
Galloway Lab Protocols

Galloway Lab

Jan 30, 2024

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This is the home of all of the Galloway Lab protocols!

Click [here](#) to access the PDF version.

**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.

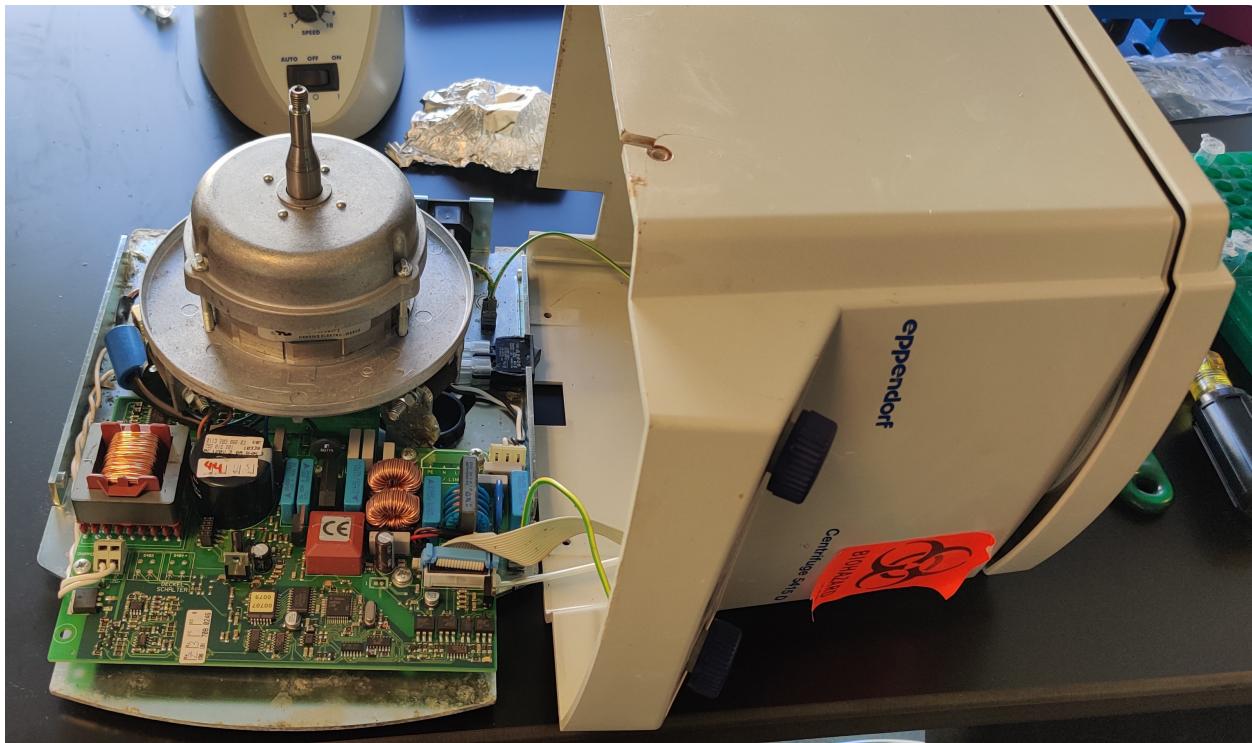


Fig. 2: The centrifuge with the shell removed. You should not disconnect the wires; just leave the shell on the right.

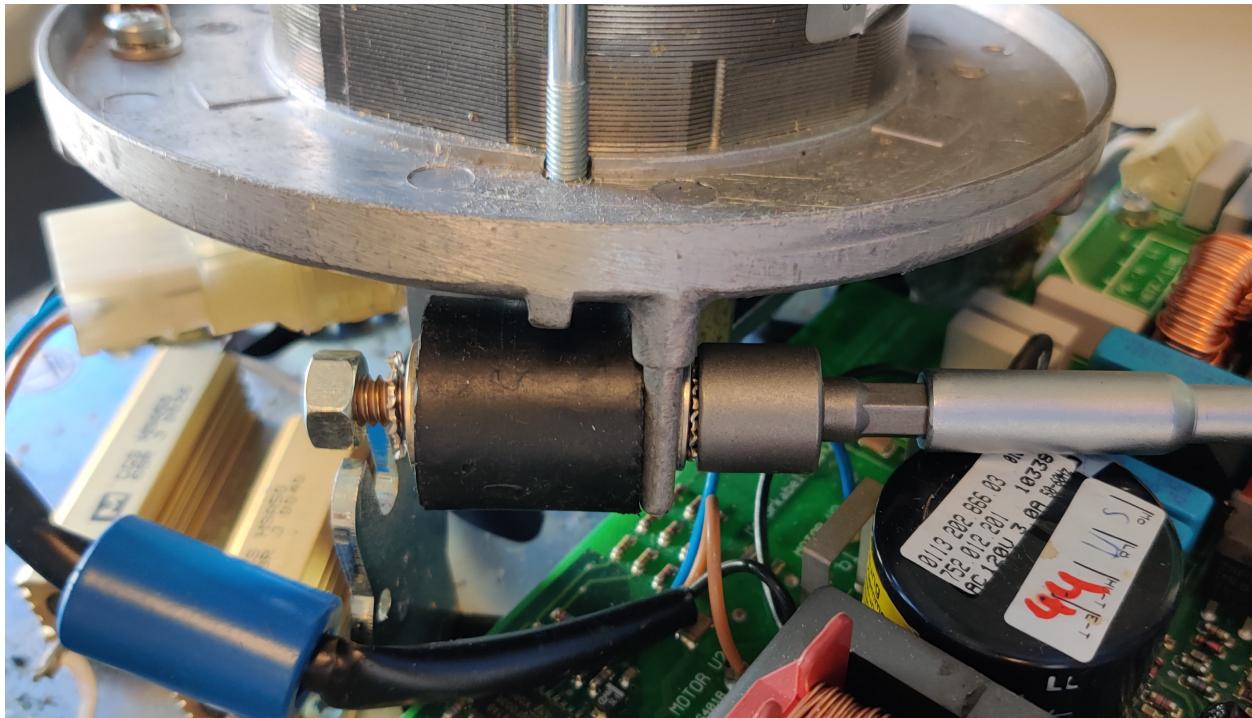


Fig. 3: A mount half-replaced. You need to attach the mount into the two slots for it. It is likely easier to attach it to the rotor first, then attach it to the base.

1.2 Cleaning and autoclaving guidelines

Important: All glassware will now be dishwashed. Place glassware to be washed to the left of the Elga water dispenser. All bacteria-related glassware should be bleached for 20 minutes and rinsed with water before being placed in the dishwashing locations. All the glassware on the wooden shelves should already be dishwashed and ready to be used.

1.2.1 Washing glassware and tools

We currently use Alconox as our soap. Alconox is stored as a powder underneath the sink. Dilute this powder in water in the squirt bottle labeled “Alconox”. There is no precise measurement for this.

There is also a dishwasher located in the autoclave room (56-414). If you use this, fill out your information (date, time, name, phone number, office number, and PI initials) in the dishwasher log on the metal racks to the left of the autoclave.

Glass bottles and flasks

Note: If you empty a glass bottle or flask (media, PBS, gelatin, ELGA water, etc.), *you are responsible for washing it*. If you cannot wash the bottle immediately, keep the bottle on your personal lab bench.

1. If the glassware had cells in it (e.g. midipreps) first add bleach to a concentration of 10% and let sit for at least 15 min.
2. Add Alconox and water, 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 DI water.
5. Put glass to dry on drying rack.
6. Move glassware when dry to the shelving with the other dry, clean glassware. To prevent dust accumulation, store beakers upside down and add foil to flask mouths.

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 DI water.
4. Let beads fully dry in strainer overnight.
5. Use funnel to put beads into glass bottle for autoclaving.

Dissection Tools

Note: Dissection tools should be washed as soon as dissection is finished. Dirty tools should not be left at 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 DI water.
4. Let dry and wrap in tin foil.
5. Place back into metal container and move to area next to ELGA water dispenser to be autoclaved.

1.2.2 Autoclaving

Glassware and plastics are autoclaved as needed. Usually, this amounts to twice a week. Clean glassware on the drying rack will be autoclaved as well as any empty tip or tube boxes.

Prep materials as described below and place in autoclave-safe bin (located on the racks above the sink). The autoclave is located in room 56-414 and requires card access. To operate the autoclave, first 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 this out and fill in the start time for your desired cycle. Open the door by turning the wheel fully counterclockwise, then turn the lever counterclockwise. Place the bins on the racks inside the autoclave and close the door, first turning the lever and then the wheel fully clockwise. 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 (~45 min)
- For wet cycles (liquids such as PBS, LB, etc.): Liquid >> 30 min / 0 min (~1 hr)

When the cycle is complete, open the door by first turning the wheel counterclockwise and then the lever. Be cautious of hot steam escaping from the open door. Remove bins from the autoclave using heat-proof gloves. 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 the empty plastic insert and autoclave tape, and 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.

2. Recycle any broken tip boxes.
3. Add inserts with new tips to empty boxes and add strip of autoclave tape where the lid meets the side of the box.

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 strip of autoclave tape where the lid meets the side of the container.

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.

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.

Flasks

1. Add strip of autoclave tape to top of clean flasks covered with tin foil.

Dissection tools

1. Make sure tools are individually wrapped in tin foil inside metal container.
2. Wrap outside of metal container with tin foil and mark with autoclave tape.

Glass bottles (containing liquid or glass beads)

1. Loosely screw on bottle cap.
2. Cover cap with tin foil and mark with autoclave tape.

1.3 Cleaning BSCs

As part of quarterly lab jobs, the biosafety cabinets (BSCs) used for TC should be thoroughly cleaned. This includes:

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.

Tip: It is convenient to clean one half of the BSC at a time, i.e., move all items to the left side and wipe the right walls and surface, then move items from the left to right side as they are cleaned, then clean the left walls and surface.

Note: Do your best to scrub away any stains or residue on the BSC surfaces, but note that the BSC in 66-219 has some permanent stains that will not come off.

1.4 Cleaning microcentrifuges

1. Remove the rotor from the microcentrifuge by unscrewing the center bolt that holds the rotor in place. An adjustable wrench can be used to initially loosen the bolt, which can then be unscrewed by hand the rest of the way.
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. Using 70% ethanol and paper towels, clean the groove where the rotor lid sits during normal operation. Dried salts tend to build here.
5. 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.
6. When the rotor is clean and dry, reattach the rotor to the centrifugre base. Tighten the screw by hand as much as possible before tightening

1.5 Checking data backups

1.5.1 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 ???

1.5.2 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](#)¹

Adding a new NGS dataset checklist

- Compress the dataset, either into a `.tgz` or a `.zip`.
- 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).
- If there is not enough room, order a new pair of hard drives. There are suggestions listed in the [backup helper instructions](#)²; 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!
- Locate and bring both fireproof safes to lab. One is likely with Katie or otherwise offsite from 66.
- Copy the dataset onto both drives, into the *data* folder.
- Follow the [backup helper instructions](#)³

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:

- Bring both fireproof safes to lab. One is likely with Katie or otherwise offsite.
- For each pair of drives, load each into the external hard drives enclosures and connect to the same computer.
- Follow the *backup helper instructions* <https://github.com/GallowayLabMIT/cold_backups> to run the auto-verification.

¹ <https://mitprod.sharepoint.com/:x/s/GallowayLab/ERLZeBrX4OVIpMRjG9Q5foIBV0iK1xhWT1DEF2Qg1dgGCA?e=0OhHi8>

² https://github.com/GallowayLabMIT/cold_backups

³ https://github.com/GallowayLabMIT/cold_backups

- Do the suggested spot-check manual check on one of the files both as practice and to make sure the automated helper is working.
- Check the desiccant packs. You can either weigh them to see how much water they have absorbed, or look at the color of the bead. You can regenerate the desiccant by microwaving the packs for the listed time (or until they lose water mass).

1.6 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.

1.6.1 Maui and Moana (Main TC)

Follow instructions on the incubator display.

1.6.2 Hei Hei and Pua (Main TC)

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

The [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.
2. Remove and empty the plastic 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.
6. 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 plastic water tray and fill with autoclaved DI water.
7. Follow the manual instructions (page 58) for starting the 90°C 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, follow manual instructions (page 45) to begin the auto-start routine. This will take **approximately 5 hours**.
9. After the auto-start routine finishes, the incubators can be used.

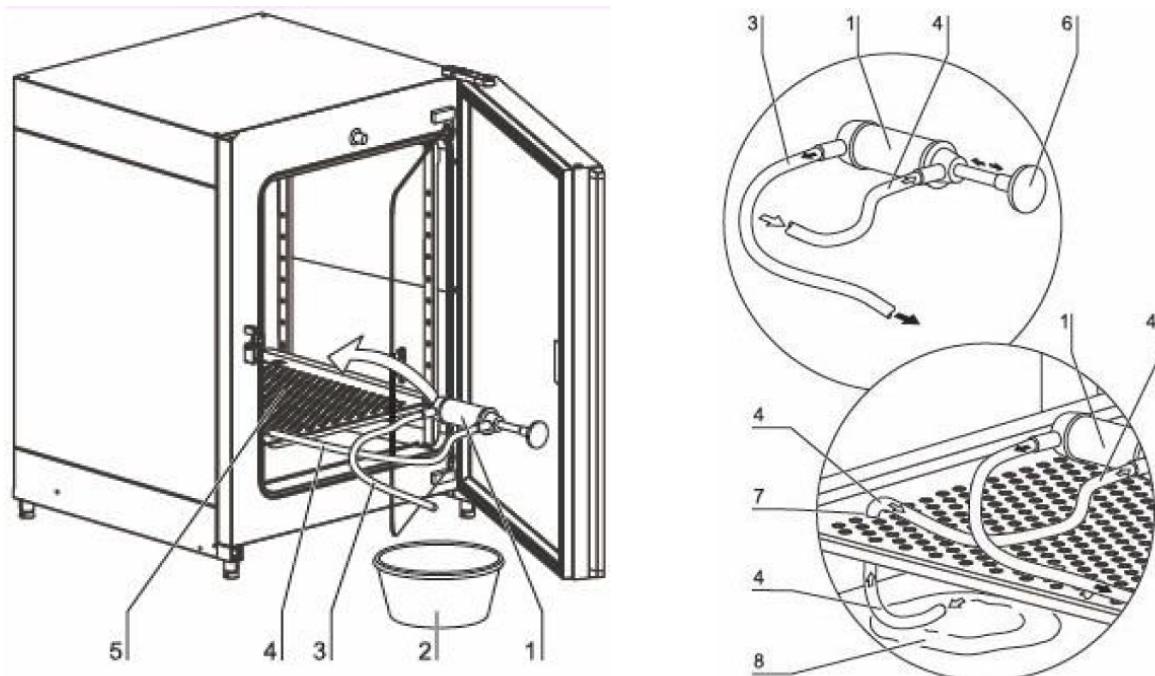


Fig. 4: Diagram of the water pump setup and parts: (1) pump, (2) bucket, (3) outlet hose, (4) inlet hose, (5) lowest incubator shelf, (6) pump hand grip, (7) incubator shelf hole, (8) water on incubator floor.

1.6.3 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, [760175⁴](#) (source from Ebay)
- Inlet filter: Thermo Scientific, [770001⁵](#)

The incubator decontamination instructions are located in a plastic sleeve on the side of the incubators. See [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
6. Disinfect the incubator components with a dilute bleach solution followed by ethanol. Also disinfect the inside of the incubator and glass door.

⁴ <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>

⁵ <https://www.fishersci.com/shop/products/thermo-scientific-midi-40-co-sub-2-sub-incubator-accessories/11687203>



Fig. 5: Carbon dioxide knob (black, center) and pressure gauge (top, currently at 0 psi).



Fig. 6: Carbon dioxide tubing connected to the back of Maui (top incubator).

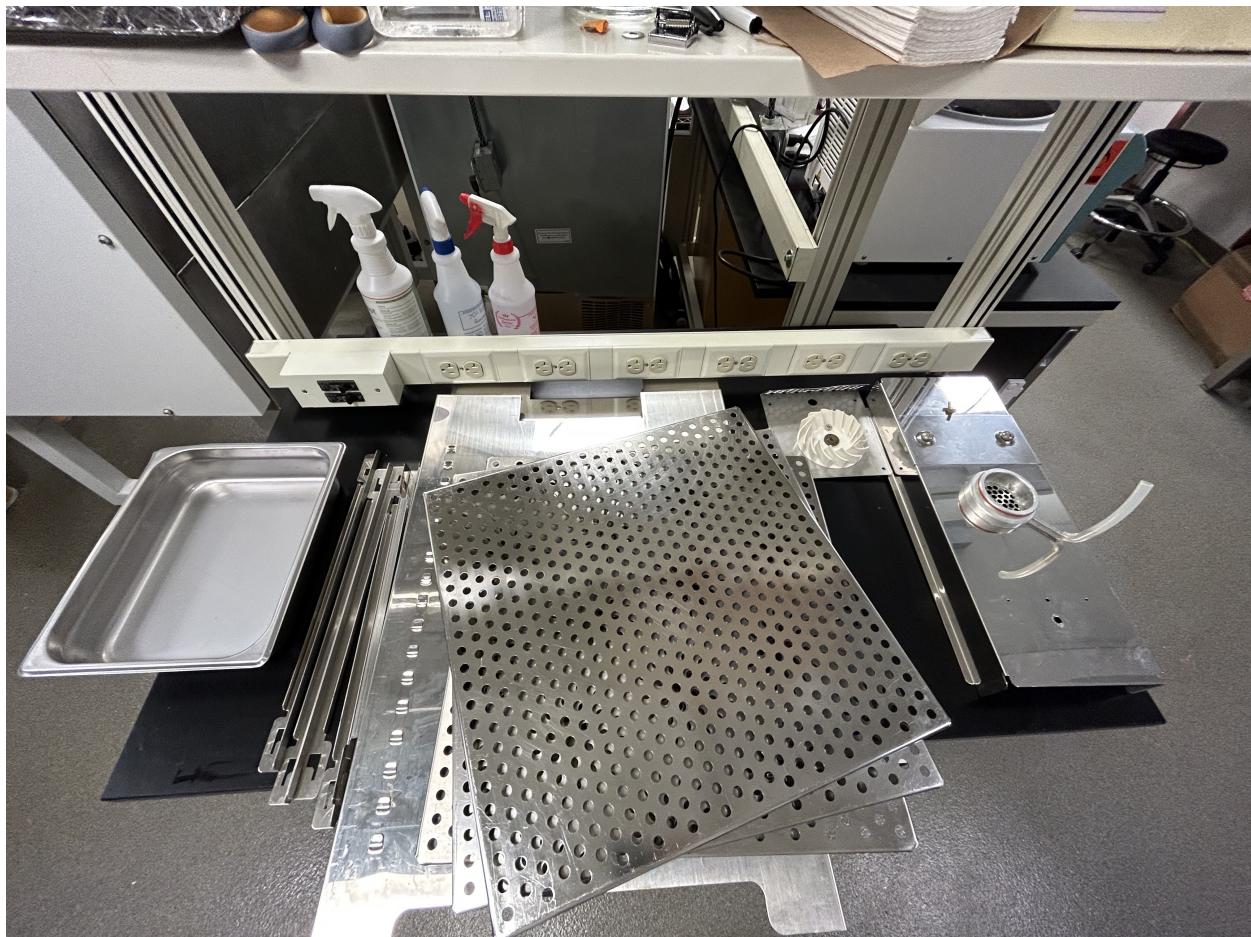


Fig. 7: Disassembled parts from one incubator.



Fig. 8: Interior of a disassembled incubator.

- 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 equilibrate for **approximately 12 hours**. If necessary, a shorter equilibration 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.
15. Turn the incubators back on. Once carbon dioxide levels reach 5%, the incubators may be used.

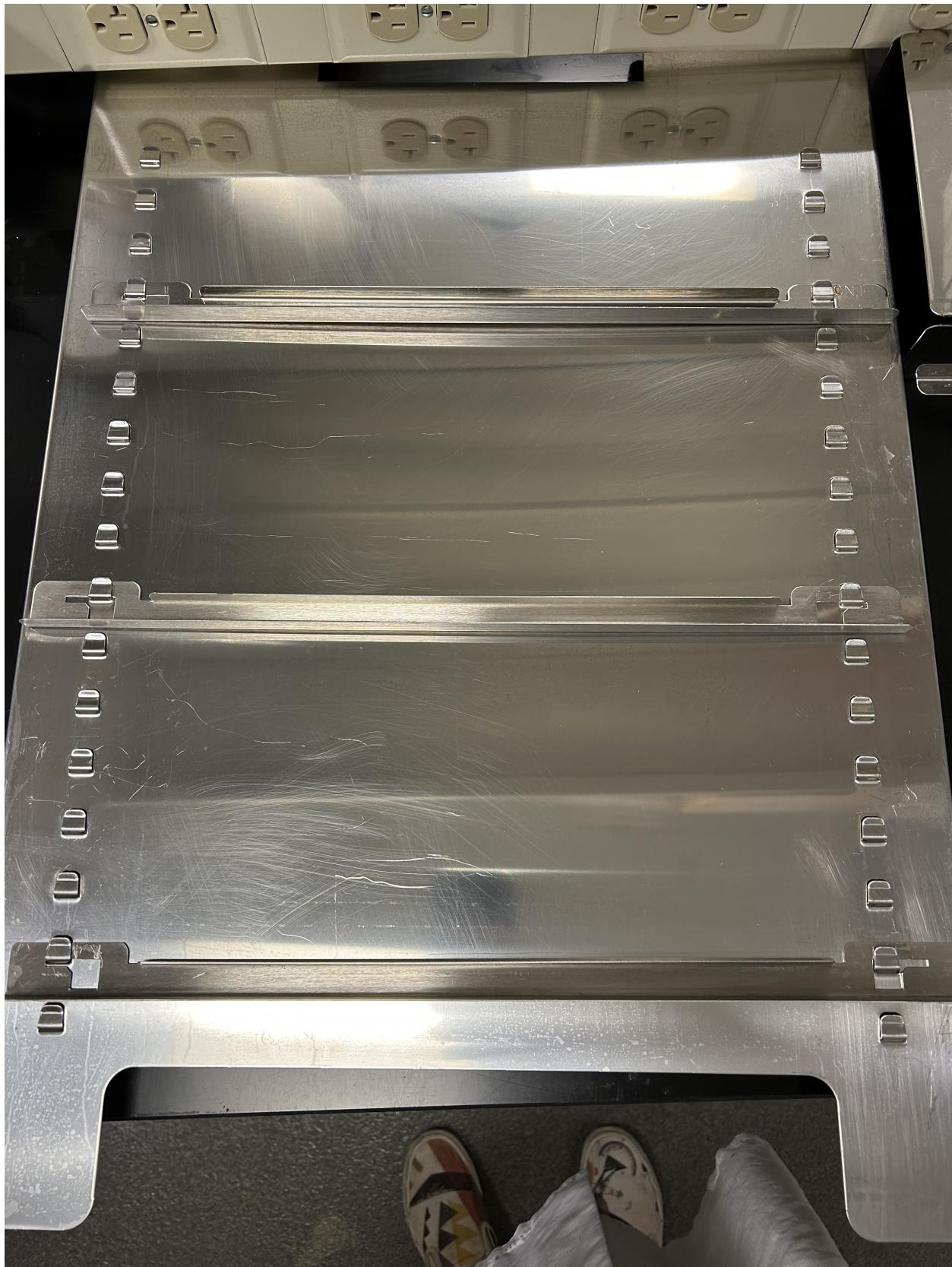


Fig. 9: Location of shelf holder bars.

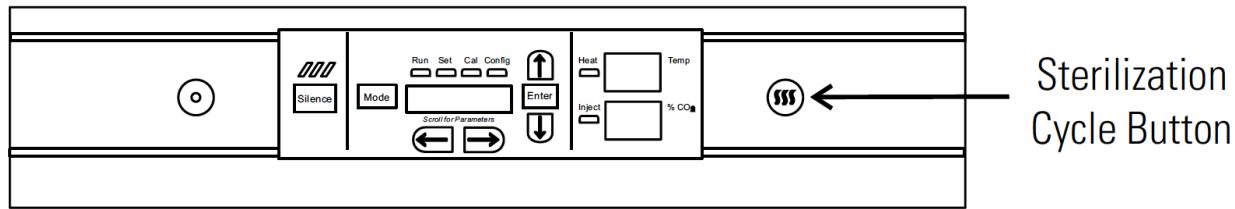


Fig. 10: Diagram of the incubator display with the sterilization cycle button indicated.

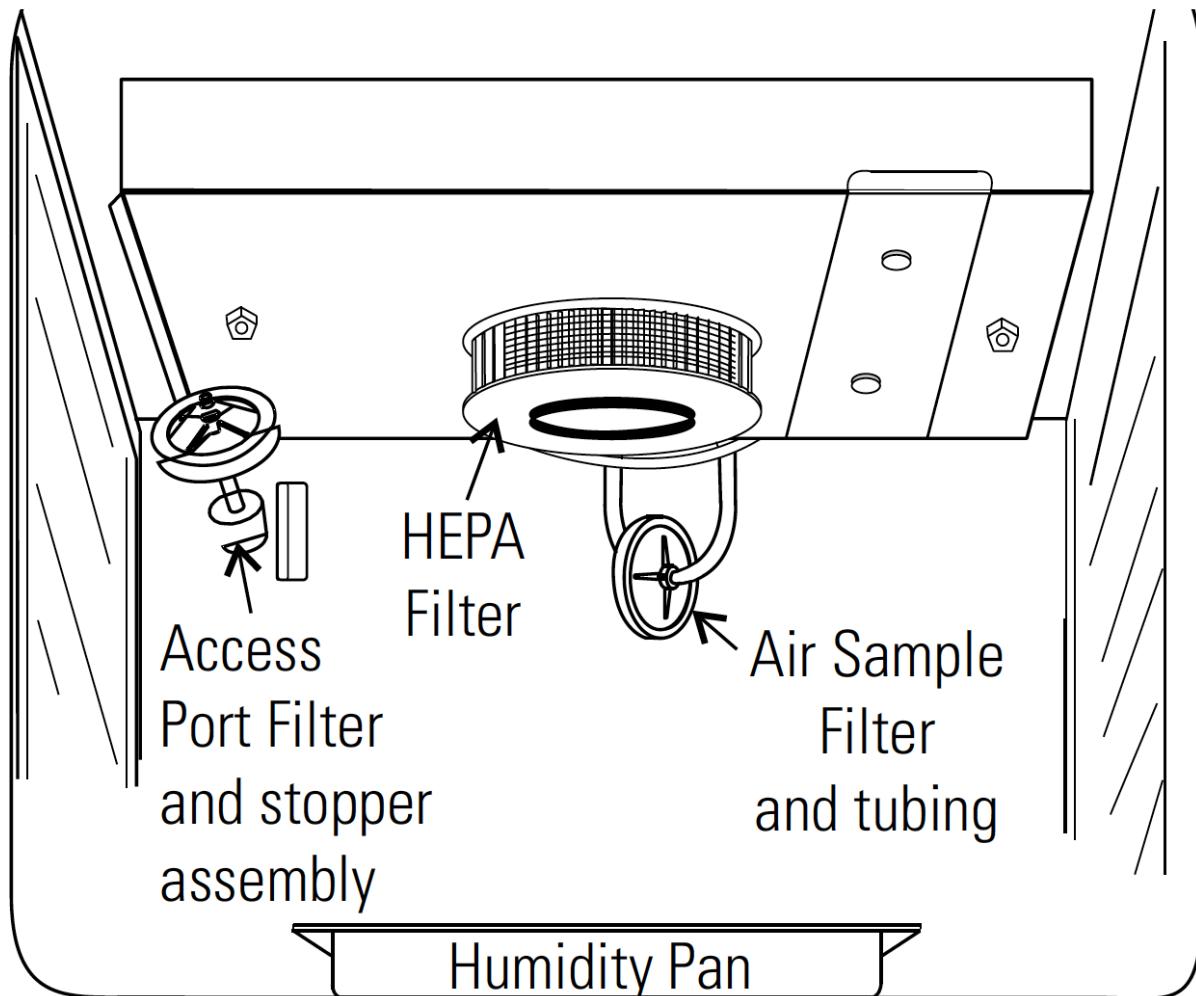


Fig. 11: Diagram of the incubator filters. The inlet filters are labeled “Access port filter” and “Air sample filter”.

1.7 Replacing the logged-in OneDrive user

IST does not provide “service users”, so one of us 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 [WinDirStat⁶](#) 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 OneDrive⁷](#) and the [timelapse OneDrive⁸](#). 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.

⁶ <https://windirstat.net>

⁷ <https://mitprod.sharepoint.com/sites/GallowayLab/Shared%20Documents/Forms/AllItems.aspx>

⁸ <https://mitprod.sharepoint.com/sites/GallowayLab-Timelapse/Shared%20Documents/Forms/AllItems.aspx>

1.8 Ordering

1.8.1 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' and select the standard delivery option. This is 32 Vassar St, the main receiving area for MIT deliveries.

Choose an Address									
Name	Line 1	Line 2	City	State	Postal Code	Country/Region	Location Code	Attention	Actions
<small>Showing results for 66 X</small>									
None	MIT	290 Albany Street	Cambridge	MA	02139	United States	None	None	<input checked="" type="checkbox"/> Choose
Bldg 3 - Overnight	MIT	33 Massachusetts Ave Rear	Cambridge	MA	02139	United States	None	None	<input checked="" type="checkbox"/> Choose
Bldg 66 - Overnight	MIT	25 Ames St	Cambridge	MA	02142	United States	None	None	<input checked="" type="checkbox"/> Choose
Bldg 66 - Standard	MIT	32 Vassar St	Cambridge	MA	02139	United States	None	None	<input checked="" type="checkbox"/> Choose
Bldg NW12 - Standard	MIT	138 Albany St	Cambridge	MA	02139	United States	None	None	<input checked="" type="checkbox"/> Choose
Bldg W32 - Overnight	MIT	120 Vassar St	Cambridge	MA	02139	United States	None	None	<input checked="" type="checkbox"/> Choose

- In the 'Attention' box, add your name with the lab address in parentheses, i.e., 'Your Name (66-219)'.

Review Cart

General Info Cart Items Approvers Comments History

[Add Tag](#)

General Info

Created By: Kasey Love

On Behalf Of:

Justification:

Attachments: Add File | URL | Text
 Attach any documentation (e.g., Selection of Source Form). If you add attachments, please check the box below.

Attachments included?

Email Address of Requester: kslove@mit.edu

Does a contract need drafting or review?
 Check box for 'yes', leave blank for 'no'

Comments to Supplier:

Notes to Buyer:
 Text here will route this req to a Buyer.

Buyer Contact: Select (VPF use only)

Ship To

* Address: MIT
 32 Vassar St
 Cambridge, MA 02139
 United States

Attention: Kasey Love (66-219)

- **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.

General Info Cart Items Approvers Comments History

Cart Items

Add Line Clear Cart View All Advanced Search Sort by Line Number: 0 → 9

Edit Selected Copy Delete

	Billing			
1	Apex Taq RED Master Mix, 2X GENESEE SCIENTIFIC CORPORATION	707.80 USD 2 x 353.90 / Each	MIT COA Laboratory Supplies 420214	
Commodity: Lab Supplies - General Supplier Part Number: 42-138B Shipping: Best Way Surface (Destination Prepay & Add)				
Add Tag				

Per page: 15 | 45

Total 707.80 USD

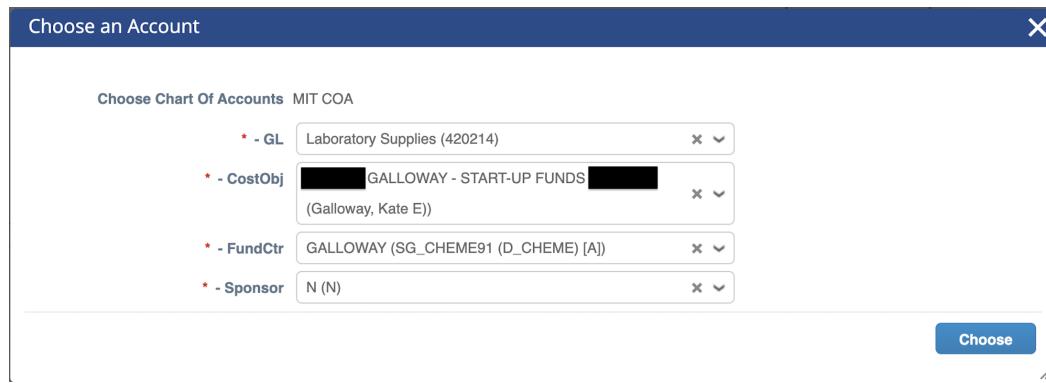
- The PO can be found by searching 'Galloway' and selecting an option from the list (e.g., 'Start-Up Funds').

Choose an Account

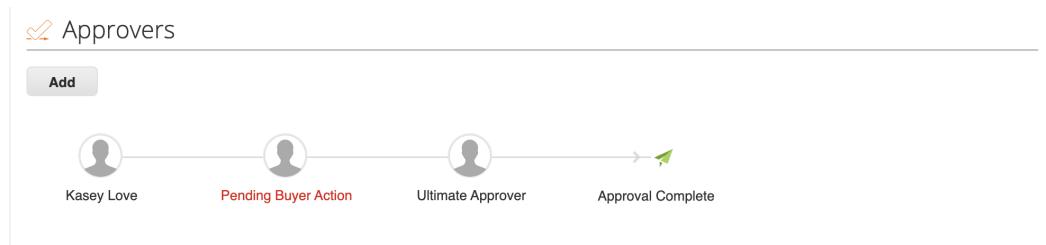
Choose Chart Of Accounts MIT COA

* - GL	Laboratory Supplies (420214)	x v
* - CostObj	GALLOWAY - START-UP FUNDS [REDACTED] (Galloway, Kate E)	x v
* - FundCtr	GALLOWAY (SG_CHEME91 (D_CHEME) [A])	x v
* - Sponsor	N (N)	x v

Choose



- 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.

1.8.2 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.

The screenshot shows the Quartzy web interface. At the top, there's a navigation bar with tabs for 'Requests', 'Inventory', and 'Shop'. Below the navigation is a search bar labeled 'Search Quartzy'. The main area displays a table of inventory items. One item, 'PB Picolyl Azide', is selected, showing its details: Vendor is 'Click Chemistry Tools', Amount is '1 unit', Location is 'TC -20C (...)', Type is 'General ...', Owner is 'Adam Beitz', and it was added on 'May 24, 2021' and last updated on 'Jun 17, 2022'. To the right of the item details is a blue 'Request' button, which is highlighted with a red box and a red arrow pointing to it.

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. 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.

The screenshot shows the Quartzy interface with the 'Requests' tab selected. In the top left, there's a large blue 'Add Request' button, which is highlighted with a red box and a red arrow pointing to it. Below the button, the status 'New 1' is displayed. Other tabs include 'Ordered 22' and 'Received'. To the right, there's a 'Purchase History' button. The main area shows a table with columns for Item Name, Vendor, Total, Details, From, and Submitted. The first row shows an item from 'Genesee Scientific' with a total of '\$80.35'.

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.

The screenshot shows the Quartzy interface with the 'Requests' tab selected. The 'New' section shows 2 items. One item, '2-inch Cardboard Box w/ Lid, White, 81-Place Divider', is listed with a vendor of 'Genesee Scientific' and a total of '\$80.35'. To the right of the item details is a blue 'Mark Ordered' button, which is highlighted with a red box and a red arrow pointing to it. Below the button, there are 'Cancel' and 'Mark Received' options.

Marking Received Items

Anytime a package arrives, be sure to mark it as received in Quartzy. To do so, navigate to the 'Ordered' section under the 'Requests' tab and click the 'Mark Received' button. Additionally, place the packing slip in the box in the entryway near 66-219.

The screenshot shows the Quartzy software interface for the Galloway Lab. At the top, there are navigation tabs: Requests (which is highlighted in red), Inventory, Shop, and a search bar. On the right side of the header, there are buttons for Invite, a notification icon (0), and user profile settings. Below the header, a summary bar indicates 'New: 2' and 'Ordered: 22'. A large red box highlights the 'Ordered: 22' count. Below this, there are buttons for Received and All Requests. To the right, there is a 'Purchase History' button. The main content area displays a table of ordered items. The columns are labeled: ITEM NAME, VENDOR, TOTAL, DETAILS, FROM, and UPDATED. A single item is listed: Liquid Bleach, vendor Genesee Scientific, total \$54.00, details 88-133, from Nathan Wang, and updated on Jun 21, 2022. A red box highlights the 'Mark Received' button at the bottom right of the item row. A red arrow points from the text in the previous paragraph to this button.

