** Black polygon outline shows the cell population, labeled "cells", on an FSC-A vs SSC-A plot. **Right:** Black polygon outline shows the single cell population, labeled "singlets", a subset of the "cells" population on an FSC-A vs FSC-H plot.

1. Load `.fcs` files.

   Open the FlowJo software and log in with the lab username and password. Do not download a new version of the software, if prompted. Then, drag the `.fcs` files from your experiment into the new workspace.

2. Draw a gate to select cells.

Double click on one of your samples. A new window should pop up with an FSC-A vs SSC-A plot. Use the **polygon gate** tool (button at the top) to gate region representing the main cell population. Draw the gate by clicking to add vertices. Call this population "cells" or something similar.

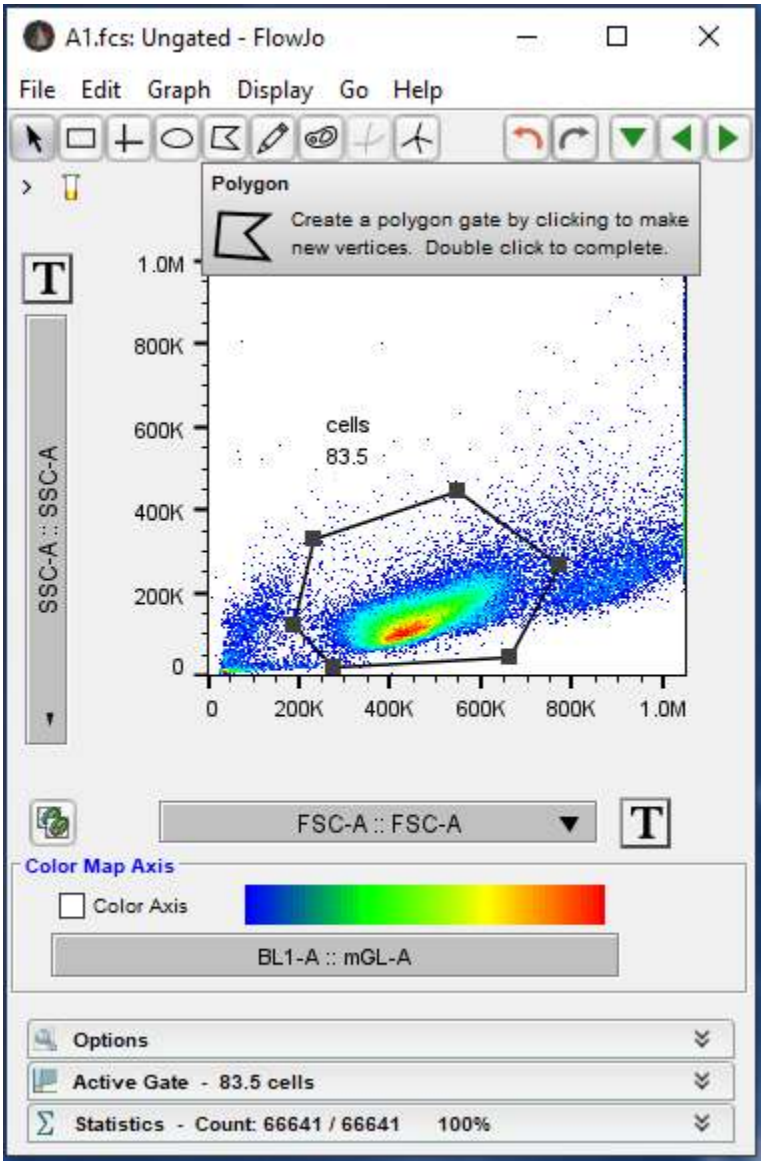


Fig. 5: Use the polygon gate tool to select cells from all events.

3. Draw a gate to select single cells.

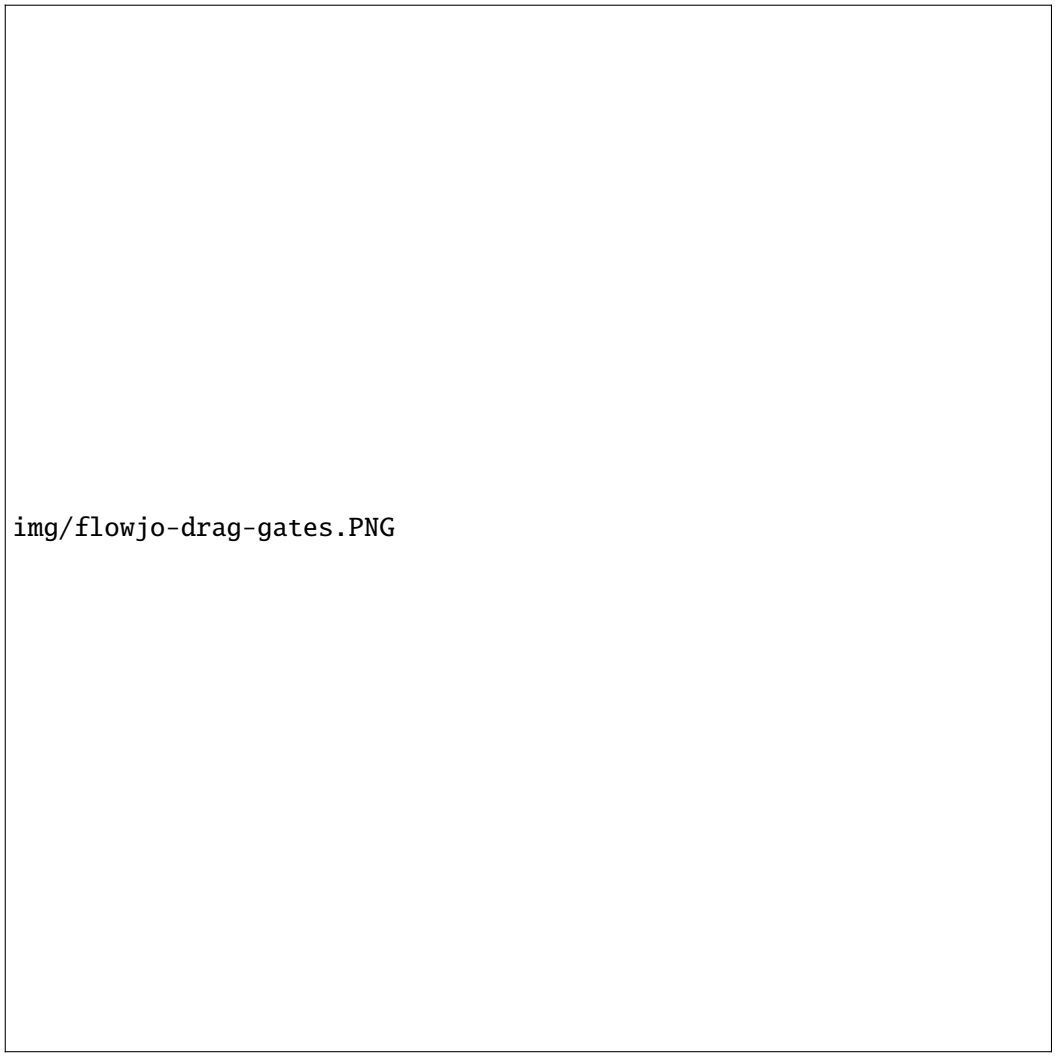
   Double click within the cells gate to select this population. A new window will appear; change the axes to plot FSC-A vs FSC-H. Use the polygon gate tool to select single cells, excluding the subpopulation of events with larger FSC-A values. (See top image.) Call this population "singlets" or something similar.

4. Apply these gates to all samples.

   To apply the same gates uniformly to all samples, select the "cells" and "singlets"

populations and drag them to the "All Samples" group at the top of the workspace.

Spot-check several samples to ensure the gates capture the desired populations across conditions.



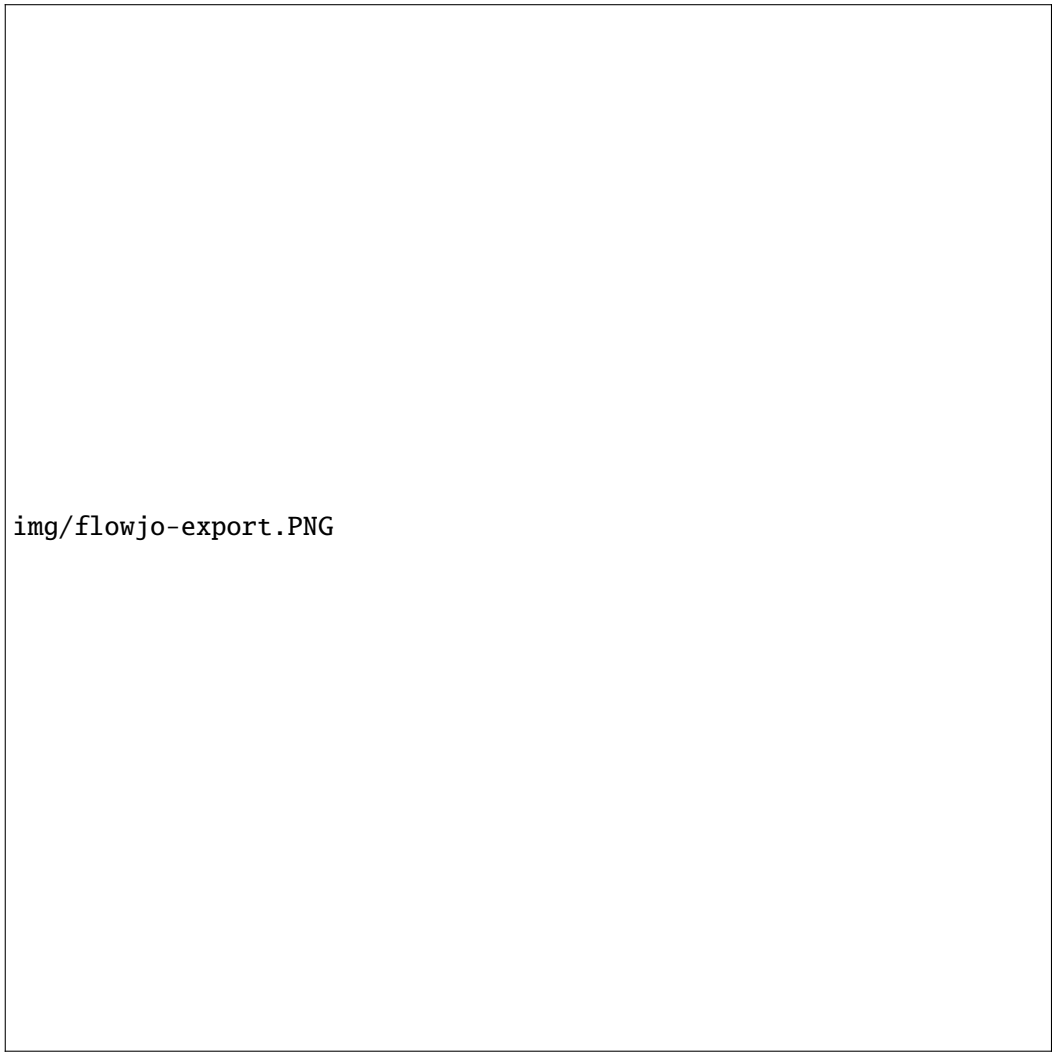
5. *Optional:* Apply compensation.