ITEM NAME	VENDOR	TOTAL	DETAILS	FROM	UPDATED
Liquid Bleach	Genesee Scientific 88-133	\$54.00 6 x 1 gal	Grant ID: PO:	Start-up Nathan Wang	Jun 21, 2022

1.8.3 Bulk Plastics Order

The following items are ordered in bulk biannually from Genesee Scientific:

- 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)
- Gloves

Information for the bulk order can be found in lab_jobs folder in the Sharepoint. To coordinate a bulk order, collect the tally sheets from lab, and calculate the number of units needed based on usage. Input this information into a spreadsheet with the prices from the previous bulk order. Directly email this spreadsheet to the Genesee Scientific representative and request a quote. Once the order is placed, print new tally sheets, and track the plastics as they are delivered.

1.9 Recycling

MIT has several recycling programs for various lab plastics, packaging, and so on. The up to date Facilities information site⁹ should be checked for details.

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

- **Cardboard:** fold up the box and place it near blue recycling bins, especially the one in the atrium.
- **Hard plastics #1, #2, #5:** place in the blue recycling bin.
- **Pipette tip boxes and conical racks:** place in the specific pipette tip/rack collection box. We schedule pickups through this Quickbase link¹⁰. This box goes to a local startup who recycles these.
- **Styrofoam:** Leave clean coolers/boxes/lids made of Styrofoam **next to** blue recycling bins. Bag Styrofoam into clear plastic bags for pickup.
- **Film plastic:** Collect film plastics, such as pallet wrap, the plastic around filter tips *into the labeled cardboard box under the 66-225 shelving, 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).

We do not recycle the following:

- Anything bio- or chemically-contaminated

⁹ <https://web.mit.edu/recycling>

¹⁰ <https://mit.quickbase.com/db/bq2rx8ncp?a=nwr>

- Gloves, even if they are clean.

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)
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 (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 (10 min)
 - c. If last user of the day, 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 38) 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.

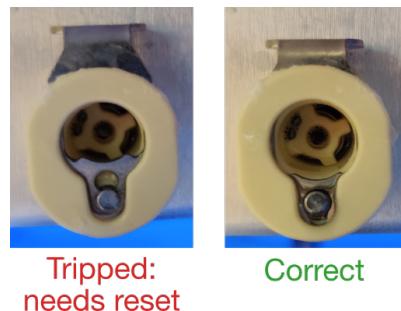


Fig. 3: Visual appearance of tripped and normal connectors.

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 [ThermoFisher¹¹](#).

¹¹ <https://www.thermofisher.com/order/catalog/product/4449754>

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 Scientific¹² version is diluted at a 1:2 ratio.

¹² <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.
- **Decontaminate 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 solution¹³. Hellmanex is an alkaline, viscous 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).
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.

¹³ <https://www.fishersci.com/shop/products/fisherbrand-hellmanex-iii-liquid-cleaning-concentrate/14385864>

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 42).

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 User-replaceable parts

Main syringe

See page 16 in the [Attune maintenance guide](#) for full details.

As detailed in [Attune startup and shutdown](#) (page 33), 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.

2.1.4 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 until 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.

2.2 Koch Flow Core

Note: TO DO

2.3 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 **68N: Mouse** trainings are complete, complete the [CAC New Personnel Addendum \(NPA\) Form¹⁴](#) and email it to cacpo@mit.edu. This will complete the CAC Certification task in the Atlas Training Center. ([Example NPA¹⁵](#))
6. Once your NPA form is approved, email dcmaccess@mit.edu to gain access to the DCM animal facility. 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.

2.4 Keck Imaging Facility

Note: TO DO

2.5 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 10am on Tuesday.

2.5.1 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 Jaime Cheah at jcheah@mit.edu. They will need to sponsor anyone outside of the KI for those who need access.

¹⁴ https://cac.mit.edu/sites/default/files/documents/NPA_current.pdf

¹⁵ https://mitprod-my.sharepoint.com/:b/g/personal/blende_mit_edu/EdA4R2z2F7tJhnkARYHe62sB7iYiGHdJKLVUz-9SHrWvew?e=70VGdT

2.5.2 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 \$25 per sample or \$50 for media-only sample.
4. Under payment information, select the desired payment number.
5. Submit the request to the core and drop off samples before 10am on Tuesday.

2.5.3 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.

2.5.4 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 MycoAlert™ 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.

2.6 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]”

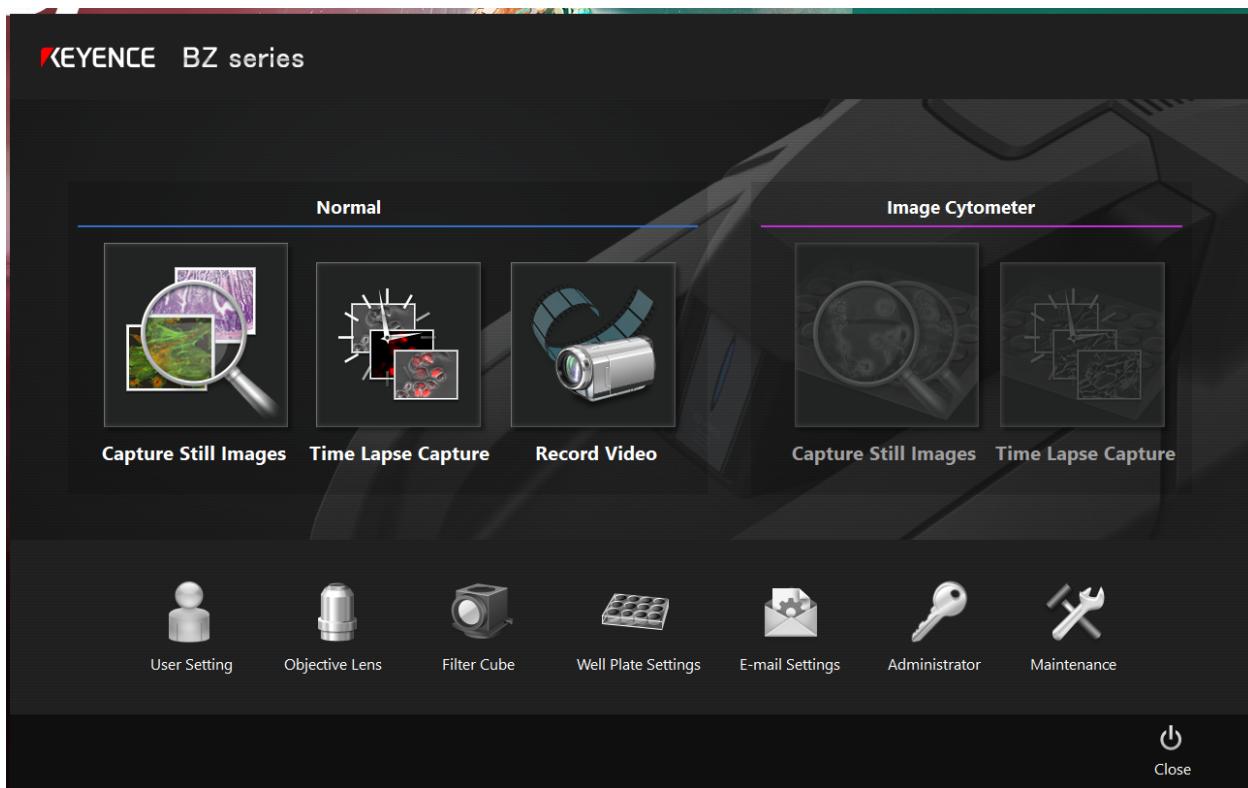
3. Once approved, you will receive an email with instructions to set up the account.

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

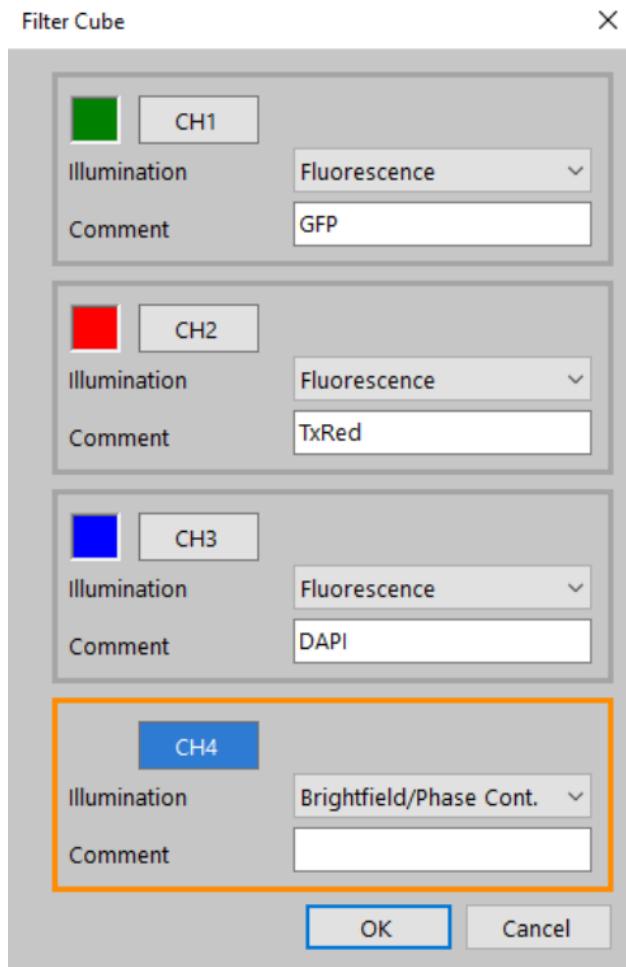
2.7 Keyence beginner’s user guide

2.7.1 Basic Operation

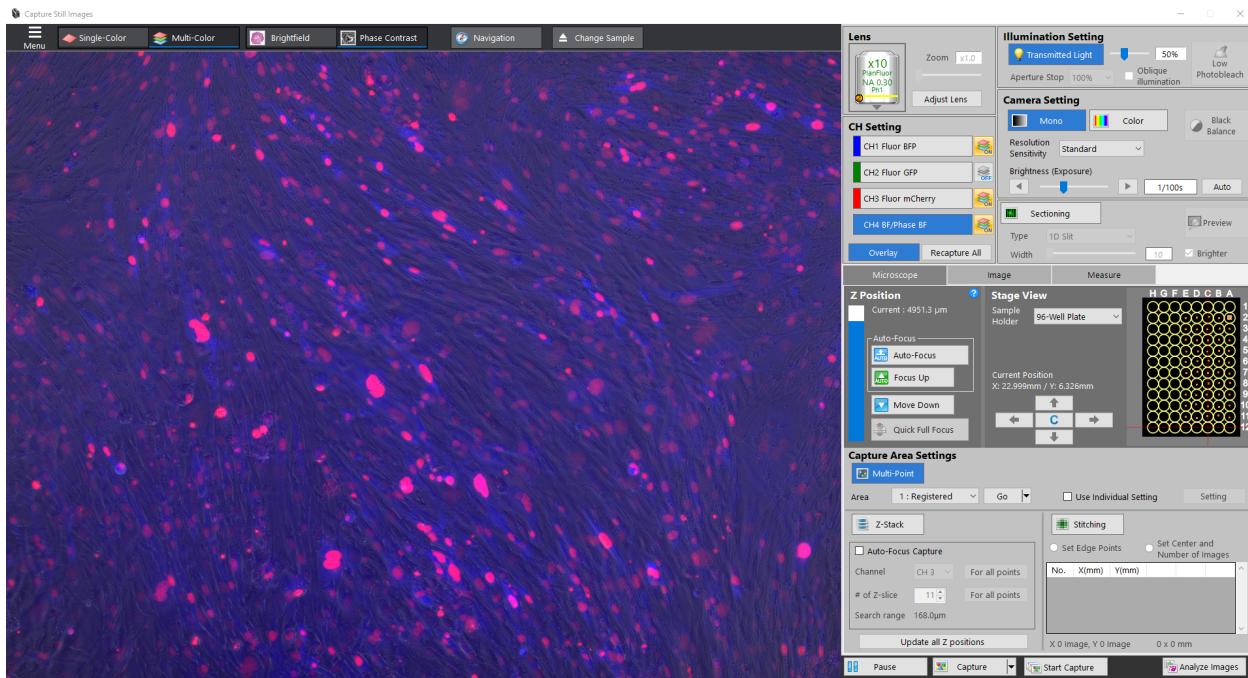
1. Turn on the Keyence using the power button.
2. Open the BZ-X800 program from the desktop shortcut. You will see the following menu.
Note: you must turn on the Keyence before you are able to navigate the software.



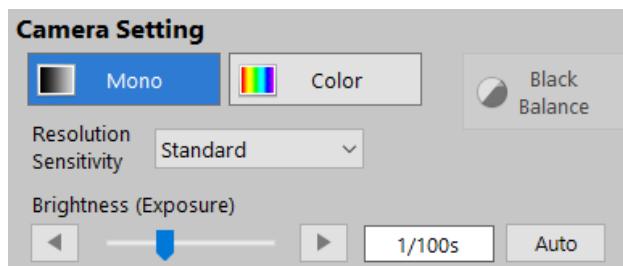
3. The **Filter Cube** button can be used to change which 4 filters are used when capturing images.



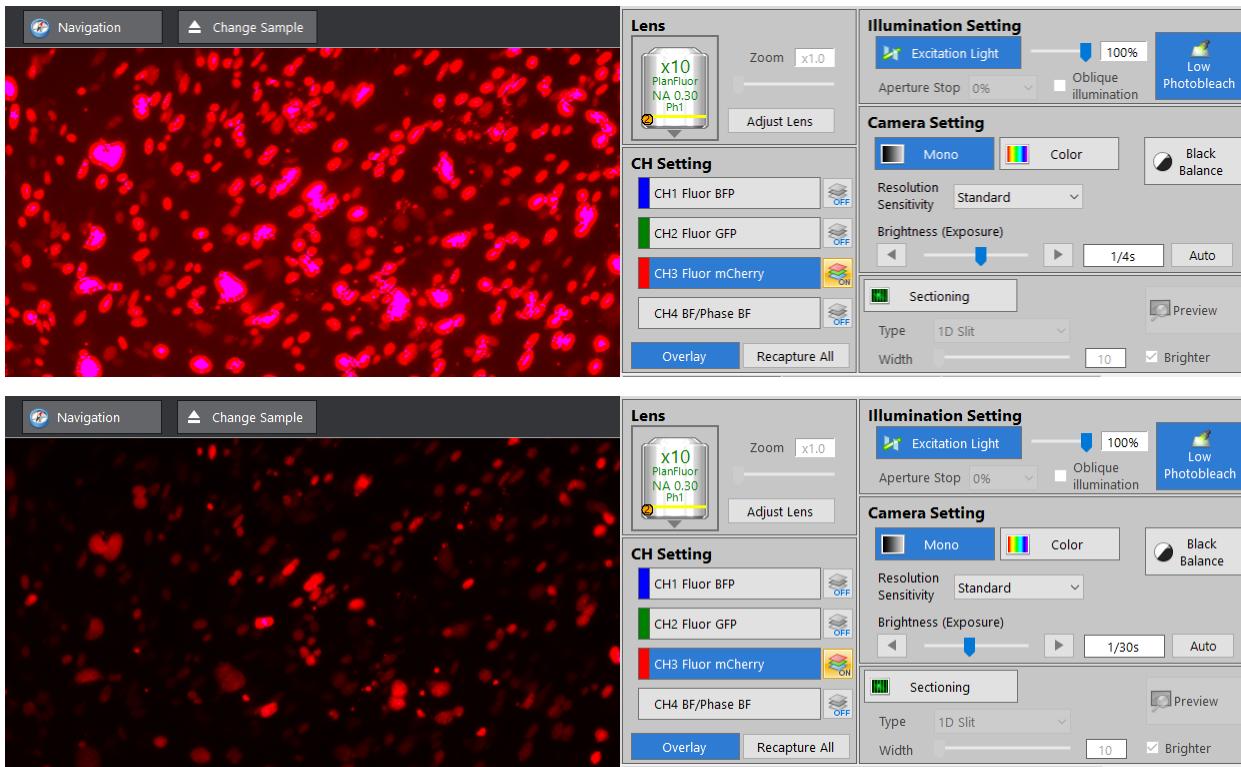
4. If you are using a plate, the **Well Plate Settings** button can be used to select the number of wells on your plate. This can also be changed within the capture window.
5. The most frequently used capture setting is **Capture Still Images**, and selecting this option leads to the window below:



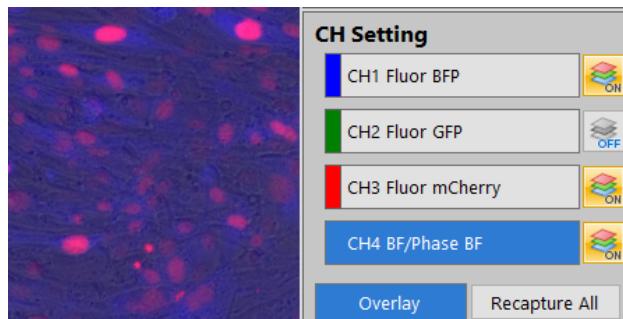
6. **Focusing Images:** To manually focus the microscope image, use the mouse scroll for fine adjustments. For moderate adjustment use **ctrl + mouse wheel**. For large adjustments use **ctrl + shift + mouse wheel**. There is also an option to auto-focus under the Z Position menu, but this method can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



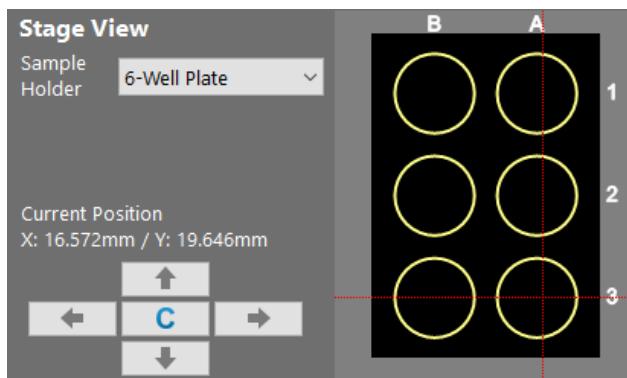
8. **Overexposure:** When the exposure time is too high, the images can be overexposed. When this happens, the image has purple spots in the areas that are overexposed. It is best to decrease exposure time until no more purple is seen.



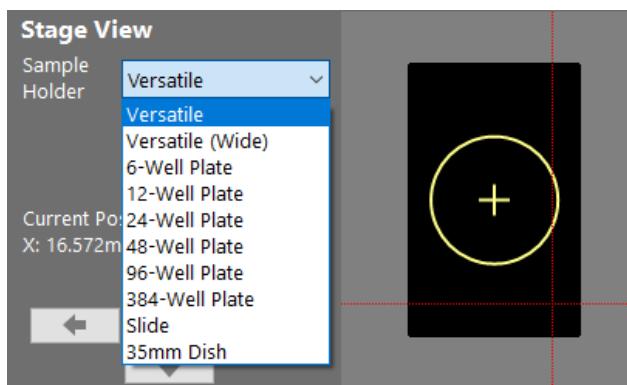
9. Channel Settings: Select which color channel to view by clicking on the buttons labeled CH1, CH2, CH3, or CH4. Only the selected channel will be captured when changing X/Y/Z position or exposure times. Multiple channels can be viewed at once by clicking the ON/OFF button next to each channel. However,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



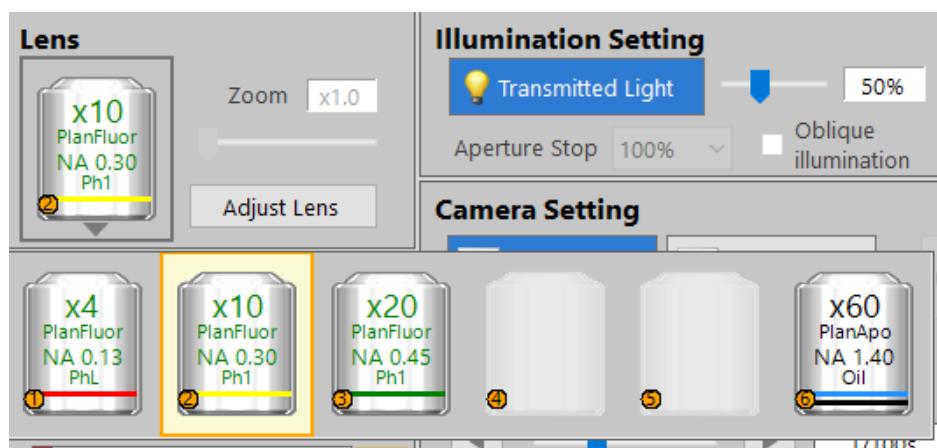
10. Navigation: There are several options to navigate a plate. (1) Click and drag on the microscope image. (2) Use the arrows in the Stage View menu. (3) Click to the desired location in the plate layout in the Stage View menu. The red perpendicular lines indicate the current position.



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



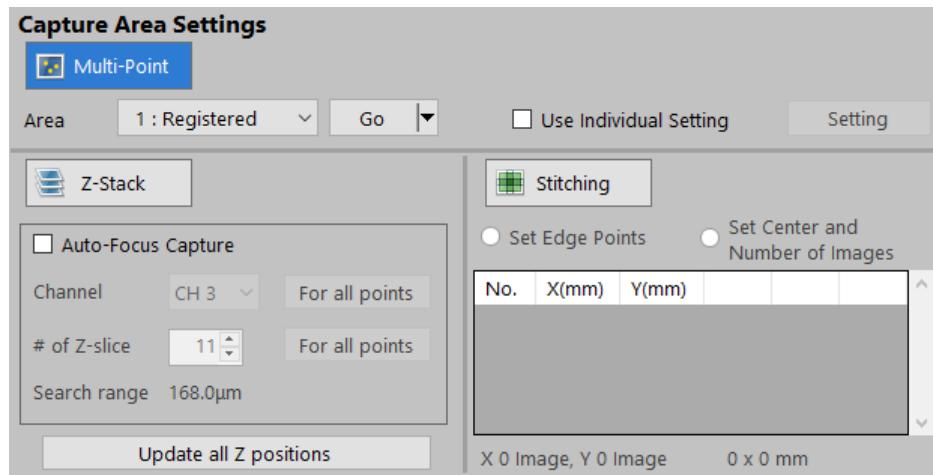
12. **Magnification:** To change the magnification, click on the objective lens button in the Lens menu on the left, and the objective lens options will appear. Select the desired magnification.



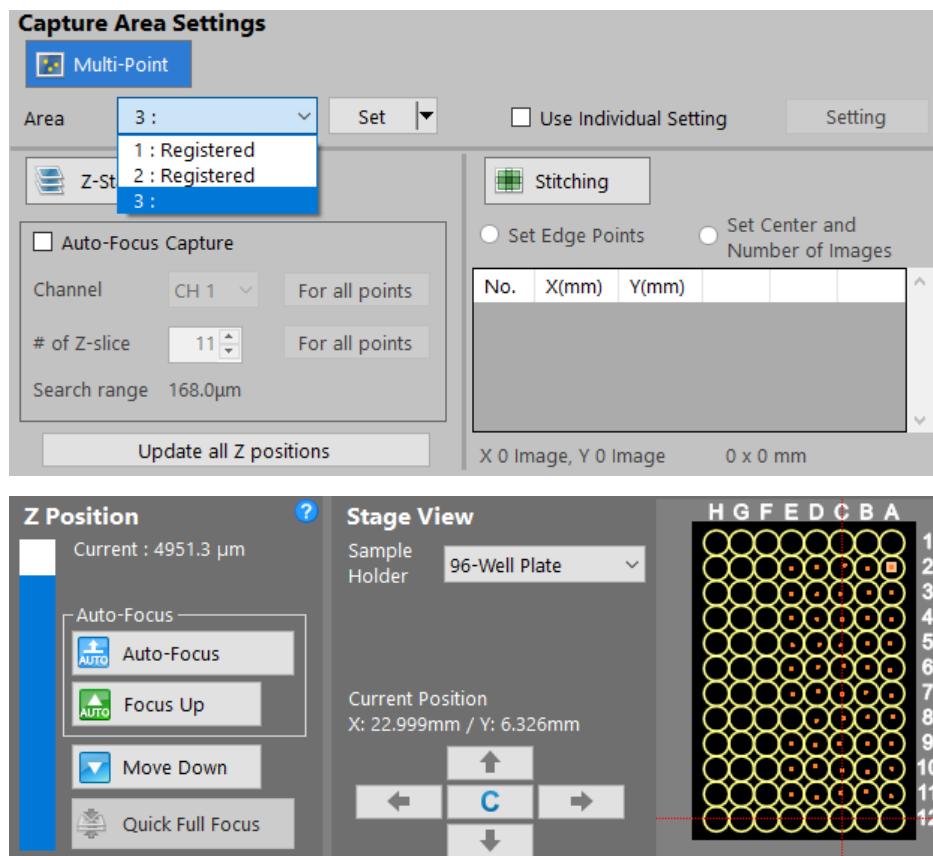
Note: The 60X lens requires adding oil to the lens.

2.7.2 Multi-point capture

- 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.



- 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.

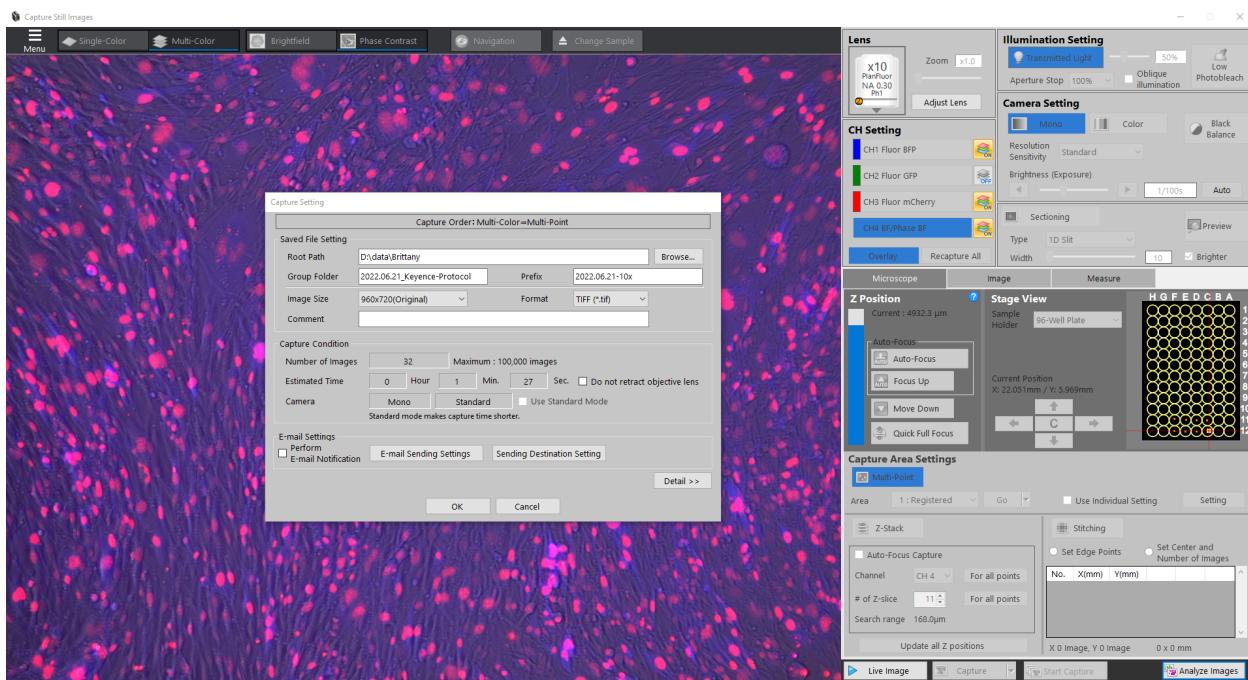


- Once all the capture points are set, turn ON all the color channels to capture and ensure the

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.

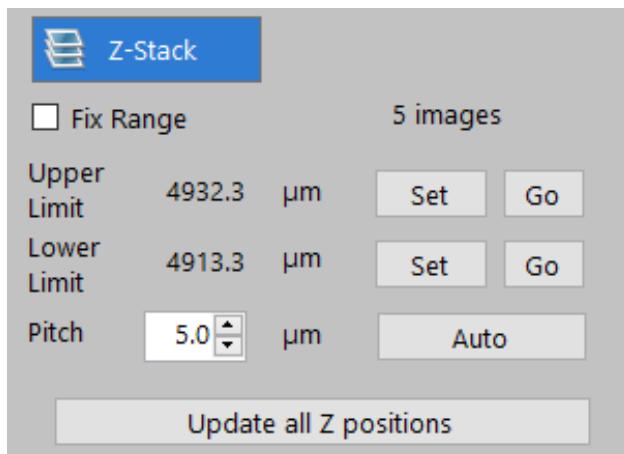
Note: Usually, you *do not* want the **Use Individual Setting** box to be selected when using Multi-Point Capture. This ensures that whichever channels are turned ON and the exposure times for those channels are the same for all the images captured. The same exposure settings between images is required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

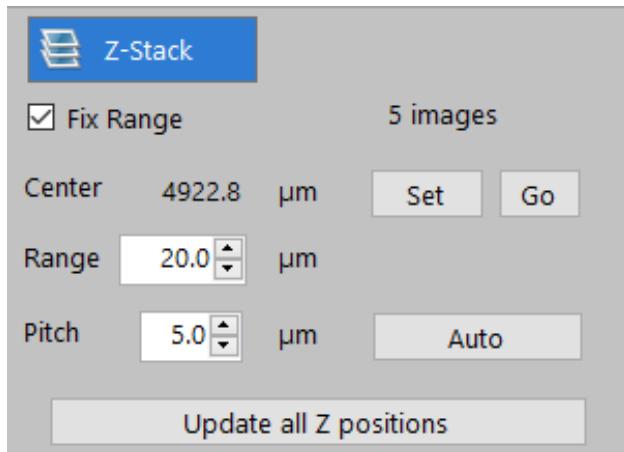


2.7.3 Z-stack

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.

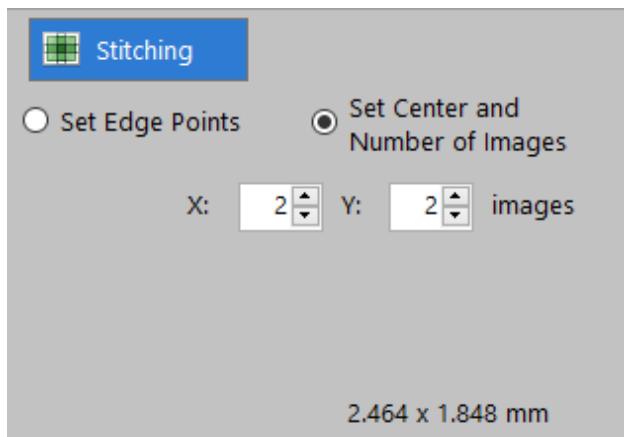


3. Alternatively, select the box next to **Fix Range**. Then set the Z position to be the center of the Z-stack by focusing the image and clicking **Set**. Enter a range to capture. For example, a range of 20um will capture 10um above and below the center, for a total range of 20um. Additionally set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

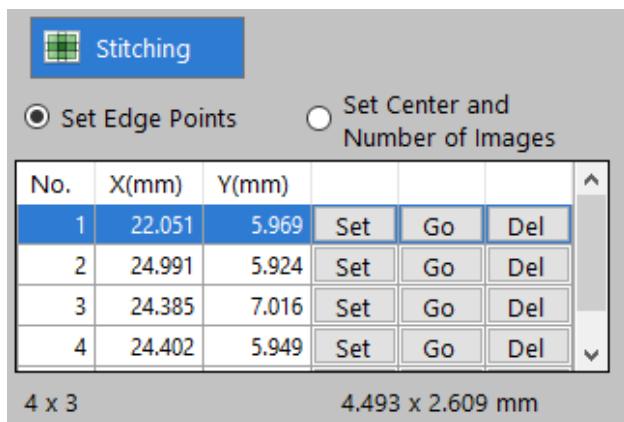


2.7.4 Stitching

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.

2.8 Keyence Time Lapse Imaging

2.8.1 Setting up the incubation chamber

The Keyence comes with a special chamber / plate holder and stage top incubator controller¹⁶ 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 valves you may need to open.
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.

¹⁶ <https://spectraservices.com/mm5/graphics/00000001/MA-STX-EN.pdf>

2.8.2 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 OneDrive that is specifically for time lapses.
5. Choose how frequently you want to capture images. The 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.

2.8.3 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.

CHAPTER THREE

PROTOCOLS

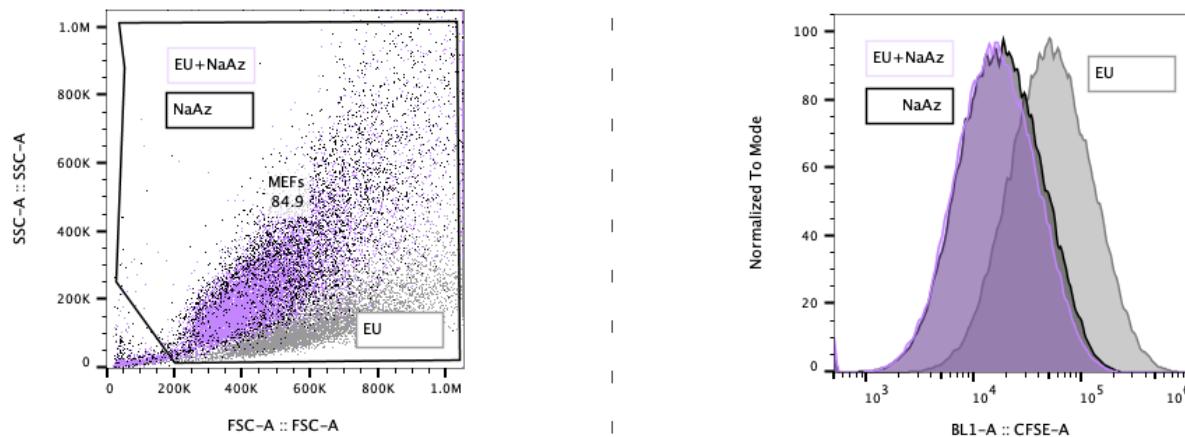
3.1 Biochemical and analytical protocols

3.1.1 Homemade EU click

1 rxn = 1 single 24-well (250 μ L EU or 175 μ L click mix).

Note that ascorbic acid should be made fresh and EU will likely be the limiting reagent.

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

Stock solutions	Stock concentration	How to make	Final concentration
EU (100X)	100 mM	5 mg EU + 186 μ L DMSO (74 rxns)	1 mM
CuSO ₄ .5H ₂ O (100X)	200 mM	250 mg CuSO ₄ .5H ₂ O + 5 mL H ₂ O (2,800 rxns)	2 mM
Ascorbic acid (10X) MAKE FRESH	200 mg/ml	120 mg Ascorbic acid + 600 μ L H ₂ O (34 rxns)	20 mg/mL

- Main azide solutions

Stock solutions	Stock concentration	How to make	Final concentration
NaN ₃ (use to make 500X soln)	4 M	250 mg NaN ₃ + 961 μ L H ₂ O	–
NaN ₃ (500X)	4 mM	1 μ L of 4 M NaN ₃ + 1,000 μ L H ₂ O (2,800 rxns)	8 μ M
Sulfo-Cy5 azide (500X)	4 mM	5 mg Sulfo-Cy5 azide + 1,638 μ L H ₂ O (4,600 rxns)	8 μ M
BDP TR azide (500X)	4 mM	5 mg BDP TR azide + 2,469 μ L DMSO (7,000 rxns)	8 μ M

- Misc/wash solutions

Stock solutions	How to make
0.5% Tween-20 in PBS	50 mL PBS + 250 μ L 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 μ g/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 μ L of 100 mM EU (100X) to 250 μ L of complete growth medium (DMEM/FBS or N3 with no neurotrophics) per 24-well. For a full 24-well plate, 60 μ L into 6 mL (90 μ L into 9 mL if need more for single color ctrls).
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 μ L 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 μ L 0.5% Tween/PBS. Let sit 15 minutes at RT in the dark (covered).
8. Wash by adding 900 μ L PBS and spin down at 4°C.

(Alt to step 8) Just add 175 μ L 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 μ L ~ 25 rxns (OR 1,000 μ L for NaN₃ ctrls)

Solution	Volume (for NaN ₃ ctrls)
PBS	4,000 μ L (or 888 μ L)
CuSO ₄ (100X, 200 mM)	45 μ L (or 10 μ L)
Azide (500X, 4 mM)	9 μ L (or 2 μ L)
Ascorbate (10X, 200 mg/mL)	450 μ L (or 100 μ L)

Label and wash

10. Add 175 μ L 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 μ L PBS and spin down at 4°C.
12. (Optional) Incubate cells 5 minutes with 175 μ L of 0.1 μ g/mL DAPI/PBS if you want to detect nuclei in flow
13. Wash by adding 900 μ L PBS and spin down at 4°C.
14. Analyze by flow.

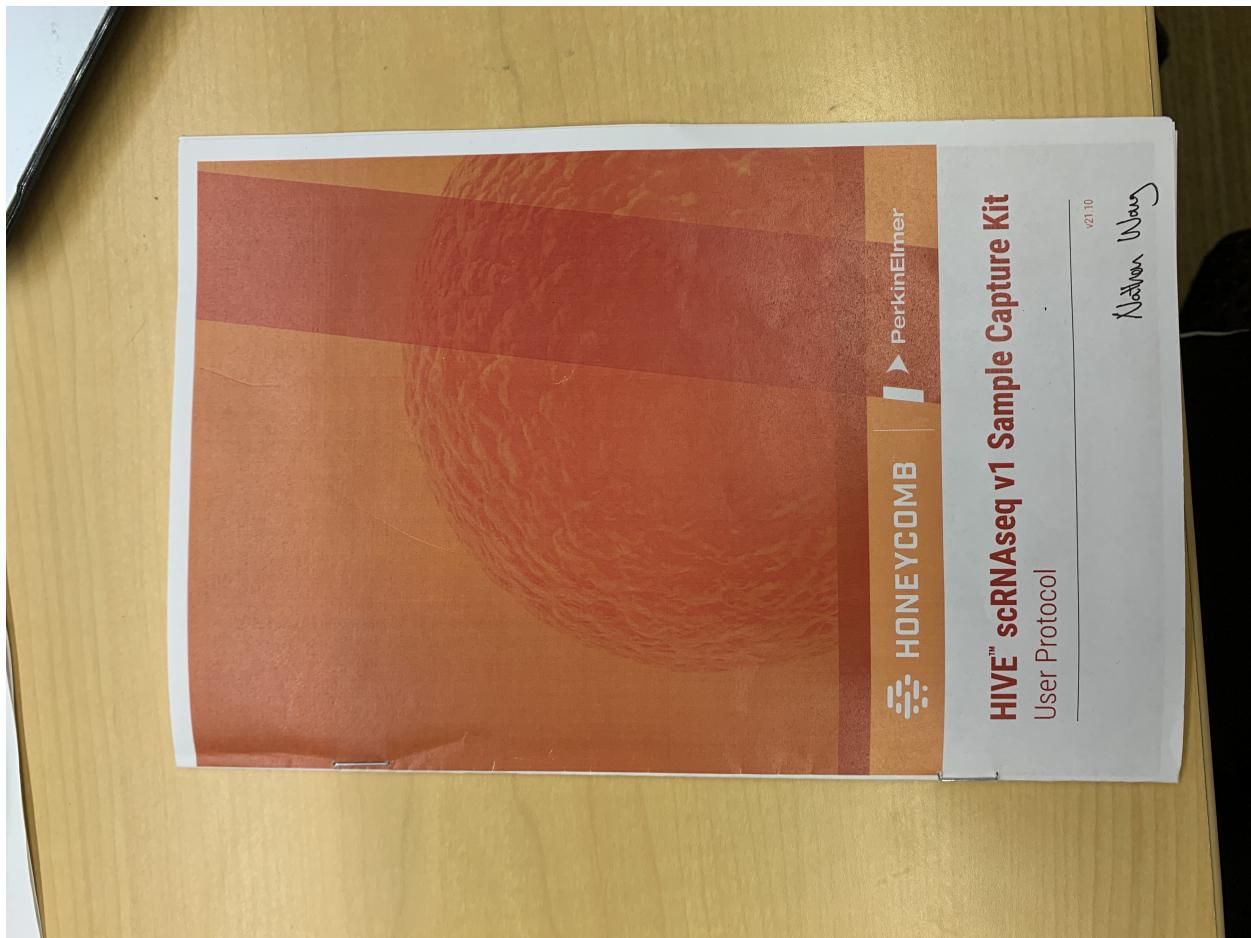
Note: All spins are performed at ~500 rcf for 5 min. Our centrifuge follows RCF = 1e-4*[rpm]² + 4e-2*[rpm] - 6e1, where **2200 rpm = 512 rcf**. It is recommended to perform all spins at 4°C once the cells have been fixed to prevent pellet loss.

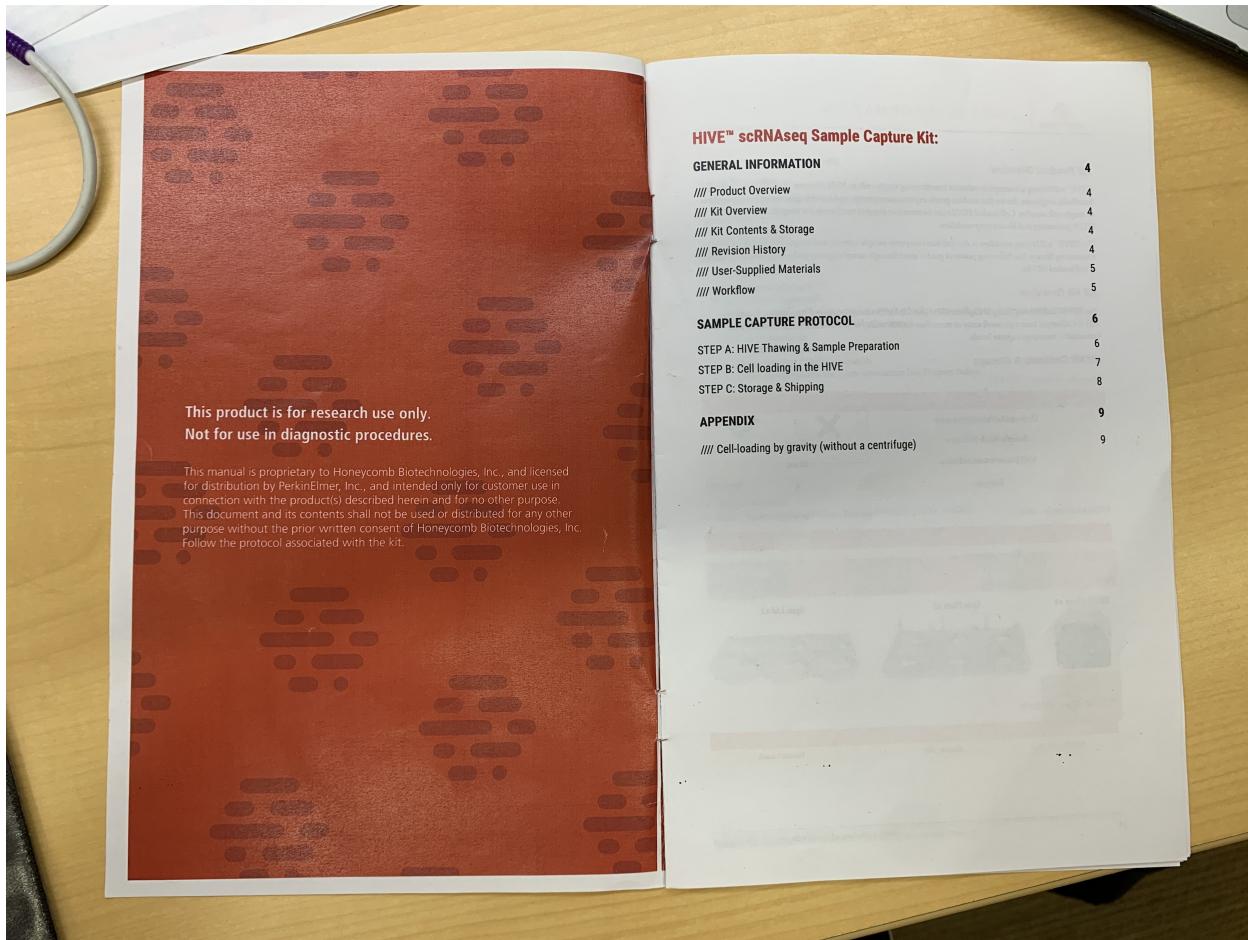
3.1.2 HIVE scRNA-seq

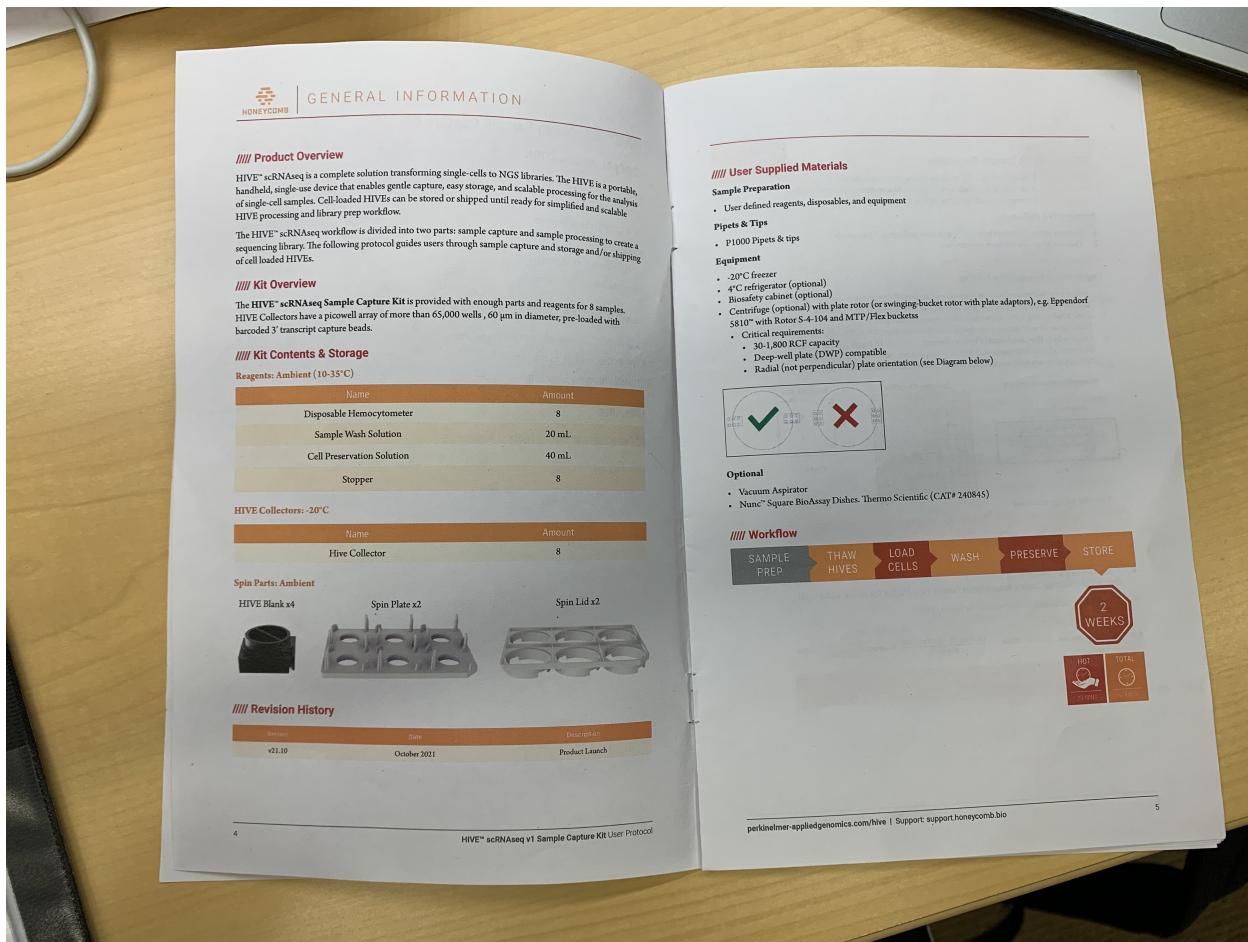
See

This protocol is from the HIVE protocols page: [\[HIVE_protocols\]](#). There are only some added annotated notes.