TODO

Description of compensation to be added.

6. Export the single cell populations as `.csv` files.

   Right click on the "All samples" group and select "Export / Concatenate Group".



   In the pop-up menu, select the "CSV – scale values" format and a destination folder in Smithsonian, somewhere near the original `.fcs` files.

   The default filename will be `export_[wellID]_singlets.csv`, which is convenient for loading into Python later. However, you can change this in the "Advanced Options" tab if you added other metadata when flowing (usually only relevant for tube experiments—additional metadata can be easily mapped later based on well IDs).

   The file structure in Smithsonian might look something like this:

```
data/
├── attune/
│   ├── your-name/
│   │   ├── 2026.01.01_exp001/
│   │   │   ├── fcs/
│   │   │   │   ├── A1.fcs
│   │   │   │   ├── A2.fcs
│   │   │   │   └── ...
│   │   │   ├── csv/
│   │   │   │   ├── export_A1_singlets.csv
│   │   │   │   ├── export_A2_singlets.csv
│   │   │   │   └── ...
│   │   │   ├── wells.yaml
│   │   │   └── exp001.wsp
│   │   └── ...
│   └── ...
└── ...
```

7. Save the FlowJo workspace.

   To keep a record of your gating, save the FlowJo workspace (`.wsp` file) near the related files. Then, quit the software.

Note

Because the FlowJo software requires a paid subscription, it is currently only available on the lab computers (e.g., Attune computer). If possible, it is convenient to perform this single cell gating immediately after finishing your Attune run. You can then do all subsequent data analysis steps on your own computer, beginning with *Loading flow cytometry data* (page **??**).

7.2.2 Loading flow cytometry data

For best transparency and reproducibility, we do most of our data analysis in Python. While it is possible to do all steps in Python, practically it is (currently) easier to gate single cells using FlowJo, a paid flow cytometry analysis software. This tutorial describes a workflow that starts with single cells previously *gated in FlowJo* (page **??**) and exported as `.csv` files. It describes **how to load all samples into one Pandas DataFrame (in a Python Jupyter notebook) with associated experimental metadata**. From here, the data can be plotted and analyzed in Python.

Step 0: Set up your computational environment

Before starting to analyze your data, make sure your computational environment is set up. The first two steps only need to be performed once, and setting up the Git repo occurs only at the beginning of a project.

1. Set up lab software as described in *Day 0: Software and training setup* (page **??**).
2. Perform the *Computational environment check* (page **??**) to confirm you can use Git properly.
3. Create a new Git repository for your project or clone an existing one by following the *Startup checklist when working with repositories* (page **??**).

A file layout for a project Git repository may look something like this:

```
your-repo/
├── analysis/
│   ├── exp001.ipynb
│   ├── exp002.ipynb
│   └── ...
├── inputs/
│   └── plasmid-metadata.csv
├── output/
│   ├── exp001/
│   │   ├── data.gzip
│   │   ├── gates.svg
│   │   ├── scatter.png
│   │   └── ...
│   ├── exp002/
│   └── ...
├── .gitignore
├── datadir.txt
├── README.md
└── requirements.txt
```

The `analysis` directory contains Jupyter notebooks or scripts for data analysis and plotting, the `inputs` directory contains small files needed for analysis (e.g., project-wide metadata), and the `output` directory holds processed data, plots, and other files generated by analysis scripts. The `datadir.txt` file contains the local path to the raw data, as explained below.

`datadir`: Where is your data located?

To load your data in Python, you'll need to specify the path to where the data is stored. We store all raw data generated by the lab on the *Smithsonian server* (page **??**). If you have the Nextcloud client synced locally on your computer, you can look up the absolute path to the `data` folder and use this as the base path for loading the data from your experiment.

However, using this absolute path means that others can't run your notebook as-is: they'd have to replace the path with one specific to their computer. For reproducible analysis and easy collaboration, you should instead specify a relative path that does not depend on the exact location of the data on your computer. Ideally, your notebook would contain paths relative to the location of the Smithsonian file sync (or, after publication and deposit on Zenodo, the location of a local download). That way, future users on other computers don't need to edit any paths for your code to run.

While there are several ways this could be achieved, the Python package `rushd` (documentation here[306]) offers an elegant solution. `rushd` **was created by members of the Galloway lab for this exact purpose: to make data management easier, robust, and reproducible.** Using this package makes it much simpler to collaborate on data analysis, and for the broader scientific community to reproduce analysis and plots in published manuscripts.

Specifically, `rushd` offers the variable `datadir`, which represents the path to your main data directory, or the folder where all of your data lives. This should be the highest level directory you'll ever need for your project, i.e., the `data` folder on Smithsonian for the Galloway Lab. You'll need to set up this path just once per repo.

To do so, create the file `datadir.txt` in the root of your repo (as shown in the file structure above). In the file, paste the full, absolute path to your main data directory. *You do not need quotes or any other characters around this path, and this should be the only line in the file.* For example, on macOS, the path might look something like:

```
/Users/username/Library/CloudStorage/Nextcloud/kerberos@mit.edu@smithsonian.mit.
↪edu/data
```

Tip

Be sure to add `datadir.txt` to the `.gitignore` file in your repo, so it isn't tracked by Git. Additionally, in your repo's `README.md`, add a description of `datadir.txt` and how to create it, so that anyone who clones your repo can follow these same steps.

[306] https://gallowaylabmit.github.io/rushd/en/main/

Note

If you edit `datadir.txt` while running a Jupyter notebook, you must restart the kernel for the changes to take effect.

Using `rushd` paths

Now you can use `datadir` as you would a normal path. For example, the final directory `csv` contains data files for one experiment:

```
/Users/username/Library/CloudStorage/Nextcloud/kerberos@mit.
↪edu@smithsonian.mit.edu/
├── data/
|   ├── attune/
|   |   ├── your-name/
|   |   |   ├── 2026.01.01_exp001/
|   |   |   |   ├── fcs/
|   |   |   |   |   ├── A1.fcs
|   |   |   |   |   ├── A2.fcs
|   |   |   |   |   └── ...
|   |   |   |   ├── csv/
|   |   |   |   |   ├── export_A1_singlets.csv
|   |   |   |   |   ├── export_A2_singlets.csv
|   |   |   |   |   └── ...
|   |   |   |   ├── wells.yaml
|   |   |   |   └── exp001.wsp
|   |   |   └── ...
|   |   └── ...
|   └── ...
└── ...
```

```
data_path = rushd.datadir/'attune'/'your-name'/'2026.01.01_exp001'/'csv'
```

`rushd` also provides the path `rootdir`, which points to the top-level directory of your Git repo. This is defined as the directory where `datadir.txt` is directly located. `rootdir` allows you to write relative paths within the repo, since this absolute path also differs across clones of the Git repo. For example, you could define a directory to save all the plots generated by your notebook:

```
output_path = rushd.rootdir/'output'/'exp001'
```

Tip

Add `output/` to `.gitignore` so that Git doesn't track your plots (since you can re-create these by running your code).

You will use these paths to load data and save plots in Step 2.

Step 1: Specify experimental metadata

Exporting your gated data from FlowJo as described *here* (page **??**) generates one `.csv` file per sample (tube or well). For plate experiments, these are named by the well ID. To analyze the data, you'll need to map well IDs to experimental conditions. `rushd` provides functions to do this easily, mapping values for multiple variables/features (e.g., plasmids, inducers) to each well. This is done using `.yaml` files containing experimental metadata.

What are `.yaml` files?

YAML is a human-readable file format (data serialization language) that is often used for writing configuration files. It is similar to JSON. Basically, it is an easy, standardized way to write nested dictionaries and arrays, which is how you'll use it here. For quick tips on syntax, check out this brief tutorial[307], from which some examples are reproduced below.

Dictionary elements are written as key-value pairs:

```
key: value
key2: value2
```

These can be nested using indentation:

```
nested_dict:
    key: value
```

You can also create lists:

```
list:
   - item1
   - item2
```

And combine lists with dictionaries:

```
nested_dict:
    list:
        - key: value
        - key2: value2
```

Numbers are interpreted as `ints` or `floats`; enclose values with quotes to treat them as strings. Letters/words are treated as strings even without quotes. Indentations are meaningful. This is the bare minimum you'll need to write metadata `.yaml` files compatible with `rushd`; see the official tutorial[308] for more.

[307] https://learnxinyminutes.com/yaml/
[308] https://www.yaml.info/learn/index.html

Writing experimental metadata

To specify metadata for experimental conditions that is compatible with `rushd`, include everything under the top-level group `metadata`. Each item in this dictionary is a list of key-value pairs, where the group/list name is the feature name, the item keys are the possible feature values, and the item values are well IDs. Conveniently, `rushd` supports ranges spanning rectangular regions, e.g., A1-H12 for the entirety of a 96-well plate.

Here's a generalized example:

```
metadata:
    feature1:
        - treatment1: A1
        - treatment2: A2-A12
        - treatment3: B1-B12
    feature2:
        - 1: A1-B1, A12
        - 10: A2-B2
        - 100: A3-B3
        - 0: A4-B11
```

In this example, well A1 has the value *treatment1* for the feature *feature1* and the value *1* for *feature2*. Since we don't specify a value of *feature2* for well B12, it will be loaded as *<NA>*.

In a more realistic example, here are the contents of a `.yaml` file for an actual experiment. In this experiment, a reporter plasmid (*reporter*) was transfected with different transcriptional activator plasmids (*activator*) and treated with different small-molecule inducers (*inducer*). Here, the value *NT* refers to the non-transfected (aka untransfected) condition. Each experimental condition is defined by a combination of reporter, activator, and inducer, and there are multiple wells per condition (technical replicates).