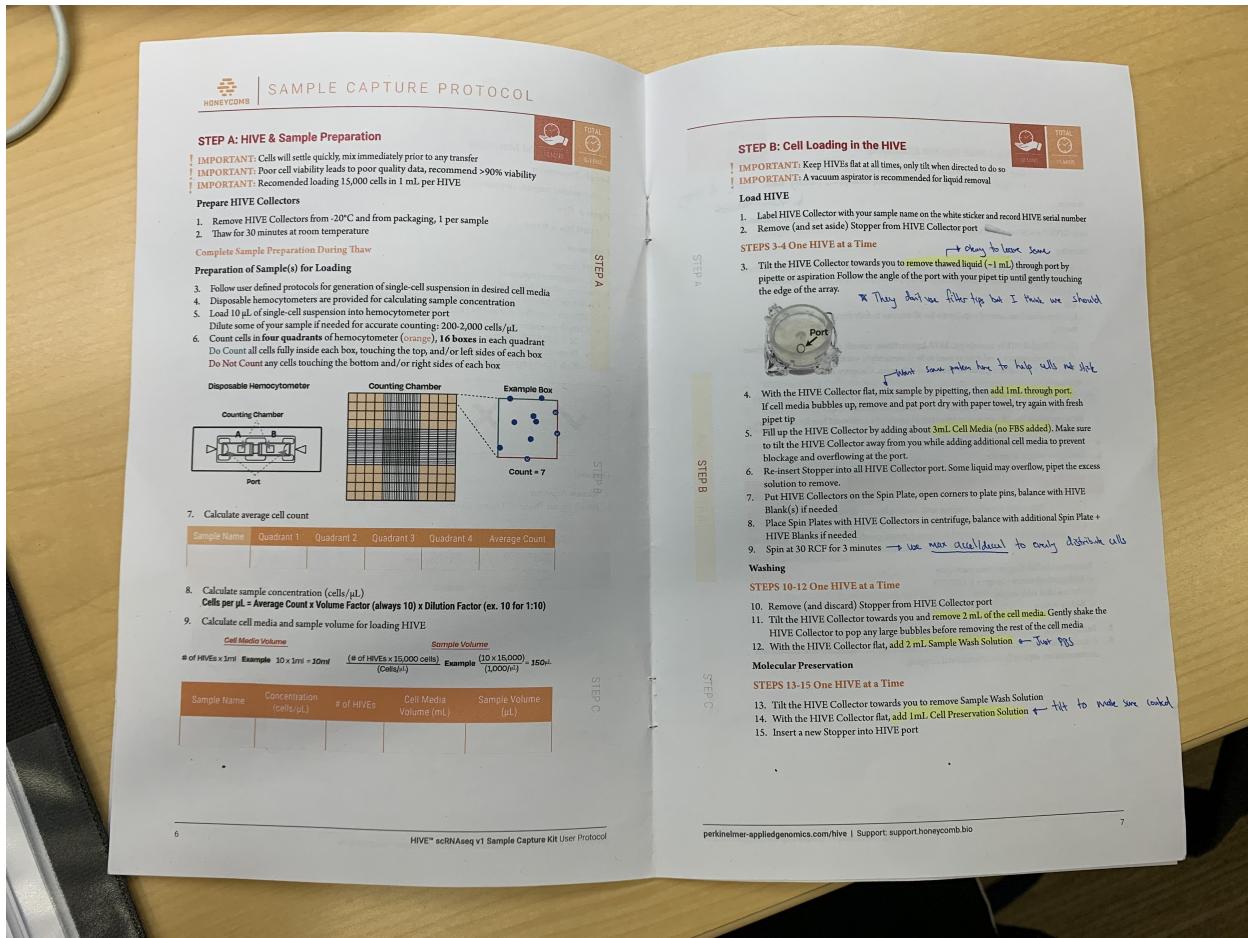
Sample capture

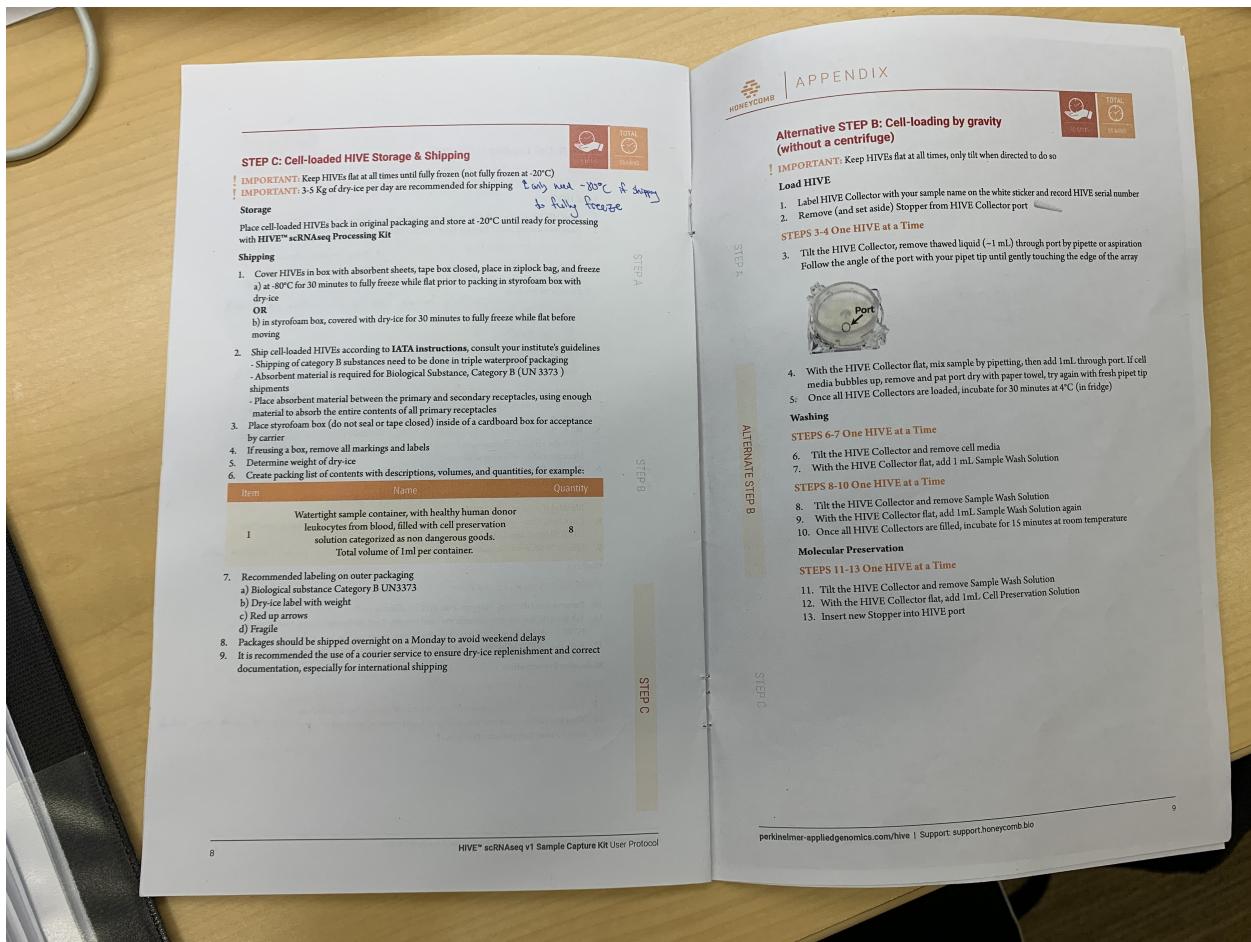




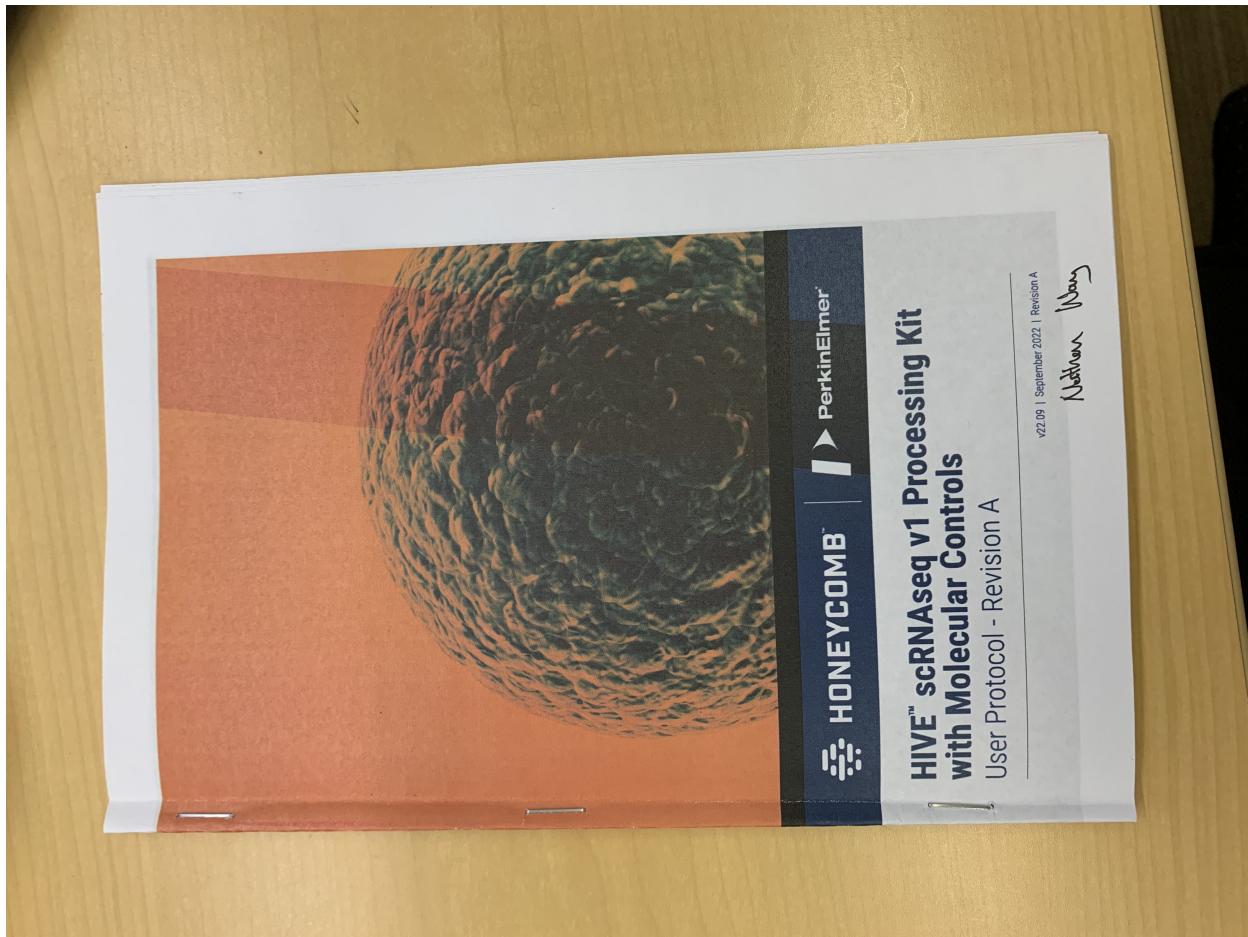


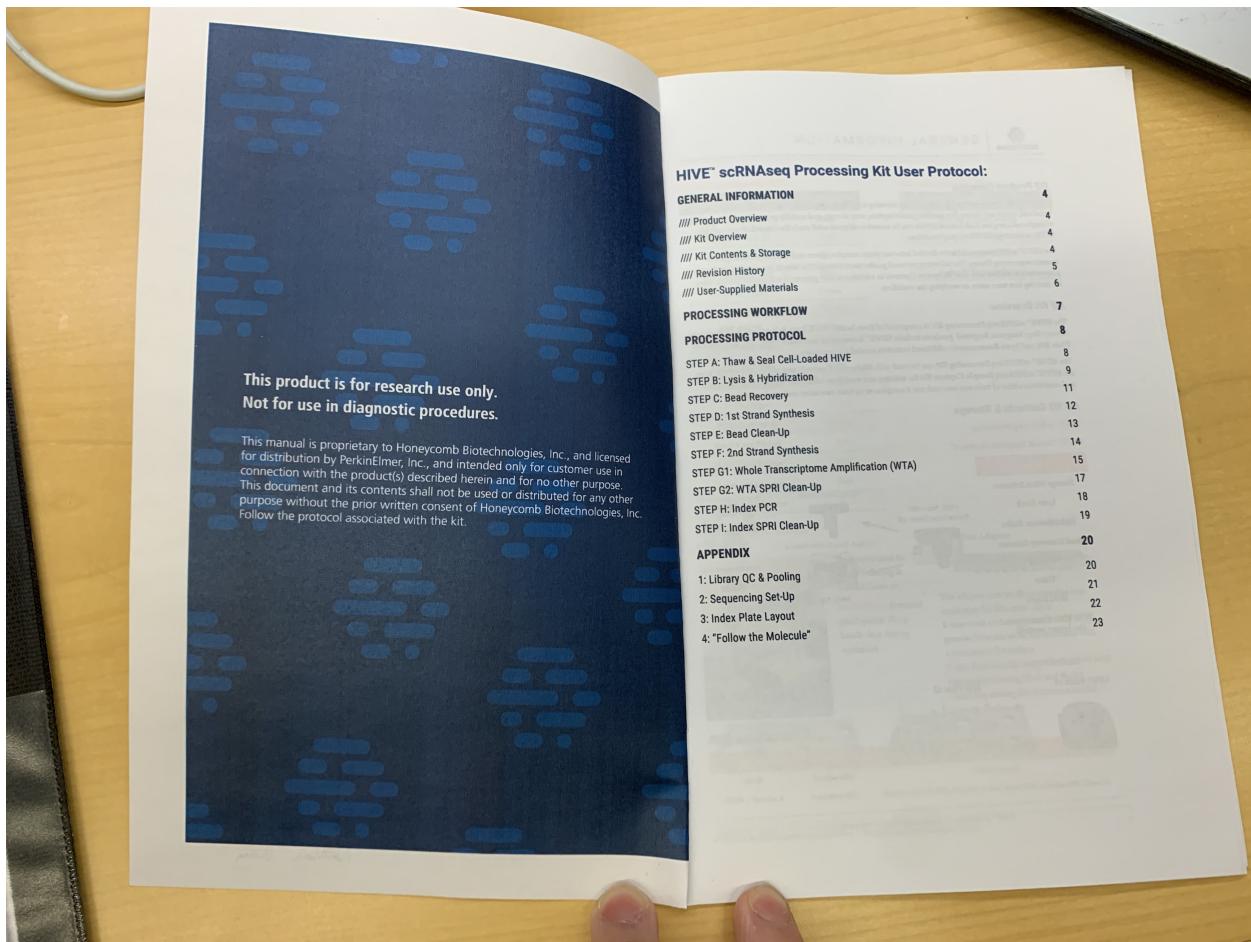
Galloway Lab Protocols

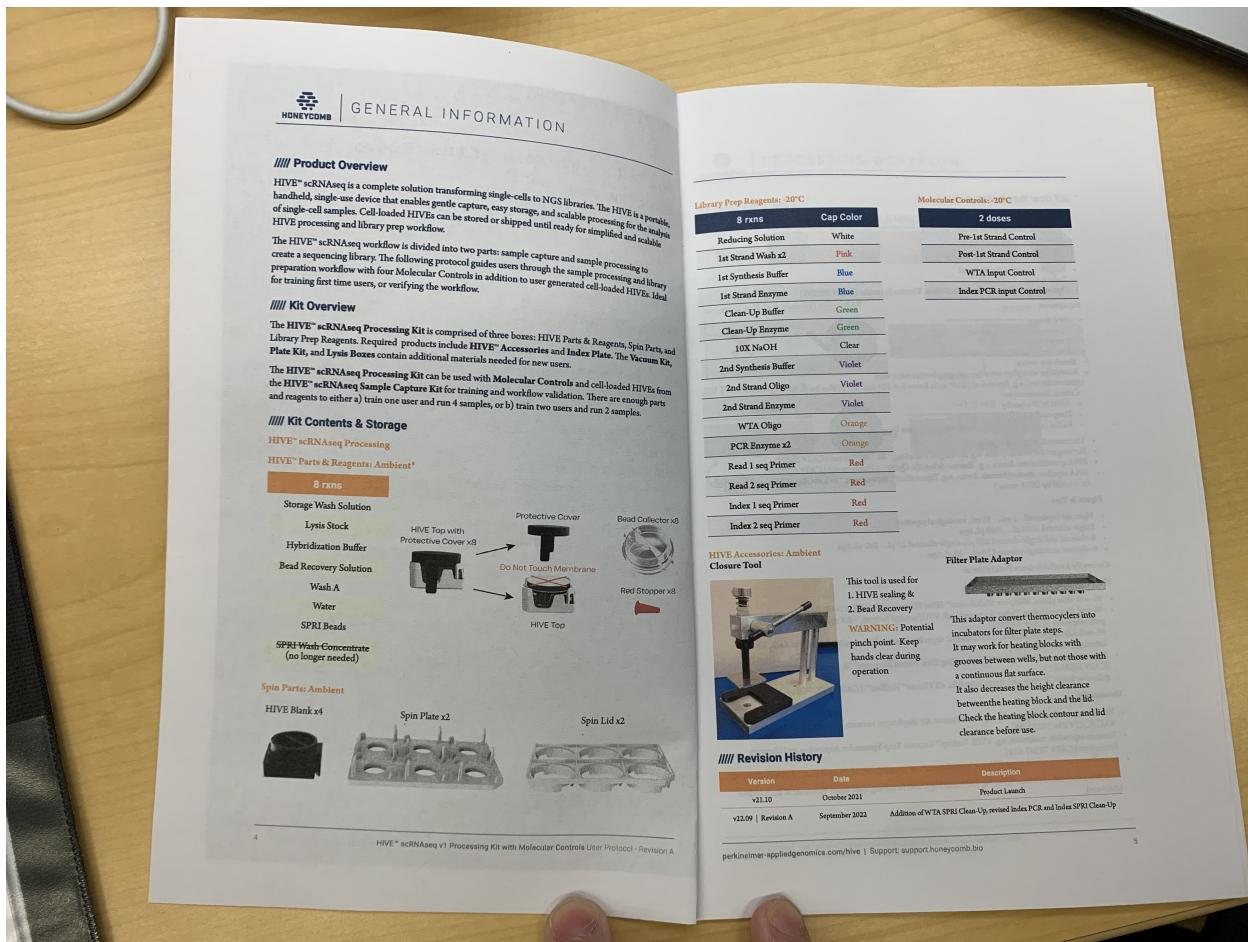


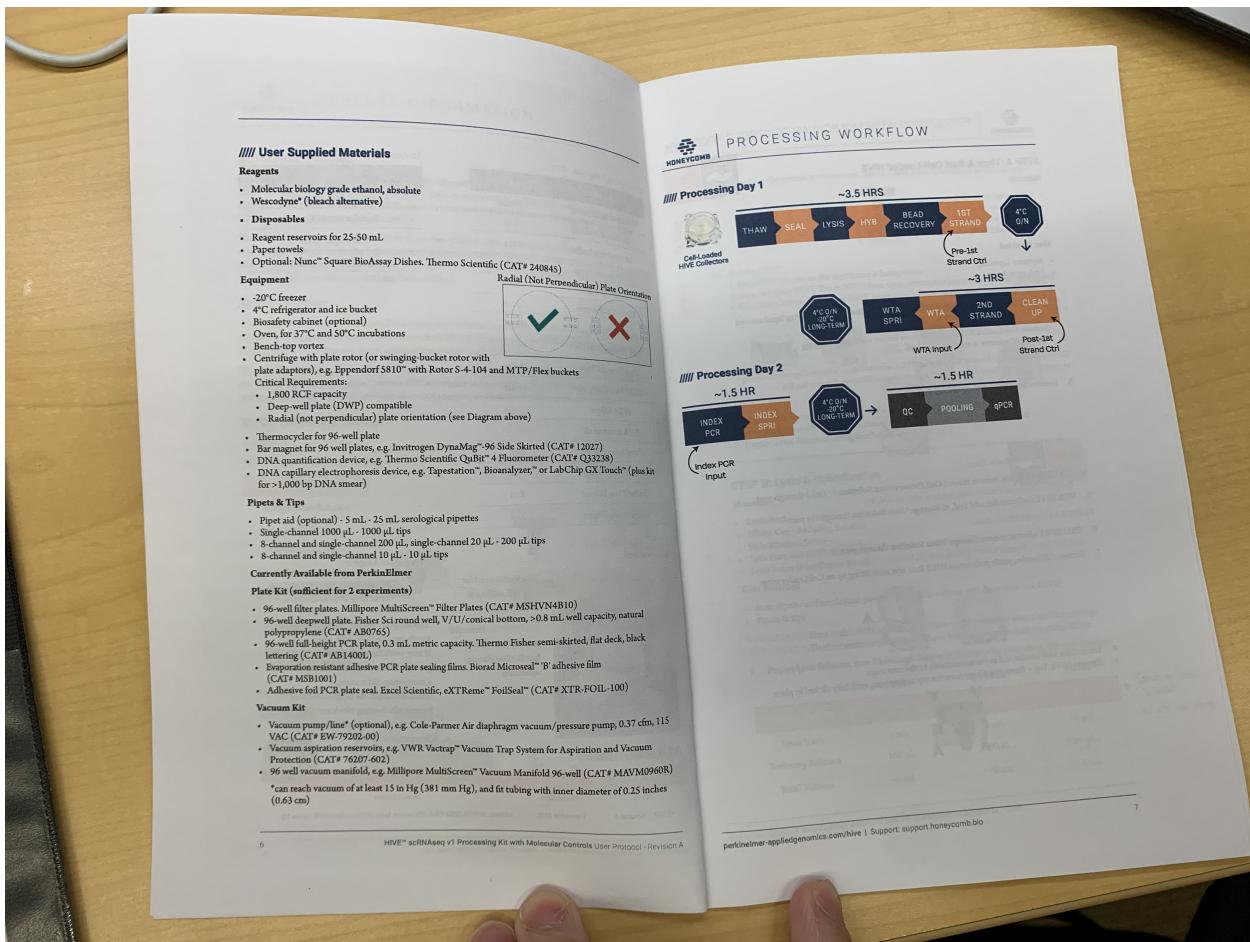


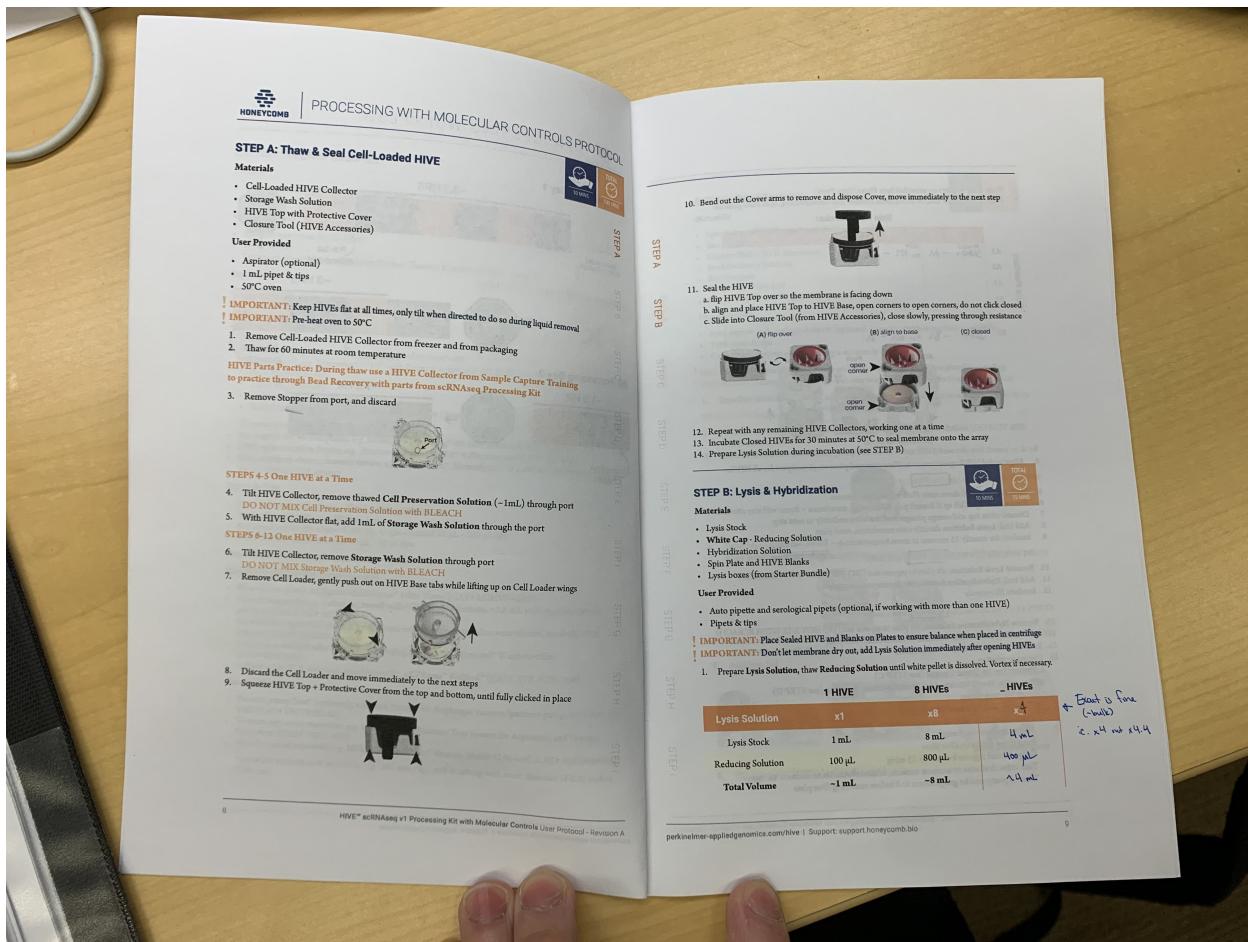
HIVE processing

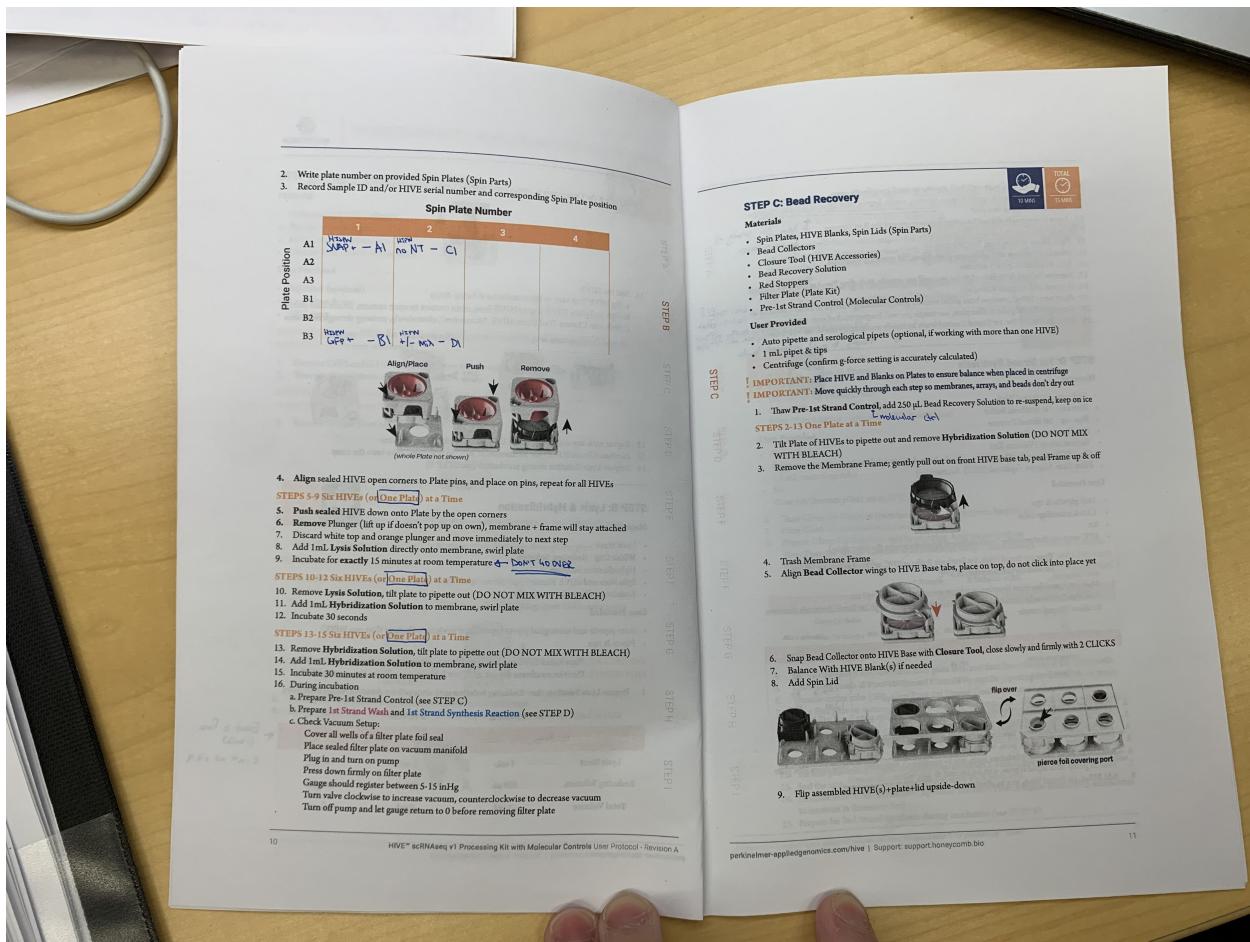


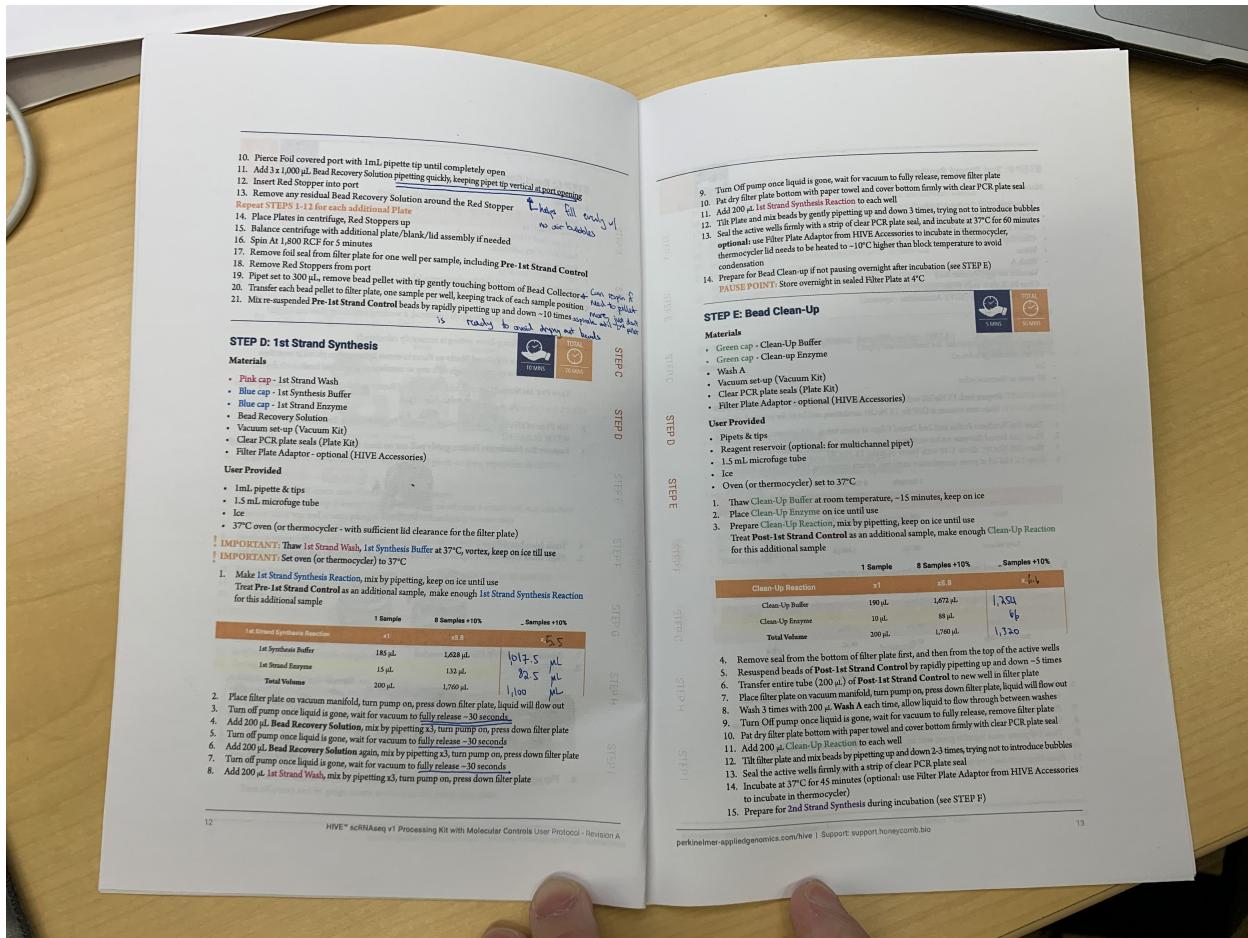


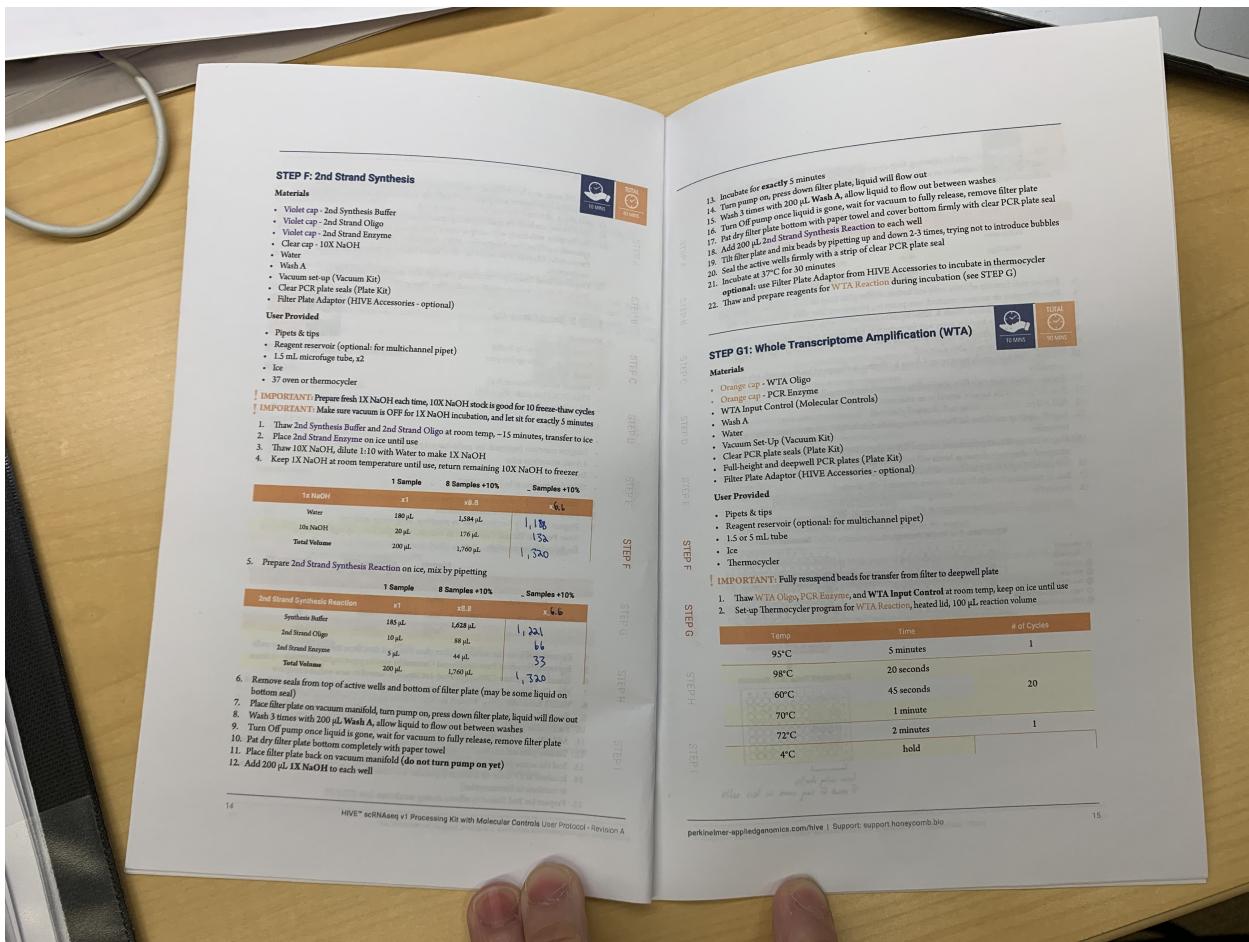


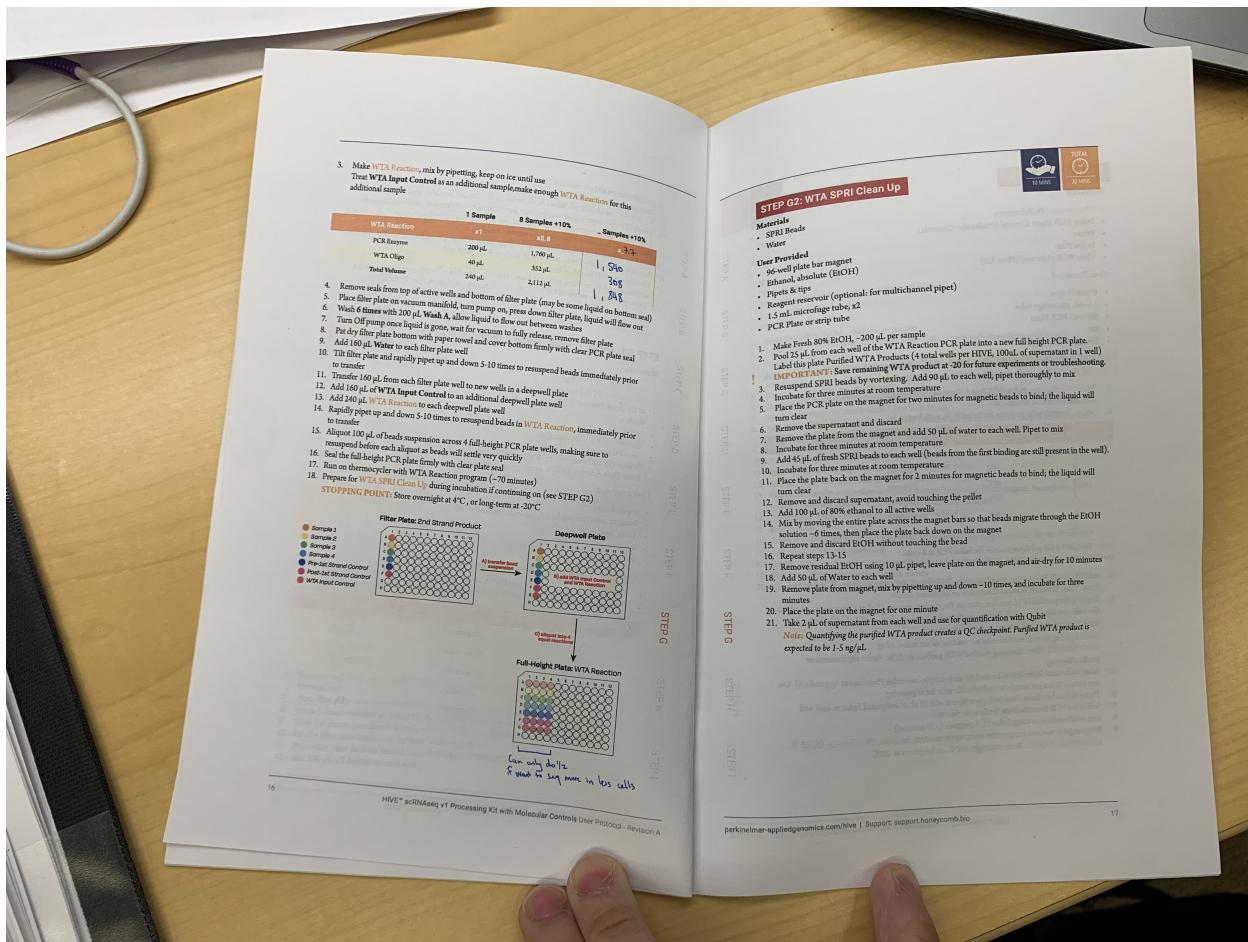


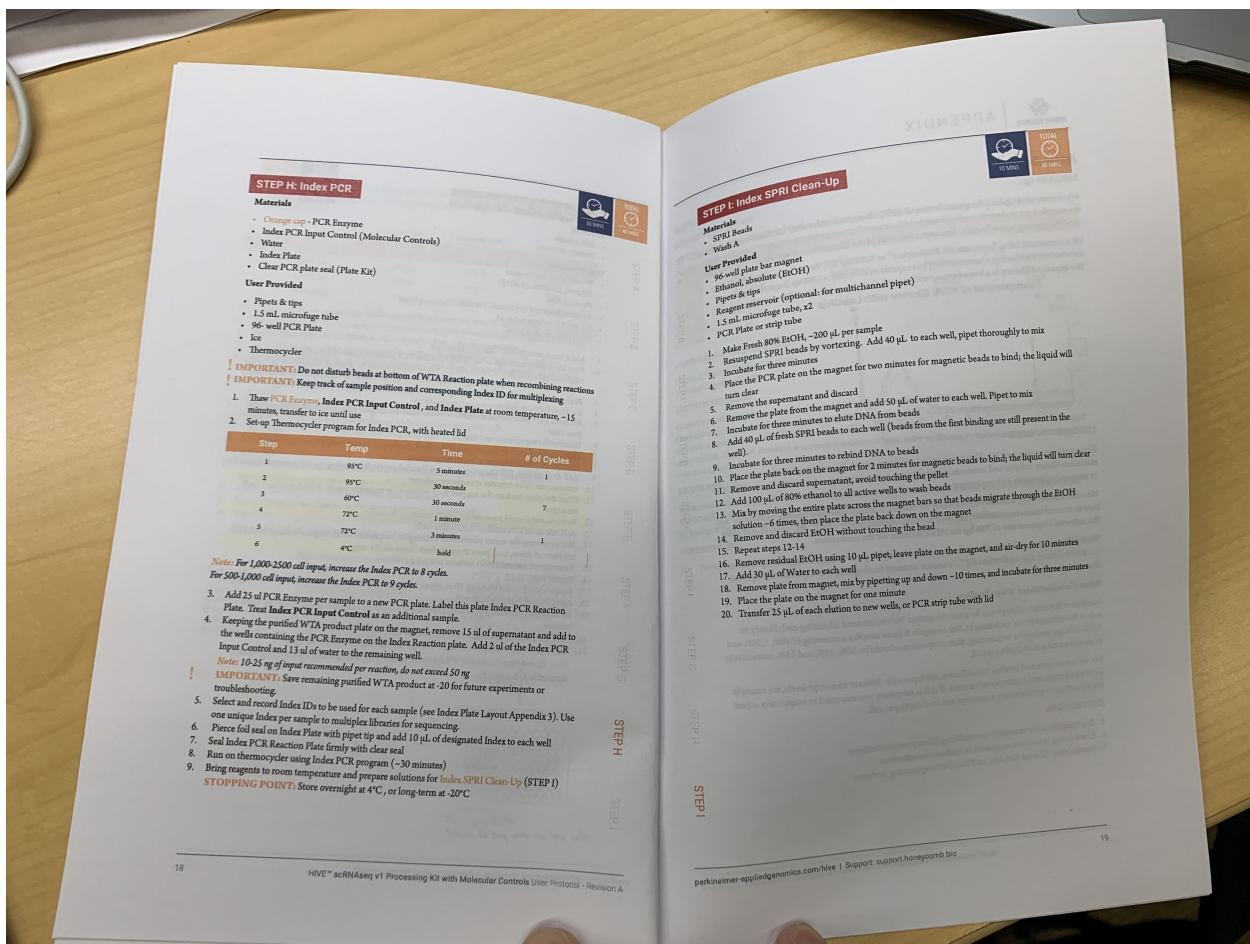


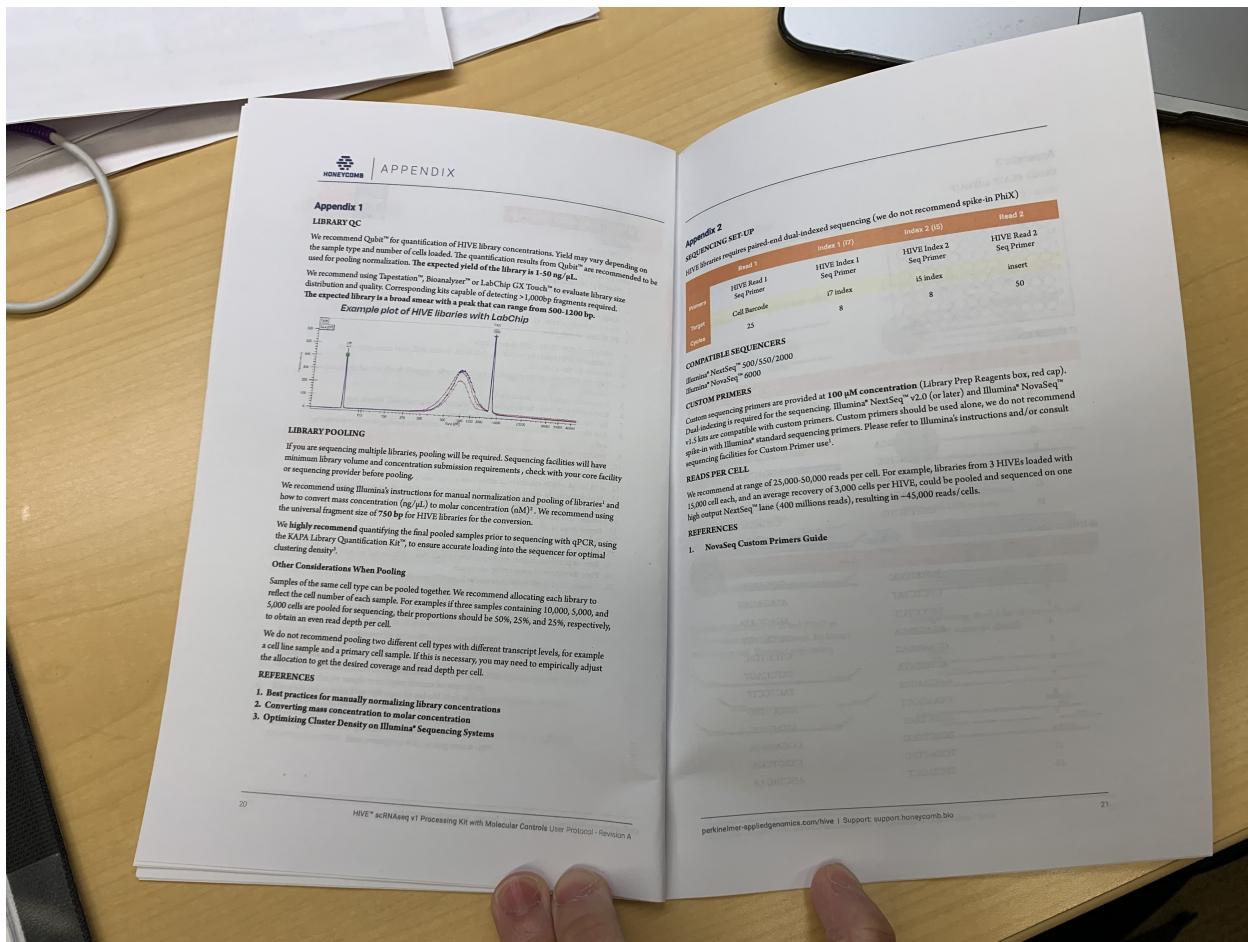












3.1.3 Immuno-Staining

Solutions Required

Stock solutions	How to make
4% PFA in PBS	8.75 mL PBS + 1.25 mL 32% PFA
0.5% Tween-20 in PBS	50 mL PBS + 250 μ L 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 So-

	lu- tion: 5% FBS, 0.1% Tween in PBS
Adherent Cell Staining	Ex- pected time: 3 days
•	Day 1: Fix cells, per- me- abi- lize overnight
•	Day 2: In- cu- bate with pri- mary an- ti- body overnight
•	Day 3: In- cu- bate

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Note:
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tion
step

6. Change solution and incubate cells in blocking solution for 1 hr

- *Cells may be left in blocking solution overnight at*

4°C

7.

Change
so-
lu-
tion
and
in-
cu-
bate
cells
in
pri-
mary
an-
ti-
body
(di-
luted
in
block-
ing
so-
lu-
tion)
overnight
at
4°C

8.

Change
so-
lu-
tion
and
wash
cells
with
0.1%
Tween/PBS
quickly
three
times.

9.

In-
cu-
bate

cells
in
0.1%
Tween/PBS
for
20
min

- *Cells
may
be
left
in
0.1%
Tween/PBS
overnight
at
4°C*

10.
Change
so-
lu-
tion
and
in-
cu-
bate
cells
in
sec-
ondary
an-
ti-
body
for
1
hr
(sec-
ondary
an-
ti-
body
is
di-
luted

1:300-500 in blocking solution)

• You can incubate your cells in secondary anti-body overnight at 4°C

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
min-
utes
at
room
temp,
then
wash
three
times
with

PBS

13.
Ei-
ther
store
in
PBS
or
re-
move
so-
lu-
tion
and
add
vec-
tashield
so-
lu-
tion.
Your
cells
can
stay
in
the
so-
lu-

tion 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
re-

skewing your results

move
all
of
the
me-
dia
from
the
cells-
they
will
dry
and
ab-
sorb
an-
ti-
body
in
a
non-
specific
way,
thereby

2.
Un-
less
oth-
er-
wise
noted,
ev-
ery-
thing
is
per-
formed
at
room
temp

3.
Keep
your
an-
ti-
bod-

ies
on
ice
at
all
times

4. Gen-
er-
ally,
50
 $\mu\text{L}/96$ -
well
for
all
steps
is
good
(can
go
higher
for
PBS
wash
steps)

5. If
mount-
ing,
try
to
avoid
bub-
bles
in
the
fi-
nal
step.
Leave
some
so-
lu-
tion
still
on
your

not dry and carefully dispense 2-3 drops of the solution, very carefully putting the coverslip on your slide

slides
so
the
cells
do

6.
Be
very
gen-
tle
with
neu-
ronal
cul-
tures,
they
are
not
very
ad-
her-
ent

Cell Staining for Flow

Ex-
pected
time:
1-
2
days
(only
2
if
overnight
pri-
mary
an-
ti-
body)

1.
Dis-
so-

ci-
ate
cells
(trypsin
or
DNase/Papain
if
MNs)
and
spin
down

2.
Re-
move
su-
per-
natant
and
in-
cu-
bate
cells
in
3.7-
4%
paraformalde-
hyde
(PFA)
(in
fume
hood)
for
15
min

- EU
calls
for
3.7%
PFA

3.
Add
1
mL
PBS
and

spin
cold
at
4°C
to
pel-
let
cells

- 4.

As-
pi-
rate
so-
lu-
tion
and
in-
cu-
bate
cells
in
0.5%
Tween/PBS
for
15
min
to
per-
me-
abi-
lize

- 5.

Add
1
mL
PBS
and
spin
cold
at
4°C
to
pel-
let
cells

- 6.

tion) for 1 hr in the rotator at 4°C

As-
pi-
rate
so-
lu-
tion
and
in-
cu-
bate
cells
in
200
 μ L
pri-
mary
an-
ti-
body
(di-
luted
in
block-
ing
so-
lu-

Note:
Some
an-
ti-
bod-
ies
(e.g.
Ki67)
work
bet-
ter
overnight
in
the
ro-
ta-
tor
at

4°C

7.
Add
1
mL
PBS
and
spin
cold
at
4°C
to
pel-
let
cells

8.
As-
pi-
rate
so-
lu-
tion
and
in-
cu-
bate
cells
in
200
 μ L
sec-
ondary
an-
ti-
body
(di-
luted
in
block-
ing
so-
lu-

tion) for 30 min in the rotator at 4°C **in the dark**

9.
Add

1
mL
PBS
and
spin
cold
at
4°C
to
pel-
let
cells

10.
If
do-
ing
a
DNA
stain,
add
Hoechst
or
DAPI
di-
luted
in
PBS
for
10
min-
utes
at
room
temp,
then
wash
with
PBS

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.

3.1.4 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	25nmol	DSL	Phosphate	<i>CTGTCTCTTATACACATCT</i>
Tn5ME_A	50nmol	HPLC	Cy5	<i>TCGTCGGCAGCGTCAGATGTG-TATAAGAGACAG</i>
Tn5ME_B	50nmol	HPLC	Cy5	<i>GTCTCGTGGCTCGGAGATGTG-TATAAGAGACAG</i>

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 H ₂ O	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 H ₂ O	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
MgCl ₂ (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 paper¹⁷](https://www.nature.com/articles/nprot.2013.118)):

Component	Concentration	Amount/L final
Tris	20 mM	3.264 g
MgCl ₂	10 m	0.95 g
DI H ₂ O	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:

¹⁷ <https://www.nature.com/articles/nprot.2013.118>

Component	Volume fraction
2x TD buffer	0.5
5 mM assembled Tn5	0.02
DI H ₂ O	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.

3.1.5 CellBaum User's Guide

A practical guide to CellBaum for those who want to live track cells but know absolutely nothing about computers.

Note: You can use CellBaum either on your computer or the MIT Supercloud.

Local Usage

Getting started

1. Follow Chris's instructions for getting CellBaum set up on your computer and update this protocol with those instructions. (I don't remember how to do this - I think you need to clone the CellBaum repository or something?)
2. Make sure you have CellProfiler and Fiji installed on your computer.
3. 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".
4. Complete the `cellbaum_config.yml` file for your specific experiment. This can be done using VS Code. See more detailed instructions below.
5. Open up a terminal in the CellBaum folder. You may open the terminal using VS Code.
6. Activate the virtual environment by typing `conda activate ./cenv` in the terminal:

```
conda activate ./cenv
```

7. To do a dry-run the CellBaum pipeline, type `snakemake --dry-run` in the terminal. This is to double check that everything is configured correctly:

```
snakemake --dry-run
```

8. To run CellBaum, type `snakemake` 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, type `snakemake -j7`:

```
snakemake -j7
```

9. 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 [GitHub¹⁸](#) and their [User's Guide¹⁹](#))
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 part-way 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:

- 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:

¹⁸ <https://github.com/quantumjot/BayesianTracker>

¹⁹ <https://btrack.readthedocs.io/en/latest/>

```
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 (I think)
 - ‘avg’ uses the average of all three RGB channels (I think)
 - using a list of 3 ratios lets you choose the weight of each RGB channel (I think)
 - For example:

```
gray_channels:  
    CH2: max  
    CH3: max  
    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 recommended 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 [here²⁰](#).
- 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 website²¹](#) 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 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:sourcopath dest:destpath
```

3. It is recommended to first do a dry run to ensure everything will transfer correctly:

²⁰ https://btrack.readthedocs.io/en/latest/user_guide/configuration.html#

²¹ <https://supercloud.mit.edu/requesting-account>

```
~/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 authentication.
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`²², 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
```

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
```

(continues on next page)

²² <https://supercloud.mit.edu/accessing-and-transferring-data-and-files>

(continued from previous page)

```
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
```

Data Analysis

To add later

How to open HDF files and understand LBEPR format.

3.1.6 CFSE 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

Note: For MEFs and reprogramming experiments, it's recommended 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 + DDDR at 4 dpi will have ~10-100k cells. Be careful to remember if you're doing 6F you will need even more.

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 μ L of stock dye per plate being stained.
2. Dilute stock solution of dye into PBS to make a working solution. Stocks are at 1000x concentration.

Wash cells and add CellTrace

3. Working in a BSC hood, aspirate the media and wash with PBS.
4. Aspirate PBS and replace with working solution of CellTrace. For control cells that do not get stained with CellTrace, incubate in PBS + 1:1000 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 PBS solution and wash once with FBS containing media.
7. Return cells to incubator and treat cells according to your experiment. For reprogramming, cells are harvested at 4 dpi and assayed via flow cytometry.

3.1.7 HCR DNA-FISH

Prepare necessary buffers as described in *FISH buffer recipes* (page 260) Expected time: 2 days

Fixation

1. Steps 1-5 of *Immunofluorescent Staining* (page 82)

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 Preparation

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

3.1.8 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 website²³ in the “Focus on Imaging Technical Manual.”

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

²³ <https://www.promega.com/products/protein-detection/protein-labeling/janelia-fluor-halotag-ligands/?catNum=GA1110#protocols>

Halo ligand staining

1. Working in a BSC hood, dilute resuspended Halo ligand in appropriate culture medium 1:1000 to make 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.

3.1.9 HCR RNA-FISH

Prepare necessary buffers as described in *FISH buffer recipes* (page 260) Expected time: 3 days

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 buffers) are hazardous, so all steps should be performed in the fume hood

Solution Detection Stage

1. Steps 1-5 of *Immunofluorescent Staining* (page 82)
2. Add 200 uL of probe hybridization buffer for 30 min at 37 degrees C
3. Prepare probe solution by adding 2 uL of 1 uM stock to 500 uL of probe hybridization buffer at 37 degrees C
4. Remove pre-hybridization solution and add 200 uL of probe/hybrid buffer solution
5. Incubate samples **overnight (12-16 h)** at 37 degrees Celsius
6. Remove excess probes by washing 1x 15 min with 200 uL of probe wash buffer at 37 degrees Celsius
7. Wash samples 1x 5 min with 200 uL of 5X SSCT at room temperature

Solution Amplification Stage

1. Pre-amplify samples in 500 uL of amplification buffer for 30 min at room temperature While waiting, begin Hairpin Preparation:
 - a. Prepare 30 pmol of hairpin h1 and h2 by snap cooling 10 uL of 3 uM stock at 95 degrees Celsius for 90 seconds
 - b. Cool hairpins in the dark at room temperature for 30 minutes
 - c. Prepare hairpin solution by adding h1 and h2 hairpins to 500 uL amplification buffer
2. Remove pre-amplification solution and 200 uL of the hairpin/amplification buffer solution
3. Incubate samples **overnight (12-16 h)** in the dark at room temperature
4. Remove excess hairpins by washing with 200 uL of 5X SSCT at room temperature for:
 - a. 1x 30 min
 - b. 1x 5 min

5. Store at 4 degrees C in the dark until imaging

3.1.10 Live nuclear Staining using Hoechst

Solutions Required

Stock solutions	Working solution
Hoechst 33342 Solution (20 mM)	Dilute 1:10000 in media (DMEM+10%FBS) or (N3 media)

Live Hoechst staining

Expected time: 30 mins

1. Remove media and add Hoechst working solution, diluted using the media of the cells in culture.
2. Incubate cells back in 37°C incubator for 20mins.
3. Wash off the Hoechst solution and replace with appropriate culture media.
4. Image stained nuclei using Keyence microscope or analyze cells by flow in the Attune.

3.1.11 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.

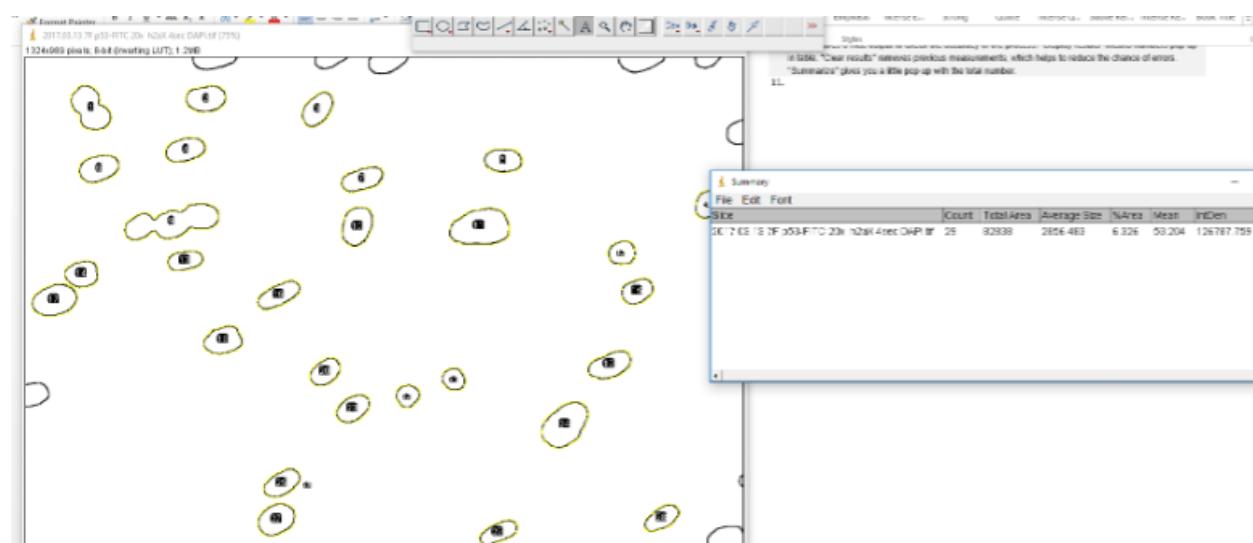
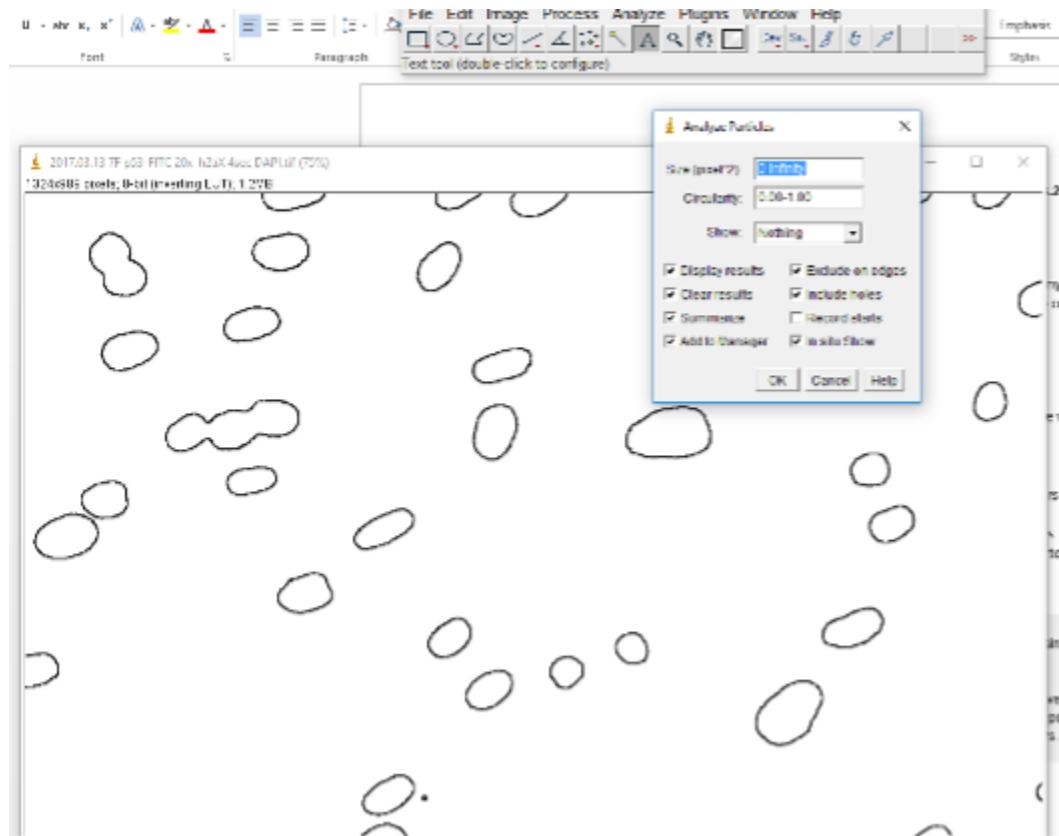
Important: To use this process, images need to be collected from the same field with DAPI and any other desired channel (e.g., green, red). Thus, this protocol only works for fixed cells. Potentially, live cell imaging with an H2B-Cerulean could allow nuclear image analysis with this method.

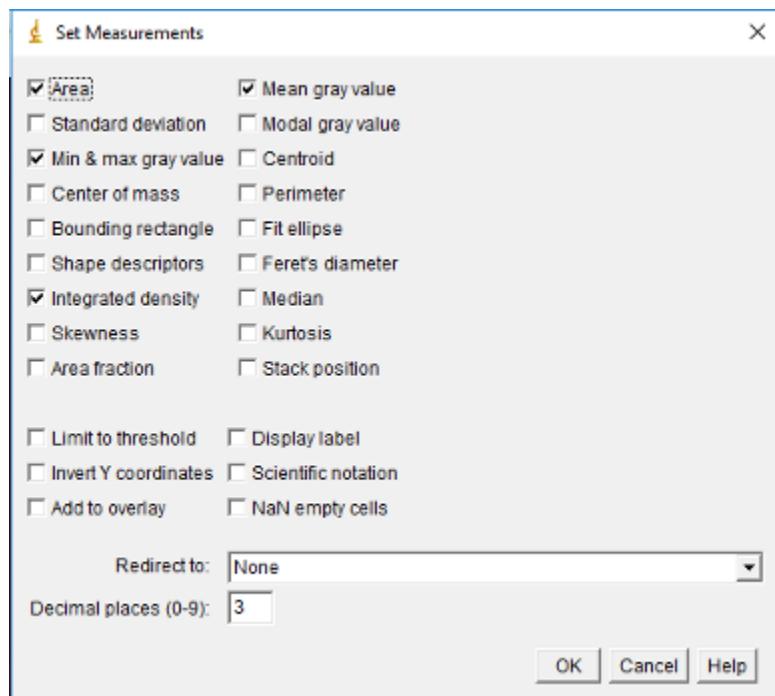
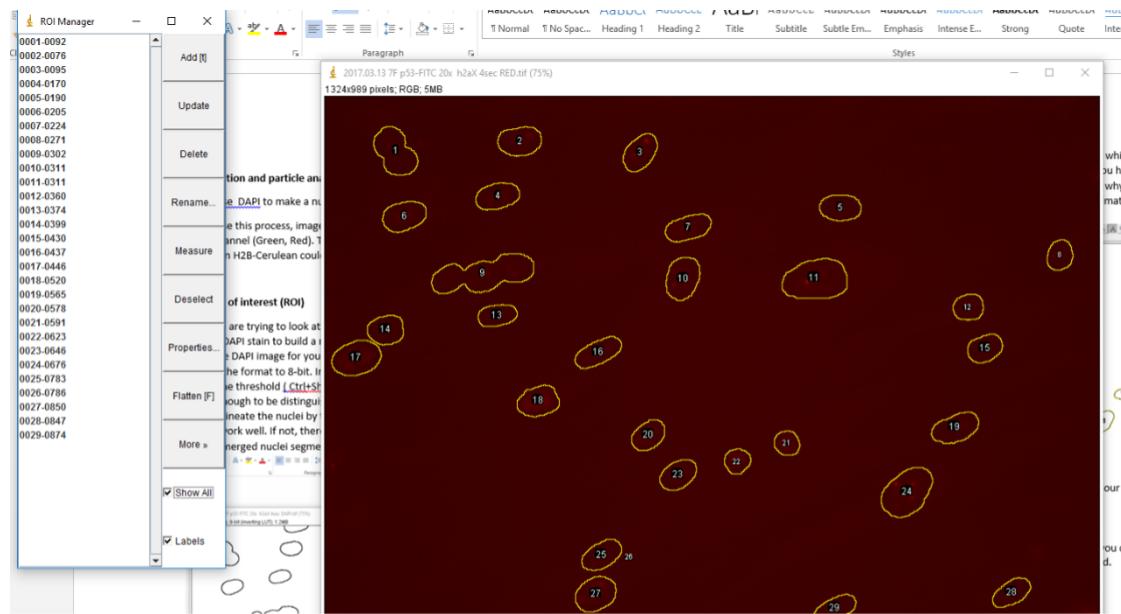
Defining regions of interest (ROI)

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 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.

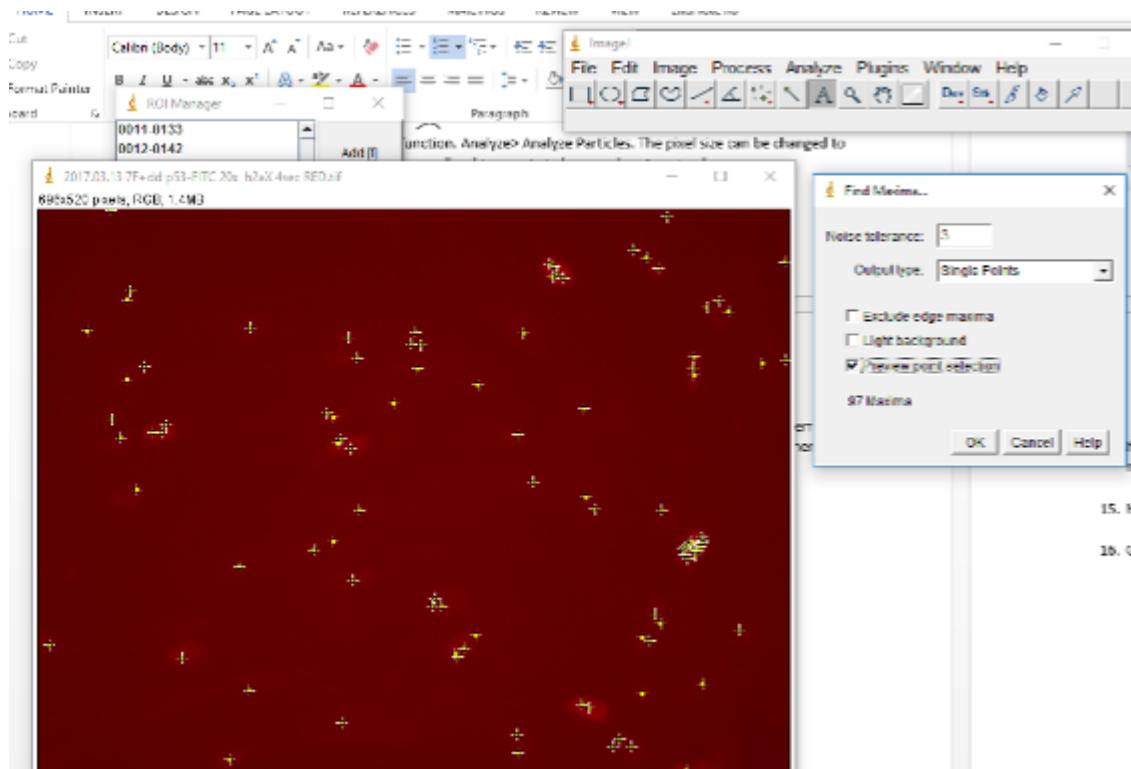
	Area	Mean	Min	Max	IntDen	RawIntDen	
1	4172	19.818	18	24	82681	82681	
2	2958	21.396	19	36	63288.000	63288.000	
3	2638	22.130	19	50	58378.000	58378.000	
4	2654	21.017	19	28	55778.000	55778.000	
5	2517	21.005	20	24	52870.000	52870.000	
6	2985	20.826	18	29	62167.000	62167.000	
7	2645	22.363	20	29	59150.000	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	

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.

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 previously 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>



	Area	Mean	Min	Max	IntDen	RawIntDen
1	906	0	0	0	0	0
2	1443	0.530	0	255	766	766
3	730	1.747	0	255	1275	1275
4	760	0.000	0	0	0	0
5	673	0.750	0	255	510	510
6	767	0.997	0	255	766	766
7	1445	0.176	0	255	255.000	255.000
8	781	0.327	0	255	200.000	200.000
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	0.000	0.000	0.000

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.

3.1.12 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](#).

1. Master Mix Preparation

1. Ensure all reaction components are properly thawed and mixed.
2. Prepare a master mix containing the appropriate volume 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.
4. Calculate the required volume of each component based on the following tables:

Component	ROX	No ROX	Final conc.
PCR-grade water	Up to 20 μ L	Up to 20 μ L	N/A
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10 μ L	10 μ L	1X
10 μ M forward primer	0.4 μ L	0.4 μ L	200 nM
10 μ M reverse primer	0.4 μ L	0.4 μ L	200 nM
Template DNA	As required	As required	<20 ng
50X ROX High/Low (as required)	0.4 μ L	--	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.

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 μ L	
	384 well	3 - 20 μ L	
Program Name	Cycles	Analysis Mode	
Pre-incubation	1	None	
Amplification	40	Quantification	
Melting Curve	1	Melting Curves	
Cooling	1	None	
Program Name	Target (°C)	Aquisition 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.

3.1.13 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 130) 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 + 300-500 μ L 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 200 μ L 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 overdries & 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's), EtOH wash 2x
3. Allow to air dry & elute back into desired vol of diH₂O or EB.

3.1.14 Nuclei Isolation for Downstream Genomics Applications

Nuclear Isolation

Expected time: 1 - 1.5 hrs depending on the # 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.
2. Obtain 50,000 cells for MEFs by trypsin and count accordingly. Optional if using a Spike: Add 1000 cells from HEK293Ts.
3. If using spike, add appropriate cell volume of Spike cells (HEK293Ts) to each MEF tube.
4. Pellet at 500xg for 5 mins at 4C. Aspirate supernatant gently using P1000.
5. Resuspend the cell pellet in 50 μ L of Lysis Buffer by pipetting up and down three times.
6. 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.
7. Add 1 mL of Wash Buffer to dilute the lysis reagents. Invert the tube five times to mix.
8. 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.
9. 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

Resuspension Buffer (RSB)- 100mL, scale as needed

Stock solutions	Working stock
1M Tris-HCl pH 7.50 - 1mL	10mM Tris-HCl pH 7.50
5M NaCl - 200uL	10mM NaCl
1M MgCl2 - 300uL	3mM MgCl2
UltraPure distilled H2O - 98.5mL	

Cell Lysis Buffer per 50 uL, scale as needed

Stock solutions	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

3.1.15 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 μ M aphidicolin for 1.5 to 2 hours prior to cell harvest and DNA extraction.

Chemical	Concentration	Molar mass	mg / mL
Aphidicolin	1 μ M	338.48 g/mol	

3.1.16 sci-ATAC-seq

This protocol is effectively copy and pasted from the STAR Protocols paper, [delPriore2021], with reference to the original Tn5 expression and purification paper, [Picelli2014].

3.1.17 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
<i>Total</i>	<i>4 uL</i>	<i>400 uL</i>

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
<i>Total</i>	<i>4 uL</i>	<i>400 uL</i>

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 H2O 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 reagent	0.25 uL	48 uL
RNAse-free water	2.75 uL	264 uL
<i>Total</i>	<i>5.5 uL</i>	<i>528 uL</i>

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
<i>Total</i>	<i>5.0 uL</i>	<i>20 uL</i>

7. Add 5 uL of each mix to each inlet. Place samples in well corresponding 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)

3.1.18 SingleShot RNA isolation and Reverse Transcription

This protocol for RNA isolation uses the Bio-Rad SingleShot Cell Lysis Kit²⁴ and Maxima H Minus Reverse Transcriptase²⁵.

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)

Materials needed for cDNA synthesis

Solution	Location
Oligo(dT) 18	-20°C freezer (Anna)
dNTP mix	-20°C freezer (Anna)
DEPC-treated Water	shelf above Katie's bench
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
- Aluminum foil
- 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.

²⁴ <https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000112808.pdf>

²⁵ 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

Dedicated RNase-free tubes and tips should be used, all pipettes and working surfaces should be wiped down with the RNase-free surface decontaminant spray. Aluminum foil can be placed on the benchtop and wiped with RNase-free surface decontaminant to create an “RNase-free” zone. RNase contamination before cDNA synthesis will destroy your RNA and your data.

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 μ L 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, μ L
Oligo(dT) 18	1
dNTP mix	1
DEPC-treated water	12.5 - (μ L of lysate to be used, max of 9 μ L lysate)
total	at least 5.5 μ L if maximum 9 μ L of lysate and 1 μ L of RT is to be used

11. Add (14.5 μ L - μ L of lysate to be used) of RT master mix 1 to a new PCR tube for each reaction to be carried out. Normally, 1 reaction per lysate. Additionally, include an extra PCR tube to use as a no-reverse transcriptase control.
12. Add up to 9 μ L of a cell lysate to a PCR tube containing RT master mix 1. 1 lysate per reaction. Mix gently.
13. Incubate lysate + master mix 1 at 65°C for 5 minutes, then cool on ice to melt secondary structures of RNA.
14. Prepare RT master mix 2. 0.5-2 μ L of RT may be used per reaction (50-200 Units). AMB has used 1 μ L and obtained satisfactory results. If using different amount than 1 μ L, you may need to adjust the water amount in step 10.

Component	volume per reaction, μ L
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 μ L

15. Add 5.5 μ L of RT master mix 2 to each PCR tube. Mix gently. If doing a no RT control, replace the RT with water.
16. Transfer to thermocycler, incubate at 50°C for 30 minutes, followed by 85°C for 5 minutes for heat inactivation.
17. 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.

3.1.19 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 website²⁶.

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 be used and labeling is still sufficient. AMB has used 400x dilution (2.5 µL dye / mL media) to label and had detectable signal.

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 make 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.)

²⁶ <https://www.neb.com/protocols/0001/01/01/cellular-labeling-s9105>

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

3.1.20 Titration of Tn5 transposition in MEFs

Part 1. Assembling the Transposase

1. Resuspend primers to 100uM stock using Annealing 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 MgCl ₂	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 μ L 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 μ L (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 μ L 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 μ L 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 μ L of product.

Pause Point: This can be Stored at -20C for as long as necessary or processed immediately for Barcode amplification followed by sequencing.

3.1.21 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 -20°C 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.

Note: We usually use the 10X cell lysis buffer

1. Cool microcentrifuge to 4°C for 20-30 minutes.
2. Dilute 10X Cell Lysis Buffer to a 1X solution using Elga water
 - Can use 10X 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 μ L	1,170 μ L
10X cell lysis buffer	40 μ L	130 μ L
200 mM PMSF	2 μ L	6.5 μ L

6. Tilt to coat, then incubate on ice for 5 minutes.
7. Scrape cells with a cell scraper and transfer lysed solution to a 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 μ L/sample
 - **~50-60 μ L/aliquot is good because you can load 25 μ L/well (12.5 μ L lysate + 12.5 μ L 2X Laemmli)**
so 50 μ L will give enough for 4 wells (i.e. 4 antibodies)

Note:

- 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.
-

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 Documentation²⁷](#) for the full list of compatible reagents.

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:

²⁷ 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

1. Combine each standard and unknown sample with the Bradford Reagent.
 - For a working range of 100-1500 ug/mL, pipette 1 uL of each standard or unknown sample into a labeled tube and add 20 uL of the Bradford Protein Assay Reagent and mix well.
 - For a working range of 1-25 ug/mL, pipette 10 uL of each standard or unknown sample into a labeled tube and add 10 uL of the Bradford Protein Assay Reagent and mix well.
2. Incubate at room temperature for 10 minutes.
3. On the NanoDrop, select the Proteins tab and then Bradford Assay.
4. Enter the concentrations of each BSA standard and select the number of replicates.
5. Measure the absorbance of each BSA standard as directed by the NanoDrop to construct the standard curve.
6. Measure the absorbance of each sample. The NanoDrop will automatically calculate the protein concentration for you based on the standard curve.

Protein Gel Casting

Modified for a Western Blot from [this protocol²⁸](#).

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	

²⁸ https://gallowaylabmit.github.io/protocols/en/latest/protocols/protein_production/bis_tris_protein_gels.html

- **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.

Component	Concentra-tion	g/L to final concentration
Ammonium persul-fate	10%	100 (For example: 10mg/100uL or 100mg/1mL)

Casting protocol

Warning: The acrylamide monomers used here are toxic. Read the SDS²⁹.

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 Wa-ter	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)

²⁹ <https://www.fishersci.com/store/msds?partNumber=BP14081&productDescription=ACRYLAMIDE%3ABISACRYLAMIDE+29%3A1&vendorId=VN00033897&countryCode=US&language=en>

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.

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.

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 μ L (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 μ L 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 μ L of **10% APS**, mix well.
3. Add 10 μ L **TEMED**, mixing quickly (don't pipette mix, just flip it x3 manually to mix).
4. Add 1,000 μ L 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 [this³⁰](#) 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.

³⁰ https://gallowaylabmit.github.io/protocols/en/latest/protocols/protein_production/denaturing_protein_gel.html

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	

- **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 μ L is good for ~4 lane (need 12.5 μ L 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 1% 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 1% MES-SDS running buffer.
5. Carefully load equal amounts of protein samples, including 5 μ L of a protein ladder, into the wells of the gel. Each well can be loaded with a maximum of 25 μ L. 20-30 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

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
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.

Note: BAL tried freezing Laemmli buffer-denatured lysate at -20°C and it worked for Western

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 your 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 video³¹](https://www.youtube.com/watch?v=g7DT5xGheCE) to learn how to use this device. The [iBlot2 manual³²](https://tools.thermofisher.com/content/sfs/manuals/iblot2_device_man.pdf) 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.
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. 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.

³¹ <https://www.youtube.com/watch?v=g7DT5xGheCE>

³² https://tools.thermofisher.com/content/sfs/manuals/iblot2_device_man.pdf

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
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 manual³³](#) 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.

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.

³³ 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 with agitation.
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)
 - Can even go down to 1:50,000 for 2nd ab HRP-conjugate
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 Substrate³⁴ working solution by mixing equal parts of the Substrate and Stable Peroxide components (e.g. 5 mL substrate with 5 mL stable peroxide). Use a sufficient volume (~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.

³⁴ <https://www.thermofisher.com/order/catalog/product/34094>

- Blot quantification can be done using the Gel Analyzer tool³⁵ 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

3.2 Cloning protocols

3.2.1 Gibson assembly

Gibson assembly allows us to create a construct out of overlapping fragments. During a Gibson reaction, an exonuclease chews back the 5' ends of both the backbone and the inserts. The complementary ends can then anneal, with nucleotides added by a DNA polymerase to fill in gaps in the annealed fragments. Finally, a ligase enzyme removes nicks to generate one contiguous plasmid. Since this method makes use of an exonuclease, it is important for fragments to have at least 30 bp of overlap.

Protocol

- Design the assembly. Online tools such as NEB Builder³⁶ and built-in tools such as those in SnapGene are helpful for designing this.

Note: If designing Gibson primers manually, having an overlap region of at least 30bp is recommended. Each fragment should also be over 200bp.

- Generate fragments by either PCR followed by DpnI digest, or by restriction digest of a target template. Measure concentration of fragments via Nanodrop.

Note: Skipping the DpnI digest step will often still work, but will tend to increase the number of background colonies because the source plasmid is still around. If the cloning strategy selects using a different antibiotic resistance cassette, it may be possible to skip this digest step.

- Calculate volumes of each fragment required for Gibson reaction. NEBioCalculator³⁷ is a helpful tool. Generally, 150 ng of the vector backbone fragments should be used. For inserts, a 2:1 molar ratio is usually successful, although a 3:1 ratio may be used for smaller fragments.

³⁵ <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>

³⁶ <http://nebbuilder.neb.com/>

³⁷ <https://nebiocalculator.neb.com/#!/ligation>

4. Setup a reaction with the calculated volumes of each fragment and 2X Gibson master mix/HIFI assembly mix solution. The master mix is 2X by volume, so add amount equal to the sum of all vector/insert volumes added.

Hint: If your backbone vector is 5 kb at a concentration of 50 ng/uL, you need to add 3 uL to the reaction. If your insert is 1 kb at a concentration of 30 ng/uL, you need to add 2 uL (2:1 molar ratio) to the reaction. Finally, the sum of vector and insert volumes is 5 uL, so 5 uL of the 2X master mix should be added.

5. Incubate at 50°C for 15 minutes (2-3 fragments) or for 60 minutes (4-6 fragments).
6. *Transform competent cells* (page 175) with 2 uL of the Gibson mixture.

Note: If you're skipping outgrowth step, adding water up to 300 μ L total volume helps spread the transformed bacteria.

6. Store unused Gibson products at -20°C

3.2.2 Glycerol Stocking

After successfully sequencing a bacterial plasmid, we must stock it for storage.

1. Add pKG # to Quartzy database. Add in a new item to the plasmid database, with the criteria below. The pKG number is XXXXX, use the next pKG number available.

Criteria	Label
Item Name	pKGXXXX (put leading zero if necessary)
Type	bacterial stock
Date Stored	date of storage
Plasmid	description of construct (ex. LentiX-EF1a-mGL-BGH)
pKG#	XXXX
Plasmid type	Selection from options
Resistance markers	Selection from options
Species	Bacterial cell type (ex. NEB Stable)

2. Culture desired 4 mL of bacteria clone with the plasmid in LB Broth with the correct resistance antibiotic (overnight).
3. Add 2 mL of 60% glycerol to the 4 mL of culture. Mix well.
4. Aliquot 2 mL of the resulting mixture to three cryovials.
5. Label each cryovial with pKG#, date, initials, and description of the construct.
6. Put cryovials in -80 C. Put one cryovial per box.

3.2.3 Golden Gate assembly

Golden Gate assembly allows us to create a construct using Type IIIs restriction enzymes (REs). These enzymes can generate custom connector sequences, enable scarless assembly, and enhance reaction efficiency through multiple rounds of digestion and ligation.

Protocol for PCR fragments

This protocol is derived from the standard NEB protocol (see *citations* (page 168) below).

1. Design the assembly. Online tools such as [NEB Golden Gate](#)³⁸ are available for designing primers, choosing connector sequences, etc.
2. Recipe for Golden Gate reaction (total volume = 10 μ L):

Component	Amount
75 ng/plasmid	
2:1 mol ratio for fragments	
10X T4 DNA ligase buffer	1 μ L
T4 DNA ligase (400 U/ μ L)	1.25 μ L (500 U)
Type II RE	0.5 μ L (increase to 1 μ L if >10 inserts)
H2O	to 10 μ L

Note: Make sure to use 500 U T4 DNA ligase. NEB sells 400 and 2,000 U/ μ L T4 DNA ligase so scale volume accordingly.

3. Use the appropriate assembly protocol

Insert number	Assembly protocol	Estimated time
For 1 insert	37°C, 5 min (cloning) or 37°C, 1 hr (library preparation) -> 60°C, 5 min	10 min - 1 hr
For 2-10 inserts	(37°C, 1 min -> 16°C, 1 min) x 30 -> 60°C, 5 min	~1.5 hrs
For 11-20 inserts	(37°C, 5 min -> 16°C, 5 min) x 30 -> 60°C, 5 min	~5.5 hrs

4. *Transform competent cells* (page 175) with 2 μ L of the Golden Gate mixture.
5. Store unused Golden Gate assembly products at -20°C

³⁸ <https://goldengate.neb.com/>

Protocol for pPV plasmids

This protocol is specifically for Golden Gate assembly using the lab's positioning vector (pPV) cloning scheme and contains general best practices.

1. Design the assembly using the *pPV cloning scheme* (page 166) below.

Note: It is okay to combine pPV plasmid fragments with PCR fragments in the same reaction. However, reactions with only plasmid fragments tend to be more efficient.

Important: The Golden Gate reaction will still work if one of the fragments has an internal RE cut site, but it will be less efficient. Be sure that the connector sequence generated by the internal RE cut site does not match any of the pPV connector sequences! If you run into issues with assemblies of this type, try the maximum number of cycles (99x on the thermocycler) and run the reaction overnight.

2. Recipe for Golden Gate reaction (total volume = 10 μ L):

Component	Amount
75 ng/plasmid	
10X T4 DNA ligase buffer	1 μ L
T4 DNA ligase (400 U/ μ L)	1.25 μ L (500 U)
Type II RE	0.5 μ L (increase to 1 μ L if >10 inserts)
H ₂ O	to 10 μ L

Note: Smaller 5 μ L (instead of 10 μ L) reactions also work, halving the volume of each component. This is best done with multiple, similar reactions by making a master mix containing the ligase buffer, ligase, RE, H₂O, and any common pPV fragments to avoid pipetting small volumes. However, be sure to use a 10 μ L reaction for difficult assemblies (e.g., fragment contains an internal RE cut site).

3. Use the guidelines below to choose an appropriate assembly protocol. In general, longer assemblies (more cycles) are preferred, especially if reactions are run overnight.

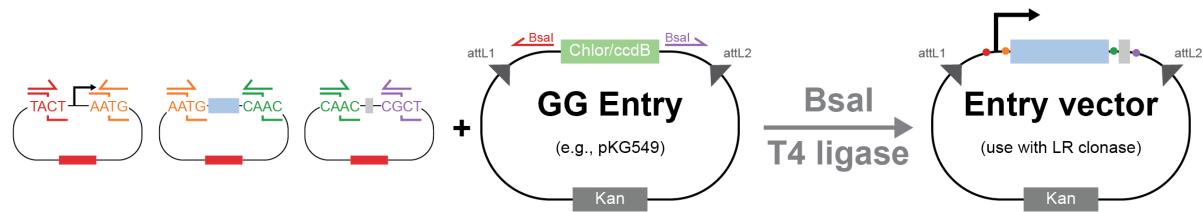
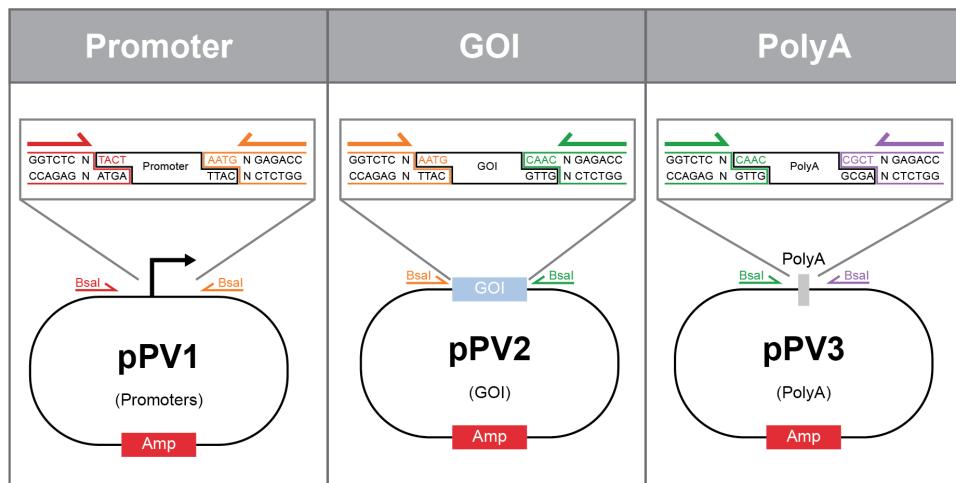
Insert	Assembly protocol	Estimated reaction time
<5 plasmid fragments (fast)	(37°C, 1 min -> 16°C, 1 min) x 30 -> 60°C, 5 min	~1.5 hrs
5+ plasmid fragments (fast)	(37°C, 1 min -> 16°C, 1 min) x 60 -> 60°C, 5 min	~3 hrs
5+ plasmid fragments (long), 1 PCR fragment	(37°C, 1 min -> 16°C, 1 min) x 99 -> 60°C, 5 min	~5 hrs
>1 PCR fragments, fragments with internal RE cut site, other difficult assemblies	(37°C, 5 min -> 16°C, 5 min) x 99 -> 60°C, 5 min	~18 hrs

Reactions can be held at 4°C or 16°C overnight.

4. If a fragment contains an internal RE cut site, perform an extra ligation step to improve efficiency.
1. Clean and concentrate the assembly reaction using the DNA Cleanup Kit. Elute in 8.5 μL H2O.
2. Add 1 μL 10X T4 DNA ligase buffer and 0.5 μL T4 DNA ligase (400 U/μL), according to the *ligation protocol* (page 169).
3. Let the reaction run at room temperature (or 16°C) at least 1 hr, preferably overnight.
5. *Transform competent cells* (page 175) with at least 2 μL of the Golden Gate mixture.
6. Store unused Golden Gate assembly products at -20°C

Reference for pPV connector sequences

Part library example



Golden Gate pPV Cloning Scheme (Bsal)

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	pPV3a,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

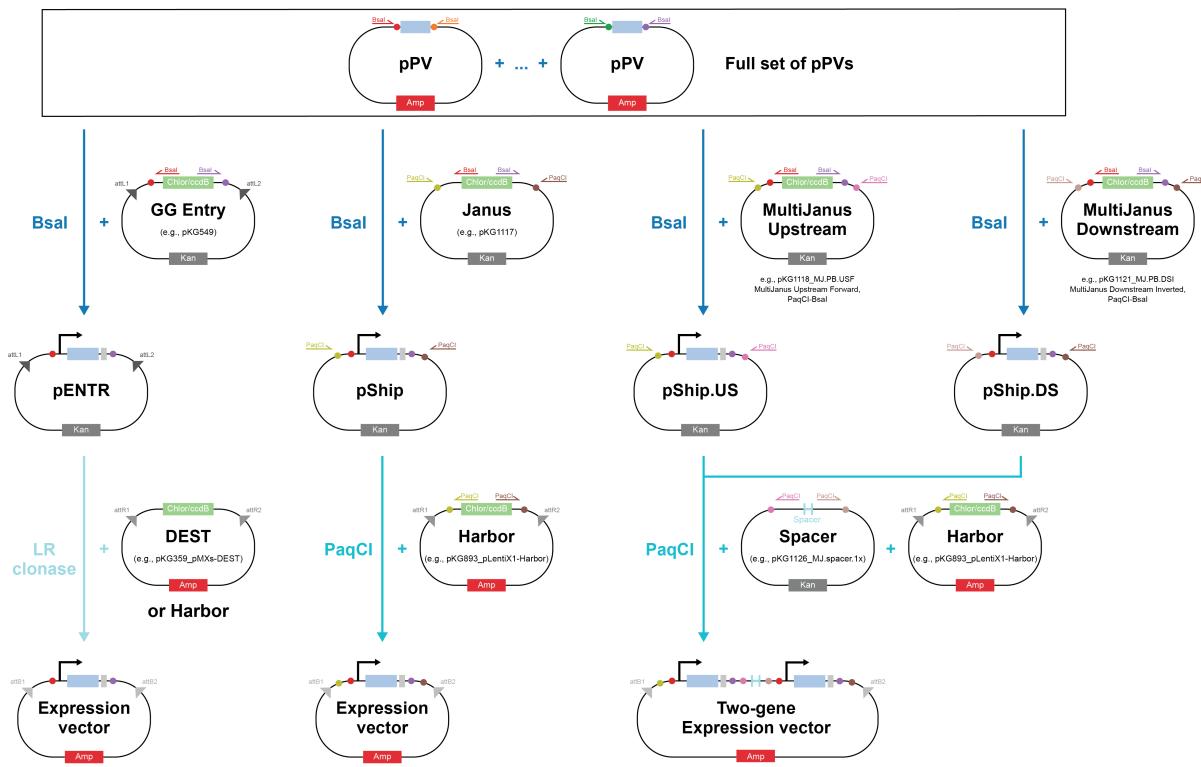
Overhang sequences are all written 5' to 3' for the sense (top) strand.

"Ship US" refers to the transcription unit formed by a Golden Gate reaction (Bsal) with pPVs and MultiJanus Upstream Forward/Inverted.

"Ship.DS" refers to the transcription unit formed by a Golden Gate reaction (Bsal) with pPVs and MultiJanus Downstream Forward/Inverted.

Reference for entire Golden Gate workflow

Full Golden Gate expression vector assembly



Citations

- [1] <https://www.neb.com/protocols/2018/06/05/golden-gate-24-fragment-assembly-protocol>
- [2] <https://www.neb.com/-/media/nebus/files/manuals/manuale1601.pdf>

3.2.4 Ligation assembly

Estimated time: At least 1 hour, or overnight for best efficiency

Ligation³⁹ assembly is used to assemble DNA fragments by creating phosphodiester bonds between different fragments to generate a larger linear fragment or plasmid. Ligation reactions can be performed with restriction digest products and/or oligos that contain compatible sticky ends.

Protocol

1. Design the assembly such that each fragment has compatible sticky ends. Sticky ends allow for DNA fragments to bind each other for efficient ligation. Built-in tools such as those in SnapGene are helpful for designing this.
2. Generate the fragments for ligation. Perform a restriction digest of the recipient vector plus any desired inserts to generate sticky ends. If ligating using oligos, *anneal and phosphorylate* (page 172) oligos prior to assembly, as 5' phosphorylation is required for proper ligation.

Tip: Performing the vector digest with rSAP to dephosphorylate the backbone has sometimes led to reduced background colonies and greater efficiency when assembling using oligos.

Tip: Gel extraction of digests can also be useful to decrease background ligation products.

3. Setting up the reaction mix is dependent on whether you choose to ligate digestion products or ligate using oligos.

Ligation with general digest products: Mix 150 ng of digested vector and 3x molar ratios of desired inserts. NEBioCalculator⁴⁰ 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). Add 2 uL of T4 ligase buffer, 1 uL of T4 ligase enzyme, and water to bring the total volume of reaction mix to 20 uL.

Ligation with oligos only: Mix the reagents as follows if performing ligation assembly using one set of annealed oligos:

Reagent	Amount (uL)
Vector digest (150 ng)	X
Oligos (annealed/phosphorylated)	1
T4 ligase buffer	0.5
T4 ligase enzyme	1
Water	10 - X

³⁹ [https://en.wikipedia.org/wiki/Ligation_\(molecular_biology\)](https://en.wikipedia.org/wiki/Ligation_(molecular_biology))

⁴⁰ <https://nebiocalculator.neb.com/#!/ligation>

Multiple oligos with compatible sticky ends can be assembled to create longer fragments for insertion. In this case, add 1 uL of each set of oligos while maintaining the total volume of 10 uL.

4. Incubate at 16 C for one hour or at room temperature overnight for best efficiency.
5. *Transform competent cells* (page 175) with 5 uL of the ligation product.

Note: If you're skipping recovery, adding water up to 300 μ L total volume helps spread the transformed bacteria.

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/uL.

A quick calculation shows that 2 uL 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.

3.2.5 LR cloning

Estimated time: 1 hour to overnight for best efficiency

Protocol

Note: In most instances, a 5 uL reaction is sufficient for effective colonies.

1. Add 50-150 ng of entry plasmid and 150 ng of destination plasmid. Dilute with 1X TE buffer to 4 uL or 8 uL depending on whether you choose to perform a 5 uL or 10 uL reaction respectively.
2. Thaw LR Clonase II enzyme mix on ice for two minutes. Vortex the enzyme mix briefly (2 seconds each time).
3. Add 1 uL/2 uL clonase mix for a 5 uL/10 uL reaction.
4. Incubate reaction at room temperature for 1 hour to overnight.
5. **Optional, if transforming immediately:** Add 0.5/1.0 uL Proteinase K to each sample to terminate the reaction. Incubate at 37 degrees C for 10 minutes.
6. Transform competent cells with the reaction product.