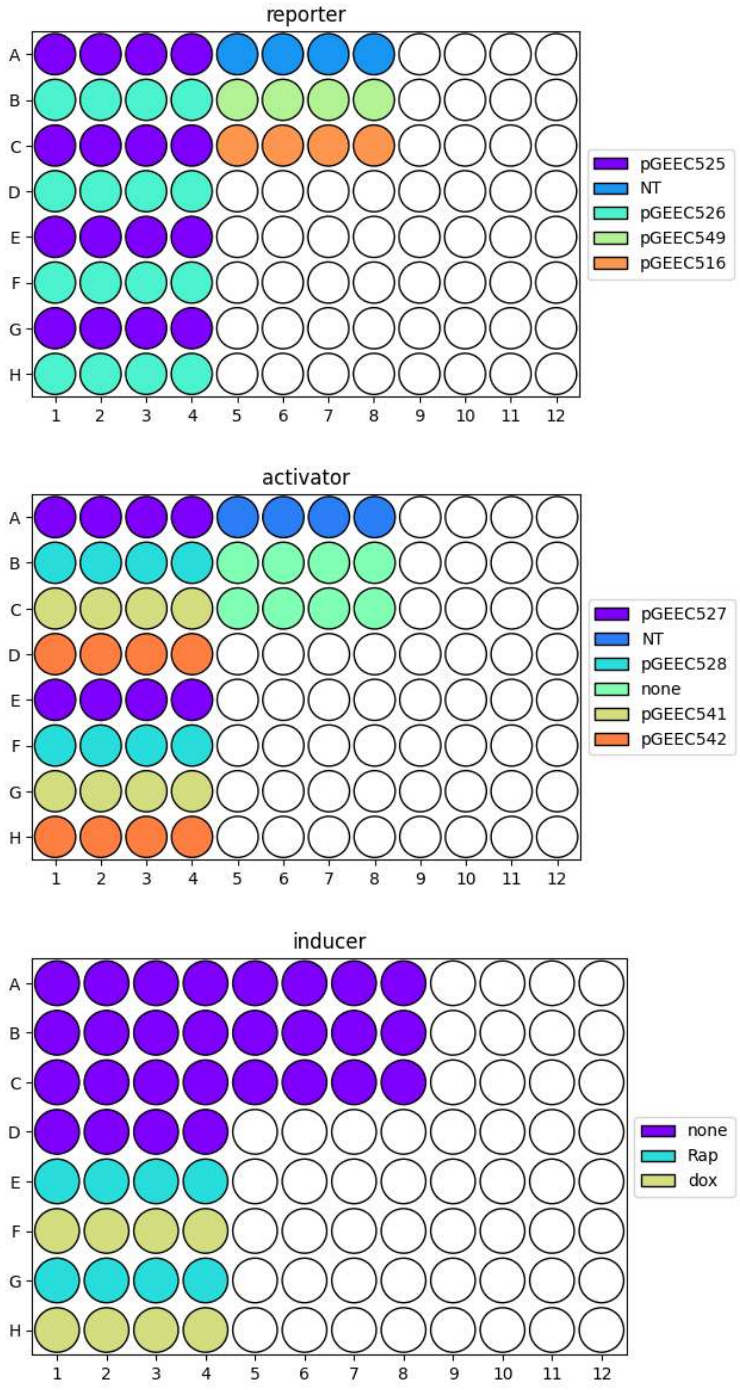
```
metadata:
    reporter:
        - pGEEC525: A1-A4, C1-C4, E1-E4, G1-G4
        - pGEEC526: B1-B4, D1-D4, F1-F4, H1-H4
        - NT: A5-A8
        - pGEEC549: B5-B8
        - pGEEC516: C5-C8
    activator:
        - pGEEC527: A1-A4, E1-E4
        - pGEEC528: B1-B4, F1-F4
        - pGEEC541: C1-C4, G1-G4
        - pGEEC542: D1-D4, H1-H4
        - NT: A5-A8
        - none: B5-C8
    inducer:
        - none: A1-D4, A5-C8
        - Rap: E1-E4, G1-G4
        - dox: F1-F4, H1-H4
```

As you can see, this format is a lot easier than writing out the full condition for each well!

Save the `.yaml` file near the `.csv` files, as shown in the file structure above.

To check that you labeled the wells properly, you can visualize the contents of the `.yaml` file using `rushd`:

```
base_path = rushd.datadir/'attune'/'kasey'/'2024.07.16_exp099'/'export_comp'
rushd.plot.plot_well_metadata(base_path/'wells.yaml')
```



Step 2: Load data with metadata in Python

Now that you have a directory of `.csv` files for your samples and a `.yaml` file with experimental metadata, you're ready to load the data and metadata into a single Pandas DataFrame in Python. You can do this in a Jupyter notebook in your project Git repo from Step 0.

The Pandas DataFrame[309] is a data structure like a long-form array, where each row is a data point (single cell), and each column is a feature of that data (metadata feature or cytometer channel). The Seaborn[310] plotting package works well with this data structure and contains a nice explanation[311] of long-form data.

One of the goals of `rushd` is to make this loading easy. The lab has written several functions to do this:

- `rushd.flow.load_csv`

  Loads a directory of `.csv` files into a single DataFrame with filename metadata only. Useful for loading experiments run as **tubes** on the cytometer, or any data where the sample names do not contain well IDs.

- `rushd.flow.load_csv_with_metadata`

  Loads a directory of `.csv` files into a single DataFrame with additional well metadata encoded by the given `.yaml` file. Useful for loading **one plate (most common)**.

- `rushd.flow.load_groups_with_metadata`

  Loads multiple directories of `.csv` files into a single DataFrame with directory-level metadata, as well as well metadata encoded by the given `.yaml` files. Useful for simultaneously loading **multiple plates** (from multiple experiments, replicates, or one large experiment).

See the relevant section[312] of the `rushd` documentation for more details on these functions.

In the simplest form, you only need to pass a path to the directory with `.csv` files and a path to the `.yaml` file. Assuming a file structure in Smithsonian like the one above, a call might look like:

```
base_path = rushd.datadir/'attune'/'your-name'/'2026.01.01_exp001'
df = rushd.flow.load_csv_with_metadata(base_path/'csv', base_path/'wells.yaml')
```

Check out this tutorial[313] for more examples using `rushd` to load files, including zipped data, non-default filenames, specific columns, and multiple groups.

[309] https://pandas.pydata.org/docs/reference/api/pandas.DataFrame.html
[310] https://seaborn.pydata.org/
[311] https://seaborn.pydata.org/tutorial/data_structure.html
[312] https://gallowaylabmit.github.io/rushd/en/main/api/rushd.html#rushd.flow.load_csv
[313] https://gallowaylabmit.github.io/rushd/en/main/tutorial/flow/loading_data.html

Adding condition-level metadata

When writing a `.yaml` file, it is easiest to define conditions based on plasmid IDs. However, when analyzing data, you may wish to subset or group condition based on features of those plasmids, or other information about a condition beyond its short name. This information is likely to remain constant across an entire project, so it would be nice to only define this once and apply it when analyzing individual experiments. Of course, you could copy and paste a dictionary that maps additional metadata to plasmids, but as the number of plasmids or metadata features increases, this can become unwieldy.

Instead, it can be convenient to make a spreadsheet of metadata, load this metadata, and add it to your data DataFrame. The spreadsheet should contain a column for the plasmid ID (or however you label conditions in your `.yaml` file) and additional columns for each new metadata feature. For example, a plasmid metadata file could look something like this:

plasmid	backbone	promoter	gene	pas
pGEEC501	pShip	EFS	mRuby2	bGH
pGEEC502	pShip	EFS	mRuby2	SV40
pGEEC503	pShip	EFS	mRuby2	WPRE
...	...	...	...	...
pGEEC737	pPB	CAG	mRuby2-P2A-PuroR	bGH

Save this file as a `.csv` or `.xslx` file in the `inputs` directory in your project Git repo.

To load this as a DataFrame in Python, use Pandas (see the relevant section of the documentation here[314]):

```
# for .csv
metadata_path = rushd.rootdir/'inputs'/'plasmid-metadata.csv'
df_metadata = pandas.read_csv(metadata_path)

# or, for .xslx
metadata_path = rushd.rootdir/'inputs'/'plasmid-metadata.xlsx'
df_metadata = pandas.read_excel(metadata_path)
```

To apply this to your loaded data, use the Pandas `merge` function. Note that this will add all the new metadata columns to your data DataFrame, which may significantly increase its size. You could consider only adding a subset of the metadata columns to the DataFrame.

In the simplest form, where the column names match, this looks like:

```
df = df.merge(df_metadata, on='plasmid')
```

However, as in the experiment example above, multiple plasmids were added to each condition, so we might want to map metadata for each plasmid. To do this, we can call `merge` multiple times:

[314] https://pandas.pydata.org/pandas-docs/stable/reference/io.html

```
df = df.merge(df_metadata, left_on='reporter', right_on='plasmid')
df = df.merge(df_metadata, left_on='activator', right_on='plasmid', suffixes=('_
↪reporter','_activator'))
```

Note

If you re-run this code, the merge function will duplicate the columns, appending the suffixes `_x` and `_y` to the old and new columns, respectively. To avoid this, you may wish to add metadata in the same cell as you load your data, or just re-load the data before re-merging the metadata.

In our running example, the DataFrame `df` now has the following columns:

- data: `FSC-A`, `FSC-W`, `FSC-H`, `BL1-A`, `...` , `VL1-H`, `Time`
- filename metadata: `well`, `population`
- well metadata: `reporter`, `activator`, `inducer`
- condition metadata: `backbone_reporter`, `promoter_reporter`, `gene_reporter`, `...` , `pas_activator`

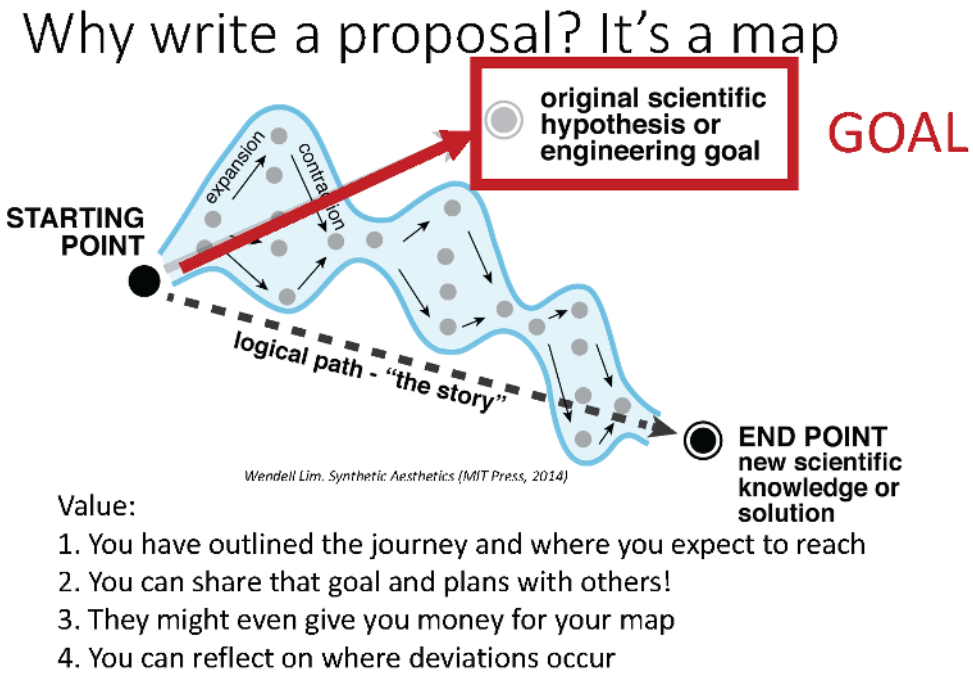
The number of rows corresponds to the number of single cells in the entire experiment.

Now you're ready to visualize and analyze data! See the example Jupyter notebook for analyzing flow cytometry data in the `example-training` Git repo here[315]. Start by exploring your data: draw gates to define expressing cells in each channel, visualize these gates on untreated and single-color control conditions, plot 1D and 2D distributions for relevant channels for all conditions, and plot distributions for subsets of conditions to facilitate comparison. Then, calculate and visualize summary statistics (e.g., geometric mean) for each condition as well as additional metrics relevant to your experiment (e.g., fold change). Summarize your findings in concise plots, and perform statistical tests on replicates. Good luck, and may your data be interesting!

[315] https://github.com/GallowayLabMIT/example-training/blob/main/analysis_tutorials/

7.3 "How to" training series

7.3.1 Tutorial 1: How to Write a Proposal

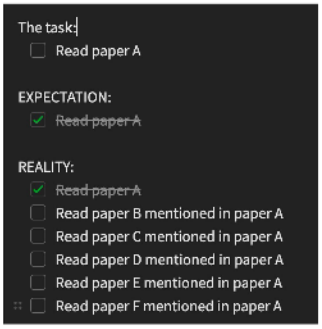


- View these slides.