Expected results

An efficient reaction will produce > 5000 colonies, if the entire volume is transformed and plated.

3.2.6 Oligo annealing for ligation cloning

This protocol describes how to anneal and phosphorylate oligos 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 and reconstitute as usual (e.g., order from Genewiz)
2. Combine the following in a PCR tube:

Reagent	Amount
Oligo (100 uM)	1 uL
10X T4 DNA ligase buffer	1 uL
T4 PNK	0.5 uL
Water	7.5 uL

T4 PNK = T4 polynucleotide kinase

Important: Use *original 100 uM stock* of oligos, not 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: 37°C for 1 hr, then heat inactivate the PNK at 65°C for 20 min.

Note: Phosphorylated oligos can be stored at -20°C

Oligo annealing

4. Combine 1 uL of each phosphorylated oligo (i.e., forward and reverse oligos from reaction above) in a PCR tube with 18 uL water, for a total volume of 20 uL.
5. 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.)

Note: Annealed oligo fragments can be stored at -20°C

6. Nanodrop annealed fragments and use in ligation reaction.

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 and reconstitute as usual (e.g., order from Genewiz)
2. Combine the following in a PCR tube:

Reagent	Amount
Forward oligo (100 uM)	1 uL
Reverse oligo (100 uM)	1 uL
10X T4 DNA ligase buffer	2 uL
T4 PNK	1 uL
Water	15 uL

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. Nanodrop annealed fragments and use in ligation reaction.

Note: When combined protocol is used, a 10X dilution of annealed oligos may be more effective in ligation reaction.

Note: Measuring the concentration of annealed oligos is not always necessary. 1 uL of the final annealed + phosphorlyated product (~0.5 uM) can be used in ligation asssemblies.

3.2.7 Touchdown PCR

This protocol describes one method to PCR a difficult to product. The idea is to start at a high annealing temperature (Ta) and go down so you start at the most specific product and amplify at less and less specific Ta's.

Touchdown PCR

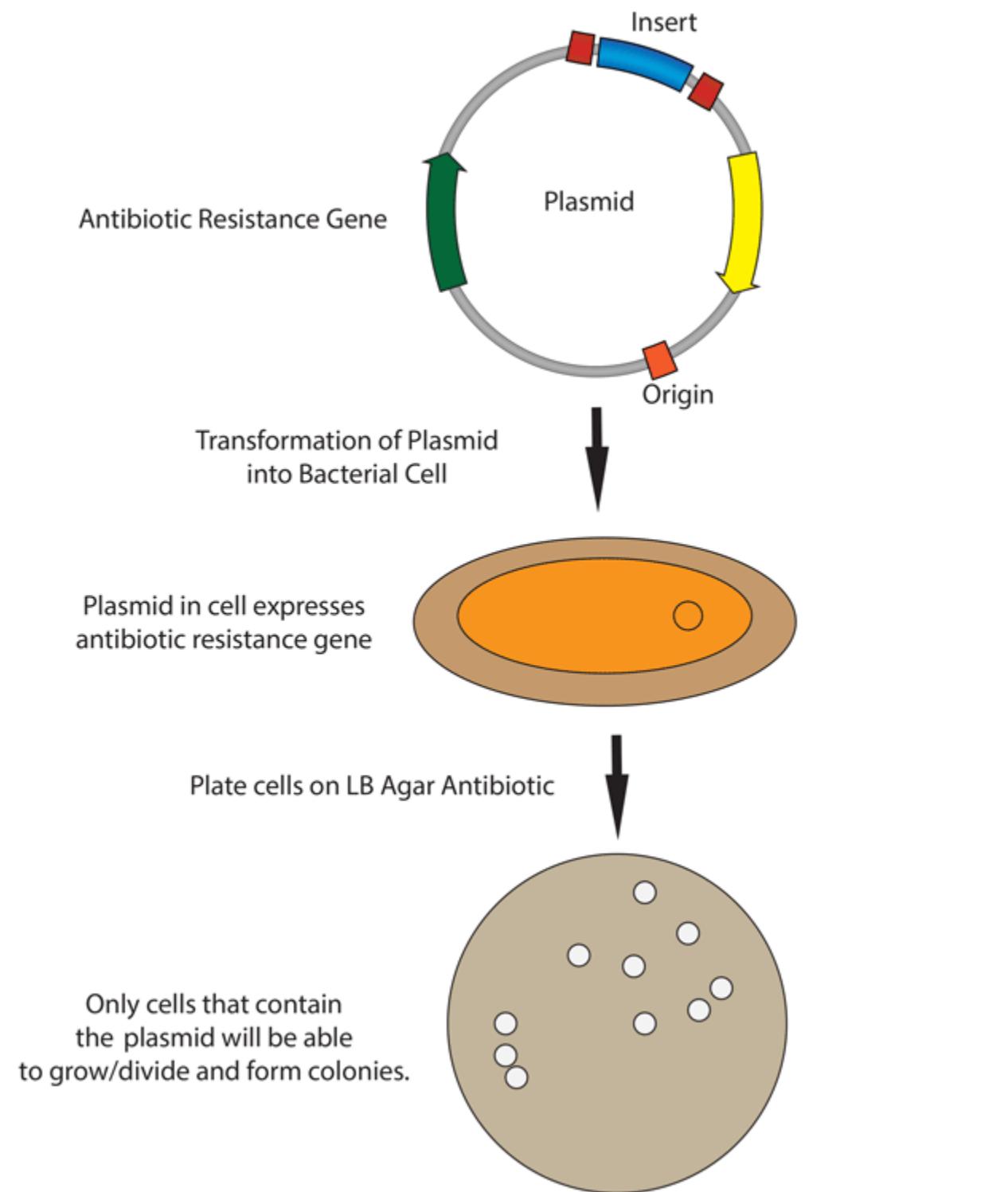
Cycle #	Ta
1	72°C
2	71°C
3	70°C
4	69°C
...	...
n	Predicted Ta
Repeat last cycle (30-n) times	Predicted Ta

Note: You could go even lower than the predicted Ta (we've gone down to 57°C as a robust protocol)

3.2.8 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 (Chemically Competent Bacteria)

Estimated time: 1 to 2 hours

1. Take chemically competent cells out of -80°C and thaw on ice (approximately 10 mins).
 2. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.
 3. Mix 1 - 5 μ l of DNA (usually 10 pg - 100 ng) into 20-50 μ L of competent cells in a microcentrifuge tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
-

Note: Transformation efficiencies will be approximately 10-fold lower for ligation of inserts to vectors than for an intact control plasmid.

4. Incubate the competent cell/DNA mixture on ice for 10 mins.
 5. (Optional) Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
-

Note: With the competent cells used in our lab, a heat shock step is not required but may increase transformation efficiency. For lower efficiency cloning strategies, such as ligation reactions, this can increase the resulting number of bacterial colonies.

6. Put the tubes back on ice for 2 min.
 7. Add 250-1,000 μ l LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 1 hour.
-

Note: This outgrowth step allows the bacteria time to generate 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 critical for Ampicillin resistance but is much more important for other antibiotic resistances. If skipping the outgrowth step, 300 μ L of Elga water can be added to help spread the mixture.

8. Add some or all of the transformation mixture along with sterile glass beads onto a 10 cm LB agar plate containing the appropriate antibiotic. Spread the mixture by gentle shaking in a lateral motion (shaking in a circular motion will cause bacterial colony growth only around plate edges). Discard used glass beads into designated box.
-

Note: If the culture volume is too big, gently collect the cells by centrifugation and resuspend in a smaller volume of LB so that there isn't too much liquid media on the agar plates. If the agar plate

doesn't dry adequately before the cells begin dividing, the bacteria diffuse through the liquid and won't grow in colonies.

9. Incubate plates at 37°C overnight.

Note: Use 30 °C if you are working with unstable vectors (e.g. repeats and/or viral vectors).

Tip: For most cloning strategies, 2 uL of the reaction mixture is sufficient to add. Ligation reactions are less efficient and may require up to 5 uL. Usually, using 30 uL of competent cells and 300 uL of SOC media (or Elga water if plating on 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 two harbors (pHAs) for IVT: one contains human beta globin 5' and 3' UTRs (pKG01864) and the other contains the 5' and 3' UTRs from the Pfizer-BioNTech COVID-19 vaccine (pKG01834).

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 Creating a new IVT template plasmid

As a pHA, pKG01864 is compatible with existing pPV2s for BsaI golden gate cloning.

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'-ctcgagctcaagttcgaattcac-3' VECTOR-REV: 5'-gaccggtagcgtgtttt-3'

3.3.3 Generating IVT linear DNA template from an IVT template 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 5' and 3' beta globin UTR, respectively. The forward and reverse primers also encode the T7 polymerase promoter and the polyA tail, respectively.

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
5x Q5 buffer	5
dNTP mix	0.4 μ L
CleanCap AG F-primer (10 μ M)	0.2 μ L
R-primer (10 μ M)	0.2 μ L
Plasmid Template DNA (1 ng/ μ L)	1.0 μ L
Q5 polymerase	0.1 μ L
Water	to 20 μ L

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.

3.3.4 modRNA synthesis

This procedure outlines modRNA synthesis with Cap 1 structure, where IVT and capping is conducted simultaneously using [CleanCap AG reagent⁴¹](#). 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](#))⁴², 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 RNase⁴³](#). Although I haven't encountered noticeable RNA degradation, it's probably a good idea to adhere to some of these general guidelines. I recommend using a new pair of gloves each time you enter/exit lab. Also, use a fresh barrier pipette tip when handling any of the reagents (nucleosides, buffers, enzymes) to prevent RNase contamination for other users.

⁴¹ <https://www.neb.com/protocols/2019/09/23/co-transcriptional-capping-using-cleancap-reagent-ag-from-trilink-and-hiscribe>

⁴² <https://www.neb.com/products/s1411-3-o-me-m7g5ppp5g-rna-cap-structure-analog#Product%20Information>

⁴³ <https://www.neb.com/tools-and-resources/usage-guidelines/avoiding-ribonuclease-contamination>

3.3.5 IVT reaction

The protocol for IVT is adapted from the NEB HiScribe protocol⁴⁴.

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 μ L):

Component	Amount
nuclease-free water	10.8 - X μ L (for 20 μ L final volume)
10X T7 IVT reaction buffer	2.0 μ L
ATP (100 mM)	1.2 μ L (6 mM final)
CTP (100 mM)	1.0 μ L (5 mM final)
GTP (100 mM)	1.0 μ L (5 mM final)
m1 ψ -UTP (100 mM)	1.0 μ L (4 mM final)
CleanCap AG (100 mM)	0.8 μ L (4 mM final)
Template DNA	100 ng, X μ L
Pyrophosphatase (U)	0.2 μ L
T7 RNA Polymerase Mix	2.0 μ L

Note: If performing multiple IVTs, one can prepare a mastermix. Dilute each template DNA to 5 μ L total volume, prepare mastermix using 5.8 μ L Nuclease-free water (based on one 20 μ L reaction), and distribute 15 μ L of master mix to each 5 μ L 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 your sample with DNase I if your application cannot tolerate residual amounts of DNA template. Add nuclease-free water to 50 μ L, add 1 μ L DNase I, and incubate at 37 C for 15 min.
5. Purify modRNA with NEB Monarch RNA Cleanup Kit⁴⁵ following manual provided in kit.
6. Keep the purified modRNA on ice to perform quality control analysis. Nanodrop to determine concentration (for a 20 μ L 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 μ L 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 ladder⁴⁶ to serve as a standard.
9. Run your denatured RNA samples on a gel to confirm product size.

⁴⁴ <https://www.neb.com/protocols/2021/10/12/mrna-synthesis-protocol-with-modified-nucleotides-using-the-hiscribe-t7-mrna-kit-with-modified-nucleotides>

⁴⁵ <https://www.neb.com/products/t2050-monarch-rna-cleanup-kit-500-ug#Protocols,%20Manuals%20&%20Usage>

⁴⁶ <https://www.neb.com/products/n0362-ssrna-ladder#Product%20Information>

Important: Ethidium bromide does not stain ssRNA very well, prepare your gel using SYBR safe!!

9. Aliquot modRNA into single-use tubes (scale appropriately for your experiment) and store in -80 C.

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.

3.3.6 mRNA transfection

We have used Thermo Lipofectamine MessengerMax⁴⁷ and Milenyibiotec StemMACS⁴⁸ reagents with good success in both immortalized cell lines and primary cells.

For each, we adapted the protocol from the manufacturer's protocol.

⁴⁷ https://www.thermofisher.com/order/catalog/product/LMRNA001?gclid=Cj0KCQiAm5ycBhCXARIsAPIdzoUQKASct6CpctkEyzxfPpOdj1xPJys2Td34cCGrxLcwaAseqEALw_wcB&ef_id=Cj0KCQiAm5ycBhCXARIsAPIdzoUQKASct6CpctkEyzxfPpObolE2SZKRp_dj1xPJys2Td34cCGrxLcwaAseqEALw_wcB:G:s&s_kwcid=AL!3652!3!535167329917!!!g!!!398236708!127976663191&cid=bid_clb_tfx_r01_co_cp0000_pjt0000_bid0000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!535167329917!!!g!!

⁴⁸ <https://www.miltenyibiotec.com/US-en/products/stemmacs-mrna-transfection-kit.html#130-132-949>

3.3.7 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 μ L MessengerMax per 100 ng mRNA) Scale up amounts of mRNA, reagent, and KO-DMEM solutions 5x for a 24-well (25 μ L total volume) and 12.5x (125 μ L total volume) for a 6-well.

1. Dilute .2 μ L MessengerMax Reagent in 4.8 μ L KO-DMEM, mix well, and incubate 10 minutes at room temperature.
2. Dilute 100 ng mRNA in KO-DMEM to a total volume of 5 μ L, and add to the MessengerMax/KO-DMEM mixture.
3. Incubate for 5 minutes at room temperature.
4. Add the 10 μ L of mRNA-lipid complex to desired 96 well.

3.3.8 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 μ L StemMACS per 100 ng mRNA) Scale up amounts of mRNA, reagent, and KO-DMEM solutions 5x for a 24-well (25 μ L total volume) and 10x (100 μ L total volume) for a 6-well (their recommendations are different from MessengerMax)

1. Add .3 μ L StemMACS to 4.7 μ L 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 μ L.
3. Add the diluted StemMACS reagent/buffer solution to the diluted mRNA and incubate for 20 minutes at room temperature.
4. Add the 10 μ L 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](#)⁴⁹.

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	

⁴⁹ https://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best

- **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

Casting protocol

Warning: The acrylamide monomers used here are toxic. Read the SDS⁵⁰.

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.

⁵⁰ <https://www.fishersci.com/store/msds?partNumber=BP14081&productDescription=ACRYLAMIDE%3ABISACRYLAMIDE+29%3A1&vendorId=VN00033897&countryCode=US&language=en>

3. Assemble them in the green alignment device.
4. Lock the two gels into the transparent gel pouring device.

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 P3143⁵¹.

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.

⁵¹ <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 186) 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 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 258) in Erlenmeyer flasks. For proper aeration, flasks should be filled to at most 30% the listed volume.
 3. Before leaving for the day, inoculate **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 an 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.

3.4.5 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%		
NaH ₂ PO ₄	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 Safety

Key to in-lab safety is everyone understanding what hazards.

3.5.1 Virus safety

The highest hazard that we have in lab is likely human-infectable lentiviruses. The lentiviruses we make are *replication-incompetent*, e.g. 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 cancer.
2. Oncogene expression: expressing known oncogenes from your constructs can cause cancer. Known or suspected oncogenes made in human-infectable lentiviruses falls under BSL2+. Only graduate student 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-infectable 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 us using engineering controls is 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.

You can reuse your sleeves through 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.

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** two days and three media changes have passed.

Specific protocols are:

Virus BSC setup

Important: The **most important** rule to follow when doing virus work is **double glove and use 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. Add them now if needed.
2. Add a secondary plastic bag to the waste container, to catch any liquid on tips or pipettes.
3. Make sure that you have all of the plates, tubes, filters and other things that you need. You may want to do the gelatin coating before you add any virus to your hood.
4. Make sure you have enough media in the hood. You may want to aliquot out of your 500mL container.

Virus BSC cleanup

1. For any liquid waste you have not aspirated (e.g. 10cm dishes you are done with), use a seriological pipette to add bleach to any waste plates/containers/tubes.
2. Spread the waste out slightly so all waste will get UV'd.
3. Remove your second pair of gloves, putting them in the biotrap trash container.
4. 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 not be contaminated)
5. Leaving all trash inside, close the sash on the BSC. Set a fifteen minute alarm!
6. Fifteen minutes later, with full PPE (double gloves and sleeves), 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 red waste containers.
7. Spray Preempt on the surface of the BSC. Wait one minute or longer, and then wipe up the Preempt by spraying ethanol. on the surface. Once the surface is looking nice, close the hood.
8. 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, especially if e.g. 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 e.g. 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 are going to urgent care / somewhere else to get it proscribed, then getting 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 are exposed, you should:

1. Make sure the BSC is in a safe state, e.g. nothing in precariously perched on something else.
2. Wash your hands and/or the exposed area of skin for ten to fifteen minutes with bleach.
3. If possible while washing your hands, inform someone else in lab to contact.
 - a. the EHS rep, Katie, or 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 inside 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 for an oncogene.
5. The prescription should be covered under even the base insurance that all students get.
6. Follow up with other people in lab to handle e.g. paperwork filling, 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-infectable lentivirus encoding for 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 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 Preempt 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 transfer the remaining virus to filter back into a separate tube, and use a second syringe. Do not apply more force than you can control.

3.6 TC protocols

3.6.1 TC Basics

TC Best Practices

When handling mammalian cells (aka tissue culture) always work in the Biosafety cabinet (BSC). 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.
- 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 (Sven).
- 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 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 run) 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 206).
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.

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 your cells with the media in them. Aspirate all liquids before putting plastics in the biohazard waste.

Finishing up work in the BSC

1. Put away all reagents, cultures, and other materials you brought into the BSC.
2. Discard trash in the biohazard waste bin (**NOT for BL2+ work**, see step 5 below); 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 ethanol or bleach through the aspirator tip after use.

Note: If you run concentrated (>10%) bleach through the aspirator, run sterile water through afterwards. Bleach residue can degrade the aspirator, especially the multichannel ones.

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: Pre-Empt is required after BSL2+ work, and should otherwise be used at least once daily.

5. Pull the sash down and allow the UV light to run for one cycle (minimum 15 min). Following the UV cycle, BSL2+ trash can be removed and placed in the biohazard waste bin.

6. Again, wipe the surfaces with Pre-Empt and ethanol following the removal of trash.
7. Check the liquid biowaste collection bottle. If contents looks pink, add more bleach.
8. Remove lab coat.
9. Discard gloves in biohazard waste containers (NOT the regular trash can).
10. Wash hands.

Note: If working with BSL2 (NOT BSL2+), you can skip step 6, wiping down with Pre-Empt after the UV cycle.

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. 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 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 μ L (500-1,000 μ L)
48-well	250 μ L (200-400 μ L)
96-well	100 μ L (100-200 μ L)
T-75	10 mL (8-15 mL)
T-182	25 mL (20-25 mL)

Source: General culture volumes⁵²

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.

⁵² <https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html>

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 μ L/well
12-well	300 μ L/well
24-well	150 μ L/well
96-well	50 μ L/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 209) 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 μ L 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 213).
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

- 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 207) steps 1-9).

- 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 μ L cells to 90 μ L media.

- Prepare the hemocytometer by placing the glass coverslip on top of the chamber.
- Pipet 10 μ L diluted cells into the notch on the chamber, such that the liquid is held between the metal and the coverslip.
- 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:

Galloway Lab Protocols

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.

$$\text{Avg # cells counted} / 100 \text{ nL} * 1e9 \text{ nL} / 1e3 \text{ mL} * 10x \text{ dilution} = \text{Avg # cells counted} * 1e5 / \text{mL}$$

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 212) 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 207) steps 1-9).
2. *Count the cells* (page 209).

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 μ L/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 μ L into each well of a 96-well plate).
5. Any excess cells can be *passaged* (page 207) to a new flask or *cryopreserved* (page 213).

Laminin Coating

Materials

- 100x Laminin (1 mg/mL; stored -20 deg C)
- PBS

Protocol

1. Add laminin to PBS.
2. Add 2 mL laminin-PBS to each 6 well plate.
3. Allow wells to set for 2-4 hours before removing excess liquid and plating cells.

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.

Culturing T-Rex 293 Cells

T-REx™ Cell Lines stably express 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/TOlacZ. 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/documents-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fflpinsystem_man.pdf>.

Culture the cells in 100ug/mL zeocin (1:1000 dilution master stock) and 5ug/mL blasticidin (1:2000 dilution master stock). Gelatin coating is not needed.

Mammalian cells cryopreservation and plating from frozen stock

Cryopreservation

1. Make cryopreservation (“freezing”) Media (should always be made fresh, lasts approx. 1 week)

Materials	Percent	Purpose
FBS	90%	FBS binds toxic materials released if some cells are lysed during the freezing or thawing process.
DMSO or sterile glycerol	10%	cryopreservative

- 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 absorption potentially toxic materials from the air.
2. Trypsinize, centrifuge, and resuspend cells in growth media with FBS (see Adherent Cell Culture)
 - Typically resuspend in 1-3 mL for a T75 or T185.
 - Optional: count cells with a hemacytometer to know how many cells are frozen per cryovial.
 3. Add cell suspension and freezing media to cryovial in a 1:1 ratio (typically, 0.5mL resuspended cells, 0.5 mL freezing media)

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 -80C 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

Materials

- Frozen vial of cells (i.e MEFs or HEK293Ts)
 - Growth Media (ex. DMEM with 10% FBS)
1. Resuspend 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 3-5 mL of growth media.
 4. Use 10 μ L 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 37C. These cells will take 1-2 days to recover.

Transient HEK293T Transfection

Note: If performing transient transfection for virus production: please use the updated *Virus production in HEK293T* (page 229) protocol for lentivirus production via HEK293Ts, and the *Plat-E Retroviral Production* (page 235) 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 folder⁵³ 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/well	1.33 mL/plate
DMEM/plasmid/well	

Example MM calculation (make 120% for extra):

Value	Example in a 96-well plate
Number plasmids/well	2 plasmids (<i>example</i>)
Total wells	50 wells (<i>example</i>)
Titrated PEI ratio	5 ug PEI : 1 ug DNA (<i>example</i>)
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):

⁵³ <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 (<i>example</i>)
Wells/condition	3 wells (<i>example</i>)
Plasmid volume/condition (110%)	$(10.8 \text{ ug/plate}) / (96 \text{ wells/plate}) / (0.100 \text{ ug/uL}) * (3 \text{ wells})$ $* 1.1 = 3.71 \text{ uL}$
Master mix/condition (110%)	$(\sim 27.7 \text{ uL/well KO DMEM}) * (3 \text{ wells}) * 1.1 = 91.41 \text{ uL}$

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 215).

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 206) (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.

Flow Cytometry

96-well plate spinning

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 trituriating, 1 for transferring to U-bottom). For a full 96-well, that means at least 2x200 μ L 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* (page 218).
7. Resuspend in ~200 μ L 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.

Cell staining (Live/Dead & surface markers)

Note: 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).

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 fluorescence intensity.

1. Begin with cell pellets, i.e. wash, dissociate, triturate, spin, and aspirate samples (e.g. *steps 1-5 above* (page 217) for a 96-well plate).
2. Resuspend cells in 100 μ L of staining solution.
 - Staining solution is made in FACS buffer (2% FBS in PBS).
 - 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.
3. Incubate for ~30 min at 4°C in the dark, on ice or in 4°C fridge.
 - Timing will require optimization.
4. Wash plate 3 times by centrifugation at 400 rcf for 5 min. If stained with unconjugated antibodies, repeat steps 1-3 but with staining solution containing secondary antibodies.

If staining for intracellular/nuclear protein targets, proceed with the following. If not, skip to step 14.

5. Remove supernatant and incubate cells in 3.7-4% paraformaldehyde (PFA) (in fume hood) for 15 min
 - EU calls for 3.7% PFA
6. Add 1 mL PBS and spin cold at 4°C to pellet cells
7. Aspirate solution and incubate cells in 0.5% Tween/PBS for 15 min to permeabilize
8. Add 1 mL PBS and spin cold at 4°C to pellet cells
9. Aspirate solution and incubate cells in 200 μ L primary antibody (diluted in blocking solution) for 1 hr in the rotator at 4°C .. note:: Some antibodies (e.g. Ki67) work better overnight in the rotator at 4°C
10. Add 1 mL PBS and spin cold at 4°C to pellet cells
11. Aspirate solution and incubate cells in 200 μ L secondary antibody (diluted in blocking solution) for 30 min in the rotator at 4°C in the dark
12. Add 1 mL PBS and spin cold at 4°C to pellet cells

13. 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 centrifuge follows RCF = $1e-4 * [\text{rpm}]^2 + 4e-2 * [\text{rpm}] - 6e1$, 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.

14. For immediate analysis:

a. Resuspend in ~200 μL FACS buffer (or PBS), 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)

b. Load plate into the Attune CytKick autosampler and run.

15. To preserve cells for several days:

a. Resuspend in 1-4% paraformaldehyde and incubate for 10-15 min at room temp.

b. Centrifuge your samples at 400 rcf for 5 min and resuspend in 200 μL of PBS.

c. Store plate at 4°C in the dark until analysis.

Glass slide cell culture

Warning: NBW hasn't gotten this to work well yet, the DRRR 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 212) to coat coverslip, incubate 10 min at RT
4. Aspirate excess gelatin and seed cells

Mounting glass coverslip

1. Carefully place a small drop ($\sim 10 \mu\text{L}$) 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 tweezers
 - Once the slide coverslip lifts a bit, **carefully** push the tweezers 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 Human iPSCs

Reagents

Media

Human iPSCs can be cultured in a range of media; in this lab, we mainly use *mTeSR-Plus*⁵⁴. The medium has two components: the basal medium and the 5X supplement. The supplement is stored at -20°C in 10mL aliquots in 50mL concials; the basal medium is stored at 4°C. To make the complete medium, add 40mL basal medium to the 10mL supplement aliquot; alternatively, add the

⁵⁴ <https://www.stemcell.com/products/mtesr-plus.html#section-protocols-and-documentation>

entire 100mL supplement to the entire 400mL basal medium. The complete medium is stored at 4°C; long-term stability at this temperature has not been tested, so aliquotting is preferred.

Some protocols recommend warming media to room temperature before use, but this does not seem to be necessary.

Dissociation Reagents

There are two main dissociation reagents we use:

1. [ReLeSR⁵⁵](#): non-enzymatic dissociation of cells in clumps; better for regular passaging so as not to disturb the cells too much
2. [Accutase⁵⁶](#): enzymatic dissociation to single cells; necessary for counting and seeding

Other Reagents

- [Geltrex⁵⁷](#): basement membrane for coating plates; 50X stored at -20°C in 120 μL aliquots, enough each for one 6-well plate (see [GelTrex Aliquoting](#) for more information)
- [ROCK inhibitor⁵⁸](#): Y-27632, a chemical inhibitor of ROCK activity used to prevent differentiation upon thawing and passaging; 1000X stored at -20°C
- DMEM/F12 for coating plates and spinning down cells
- PBS

Geltrex coating plates

Important: Plates require at least 1 hour to coat, so start early or make extras up to a week ahead of time!

1. Thaw Geltrex on ice (use the designated TC styrofoam for ice).
 - Thaw one tube (50X, 120 μL aliquot) per 6-well plate to coat. It is recommended to only do ~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 at 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.

⁵⁵ <https://www.stemcell.com/products/relesr.html>

⁵⁶ <https://www.sigmaldrich.com/US/en/product/sigma/a6964>

⁵⁷ <https://www.thermofisher.com/order/catalog/product/A1413301>

⁵⁸ <https://www.caymanchem.com/product/10005583>

4. Let the plate sit to coat:

- *If using same-day:* leave the plate at 37°C for at least 1 hour
- *If preparing ahead:* parafilm the plate, then leave it at room temperature overnight. Plates can be stored at room temperature for at least a week.

Note: The official recommendation is to store coated plates at 4°C for up to 2 weeks, and then equilibrate them at room temp before plating.

5. Immediately before seeding cells, aspirate the liquid from the coated well(s). Leave the liquid on any unused wells; these can be aspirated and used at a later date.

Thawing

Estimated time: ~30 min

1. Remove cryovial(s) of cells from liquid nitrogen and thaw quickly in the 37°C bead bath.

Important: Thaw cells quickly and spin down as soon as all ice is gone to limit the cells' exposure to DMSO.

2. Pipet the contents of the cryovial into a 15 mL conical with 10 mL of DMEM/F12. Optionally, pipet 1 mL of liquid from the conical around in the cryovial to remove any residual cells, then return the liquid to the conical.
3. Spin down cells at Xg (*speed TBD, 400g seems fine but ~210g has also been recommended*) for 4 min.
4. Aspirate the media and *gently* resuspend in 10 mL fresh DMEM/F12 to wash the cells again (iPSCs are sensitive to trace DMSO and may differentiate otherwise).
5. Spin down the cells and then aspirate the media. Aspirate the coating liquid from the wells to plate onto.
6. *Gently* resuspend the cells in the culture medium (i.e., mTeSR) with ROCK inhibitor (1000X stock) and plate. Typically, iPSCs are frozen such that 1 vial goes to 1 well of a 6-well plate.
7. Within 24 hours (e.g., the next day), media change to fresh mTeSR to remove the ROCK inhibitor. Then, culture as normal.

Note: Addition of ROCK inhibitor leads to cytoskeletal remodeling, so cells will have a spiky morphology the day after thawing. Let the cells recover for at least a day without ROCK inhibitor (i.e., 2+ days post-thaw) before passaging.

General Culturing Tips

- Always culture human iPSCs on Geltrex-coated plates (or other similar matrix coating).
- Typically, all culturing is done in 6-well plates. Use at least 2 mL of media per well.
- Change to fresh media *every 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 (e.g., 3 mL).
- Some amount of cell death is expected, but media changes every 24 hrs with enough fresh media should mitigate this.
- To maintain optimal cell health, be sure to passage cells before they are 100% confluent. Typically, healthy cells that are passaged 1:6 will need to be passaged again 2 days later.
- Splits of 1:6 to 1:12 are recommended. Larger dilutions may impede growth if the cells are too sparse, and lower dilutions will require very frequent passaging.
- iPSCs grow in colonies with a cobblestone-like cell morphology. The edges of the colonies should be smooth, not spiky. However, ROCK inhibitor remodels the cytoskeleton, so changes to spiky morphology are expected the day after thawing and passaging.
- Be extra attentive with sterile technique (i.e., don fresh gloves before beginning, use separate glass pipet aspirators for different cell lines) to avoid contamination.

Passaging with ReLeSR

Estimated time: ~15 min — don't forget to coat plates at least 1 hr ahead of time!

To passage for general cell culture maintenance, use ReLeSR to dissociate cells in clumps. This is less disruptive and may improve the long-term integrity of the iPSCs. For seeding or other applications that require counting, dissociate with Accutase to achieve a single-cell suspension (see *Dissociating with Accutase* (page 224) below).

1. Aspirate the old media. Gently wash with 1 mL PBS and aspirate.
2. Add 1 mL ReLeSR and incubate at 37°C for 1 min.
3. Immediately after the 1 min is up, aspirate to remove the ReLeSR. At this point, *no cells should be lifting off from the plate*.
4. Incubate the empty plate (it essentially has a thin film of liquid) at 37°C for 5 min.
5. Optional: while the plate is incubating, prepare fresh media with ROCK inhibitor (i.e., mTeSR complete medium + 1000X ROCK inhibitor). It is up to you to decide how/when to mix in the ROCK inhibitor, but it is convenient to prepare the media in a new conical.
6. When the plate is done incubating, add 1-3 mL media to each well and tap the plate to dislodge the cells.

- It is convenient to add 0.5 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.
 - 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). Pipetting up and down here is not necessary; rocking back and forth achieves sufficient mixing.
 - 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.
 - After dissociation, avoid excessive pipetting to maintain cell clumps.
 9. Within 24 hours (e.g., the next day), media change to fresh mTeSR to remove the ROCK inhibitor. Then, culture as normal.

Note: Addition of ROCK inhibitor 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 the ROCK inhibitor is removed.

Dissociating with Accutase

Warning: TODO

Freezing

1. Passage cells with ReLeSR through step 4 - incubate empty plate at 37 °C for 5 min.
2. Add 0.9 mL of media to each well, tap to dislodge cells.
3. Gently pipet up the liquid with a serological and dispense into a labeled cryovial. 1 well of a 6-well plate per cryovial.
 - Alternatively, dissociated cells from multiple wells can be pooled into a conical tube before aliquoting for freezing.
 - After dissociation, avoid excessive pipetting to maintain cell clumps.
4. Add 100 uL of DMSO to cryovial to achieve a final concentration of 10% DMSO.
 - If multiple wells were pooled in the previous step, add 100 uL of DMSO per well pooled, and then aliquot 1 mL of final mixture into labeled cryovials.

5. Transfer tubes to styrofoam boxes in -80 °C freezer for overnight freezing.
6. The following day, transfer frozen tubes to liquid nitrogen for longe term storage.

Plat-E Retroviral Transduction of MEFs

Plat-Es are a retrovirus packaging line that produce retroviruses that can only infect mouse 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.

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 212) 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 4X-6X every 2-3 days when culture reaches 70-90% confluency.

Tip: **Start growing MEFs today (2 days before seeding).** If they don't do well by the next day, this gives you 1 more day to defrost more.

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 5 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)

Component	Volume/ 1 rxn	Volume / 3.5 rxn	Total Vol/well*
Brn2 (4.49 $\mu\text{g}/\mu\text{L}$)	0.40 μL	1.40 μL	189.40 $\mu\text{L}/\text{well}$
Ascl1 (2.78 $\mu\text{g}/\mu\text{L}$)	0.65 μL	2.27 μL	189.65 $\mu\text{L}/\text{well}$
Myt1l (2.1 $\mu\text{g}/\mu\text{L}$)	0.86 μL	3.00 μL	189.86 $\mu\text{L}/\text{well}$
Ngn2 (4.4 $\mu\text{g}/\mu\text{L}$)	0.41 μL	1.43 μL	189.41 $\mu\text{L}/\text{well}$
Isl1 (6.2 $\mu\text{g}/\mu\text{L}$)	0.29 μL	1.02 μL	189.29 $\mu\text{L}/\text{well}$
Lhx3 (5.4 $\mu\text{g}/\mu\text{L}$)	0.33 μL	1.17 μL	189.33 $\mu\text{L}/\text{well}$
p53DD (3.93 $\mu\text{g}/\mu\text{L}$)	0.46 μL	1.60 μL	189.46 $\mu\text{L}/\text{well}$
HRasG12V (0.9 $\mu\text{g}/\mu\text{L}$)	2.00 μL	7.00 μL	191.00 $\mu\text{L}/\text{well}$
NIL (7.35 $\mu\text{g}/\mu\text{L}$)	0.24 μL	0.86 μL	189.24 $\mu\text{L}/\text{well}$

3. Add each KO DMEM + PEI + DNA mix to a single 6-well (1 WELL PER VIRUS) **DROPWISE** and evenly around the plate, rocking the plate back and forth, side to side to mix. Place back in incubator. *Rocking side to side prevents cells from clustering at the well edge.*

Day Three (Plat-E media change + seed MEFs):

1. Change with 1.25 mLs fresh media (*DMEM/HEPES + 10% FBS* (page 270)) after 24 hours.
Note: NBW transfets ~4pm and media changes ~10am next day to minimize PEI cytotoxicity.
2. 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	3	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 270)) to Plat-E plates for a second time.
2. PROCEED TO TRANSDUCTION!

Transduction of Mouse Embryonic Fibroblasts (MEFs)

Day Four (Plat-E media change + infect 1):

1. Transduce MEFs 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 μ L 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 μ m filter
 - i. For 6F, all 6 TFs (BAMNIL) will be filtered through the same syringe together to simplify adding them each individually to MM combos.
- 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 μ L
48-wells	250 μ L
96-wells	100 μ L

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 minimize filtering.

2. Examples of mixing AFTER filtering

- i. Example - 6F alone (96-well = 100 μ L total/96-well):

*For 1 rxn, 96-well: 66 μ L 6F (= 11 μ L PER FACTOR*6) + 34 μ L DMEM + 0.1 μ L polybrene (1,000X) = 100 μ L total/96-well*

For 3.5 rxn, 96-well: 231 μ L 6F + 119 μ L DMEM + 0.35 μ L polybrene (1,000X) = 350 μ L total for 3.5 96-wells

- ii. Example - 6F + DD + RR (96-well = 100 μ L total/96-well):

For 1 rxn, 96-well: 66 μ L 6F + 11 μ L p53DD + 11 μ L hRasG12V + 12 μ L DMEM + 0.1 μ L polybrene (1,000X) = 100 μ L total/96-well

For 3.5 rxn, 96-well: 231 μ L 6F + 38.5 μ L p53DD + 38.5 μ L hRasG12V + 42 μ L DMEM + 0.35 μ L polybrene (1,000X) = 350 μ L total for 3.5 96-wells

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 MEFs for a second day.

Day Six (1 dpi):

1. Change media on transduced MEFs according to transduction MM table (e.g. 100 μ L for 96-well)
2. If doing CFSE, do it now: *CFSE Labeling* (page 112)

Day Eight (3 dpi):

1. Media change plates to N3 media
 - a. N3 media = N3 base + BDNF/CNTF/GDNF (1,000X, 10 μ g/mL) + FGF (10,000X, 100 μ g/mL) + 2% FBS (*optional*)
2. **Spike in 1,000X RepSox to N3 media for RR conditions**

Day 10, 12, 14, etc:

1. 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 270)) instead of trypsin

3.6.2 Virus

Virus production in HEK293T

This protocol describes how to produce lentivirus and retrovirus in HEK293T cells, including a 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.

Scale and virus type selection

- Decide on a scale to perform the virus production. This method gives virus titers roughly high enough to infect an equal-area plate initially; each subsequent freeze-thaw cycle will reduce virus titer by roughly 50%. A 10cm dish is sufficient to infect about one and a half 96-well plates. Look up relevant values from the scale table.
- Decide on a virus type; this determines your packaging and envelope plasmids. Look up the packaging and envelope plasmids in the virus type table.
- For lentiviruses, use [this excel template](#).

Scale:

Scale	Area (cm ²)	Seeding amount (cells)	Total DNA mass (ug)	Collection media volume (mL)
10cm	56.7	7.5 x 10 ⁶	24	6.5
6-well	9.6	1.0 x 10 ⁶	4.1	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.

Virus type:

Type	Packaging plasmid	Envelope plasmid
Lentivirus	psPAX2 (pKG362)	pMD2.G (pKG096)
Retrovirus	piK-MLVgp (pKG015)	pHDMG (pKG022)

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 7.5-8.0 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) 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, but the transfection seems relatively insensitive to this ratio.
3. After waiting for 10-15 minutes, mix the following amounts 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.

Scale	Mass Transfer (ug)	Mass Packaging (ug)	Mass Envelope (ug)
Ratio	1	1	2
10cm	6	6	12
6-well	1.02	1.02	2.05

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.

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 H ₂ O	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!

Day 2, 3, ... (n - 1)

1. 24 hours after the last media change, collect the media, replacing it with the same volume of fresh HEPES-buffered DMEM. The collected media from separate days can be stored in the same 50mL conical tube. Keep the collected media at 4°C.

Note: Two collections give high enough virus titer to infect the vast majority (70%+) of cells. However, a third collection can also be taken to further increase titer.

Day (n)

1. On the last day of collection, either:
 - a. Proceed to the *Virus Concentration* (page 232) protocol if you want to store your virus long-term.
 - b. Filter the collected virus using a 0.45um filter, and 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.

For example, 100 μL of unconcentrated virus media (plus 0.1μL polybrene) can be added to each well of a 96-well plate to perform an infection.

Note: Unconcentrated virus appears to lead to more cell death than concentrated virus does. Therefore, concentrating the virus is advised.

Virus Concentration

Estimated time: 1 hour in-TC time, 1 day overnight time

1. Filter collected virus through a $0.45\mu\text{m}$ syringe filter into a 50ml conical. This eliminates any 293T cells that may have been carried over or collected in the media.
2. Add 1/3 volume of Lenti-X concentrator (e.g., for 30ml virus, add 10ml Lenti-X). Mix by inverting several times.
3. Store at 4°C for 24 hours (or overnight).

Note: If you plan to infect plated cells the next day after concentrating the virus, seed cells on gelatin-coated plates the same day as virus filtration and concentrator addition. Typically, this is 10K cells/well of a 96-well plate.

4. The next day, centrifuge at $1500 \times g$ at 4°C for 45 minutes (use the lower centrifuge). Be sure to use the caps/lids on the centrifuge buckets.
5. Aspirate the supernatant. There will be a little liquid left; this is okay.
6. Resuspend the pellet gently in the remaining liquid and transfer the entire volume to an Eppendorf tube.
7. Add media to the Eppendorf tube to reach the desired final volume (e.g., 100 or $200 \mu\text{L}$).
8. Use or store the concentrated virus. a. To use: Infect according to the *Transduction of concentrated virus* (page 232) protocol. b. To store: Store all or any extra virus remaining after infection in a cryovial at -80°C .

Transduction with concentrated virus

This protocol describes how to transduce cells with concentrated virus (see *Virus Concentration* (page 232) protocol). Virus produced in HEK293T cells can be used to transduce either human or mouse cells. The following calculations are for transduction in a 96-well plate; volumes can be scaled up accordingly for other plate sizes.

Calculate amount of virus to use

Before beginning, calculate the volume of concentrated virus to use per well.

As an initial rule-of-thumb, ~1-2% of the total virus produced in one 10cm dish (e.g., 2-4 μ L if concentrated and resuspended in 200 μ L total) may be appropriate for infection of one well in a 96-well plate. (FURTHER VALIDATION REQUIRED.)

Note: If it is important to know the titer of the concentrated virus, an initial infection (transduction) can be performed to calculate this value before conducting experiments. See the [MOI Calculation](#) (page 240) protocol for more details.

Note: Viral titer is reported to decrease during freezing and thawing. Therefore, it is recommended to double the amount of virus used for every freeze-thaw cycle. NOTE: VALIDATION IN PROGRESS.

Note: Generally, higher total virus per well increases infection efficiency.

Protocol

This protocol describes transduction of both [plated cells](#) (page 233) and [cells in suspension](#) (page 234).

Tip: Virus infection is more efficient if it is done at the same time as seeding (i.e., in suspension).

Warning: Since virus produced in HEK293T cells is able to infect human cells, be sure to use proper PPE (i.e., lab coats, disposable sleeves) and wipe down hoods with Pre-Empt after use.

Transduction of plated cells

1. The day before (18-24 hours prior) infection, seed cells on 0.1% gelatin-coated plates. Seeding densities may vary, but typically this will be 10k cells/well for a 96-well plate.
2. Dilute virus according to calculations in fresh media and polybrene (total volume = total well volume):

Component	Volume
Concentrated virus	calculated
1000X polybrene	0.1 μ L
Media	to 100 μ L

Tip: Since the amount of polybrene for a single well is so small, it is recommended to infect many wells or make a media-polybrene master mix.

3. Aspirate the media from the cells.
4. Add the virus-media mix to each well.
5. The next day (1 dpi), aspirate and replace with fresh media.

Note: Viral particles are no longer present after 3 days or 1 media change.

6. Continue culturing cells (e.g., select for integration) or proceed with end-point assay (e.g., flow cytometry).

Transduction of cells in suspension

1. Coat plates with 0.1% gelatin. Do this first to provide at least 20 min for coating (plates will be used at step 5).
2. Dissociate and count cells to infect.
3. Resuspend cells in fresh media at double the final concentration. For instance, if using 10k cells/well in a 96-well plate, resuspend at a concentration of 10k cells per 50 μ L.
4. Dilute virus according to calculations in fresh media and polybrene (total volume = **half** total well volume):

Component	Volume
Concentrated virus	calculated
1000X polybrene	0.1 μ L
Media	to 50 μ L

Tip: Since the amount of polybrene for a single well is so small, it is recommended to infect many wells or make a media-polybrene or cells-polybrene master mix.

5. Add virus-media mix (50 μ L or half well volume) to gelatin-coated plate. Then add cells (50 μ L or half well volume) and pipette to mix.

Tip: It is convenient to use a multichannel pipette to transfer the cells from a reservoir to the plate.

6. The next day (1 dpi), aspirate and replace with fresh media.

Note: Viral particles are no longer present after 3 days or 1 media change.

7. Continue culturing cells (e.g., select for integration) or proceed with end-point assay (e.g., flow cytometry).

Spinfection

After following the transduction protocols above for either plated cell or cells in suspension, additional spinning could increase infection efficiency. The combined protocol is dubbed “spinfection.”

For the spinning steps follow the below protocol.

1. Move plate into TC centrifuge.

Important: Ensure to cover the centrifuge buckets with the plate spinner tray caps.

2. Centrifuge the plate at 2500rpm for 90 min at 32C.
3. Subsequently transferred to 37oC incubator.

Plat-E Retroviral Production

Plat-Es are a retrovirus packaging line that produce retroviruses that can only infect mouse 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.

Note: Do a **10 µg/mL blast and 1 µg/mL puro selection every ~3 passages**. This is to ensure cells are expressing the retroviral structural proteins.

Plat-E Transfection

Day One (seed Plat-Es):

1. *Gelatin coat* (page 212) 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 4X-6X every 2-3 days when culture reaches 70-90% confluence.

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 μ L KO DMEM.
 - ii. SECOND, add 7.2 μ L PEI (1 mg/mL) for a 4:1 ratio (of μ g PEI: μ g DNA ratio).
 - iii. Gently flick to mix. Let sit for 5 minutes.
- b. For *3.5 rxns or wells of a 6-well plate*, same as above but use 630 μ L KO DMEM + 25.2 μ L 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 μ g DNA/well, (or 6.3 μ g DNA total for 3.5 rxns)
 - b. For example:

Component	Volume/ 1 rxn	Volume / 3.5 rxn	Total Vol/well
pMXs-mGL (500 μ g/ μ L)	3.6 μ L	12.6 μ L	190.8 μ L/well

3. Add each KO DMEM + PEI + DNA mix to a single 6-well (1 WELL PER VIRUS) **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 270)) after 24 hours.
Note: NBW transfets ~4pm and media changes ~10am next day to minimize PEI cytotoxicity.
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	3	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 270)) 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 minimize 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 is typically performed on the second day of transduction. Spinfection protocol is [here](#) (page 235).

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 5.0×10^5 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 214):

Plasmid	Amount of DNA
pVSV eGFP dG	5 μ g
pCAG-VSVN	1.5 μ g
pCAG-VSVP	2.5 μ g
pCAG-VSVL	0.5 μ g
pCAG-T7pol	5 μ g
pCAG-VSVM	4ug
pCAG-VSVG	4 μ g

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, transfet $2\mu\text{g}$ of pCAG-VSVG
 - If using a 10cm dish, transfet $10\mu\text{g}$ 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 transfer 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 $0.45\mu\text{m}$ 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.

MOI Calculation

Protocol

1. Seed 293T cells at 20,000 cells per well on a 96-well plate 1 day prior to infection.
2. Create a dilution of the virus stock in DMEM with 10% FBS

Example dilution for 1 column of 1 plate:

Row	DMEM	Added
1	30 uL	15 uL Virus stock
2	30 uL	10 uL Row 1 solution
...
8	30 uL	10 uL Row 7 solution

3. Add 30 uL of diluted virus + 70 uL DMEM with 10% FBS to each well
4. Record how many uL of the original virus stock was used in each well (can be calculated with [this excel file](#))
5. Count and record the cells in 1 well of the plate at the time of infection
6. 24 hours later, analyze the cells via [flow cytometry](#) (page 217)

Note: Rather than seeding the cells 1 day before infection, cells can instead be [infected in suspension](#) (page 234) to precisely control the number of cells mixed with the virus. In this case, it is convenient to combine diluted virus with media to a volume of 50 uL in each well, then use the multichannel to add 50 uL cells to each well of the plate.

3.6.3 SNAP circuit (Transient expression in 96-well plate version)

Materials

- Cells
- Plates
- DMEM supplemented with 10% FBS
- PEI
- Small molecule inducer such as guanine or O6-benzylguanine (check the concentration that you will need)
- Output construct: plasmid expressing reporter gene-ribozyme switch cassette (e.g, pHAGE-UBC-mGreenLantern-p2g6)
- Input construct: plasmid expressing SNAPtag conjugated with target protein (e.g, pHAGE-SNAP-tagBFP)

Procedure

Day 1: Seed cells

1. Seed the cells at a density of 2.5-4k cells/well in a 96-well plate
2. Incubate the cells at 37°C overnight

Day 2. Transfection

1. Transfect $100\mu\text{g}$ of the input and output plasmids per well with PEI by following the *general transfection protocol* (page 214). If your small molecule or the solvent has cytotoxicity, using a lower PEI:DNA ratio of 3:1 is recommended.
2. Incubate the cells overnight

Day 3: Small molecule treatment

1. Remove the media from the wells, being careful not to detach the cells, and add $100\mu\text{l}$ of fresh media that contains the small molecule inducer such as O6-benzylguanine or the solvent.
2. Incubate the cells for 48-72 hours at 37°C

Note: Be careful when changing the media for 293T cells—they easily detach, especially in a 96-well plate.

Day 5-6

Analyze the cells via microscopy or flow cytometry.

3.6.4 Cell Line Creation

Estimated time: 1 month

While the initial transfection does not take very long, the selection process takes a long time!

Note: Except for day 0, all steps are the same for the creation of PiggyBac, Crispr, or TALEN cell lines

This protocol is still in development! In particular, for PiggyBacs, the ratio of transposase to integration plasmid is a key variable that must be tuned!

An efficient selection method has also not been fully tested.

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 ng/mL (10,000x)
U2-OS	0.25 ng/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.

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.

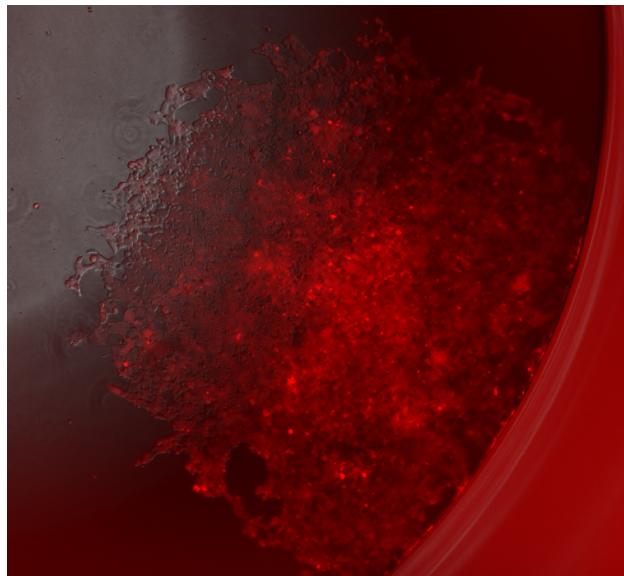


Fig. 1: Example of what outgrowth looks like when grown out from a single-cell dilution. This is one well in a 96-well dilution plate.

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).

Note: This assumes that you are sorting on the Sony in the flow core. You can review the [Sony SOP⁵⁹](#)

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

⁵⁹ https://docs.google.com/document/d/1toqMY_qnDy0_YDkcEr2ktDJWcteKe0Pj42_scukqT5s/edit?usp=sharing

- 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
 - 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-BLAST⁶⁰](#). This tool helps pick primers that anneal only to your target and not other genomic regions.

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 3a⁶¹](#).
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 μ L of Cell Lysis buffer supplemented with 0.5 μ L 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 μ L of supernatant as template for 20 μ L 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.

⁶⁰ <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>

⁶¹ <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5826598/>

3.6.5 Electroporation of plasmids into MEFs

Warning: This protocol has not been tested!

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 μ g pMax-GFP with MEF buffer 1, under setting A-23
- MEFs + 2 μ g pMax-GFP with MEF buffer 1, under setting T-20
- MEFs + 2 μ g pMax-GFP with MEF buffer 2, under setting A-23
- MEFs + 2 μ g pMax-GFP with MEF buffer 2, under setting T-20

The procedure for electroporation is as follows:

1. Add 100 μ l 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 2×10^6 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 store in the incubator at 37°C.
5. Trypsinize and count cells.
6. Divide cells into 50 mL tubes so that each tube has 2×10^6 cells. Spin cells down and remove media.
7. Resuspend cells in 100 μ l 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 μ l of pre-warmed media. Remove entire mixture and add to a 1.5 mL eppendorf tube.
10. Add 150 μ l 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% CO₂) for 24 hours, then examine cells under the fluorescent microscope to estimate confluence and percent GFP positive.
12. The experimental parameter that yields the best uptake efficiency and highest confluence 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 μ g 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 μ g pMax-GFP with MEF buffer 1, under setting T-20
- Cells + 3 μ g experimental plasmid with MEF buffer 1, under setting T-20
- Cells + 6 μ g experimental plasmid with MEF buffer 1, under setting T-20
- Cells + 10 μ g 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.

3.6.6 Transgene expression control through ribozyme switch (96-well plate version)

Materials

- Cells
- Plates
- DMEM supplemented with 10% FBS
- PEI
- DNA containing the transgene-ribozyme switch cassette
- Small-molecule inducer such as theophylline or guanine (check the concentration that you will need)

Procedure

Day 1: Seed cells

1. Seed the cells at a density of 2.5-4k cells/well in a 96-well plate
2. Incubate the cells at 37C overnight

Day 2: Transfection

1. Transfect $100\mu\text{g}$ of a plasmid expressing transgene-ribozyme switch cassette per well with PEI by following the [general transfection protocol](#) (page 214). 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 37C for 4-6 hours
3. Remove the media from the wells carefully to not lose the cells, and add $100\mu\text{l}$ 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 hpt, 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 hpt 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.

**CHAPTER
FOUR**

RECIPES

4.1 Bacterial recipes

4.1.1 LB (Amp/Kan) plate

1. Dissolve 32 g of LB agar powder in 800 mL of sterile/ELGA water. (4g of LB agar powder in 100 mL)
2. Autocleave it for 20 min at 121C.
3. Cool it to about 45-50C by letting it sit on a bench (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.

1. Add 800 uL of 1000x antibiotics to the autocleaved LB agar-water.
2. Dissolve the antibiotics by stirring with a stirrer.
3. Pour 30-35ml of the antibiotics-containing LB per a petri dish.

Tip: You can use a pipette but the LB can easily become solid so pouring LB by hand might be a good alternative method.

8. Incubate the plate at RT until the LB agar become solid (normally it takes about 30-45min).
9. After the LB agar become solid, put the plates back to a bag and store it at 4C.

4.1.2 Antibiotic Stock Recipes

1000X Ampicillin (AMP)

1. Dissolve 100 mg of Ampicillin powder in 1 mL sterile/ELGA water (Or 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 -20C

1000X Kanamycin (KAN)

1. Dissolve 50 mg of Kanamycin powder in 1 mL sterile/ELGA water (Or 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 -20C

1000X Chloramphenicol (Chlor)

1. Dissolve 34 mg of Chloramphenicol powder in 1 mL 70% EtOH (Or 340 mg of Chloramphenicol powder in 10 mL 70% EtOH)
2. No filtration step needed.
3. Make 1 mL aliquots and store at -20C.

4.1.3 Competent cells (NEB stable)

Day 1. Culture NEB stable cells for overnight.

Take the glycerol stock of NEB stable from -80C freezer (Elsa) and pick the cells and culture them with 2ml of plain LB at 30C for overnight.

Day 2. Grow the competent cells and stock the cells.

1. Take the 0.5ml of the overnighnt NEB stable cells from Day1, and put the cells into 50ml of plain LB and culture them @ 30C until the OD(600nm) reaches 0.4-0.6 (use nanodrop to measure the OD).

Note: Usually, it takes 2-2.5 hours to the OD(600nm) reaches 0.4-0.6 when the cells are cultured at 30C.

1. Once the OD(600nm) reaches to 0.4-0.6, place the cell on ice for at least 10min.
2. Prepare 5ml of 1xWash Buffer and 5ml of 1xCompetent Buffer by diluting the 2x solutions with Dilution Buffer.
3. From step 2, after 10 minutes incubation on ice, pellet the cells by centrifugation at 3000xg for 10min. at 4C.
4. After the cenritugation, remove the supernatant completely and suspend the pellet GENTLY with 5ml of ice-cold **1xWash Buffer** prepared at step 2.
5. Re-pellet the cells by centrifugation at 3000xg for 10min. at 4C.
6. After removing the 1xWash Buffer COMPLETELY by transferring the supernatant to an another 50ml falcon tube (**see below how to discard the wash buffer**), GENTLY suspend the cells with 5ml of ice-cold **1xCompetent Buffer**.

Important: Add a few ml of 10% bleach to the collected 1xWash Buffer and centrifuge the tubes at 3000xg for 3-5min.. Then, you can discard the supernatant into a sink and the ppt to a garbage box.

8. Aliquot 100ul of the cell suspenstion (ON ICE!) .
9. Store the cells @ -80C (ELSA)
10. To make sure if the competent cells are not carrying any plasmid, culture the competnet cells made at step 8. with 2ml of LB-Kan, LB-Amp, and, LB-Amp-Chlor for about 2days at 30C.

Day 4. Confirm the cell growth

1. Confirm if the competent cells were not growing in any of the media. If you see cell growth in either LB media, discard the competent cells you made and remake the cell from step 1.

4.1.4 DNA wash buffer

This wash buffer can be used for the DNA cleanup washes or the second miniprep wash.

DNA wash stock solution (5x)

Ingredient	Amount (per 80 mL)
80 mM NaCl	0.374 g
8 mM Tris-HCl	0.101 g
H ₂ O	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 258) 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
KH ₂ PO ₄	0.17 M	23.1 g
K ₂ HPO ₄	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 25 g of LB Broth powder with deionized water to a final volume of 1 L.
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 FISH recipes

4.2.1 FISH buffer recipes

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
H2O	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
H2O	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
H2O	Bring volume up to 10 mL

2. Bring the pH to 6.8

5X SSCT

1. Combine the following components

Ingredient	Amount
20X SSC	20 mL
10% Tween 20	400 uL
DEPC H2O	Bring volume up to 40 mL

1. Store at -4 degrees C, expiration in 1 month

4.3 TC recipes

4.3.1 FBS heat inactivation

- **Heat Inactivation (HI):** a process by which serum is maintained at a temperature of $56 \pm 2^{\circ}\text{C}$ 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.

4.3.2 Common aliquot sizes for TC

1,000X Neurotrophic factor stocks

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
BDNF	R&D Systems 248-BDB-050
CNTF	R&D Systems 257-NT-050
GDNF	R&D Systems 212-GD-050
FGF	PeproTech 100-18B

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 $\mu\text{g}/\text{ml}$ (10kx) master stock

For FGF only

3. Make 10 μL of 10kx master stock aliquots for long term -80°C storage
 - You'll end up with ~50 of 10kx aliquots

For BDNF, CNTF, GDNF only

4. Make 90 μL of 10kx master stock aliquots using for long term -80°C storage
 - You'll end up with ~5.5 of 10kx aliquots
5. For BDNF, CNTF, GDNF only, directly add 810 μL filtered 0.1% BSA/PBS to the 90 μL 10kx master stock to make 10 $\mu\text{g}/\text{ml}$ (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 .
6. Make 50 μL 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

Property	
Selleck Chem #	S7223
MW	287.32
1000x stock	7.5 mM
Final concentration	7.5 uM

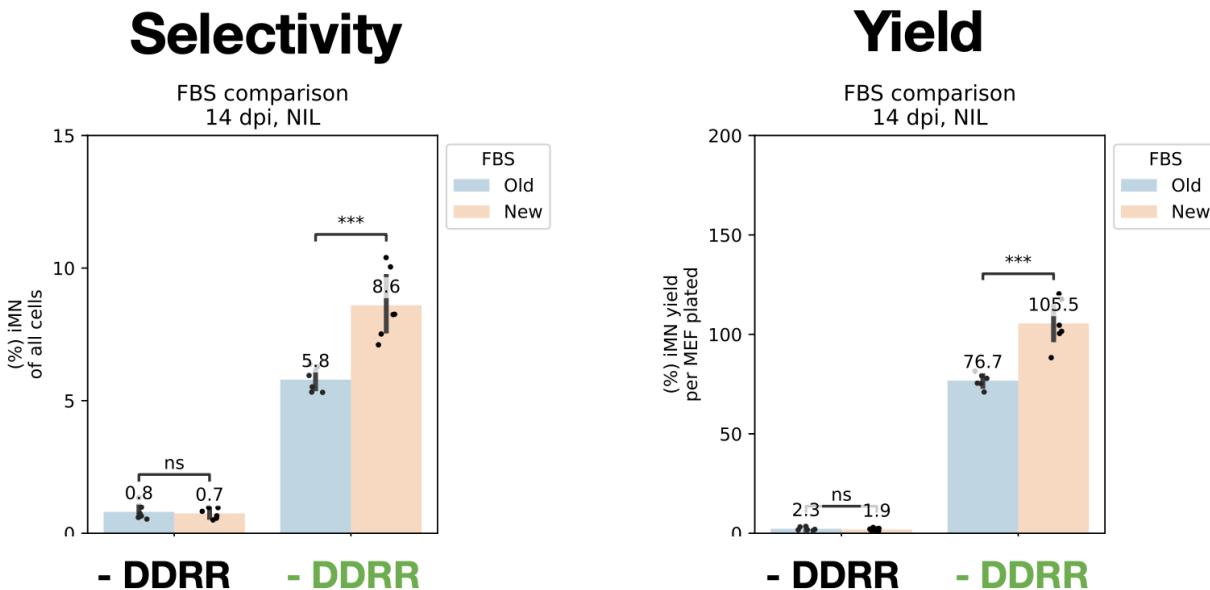
1. To make working 1,000x stock (7.5 mM), resuspend 25 mg RepSox in 11.6 mL DMSO.
2. Aliquot 50 uL in 600 uL tubes and store at -20°C in a labeled box.

Fetal Bovine Serum (FBS) Aliquots

- 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 270) (just add 3 mL DMSO)

Note: This is now a lab job. As of 3/15/23, notify Nat 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 225). Use the sample FBS to defrost the MEFs, make the Plat-E virus, and do the infection until 4 dpi. Assess 14 dpi reprogramming between the two lots. You will have to *heat inactivate* (page 261) 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 current FBS.** An example of results



100X Pen-strep aliquots

- 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 A1413302⁶²) should be stored at -80°C until aliquoting
2. Thaw the new vial overnight at 4°C
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.

⁶² <https://www.thermofisher.com/order/catalog/product/A1413301>

6. To use, further dilute a 120 μ L 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-Artifacts/LSG/Application-Notes/stem-cell-transfection-guide-app-note.pdf>

4.3.3 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%) (<i>optional</i>)	1%	5 mL
FBS (<i>optional</i>)	2%	10 mL
DMEM F12		500 mL

Add the following small molecules to small volumes immediately 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 (<i>optional</i>)	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 (<i>for collection only</i>)	10%	5 mL
DMEM/F12	89%	44.5 mL
100X Pen/Strep	1%	500 μ L

Motor neuron dissociation media

Used for dissociating iMNs or primary motor neurons (embMN) harvested from spinal cords for plating/sorting. Add 50 μ L/96-well, let sit in 37°C for ~15 min. Lightly tap plate 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 μ L	90%
DMSO	100 μ L	10%

- It is easy to keep a 4°C stock of (*80% FBS/20% DMSO* (page 264)) then use 500 μ L 80%/20% FBS/DMSO + 500 μ L 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 μ m filter after pH-ing)

Component	Concentration	Amount/50 mL
HEPES	1M	13.82 g
DI H ₂ O	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

4.3.4 Other TC Solutions

Phosphate buffered saline (PBS)

1. Dissolve 8 tablets of PBS tablets into 2L of ELGA water in a large beaker (1 tablet/250mL) 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 7.4 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.

4.3.5 PEI Stocks

Materials

- Linear PEI (located in “Chemicals” tray in Oaken 4°C)
- 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. Total acid required is ~1 mL of 2.5M 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 Elsa (-80°C, top shelf). Determine the optimal PEI ratio before use.

Test PEI Ratio

Warning: TODO

Reference

<https://www.addgene.org/protocols/transfection/>

4.3.6 Small molecule stocks for TC

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 uM 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 uM filter.
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 uM 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 uM filter.
3. Separate into 50 uL aliquots and store at -20°C.

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.

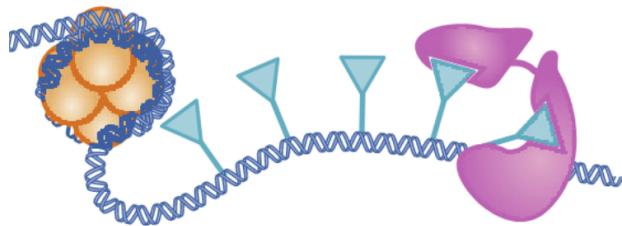
5.1.2 Problematic features

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:



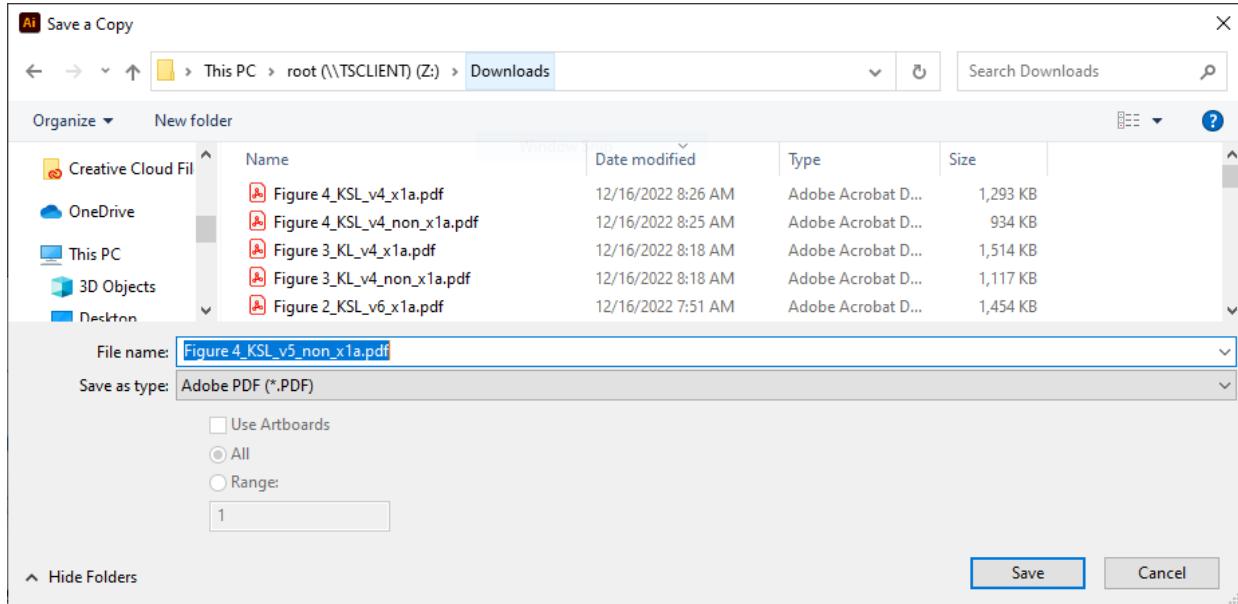
Gradients/blurs

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.

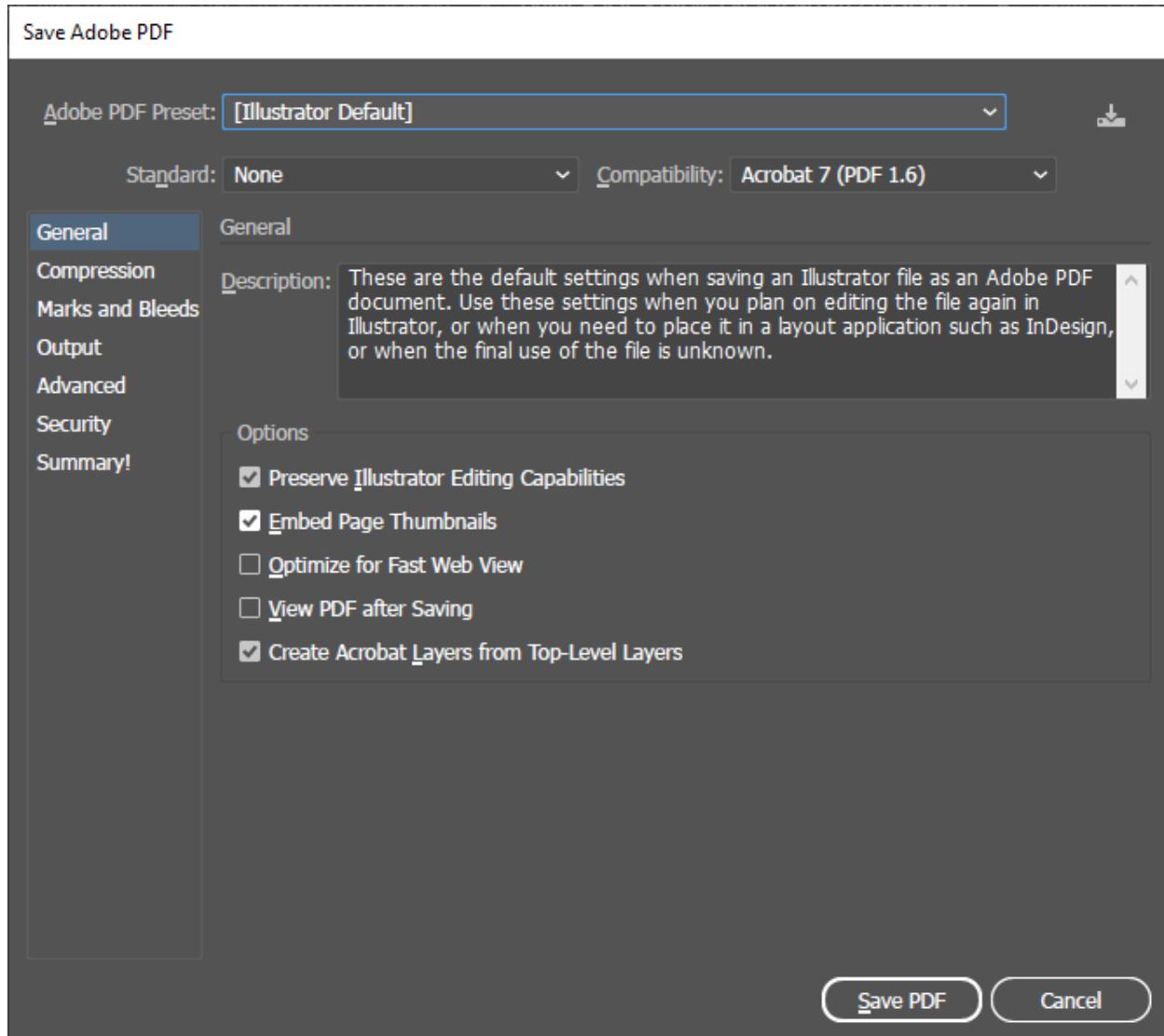


When you get to the export screen, make sure you select *[Illustrator Default]* as the output type:

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.

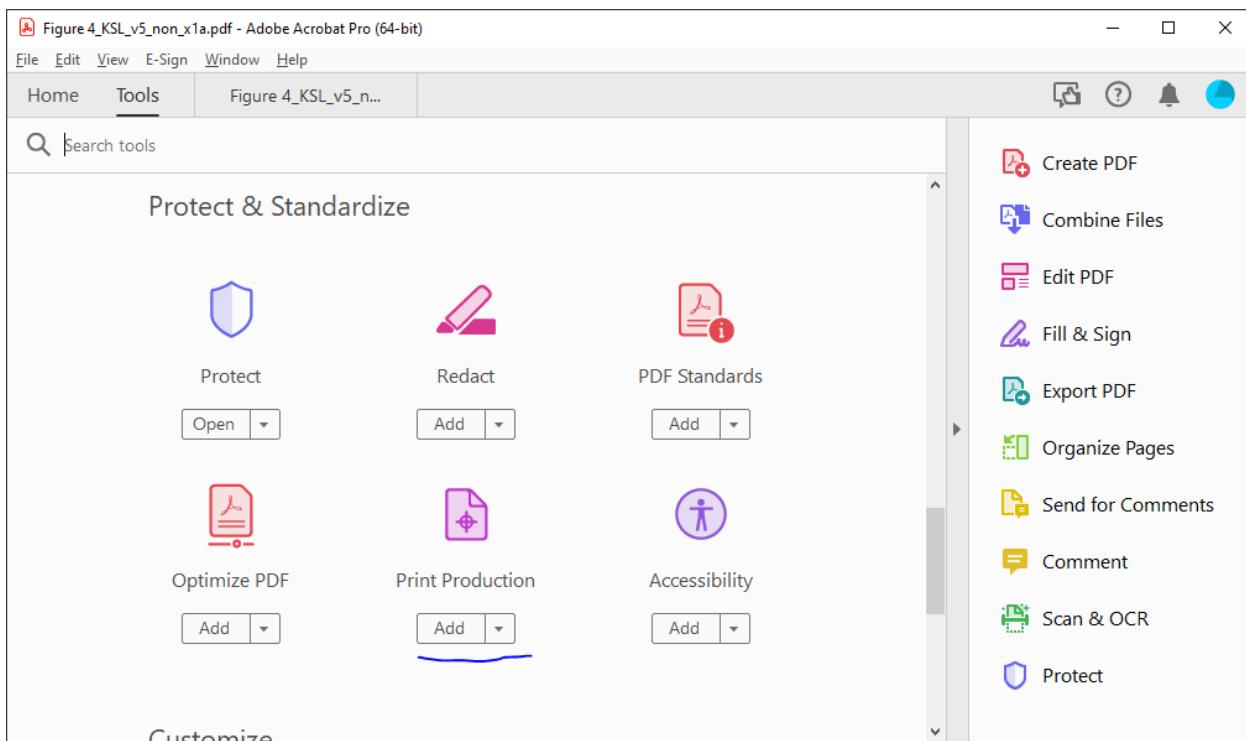
Matplotlib/Python

Doing `plt.savefig` with a `.pdf` filename is sufficient.