7.3.2 Tutorial 2: How to Read a Paper

Words of Wisdom

- Reading scientific papers is a skill that requires **practice** and **patience**
- **It gets easier** to read papers over time!
- **Google** is your friend
- Make **vocab** lists
- Don't worry if you don't understand every single thing right away
- Do what works best for **you**!



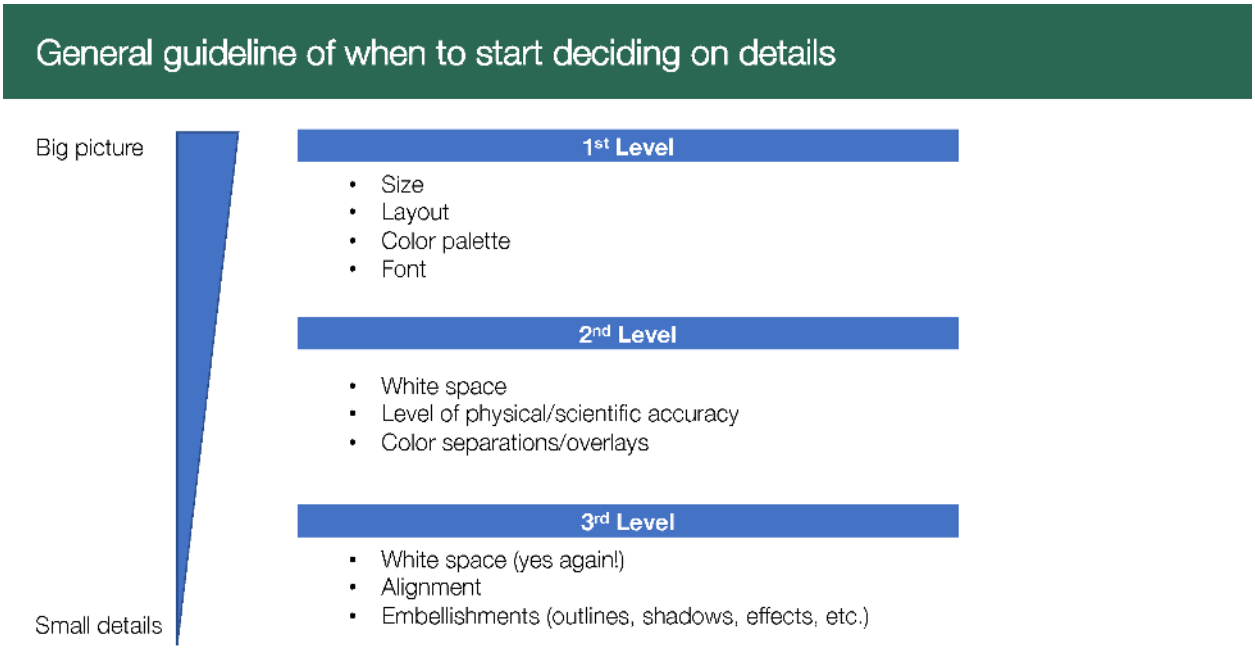
- View these slides.

7.3.3 Tutorial 3: How to Review a Paper

1. **Read** the paper, including looking at the SI
2. Write a **summary**, including highlights/limitations/etc.
3. From the discussion and intro sections, identify needed **extra context** to understand the problem/solution (engineering) or observation/hypothesis (science)
4. Decide how to **group figures** together to tell the story
5. While thinking through the story, **identify limitations** and alternative hypotheses

- View these slides.

7.3.4 Tutorial 4: Graphic Figure Design

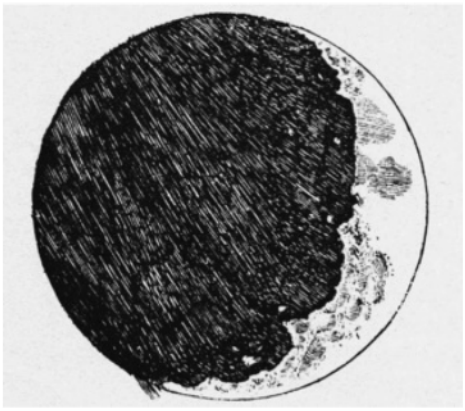


- View these slides.

7.3.5 Tutorial 5: How to Compose Figures

Purpose of figure?

1. Provide context, scale, frame the story
2. Convey complex ideas
3. Summarize data



Engraving of the moon from Galileo's *Sidereus Nuncius* (III, 53-96) 1610.

- View these slides.

7.4 Gene expression modeling with ODEs

The following tutorials outline how to model gene expression with ordinary differential equations (ODEs). These lessons use MATLAB as the primary coding software, with code files attached when necessary.

7.4.1 Tutorial 1: Modeling gene expression with differential equations

- Lesson PDF: Lesson1_Introduction.pdf
- Scripts: L1_constitutive_script.m, ode-modeling-python.ipynb

On your own:

1. Given the following parameters, calculate the steady state mRNA and protein levels for the simple model of protein expression in the notes. $alpha\_m = 5$, $beta\_m = 0.1$, $alpha\_p = 2$, $beta\_p = 0.5$
2. Use the provided MATLAB script or Python Jupyter notebook to plot mRNA and protein levels over time.

7.4.2 Tutorial 2: Examining parameters affecting our models

- Lesson PDF: Lesson2_ExaminingParameters.pdf
- MATLAB Script: L2_constitutive_param_script.m

On your own:

1. How does increasing *alpha_m* affect the steady state mRNA and protein levels? Use the provided code to plot mRNA and protein levels over time for several different values of *alpha_m*.
2. How does increasing *alpha_p* affect the steady state mRNA and protein levels? Increasing *beta_m*? Increasing *beta_p*? Choose one of these parameters (*alpha_p*, *beta_m*, or *beta_p*) and plot mRNA and protein levels over time for several different values of that parameter.
3. Optional: How does increasing *beta_p* affect the response time of the system (the time for mRNA or protein levels to reach steady state)? Hint: Plot mRNA or protein levels over time normalized to their steady state values.

7.4.3 Tutorial 3: Michaelis-Menten Kinetics

Lesson PDF: Lesson3_MichaelisMenten.pdf

On your own:

1. How does changing the amount of enzyme ([E_T]) affect the rate of product formation (dP/dt)?
2. How does changing kcat affect the rate of product formation (dP/dt) when the amount of substrate is small? When the amount of substrate is large?
3. Optional: Using MATLAB or Python, plot rate of product formation (dP/dt) as a function of the amount of substrate ([S]). How does this curve change as K_M increases?

7.4.4 Tutorial 4: Modeling repressors

- Lesson PDF: Lesson4_Repressors.pdf

On your own:

1. If you start at half activity ($X = 0.5$), how many fold do you have to increase *X/K_d* to get 90% repression? How much to get 99% repression?
2. If you need very tight repression (*P/beta* $<<$ 0.01) which parameters would you experimentally or by design be able to change?

7.4.5 Tutorial 5: Hill functions

- Lesson PDF: Lesson5_HillFunctions.pdf
- Scripts: L5_repressor_script.m, L5_repressilator_script.m

On your own:

1. How does changing n affect the shape of activation and repression Hill functions? Changing K?
2. Use the provided code to plot mRNA and protein levels of a repressor and its target over time. Describe the shape of the target protein curve. Why does it have that shape?
3. Use the provided code to plot protein levels of the three genes in the repressilator over time. Describe the pattern you observe.
4. Optional: What happens to the repressilator when there is no cooperativity ($n = 1$)? In general, when would you want high cooperativity (large Hill coefficient) in a repressor/activator?
5. Optional: What happens to the repressilator if the basal expression of each gene is non-zero ($alpha\_0 > 0$)?

CHAPTER

EIGHT

TECH DOCUMENTATION

8.1 Builder config and update

This repository is automatically built and pushed to https://gallowaylabmit.github.io/protocols using Github Actions. Github Actions[316] is a "continuous integration" (CI) service built into Github that is free for public repositories (like this one!)

It lets us programmatically define build steps, such as building the website and PDF versions of the protocols, automatically whenever someone pushes to Github.

A special YAML file defines how this build works. If you edit this file through Github's interface, it will give you nice suggested completions. The general (annoying) workflow for editing CI jobs is thus:

1. Edit in the online interface.
2. Commit your changes.
3. Wait for the Github Actions job (at https://github.com/GallowayLabMIT/protocols/actions) to complete.
4. Check for errors, and so on.

8.1.1 Current CI jobs

Github Actions runs jobs that are in the special `.github/workflows` folder. The only current one is `.github/workflows/build_helper.sh`:

```
name: Build protocols

on: [push]

jobs:
  build:

    runs-on: ubuntu-latest

    steps:
```

(continues on next page)

[316] https://docs.github.com/en/actions

(continued from previous page)

```
    - uses: actions/checkout@de0fac2e4500dabe0009e67214ff5f5447ce83dd # pinned␣
↪@v6.0.2
      with:
          fetch-depth: 0
    - name: Install dependencies
      run: |
          sudo apt-get update
          sudo apt-get install texlive-latex-recommended texlive-fonts-
↪recommended \
                               texlive-latex-extra texlive-extra-utils latexmk␣
↪tex-gyre \
                               ghostscript \
                               --no-install-recommends
    - name: Set up Python
      uses: actions/setup-python@a309ff8b426b58ec0e2a45f0f869d46889d02405 #␣
↪pinned @v6.2.0
      with:
        python-version: 3.14
    - name: Install Python dependencies
      run: pip install -r requirements.txt
    - name: Build documentation and deploy
      run: ./.github/workflows/build_helper.sh
      env:
        GITHUB_TOKEN: ${{ secrets.GITHUB_TOKEN }}
```

This job is run sequentially on a Linux virtual machine.

As an explanation of what each of these jobs are doing: 1. checkout: checks out a *full* (`fetch-depth = 0`, e.g. unlimited) version of the repo so we can render all active branches. 2. install dependencies: installs LaTeX so that we can build the PDF. 3. setup Python: installs a specific Python version. 4. Python dependencies: install the specific packages listed in the `requirements.txt` 5. Build: calls the build helper script that builds and deploys the website.

8.1.2 Updating the CI job

From time to time, you may have to update the CI job. This is good practice to do, so that the documentation jobs don't get horribly out of date.

1. Start by looking at the YAML file and see if the *setup-python* or *checkout* functions have updates by checking the "Actions Marketplace".

   - The `actions/checkout` step is viewable at https://github.com/marketplace/actions/checkout
   - The `actions/setup-python` step is viewable at https://github.com/marketplace/actions/setup-python

   Change the pinned version (the thing after the `@`) if necessary.

2. If a new stable version of Python has been released, first make sure that you are running it on your local computer. Then, change the `python-version` line to the new version.

3. If Python has been updated, update the requirements file by doing:

   - Create a **fresh virtual environment** and activate it.
   - Remove the version specifier from every package in the `requirements.txt` file (the `==1.2.3` part)
   - `pip install -r requirements.txt` to fetch the latest versions of all packages.
   - Run `python build.py --force-rebuild` and **check the output in ``output/html`` carefully**.

   If nothing in the built output is broken, then you can commit your changes!

4. Commit the changes to both the `requirements.txt` and `build_docs.yaml` files, and see if it works on Github.

5. Monitor progress through https://github.com/GallowayLabMIT/protocols/actions

8.2 Possible build/install errors

8.2.1 Virtual environment install errors

When doing `pip install -r requirements.txt`, you might (after a long time "collecting build dependencies") get an error saying that it is unable to compile / build a wheel. What's happening here?

There are two ways to distribute Python packages:

- As source code, which requires a working compiler if there are any non-Python code included (like C++ code). This is the same on all computers and operating systems.
- As a "binary wheel", e.g. a pre-compiled version of the package. Separate "wheels" have to build for every combination of Python version, operating system, and computer architecture.

Likely what happened is you are trying to install version-pinned packages that are **too old for your version of Python**. Because the package developer has to make wheels for all Python versions,

this means that old package versions will not have wheels for versions of Python released after the package version.

The solution is generally simple; just update those packages that are trying to install from source code. Newer packages are typically backwards compatible to older versions of Python.

E.g, if *pandas* is trying to install from source and failing, you can do:

```
$ pip install -U pandas # update pandas
$ pip freeze > requirements.txt # re-freeze dependencies
```

8.2.2 No PDF is generated when I push

If the PDF link is broken in the protocols page, then something broke the LaTeX build.

There are two ways to fix this:

1. Find someone with LaTeX locally installed (e.g. TeX Live). Have them run:

   ```
   $ python build.py --latex
   ```

   This should show you the LaTeX error.

2. Go to the build logs. You get to it by going to the "Actions" tab[317] in the Github repo, finding the latest "CI" run, and then scrolling through the logs to find the LaTeX error.

[317] https://github.com/GallowayLabMIT/protocols/actions

8.2.3 My screenshots aren't visible

Check that you don't have an uppercase `.PNG` either in the actual filename or wherever you are loading the image.

On Windows, filenames are case-insensitive, that is `some_file.txt` is "the same file" as `sOmE_fiLe.TXT`. This is not true on MacOS or Linux, which means if there is a mismatch between the filename case, the website build (which runs on Linux) will fail.

8.3 Slack bot (labbot)

The lab slackbot does a lot of convenient things:

1. Uploads sequencing results automatically to the sequencing channel.
2. Handles lab job reminders.
3. Runs the label printer.

This slack bot is mostly written in Python, using the convenient slack-bolt Python library to interact with the Slack API.

8.3.1 Accessing the labbot server

The lab slack bot runs on a (free) cloud server, specifically a f1.micro Google Cloud Platform server. This server is "owned" by Katie's Google account. The server is currently accessible at the domain name `gallowaylabapi.meson.us`. If the server is stopped and restarted, Google will likely change its IP address. If this happens, the subdomain has to be pointed at the new IP address.

Currently, that domain name is a subdomain owned by Christopher Johnstone. I (CPJ) am happy to keep this subdomain going, as it doesn't cost me any money. It's possible to point a different domain at this if needed in the future, but the Slack settings need to be updated, since Slack servers expect to talk to the labbot at `gallowaylabapi.meson.us`.

To have direct access to the server, you need to be added as an editor to the project by Katie, through the GCP project settings.

For Katie, the GCP console is available at https://console.cloud.google.com and permissions are editable from https://console.cloud.google.com/iam-admin/iam This view allows for "Grant access" and "Remove access" for other people. Luckily, the labbot server only needs to be connected to directly if you are updating Python dependencies; most other actions don't need direct access.

8.3.2 Updating labbot

Normal updates

Through the labbot homepage within slack, if you click on **Dev Tools**, you will open a page where you can restart the labbot. If it is misbehaving, this restart will often fix problems.

There is also a **Validate commit** button on here. This is how you normally update the labbot software. You can type in either a specific git commit (identified by a commit hash) or a specific git branch. The labbot will validate that the commit didn't change the depedencies, then give you an option to **Update and restart**. Upon clicking this, the labbot will update to the latest version without having to manually log in.

Unusual updates

If you update the dependencies (say, for adding a new feature or updating the slack-bolt package) or if you need to modify a config file that lives on the server, you need to SSH into the server.

1. Have Katie grant you access to the labbot server.
2. Log into GCP (at https://console.cloud.google.com). Go specifically to the Compute Engine tab (https://console.cloud.google.com/compute/instances)
3. Click the SSH button to spawn an SSH connection into the server.

This will log you into the server running the labbot. If you want to edit the labbot files, you need to switch to the labbot user. The server files are in a folder called `labbot` within the labbot user's home directory.

```
$ sudo su labbot
$ cd
$ ls
labbot
$ cd labbot/server
```

Inside the server folder, you can activate the `env` virtual environment, updated config files, and so on.

To start, stop, and restart the labbot from the server, you need to be using your user account. If you are currently switched to the labbot user, exit back to your user first:

```
$ whoami
labbot
$ exit
$ whoami
<whatever your google account is>
```

and then use the requisite `systemctl` command depending on what you want to do:

```
$ sudo systemctl start labbot
$ sudo systemctl stop labbot
$ sudo systemctl restart labbot
```

8.3.3 Label printing

The label printer client connects to the label print server run by the slack bot. This software is running on the computer that is behind the (paper) printer. You can connect a monitor and mouse/keyboard for debugging if needed.

That computer is running the software in the `clients/label_printer` folder of the labbot repo. The software runs in a loop, and connects to the labbot server to pull labels to print.

It is very simple; likely, this computer will never have to be touched. If weird stuff happens, you can unplug the computer and plug it back in, or force shutdown and restart. This has fixed every problem with the label printing client to date.

8.4 Self-hosted data storage

In 2025, we moved data off of the OneDrive to our own data storage system. This is because OneDrive is limited to 5TB, and MIT has no current plans to make more space accessible to labs.

Self-hosting your data comes with certain problems that need to be solved: first, you need a server and a robust way to store the data. Second, you need some way for lab members to login to access and sync the data. Third, the data needs to be robustly backed up so that we don't lose data.

We solve each of these problems in turn by 1) placing the data on a Synology NAS, which provides a (more) user friendly method for this type of data storage and redundancy than other alternatives, 2) hosting Nextcloud on the server and linking it to MIT Touchstone, and 3) backing up all of the data to TSM / Spectrum Protect, a managed backup server run by MIT. This server is accessible at https://smithsonian.mit.edu, with internal admin interfaces accessible at https://smithsonian-internal.mit.edu (only available within MIT).

Relevant passwords are stored in the Galloway Lab password database, which is in the Sharepoint, in the file `logins/gallowaylab_main.kbdx`. Install KeePassXC to access these passwords.

8.4.1 NAS setup

The NAS is a Synology DS1825+. For storage, it currently (2025-11-26) has six 12TB hard drives (Seagate IronWolf) and two 2TB SSDs (Samsung 990 Pro). The hard drives are are arranged in what is called a RAID: a redundant array of independent disks. It using a RAID layout called **SHR-2**, which means that it can tolerate two drive failures. E.g., we don't lose any data until three drives out of the six fail. However, if a drive fails, we do need to replace the failed drive as quickly as possible.

The two SSDs do not participate in the RAID; they are there as a read cache. Commonly accessed files will be automatically copied to the SSDs for faster access. If the SSDs fail, there is no data loss.

Up to two more drives can be added, and drives can also be replaced with larger hard drives. I (CPJ) recommend using Samsung SSDs and buying WD Red Pro or Seagate IronWolf hard drives. To replace a drive (either a failed one, or a in-use one in order to expand available storage), follow the Synology instructions[318].

[318] https://kb.synology.com/en-global/DSM/help/DSM/StorageManager/storage_pool_expand_replace_disk?version=7

The NAS user interface is accessible through a webbrowser at https://smithsonian-internal.mit.edu:5000 or over SSH at `gallowaylab@smithsonian-internal.mit.edu`. For both cases, the username and password are listed in the password database.

Of note, as of 2025, Synology as a company wants to squeeze profits out of users, and, with varying levels of firmness, tries to force customers to buy their (white-labeled) hard drives. Western Digital and Seagate make reliable hard drives, so we just use this Github project[319] to add any installed hard drives to the local Synology compatibility database. If you install new drives/SSDs and they come up as not recognized / incompatible in the software, you need to SSH in and re-run the script.

Note, as always, command line password interfaces do **not** show astrisks or any other sign that you are typing a password in.

```
$ ssh gallowaylab@smithsonian-internal.mit.edu
$ cd Synology_HDD_db-main
$ sudo ./syno_hdd_db.sh
Synology_HDD_db v3.6.111
DS1825+ x86_64 DSM 7.3.1-86003-1
StorageManager 1.0.0-01026

ds1825+_host_v7 version 8008

Running from: /volume1/@userpreference/gallowaylab/Synology_HDD_db-main/syno_hdd_
↪db.sh

HDD/SSD models found: 1
ST12000VN0008-2YS101,SC60,12000 GB

M.2 drive models found: 1
Samsung SSD 990 PRO 2TB,5B2QJXD7,2000 GB

No M.2 PCIe cards found

No Expansion Units found

ST12000VN0008-2YS101 already exists in ds1825+_host_v7.db
Samsung SSD 990 PRO 2TB already exists in ds1825+_host_v7.db

Support disk compatibility already enabled.

NVMe support already enabled.

M.2 volume support already enabled.

Drive db auto updates already enabled.

DSM successfully checked disk compatibility.
```

(continues on next page)

[319] https://github.com/007revad/Synology_HDD_db

(continued from previous page)

```
You may need to reboot the Synology to see the changes.
```

8.4.2 Nextcloud setup

Nextcloud is a piece of open-source software that acts like your own OneDrive / Google Drive / Dropbox. It provides sync clients that allows you to access "cloud" data seamlessly and it has a web interface where you can view files.

Our version of Nextcloud is the one installed by Nextcloud All-in-one[320].

This works by running a top-level, "coordinator" Docker container that is responsible for auto-updating Nextcloud, taking backups of the Nextcloud code, and so on.

There is a special admin account on Nextcloud that is possible to use the direct login feature with. The username and password are in the password database. The lab computers also use a special "service account" that is not tied to a Touchstone account. However, most use will login using Touchstone, using your normal MIT credentials.

The AIO interface is accessible through https://smithsonian-internal.mit.edu:8080, but you can only directly access this interface when Nextcloud is not running. While Nextcloud is running, you can access this by logging in as the admin user to https://smithsonian.mit.edu and then going to https://smithsonian.mit.edu/settings/admin/overview and clicking the "Open Nextcloud AIO interface" button at the top.

In the worst case that there is large dataloss, you may have to first restore the backup as described below, and then use the AIO interface to restore from a backup.

In a truly worst-worst case where the Nextcloud database is lost, there is still no file loss: all of the individual data files are backed up and can be downloaded / reuploaded into a fresh Nextcloud install.

[320] https://github.com/nextcloud/all-in-one

8.4.3 Network setup

To run all of this software correctly, we use the Synology web UI, as linked above (https://smithsonian-internal.mit.edu:5000).

In particular, we have setup the firewall settings to block all access except Nextcloud on the "external" network, accessible through `smithsonian.mit.edu`. The admin interfaces are only accessible through `smithsonian-internal.mit.edu`.

Within the Synology UI, we have setup 1) an automatically-renewed TLS/HTTPS certificate for smithsonian.mit.edu, and 2) a reverse proxy for this domain. The NAS itself takes care of forwarding requests to the Nextcloud docker containers.

8.4.4 Touchstone setup

To integrate with Touchstone, we broadly follow MIT IS&T's instructions[321].

Here, Touchstone is a SAML identity provider (IdP), and we are running a SAML Service Provider (SP). Our service provider needs to talk to the identity provider to get information on who is logging in.

Nextcloud has a service provider built-in! You can change the configuration at https://smithsonian.mit.edu/settings/admin/saml (when logged in as the Nextcloud admin user). Registering with Touchstone involves going back and forth with Touchstone support, configuring our Service Provider on our end to match the settings on the IdP. An important accessible file is the metadata XML file which SAML uses: https://smithsonian.mit.edu/apps/user_saml/saml/metadata

Luckily, once setup, this setup should not need to be touched.

[321] https://wikis.mit.edu/confluence/display/TOUCHSTONE/Provisioning+Steps+for+Shibboleth+SP

8.4.5 Backup setup

For server backups, MIT provides a service called Spectrum Protect / TSM. We have installed the Spectrum Protect client and packaged it as a docker container. The Docker container build repository is at https://github.com/GallowayLabMIT/spectrum-protect-container and the container image is available at `ghcr.io/gallowaylabmit/spectrum-protect:latest`.

Using this Docker container, we mount two volumes: `/volume1/NextCloudData` (all of the actual Nextcloud files) and `/volume1/NextCloudBackup` (the daily backup of the Nextcloud database and other files). The total contents of these are uploaded to MIT's backup system.

MIT sets the schedule; these backups are run every weekday at 6pm.

See the *Builder config and update* (page **??**) if you are interested in how the continuous build system works, or you'd like to fork this repository to make it your own.

CHAPTER

NINE

CONTRIBUTOR GUIDE

Protocols and recipes are written in reStructuredText, a lightweight markup language that allows us to use plain text to describe relatively complicated intent, letting us make tables, cross-links between different files, image inclusion, and so on.

The Python package Sphinx is used to automatically take this information and make a searchable website[322], in addition to a printable PDF version of all of the protocols and recipes, available here[323].

If you want to make small edits or prefer the browser interface, you can skip all local setup and go directly to *Online editing through Github* (page **??**).

If you already are familiar with text editors and have an editing/programming environment already setup, you can go directly to *Local building* (page **??**).

9.1 Local environment setup

To locally build the protocols documentation, you will need to have a text editor of your choice and a local install of Python. To contribute your changes, you will need access to **git** as well.

9.1.1 Text editor

Unlike a what-you-see-is-what-you-get (WYSIWYG) editor like Word, a *(plain)* text editor is used for editing markup and programming languages.

Note

Integrated development environments (IDEs) such as MATLAB, RStudio, TeXStudio, and others typically have a text editor integrated with debugging and other tools. These are helpful, but are often single-purpose. Having a text editor customized to your liking is especially helpful when switching between many languages or if extra customization is desired.

There are many options available:

[322] https://gallowaylabmit.github.io/protocols
[323] https://gallowaylabmit.github.io/protocols/galloway_lab_protocols.pdf

- **Built-in editors**: Every major OS comes pre-installed with a plain text editor. Windows has *notepad*, Mac OSX has *TextEdit* (while this defaults to a rich-text editor, it can also be used as a plain-text editor), and most Linux distros have *vi* or *GEdit*.
- **Terminal-based editors**: *nano* is a basic editor, whereas *vim* and *emacs* are extraordinarily customizable.
- **Standalone editors**: These editors can be customized to be nearly IDE-like. On Windows, there is the venerable Notepad++[324]. More recently, there are the commonly used editors Sublime Text[325], Atom[326], and VS Code[327].

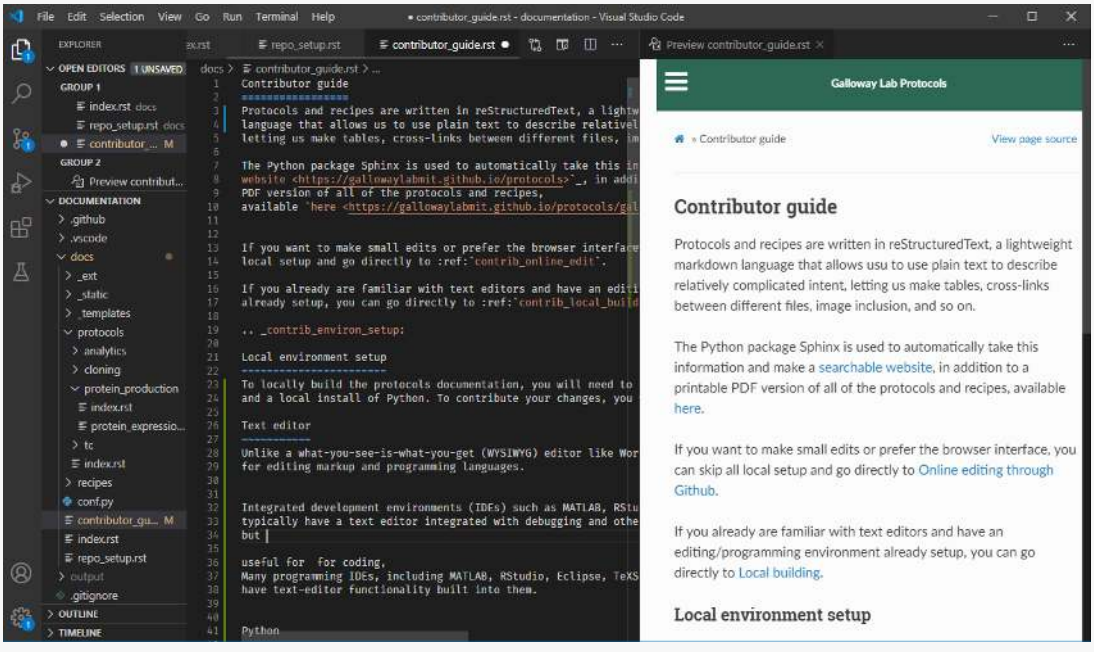
Choice of editor is largely a personal preference. If you are doing limited plain-text editing, the built-in editors may be sufficient. Becoming familiar with one of the terminal-based editors is useful when working on remote compute clusters and servers.

If you are looking for a recommendation, VS Code is an excellent, relatively lightweight text editor with plenty of helpful extensions.

Tip

Using a modern text editor is very helpful when working with markup languages such as reStructuredText, Markdown, and LaTeX, as they often have support for live document preview, intelligent spell-check (ignoring programming terms as mis-spelled), and git support (no need to use the git command line or GUI app).

As an example, this document was written in VSCode, with the Python and reStructuredText extensions installed:



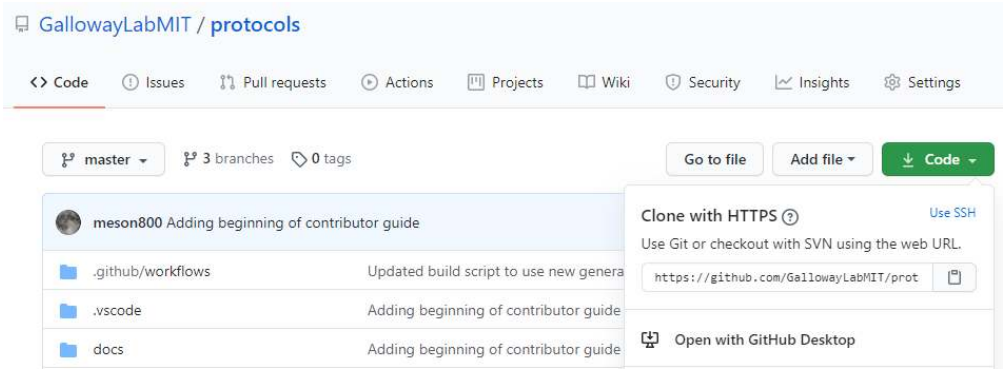
[324] https://notepad-plus-plus.org/
[325] https://www.sublimetext.com/
[326] https://atom.io/
[327] https://code.visualstudio.com/

9.1.2 git

We use `git` to manage version history, simultaneous editing, and other features. There are several[328] excellent[329] tutorials[330] elsewhere that explain how to use `git`.

If you don't have `git`, you can install it from here[331], and install a GUI tool if you wish (such as the standalone Github Desktop[332], or using one built-in to your text editor).

Once you have `git` installed, you should *clone* the protocols repository. For any Github repository, you can find the clone URL by clicking the green "code" button:



In the case of this repository, the HTTPS clone URL is https://github.com/GallowayLabMIT/protocols.git.

If you access Github using `ssh` keys, the SSH clone URL is git@github.com:GallowayLabMIT/protocols.git.

The published version of the website uses the default `latest` branch, so push to this branch to update the website.

[328] https://git-scm.com/book/en/v2
[329] https://try.github.io/
[330] https://gitimmersion.com/
[331] https://git-scm.com/downloads
[332] https://desktop.github.com/

9.1.3 Python/Sphinx setup

Sphinx[333] is used to create the rendered website and PDF. Sphinx relies on a Python version at least as new as Python 3.5.

If you do not already have a working Python version >= 3.5, use the standard Python installer[334]. Anaconda can also be used, but generally the standard Python installer is preferred.

For standardization purposes, the Python packages required to build the website is specified in a `requirements.txt` file. This means that we can use a **virtual environment** to reproducibly build the website.

To start, we need to make a virtual environment. We can do this using the `venv` Python module. The last argument is the name of the virtual environment: here we give it the customary name `env` but you can choose anything. From the root of the repository (e.g. the folder containing `README.md`) create the environment. This only needs to be performed once! If weird package errors happen later, we can always just delete the `env` folder and recreate it.

```
$ python -m venv env  # On Windows, most Linuxes
$ python3 -m venv env # On modern MacOS
```

We now need to **activate** the environment. The syntax is slightly different between Powershell (on Windows) and bash/zsh (Linux, MacOS). This typically has to be done every time you open a new terminal or when you switch between projects with different virtual environments.

```
$ source env/bin/activate # On MacOS, Linux
```

```
> .\env\Scripts\activate # On Windows
```

Now that the environment has been activated, any Python changes we do (installing packages, etc) will only affect this environment. We install all of the necessary build requirements by doing:

```
$ pip install -r requirements.txt
```

From now on, you just need to follow the virtual environment activation step.

Potential Version Issues

You may run into problems with requirement versions being incompatible with your computer's versions. In this case, you should delete all version information from requirements (control + f "==.*" and delete). This will automatically install the most up-to-date versions of the packages. After successful installation, freeze these versions by doing:

```
$ pip freeze > requirements.txt
```

[333] https://www.sphinx-doc.org/en/master/
[334] https://www.python.org/

9.2 Standard workflow

A normal workflow to update a protocol would be:

1. Do a `git pull` to receive any updated changes from others.
2. Make changes to the desired files, such as adding pictures, writing new text, and so on.
3. *Locally build* (page **??**) the protocols website, checking for any errors (e.g., incorrect reStructuredText). Before running the local build, you will likely have to create/activate your virtual environment as listed in the *Python setup* (page **??**) section.
4. When there are no build errors, add/stage the changed files and create a commit describing your changes.
5. Do a `git push` to update the website.

Common problems

- **The website isn't updating!**

  When you push your changes to Github, you start a remote build that does a full build of the project. This can take about two minutes.

  If the website still hasn't updated after two minutes, there is likely a (fatal) build error. Check to make sure that there are no errors printed when you locally build the website! You can also check the remote build log through Github actions[335].

  If there are no errors or warnings while building locally but the remote build still fails, something strange is happening.

- **I got a push rejected error!**

  If you run `git push` and get an error like:

```
$ git push
Pushing to https://github.com/GallowayLabMIT/protocols.git
  ! [rejected]        latest -> latest (non-fast-forward)
error: failed to push some refs to 'https://github.com/GallowayLabMIT/
↪protocols.git'
hint: Updates were rejected because the tip of your current branch is␣
↪behind
hint: its remote counterpart. Merge the remote changes (e.g. 'git pull')
hint: before pushing again.
hint: See the 'Note about fast-forwards' in 'git push --help' for details.
```

  this means that someone else pushed changes to the same branch while you were making edits. This is not a problem, git is just warning you that you first need to merge their changes first. As suggested by the hint, the solution is often to do `git pull` again, which will download the remote changes and attempt to auto-merge them. If there are no errors shown when you run `git pull`, the merge happened automatically and you can re-push.

- **I got a merge conflict!**

If another person has edited the **same part** of a protocol file at the same time you edited, you may get a merge conflict when you try to pull in the remote changes. This looks like this:

```
$ git pull
From https://github.com/GallowayLabMIT/protocols.git
* branch            latest -> latest
Auto-merging contributor_guide.rst
CONFLICT (content): Merge conflict in contributor_guide.rst
Automatic merge failed; fix conflicts and then commit the result.
```

This error means that `git` isn't sure how to merge two sets of changes together. Instead, it needs the user to choose. For every conflicting file, search for the *conflict markers* marked with <<<<<<< and >>>>>>> symbols, and decide how to combine the two versions. This probably involves talking with the other person who edited the protocol! An example merge conflict is:

```
Text before the merge conflict.

<<<<<<<HEAD
The conflict region! Up here is
your version of the files.
=======
Below the equal signs is whatever the remote version
of this part of the file is.
>>>>>>>a21ca24 (the git commit identifier)

Text after the merge conflict
```

It's up to you to decide how to combine the two versions of the file. When you are done editing (making sure to remove the conflict markers!), you need to `git add` the file to mark that you resolved the merge conflict, then `git commit` when you are done handling merge conflicts. You are then ready to `git push`!

9.3 Local building

The documentation can be built by calling the `build.py` script at the base of this repository. Normally, this means opening a terminal window, navigating to the repository, and calling:

```
python build.py
```

This will attempt to build both the website only. If you have a local LaTeX install, then you can build the PDF locally with:

```
python build.py --latex
```

These build functions build the website in the folder `output/html`. If you want to view your locally

[335] https://github.com/GallowayLabMIT/protocols/actions

built website, open the file `output/html/index.html`.

9.3.1 Local previewing

If using VS Code with the reStructuredText plugin installed, you can use the instant previewer to view the HTML version as it recompiles on the fly. To show the preview, open the Command palette, accessible through `Control-Shift-P` or `Command-Shift-P`, and run `Esbonio: Preview Documentation in Split Window`.

If local previewing fails, you need to check a few things:

1. Make sure that the reStructuredText language server is installed in your virtual environment. Run `pip install -U esbonio`.
2. Make sure that VSCode is using your virtual environment. To check, open the command palette and go to `Python: Select Interpreter` and make sure that the virtual environment Python is selected.
3. Make sure the reStructuredText plugin is using Sphinx, not docutils. On the left side of the statusbar, you should see `Sphinx: protocols/docs/conf.py`. If you see `Use docutils`, click the label and switch to using Sphinx.
4. If you run into `esbonio` startup errors, check for the existence of a `.vscode/settings.json` file. If any lines are marked with errors (yellow underline), delete them.

9.3.2 In case of build errors

In the case of strange build errors that seem to be because the output directory has been corrupted, you can close any program that might be using the output (a common one might be Adobe Acrobat, with the generated PDF open) and run:

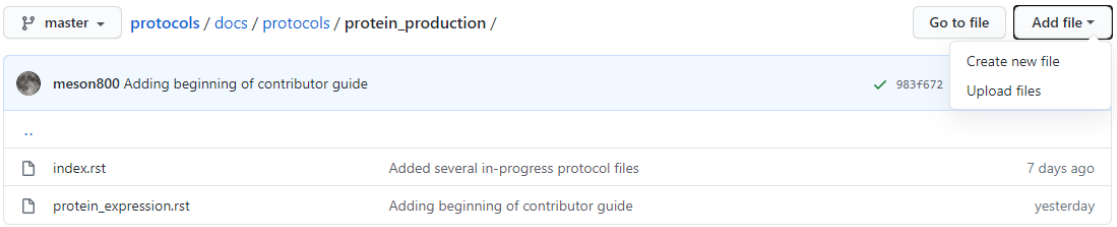
```
python build.by --force-rebuild
```

Adding this flag deletes the `output` folder and recreates it. You can also do this manually to recreate "rebuild" behavior.

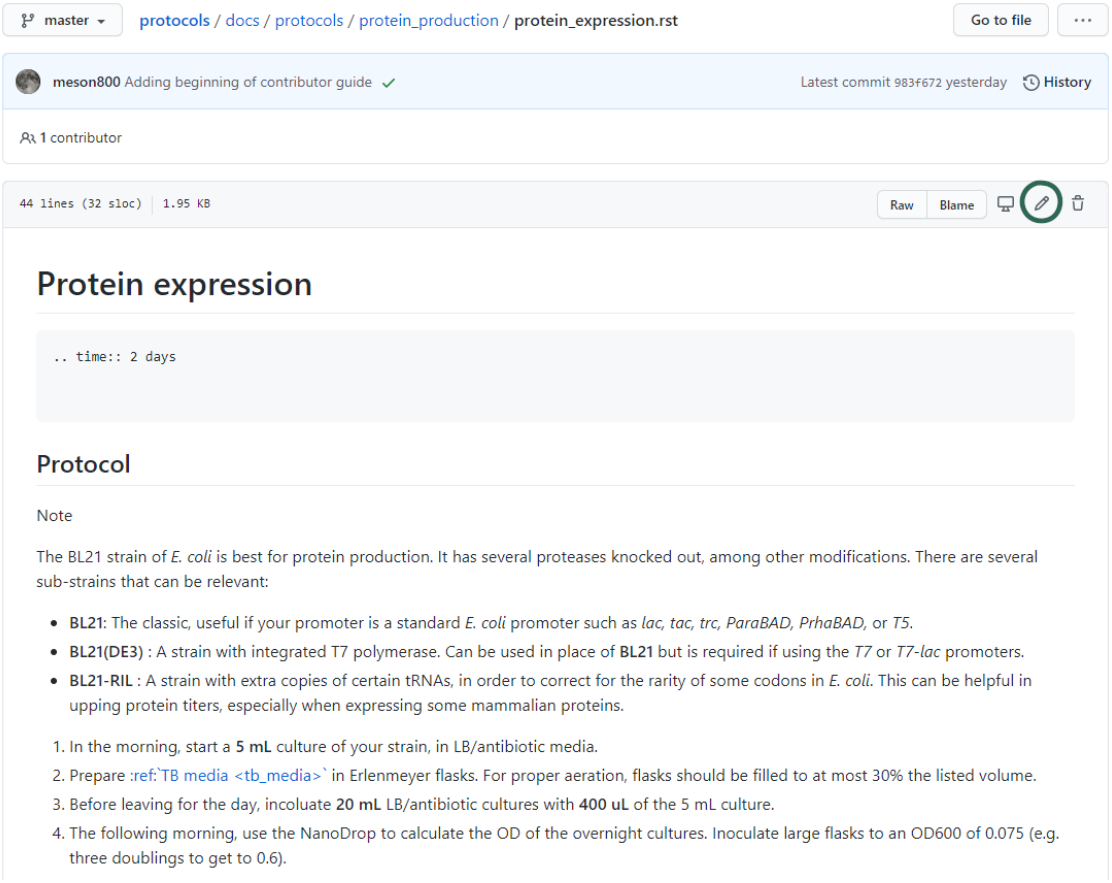
9.4 Online editing through Github

When editing directly through the Github website, you won't be able to check for Sphinx build errors or fully preview the generated PDF and website until you commit to the branch. For this reason, doing *local builds* (page **??**) is preferred.

To create a new file directly through Github, navigate to the folder you want to add the file, and click the **Add file** dropdown on the right:



To edit through Github, navigate to the file you want to edit, then click the pencil in the upper right of the file view:

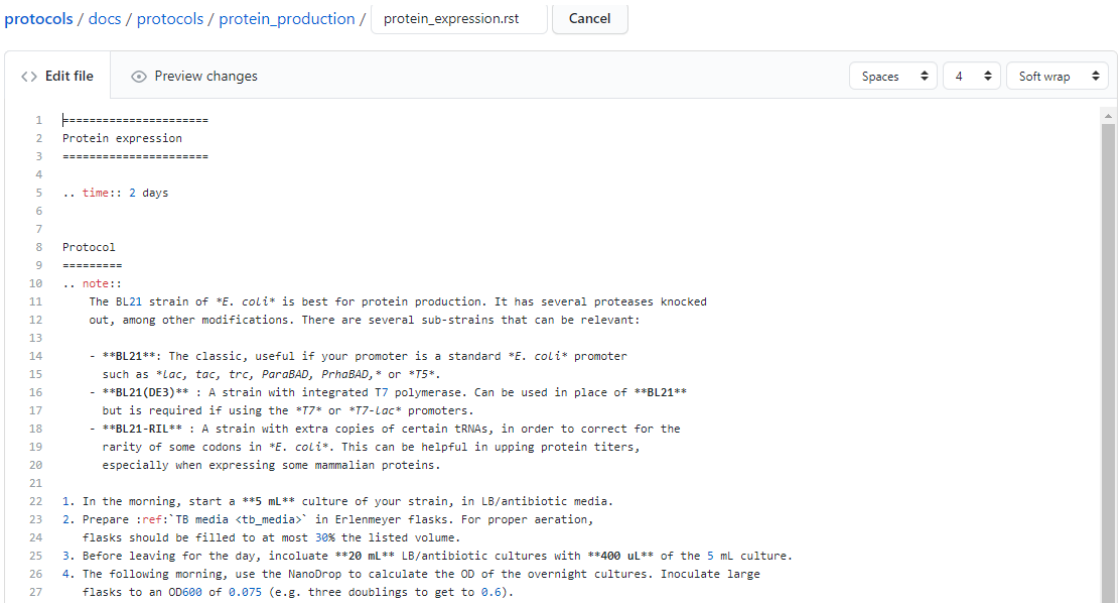

master
protocols / docs / protocols / protein_production / protein_expression.rst
Go to file
meson800 Adding beginning of contributor guide
Latest commit 983f672 yesterday
History
1 contributor
44 lines (32 sloc) | 1.95 KB
Raw
Blame
Protein expression
.. time:: 2 days
Protocol
Note
The BL21 strain of E. coli is best for protein production. It has several proteases knocked out, among other modifications. There are several sub-strains that can be relevant:
BL21: The classic, useful if your promoter is a standard E. coli promoter such as lac, tac, trc, ParaBAD, PrhaBAD, or T5.
BL21(DE3) : A strain with integrated T7 polymerase. Can be used in place of BL21 but is required if using the T7 or T7-lac promoters.
BL21-RIL : A strain with extra copies of certain tRNAs, in order to correct for the rarity of some codons in E. coli. This can be helpful in upping protein titers, especially when expressing some mammalian proteins.
1. In the morning, start a 5 mL culture of your strain, in LB/antibiotic media.
2. Prepare :ref:`TB media <tb_media>` in Erlenmeyer flasks. For proper aeration, flasks should be filled to at most 30% the listed volume.
3. Before leaving for the day, incoluate 20 mL LB/antibiotic cultures with 400 uL of the 5 mL culture.
4. The following morning, use the NanoDrop to calculate the OD of the overnight cultures. Inoculate large flasks to an OD600 of 0.075 (e.g. three doublings to get to 0.6).

Note

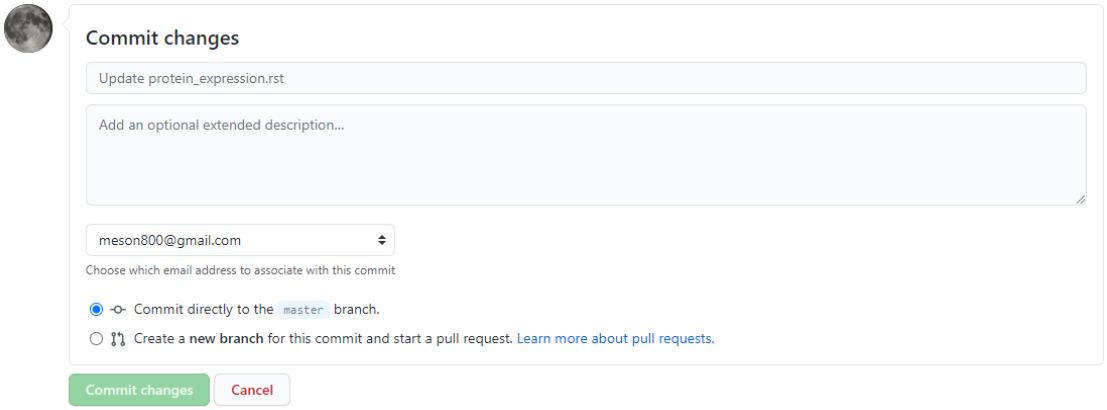
While Github does render a preview of what the reStructuredText will look like, it does not preview how Sphinx will render the final website. For example, we can see in the above image that the Github preview does not show the custom `time` directive, and it does not show the proper link destination for the cross-referenced recipe.

In general, the Github preview will give you a good idea of what tables/lists/other text will appear, but it will not properly render all Sphinx-enabled markup.

This will open an editor window:



After you are done editing, add a commit message describing your change, and (normally), commit directly to the `latest` branch. If there is need for further discussion of an added protcol, creating a secondary branch + pull request could be helpful.



Note

Make sure you make the commit message more descriptive than the default "Update <file-name>" message!

9.5 Repository layout

All of the relevant documentation files to edit are stored in the `docs` folder.

Each subdirectory is included as its own sub-level in the table of contents. This hierarchy is derived from the `index.rst` that is in each folder. Generally, each of these `index.rst` files have the following content; if you create a new subdirectory you should generally add this as the `index.rst` file:

```
============
Section name
============

.. toctree::
  :maxdepth: 2
  :glob:

  */index
  *
```

Here, `glob` means that the * is expanded as a wildcard. The first wildcard search, `*/index` means "include all subdirectories beneath this directory". The second wildcard search * means "include all other `.rst` files in this directory.

The current subdirectory layout looks like:

```
docs
├─protocols
│  ├─analytics
│  ├─cloning
│  ├─protein_production
│  └─tc
└─recipes
   ├─bacteria
   └─tc
```

The `.github` folder contains the continuous integration script responsible for updating the website on every push.

9.6 Basics of reStructuredText

reStructuredText (RST) is a *lightweight* markup language. This means that it is not as cumbersome as languages like HTML and LaTeX, but still has enough power to make nice looking documents.

There is an excellent RST primer and reference here[336] which should be your primary reference, but here we will cover some of the basics.

One nice feature of the generated website is the ability to view the page source for each page. If there is a protocol that uses some RST feature that you want to replicate, on the desktop version of the website (e.g. not mobile), you can click the "View page source" button in the upper right of **any page** on the website to see the RST code that generated that page. That includes this page, so check out this page's source to see how this guide was written!

Note

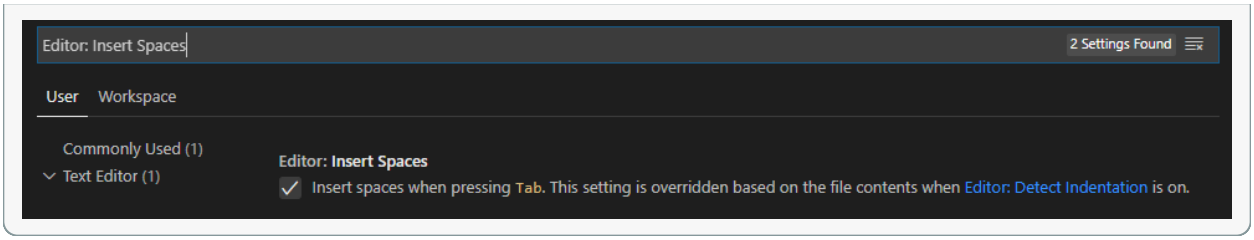
There are several whitespace-dependent features of RST. This means that you should configure your text editor to insert spaces instead of tabs when you hit the tab button (this is also true if you are programming in whitespace-dependent languages like Python).

Without wading too deeply into the holy war[337], tabs vs spaces **does not mean the difference between pressing the space bar vs hitting tab for indentation**, it refers to what character actually gets inserted into the document when you press the tab button.

Long story short, if your text editor inserts literal tab characters, there is possible inconsistency between tools and editors; some may display a single tab character as the width of two spaces, some as the width of four spaces, and so on. This causes problems. If you set your editor to insert spaces, you still hit tab, but the editor inserts some fixed number of spaces, typically four.

This setting will depend per editor. In VS code for example, you don't have to do anything; it defaults to inserting spaces, but the option looks like this:

[336] https://www.sphinx-doc.org/en/master/usage/restructuredtext/basics.html



9.6.1 Simple markup

You can add headers by surrounding the header with equal signs, hyphens, tildes, and other special characters.

Example

```
Section header
==============

Subsection header
-----------------
```

Use single asterisks to italicize text. Use double asterisks to bold text. Wrapping double backticks around text renders it in monospace.

Example

```
*italics* and **bold** and ``monospaced``.
```

renders as

italics and **bold** and `monospaced`.

You can make bullet lists by starting lines with `*` or `#`, and you can make numbered lists by starting lines with `1.`, `2.`, etc.

If you are nesting lists, you must surround nesting levels with blank lines.

Example

```
* Lab activities

  * Axe throwing
  * Pizza party
  * ?????
```

[337] https://softwareengineering.stackexchange.com/questions/57/tabs-versus-spaces-what-is-the-proper-indentation-character-for-ever

```
* Lab meme sources

  * p53
  * Cloning, so much cloning

1. Testing
2. a
3. numbered
4. list
```

renders as

- Lab activities
  - Axe throwing
  - Pizza party
  - Other?
- Lab meme sources
  - p53
  - Cloning, so much cloning

1. Testing
2. a
3. numbered
4. list

9.6.2 Explicit markup

In RST, an "explicit" block is any block that starts with `..` Explicit blocks must be surrounded on both sides by blank lines, like nested lists. This means that explicit blocks like:

```
Do the foo, then the bar
.. note::

  Make sure you don't do the bar, then the foo!
```

will not render correctly, it must be written:

```
Do the foo, then the bar

.. note::

  Make sure you don't do the bar, then the foo!
```

9.6.3 Admonitions

To call-out a specific part of a protocol, you can use one of the various admonitions.

This project includes the special time estimation admonition, which demonstrates the general principle. Writing:

```
.. time::

  2 hours
```

renders as

Estimated time

2 hours

Other directives (code blocks, tables, images, etc) can be fully nested inside these blocks.

Other options commonly used here are `hint`, `important`, `note`, `tip`, `deprecated`, and `warning`, which render as

Hint

Test

Important

Test

Note

Test

Tip

Test

Deprecated since version 2021.11.24: Test

Warning

Test

9.6.4 Code

To insert a codeblock, start an empty line with `::`, followed by an indented block that will be rendered as code.

Example

The above code example of the time estimation admonition was written as:

```
::

  .. time::

    2 hours
```

9.6.5 Tables

See the table documentation[338] for more details, but in brief, most of the time you can use the "simple" table layout.

In the simple table layout, you simply surround the desired table text with equal signs to set off different columns.

Example

```
================= ============================
Ingredient         Amount per 1L final volume
================= ============================
Tryptone                     12 g
Yeast extract                24 g
Glycerol                     4 mL
Deionized water              900 mL
================= ============================
```

renders as

Ingredient	Amount per 1L final volume
Tryptone	12 g
Yeast extract	24 g
Glycerol	4 mL
Deionized water	900 mL

[338] https://www.sphinx-doc.org/en/master/usage/restructuredtext/basics.html#tables

9.6.6 References and links

To reference a standalone hyperlink, you can just simply write it directly in the rst file, no special markup required.

If you want to add link text, use the following syntax:

Example

```
https://example.org or `Link text <https://example.org>`_
```

renders as:

https://example.org or Link text[339]

If you want to specifically link to other protocols/recipes files, you use the special *doc* syntax:

Example

```
:doc:`This <contributor_guide>` is a link to this very document!
```

renders as:

This (page **??**) is a link to this very document!

as you can see, this is very similar to the external hyperlink, except it has the special `:doc:` before it, which tells Sphinx that the document is included in this repository.

If you want to reference a specific subsection of a document, you can set a label and set a reference to it. This is best explained by the documentation[340], noting that labels that you create are global and shared across the entire repository!

[339] https://example.org
[340] https://www.sphinx-doc.org/en/master/usage/restructuredtext/roles.html#ref-role

9.6.7 Images

To include an image, you can specify it as follows. Typically, you want to align center and make the image fill the available horizontal space, so a common image call would be:

```
.. image:: image_location/image_filename.png
  :width: 100%
  :align: center
```

9.6.8 Math

You can write arbitrary LaTeX-formatted math by using the math directive. The math must be separated from the directive by a blank line, followed by an indented math block.

Example

```
.. math::

   E = mc^2
```

renders as

$$E = mc^2$$

CHAPTER

TEN

CONTRIBUTING

See the *Repository setup guide* (page **??**) if you are interested in how the continuous build system works, or you'd like to fork this repository to make it your own.

See the *Contributor guide* (page **??**) for a more detailed walkthrough of how to contribute to this protocols repository.

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