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.



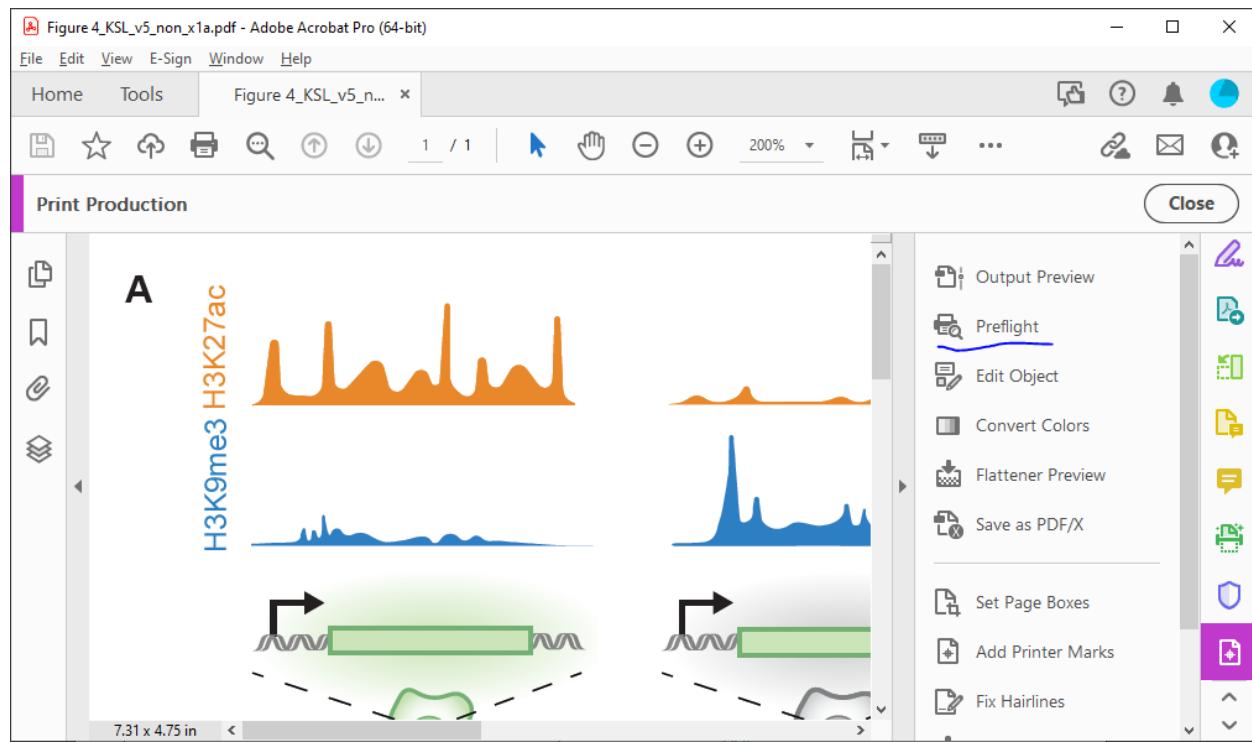
3. Click Print Production to bring up the tools, and hit “Preflight”:

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 transparency 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.

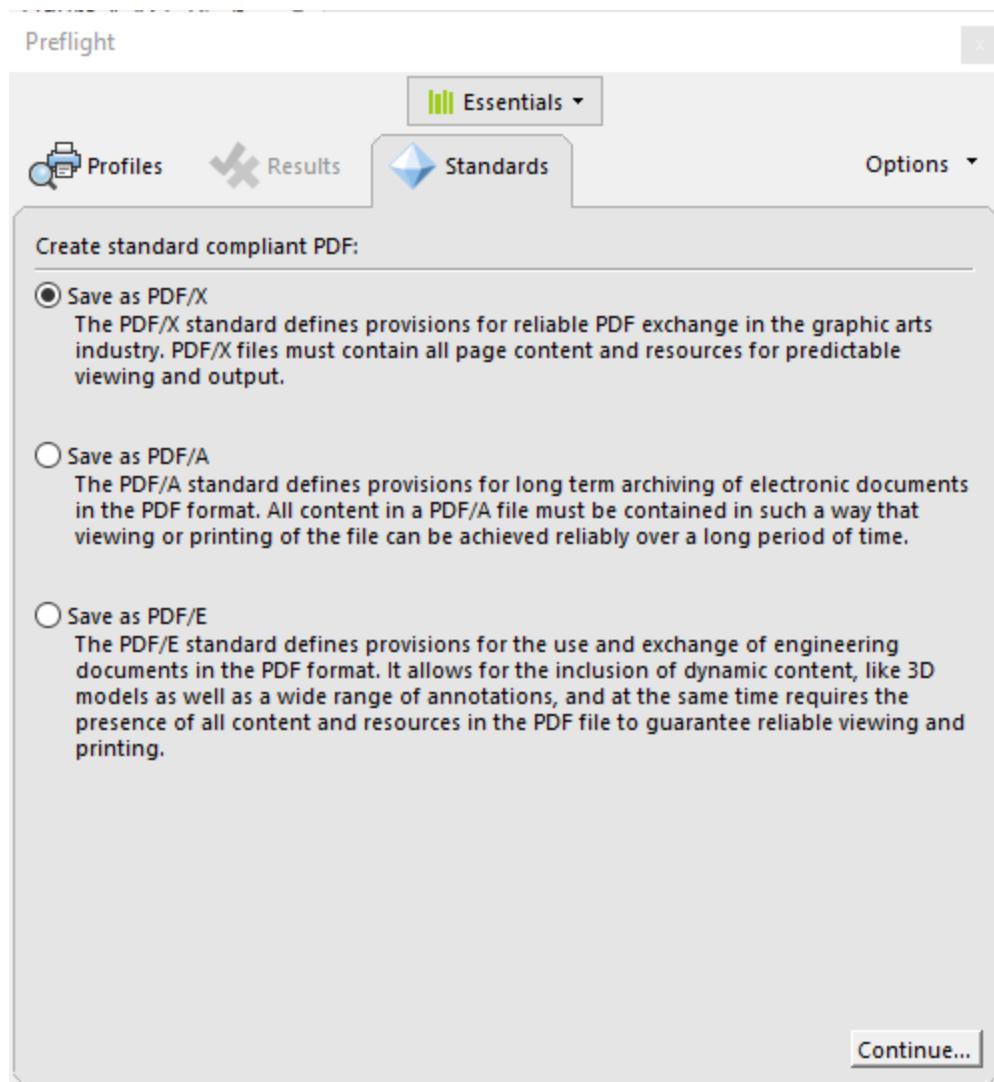
5.2 Flow plots with adjunct histograms

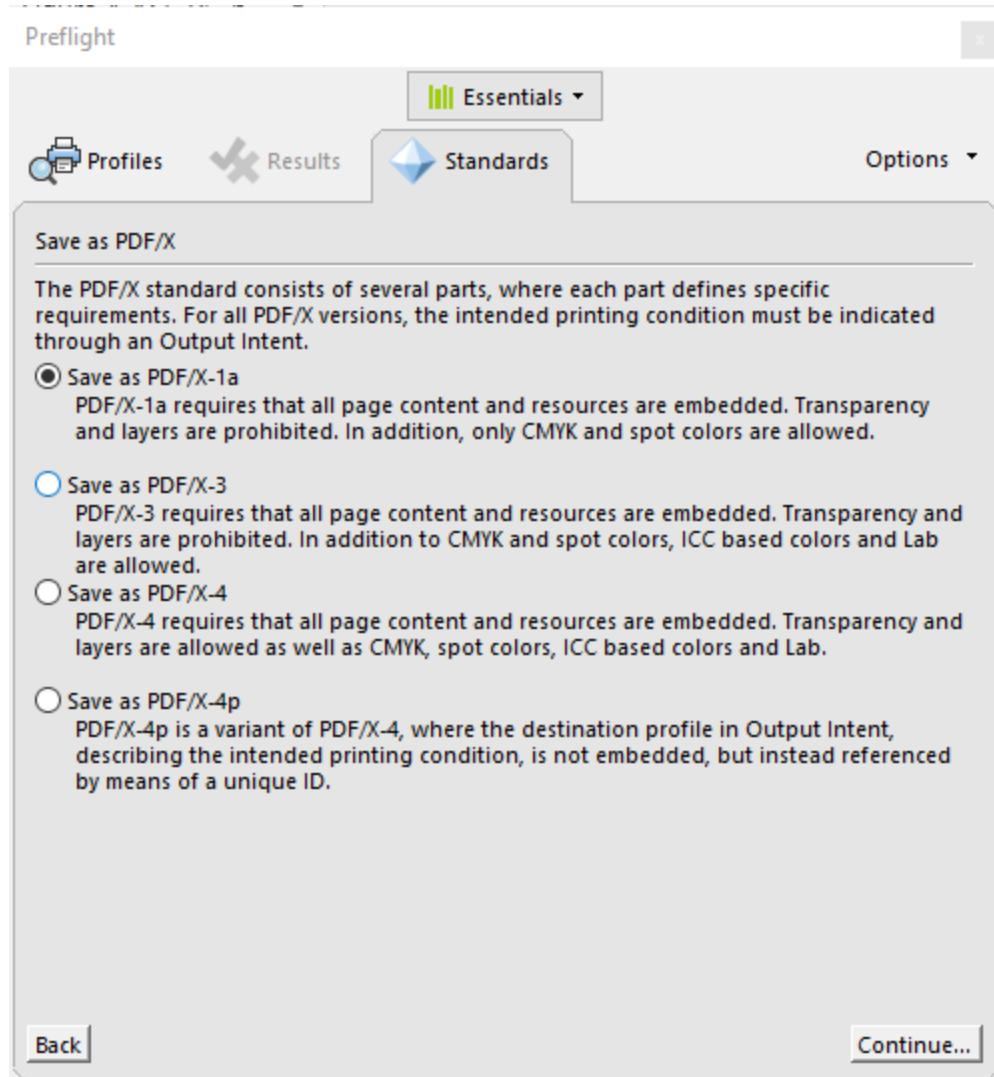
```
# Read in data
data = pd.read_csv('data/data_flow_with_adjunct_hist/data_flow_with_adjunct_hist.
˓→csv')

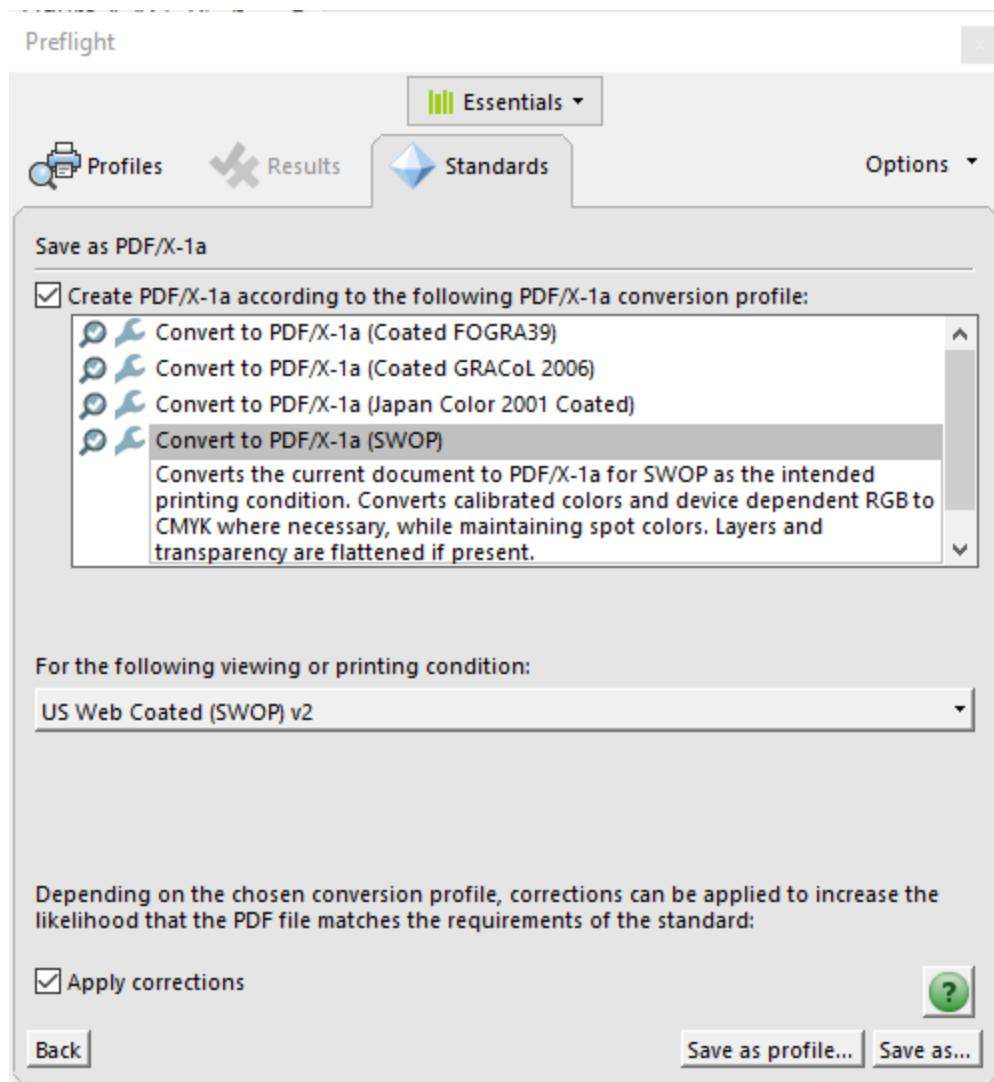
# Randomly sample 10^4 samples from each condition to make representative flow
˓→diagram
numSamples = 10**4
small_data = data.groupby(['cond']).sample(n=numSamples, random_state=1)

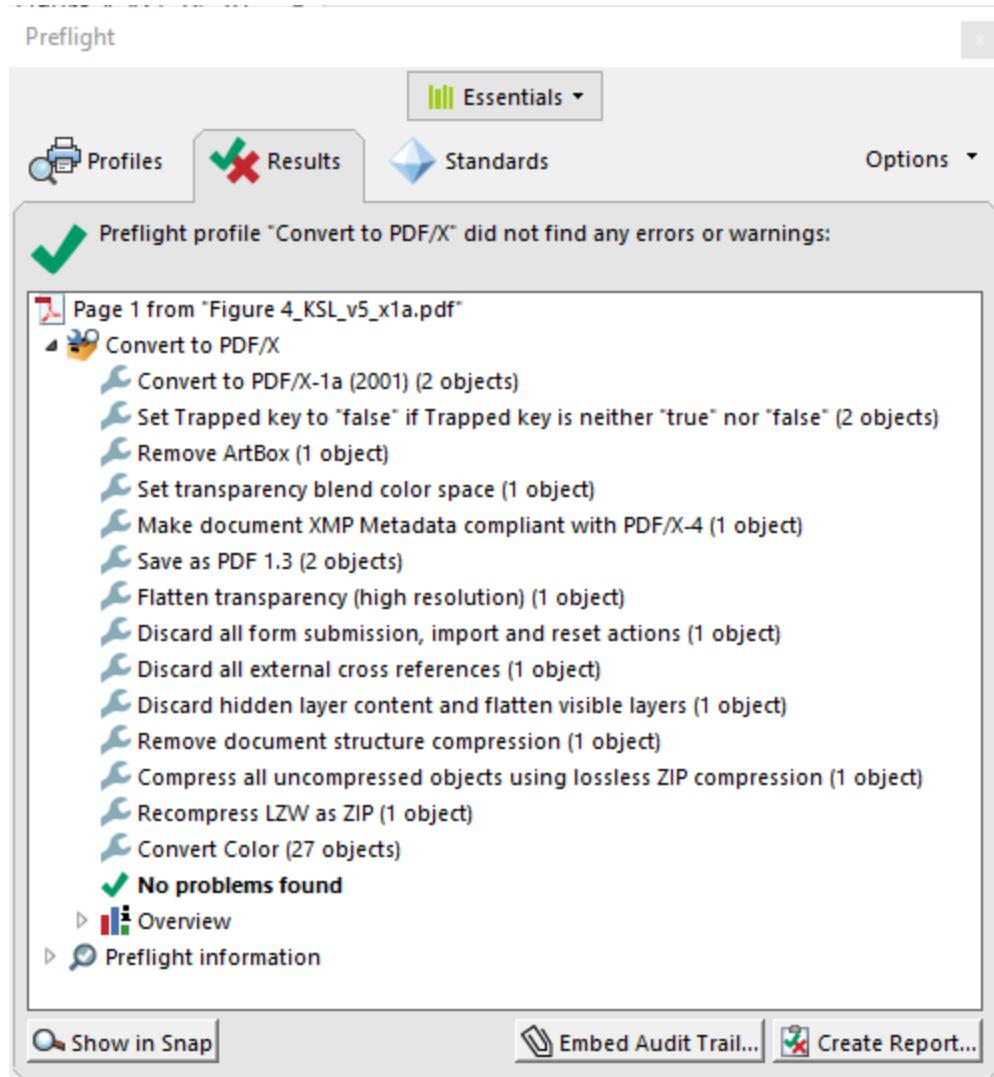
# General plotting params
```

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```

x = 'CFSE-A'
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,

```

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```

    palette=colormap, alpha=0.1, fill=True, legend=False)

# 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

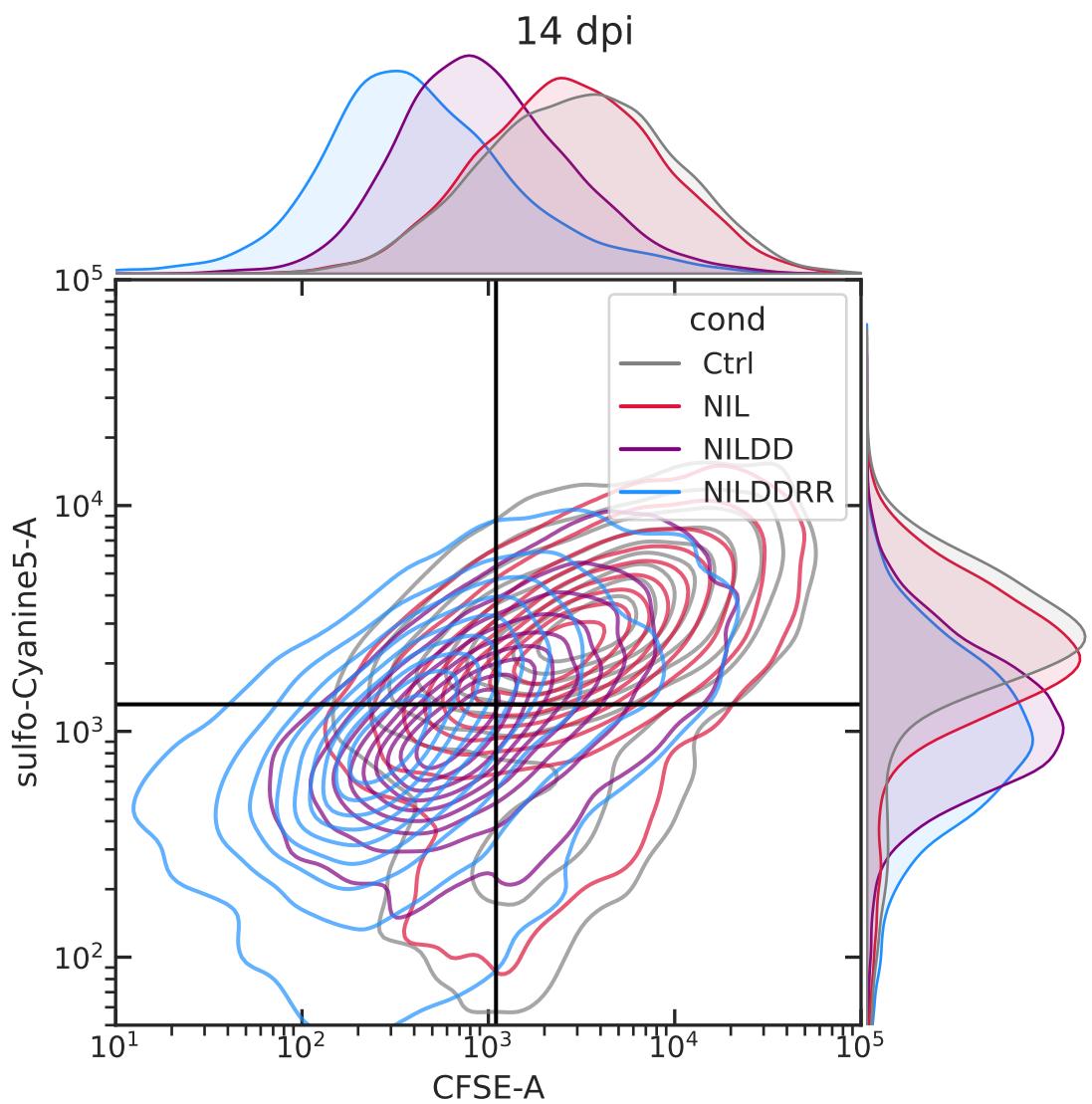
```

# ----- 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']})

```

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```

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
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 -----

```

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```

# ----- 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)

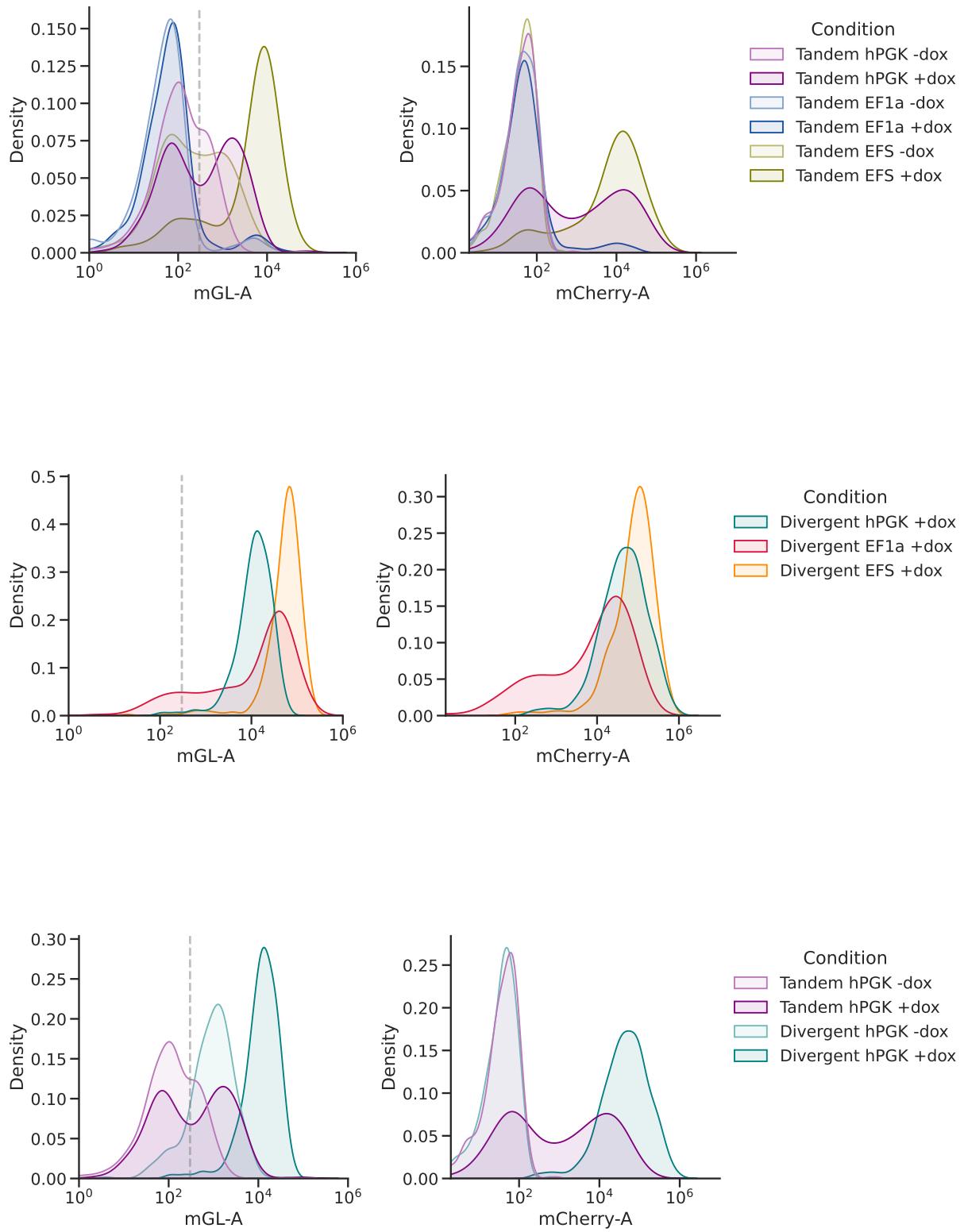
# Dictionary of plots (separate figures) to generate
# keys = short name that plots are saved under
# values = list of samples (values in 'Condition' column) to include in the plot
plot_list = {
    'tandem': ['260_False','260_True','261_False','261_True','262_False','262_'
↪True'],
    'divergent+dox': ['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_WPREGATE/data_mGL_WPREGATE.csv')

# 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)

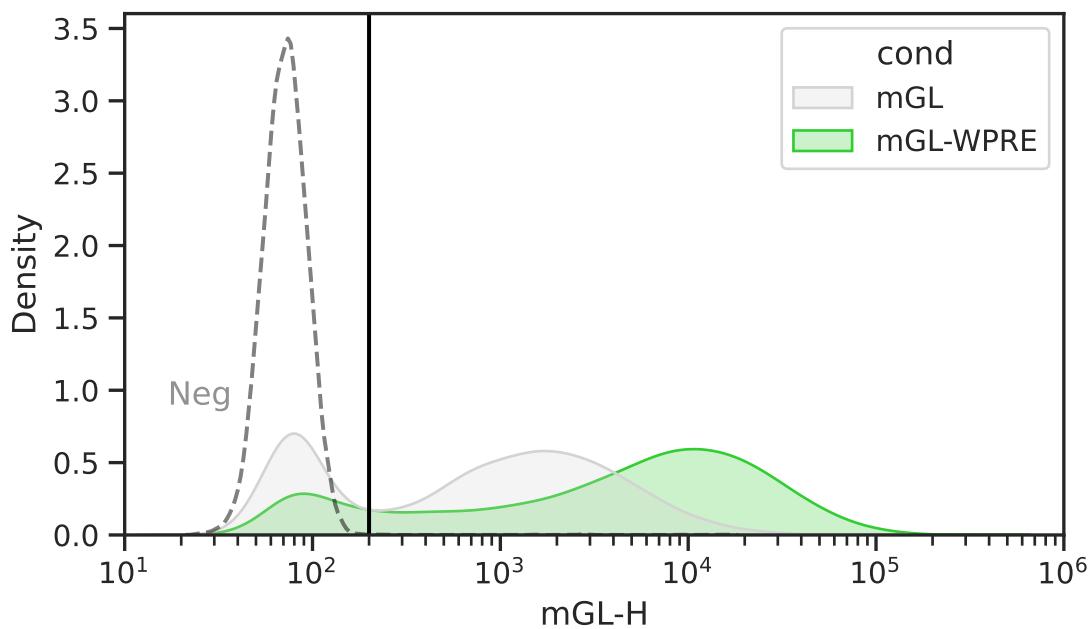
# Plot neg ctrl
sns.kdeplot(ax=ax, data=data.loc[data['cond'] == 'Neg'], x=x,
             log_scale=(True, False), common_norm=False,
             shade=False, color='black', alpha=0.5, linestyle='--')
ax.annotate('Neg', (0.08, 0.25),
            xycoords='axes fraction', alpha=0.5, ha='center')

# 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



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,

```

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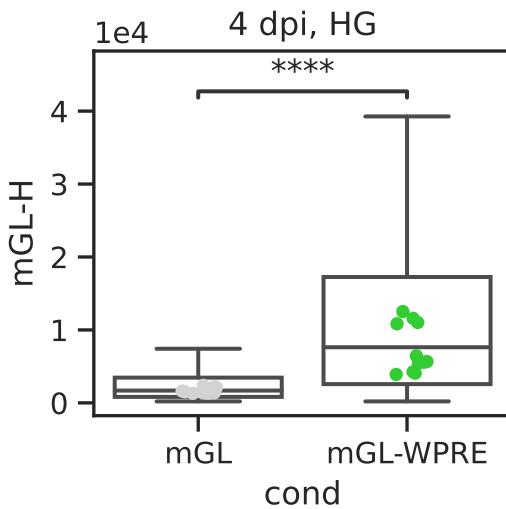
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```
x=x, y=y+' (gmean)', order=order,
dodge=True, palette=colormap, size=5)

# 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)'])

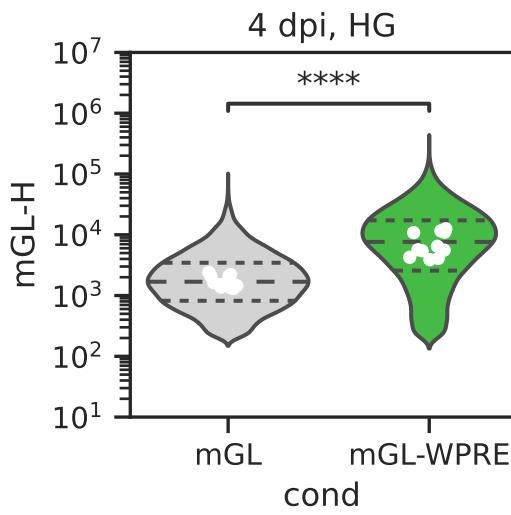
# Plot
fig, ax = plt.subplots(1, 1, figsize=(3, 3))
# Plot all points as violin
sns.violinplot(
    ax=ax, data=data_mGL,
    x=x, y='log({})'.format(y), order=order,
    palette=colormap, inner="quartile")
# Plot log10 transformed -> well geometric means of mGL-A as points
sns.stripplot(
    ax=ax, 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
# 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 = {
```

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```

'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]})

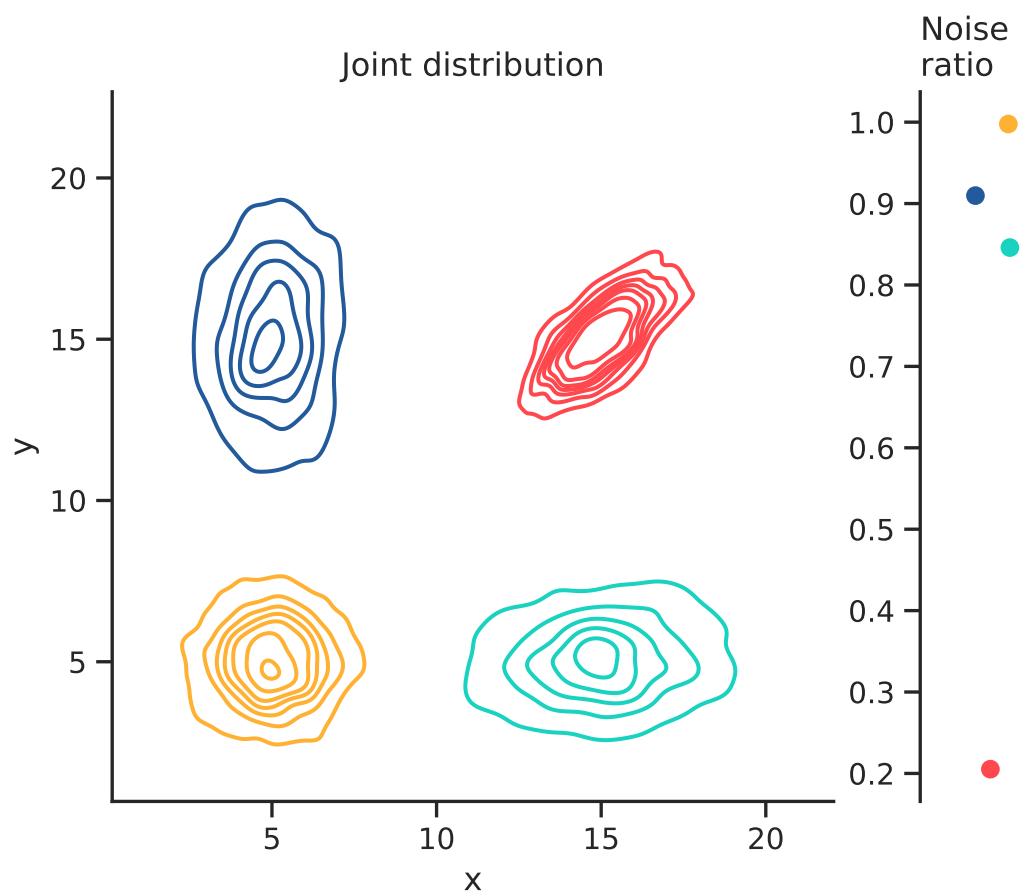
# Use Seaborn to plot the joint distributions.
sns.kdeplot(data=df, x='x', y='y',
    hue='condition', palette=beach_towel,
    levels=8,
    ax=axes[0], legend=None)

# 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]
    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])

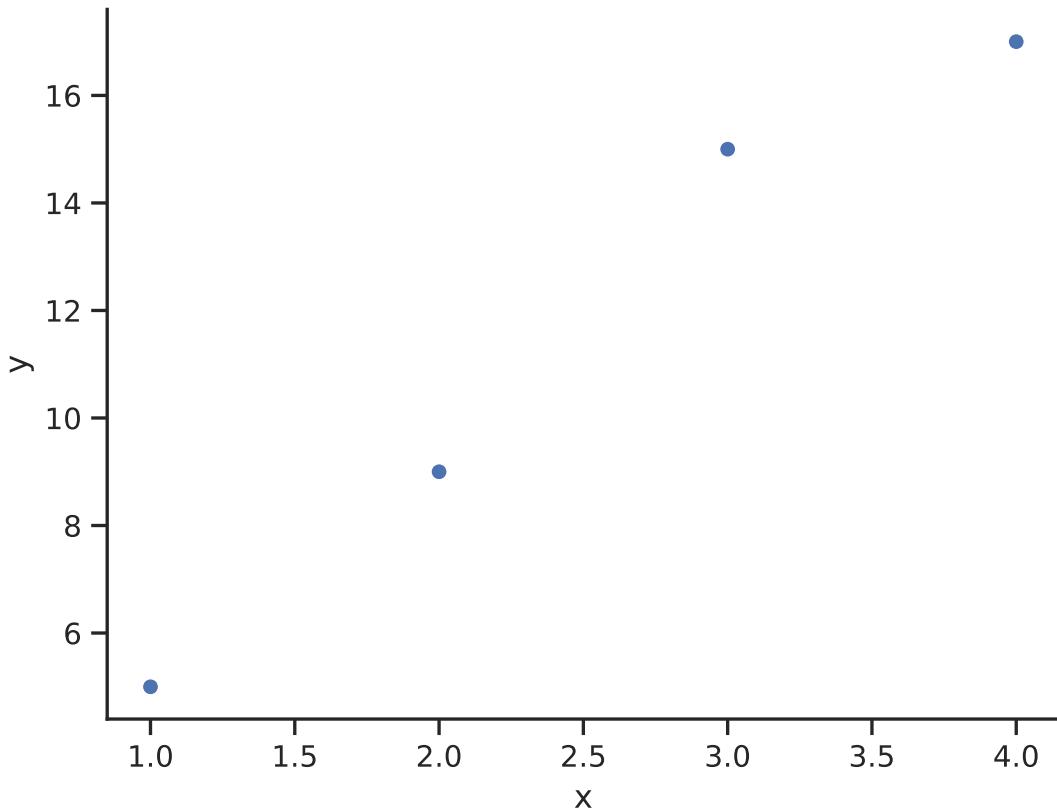
```



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
```

(continues on next page)

(continued from previous page)

```
import seaborn as sns
from statannotations.Annotator import Annotator
from matplotlib import ticker as mticker

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

Resources 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 Whitesides⁶³
- Suo group paper template by Zhigang Suo⁶⁴
- How to write a paper by Dave Weitz⁶⁵
- Ten simple rules for structuring papers by Konrad Kording⁶⁶
- Thread on how to write a paper by Marcos Morgan⁶⁷

⁶³ https://intra.ece.ucr.edu/~rlake/Whitesides_writing_res_paper.pdf

⁶⁴ <https://docs.google.com/document/d/1a3ElMO7XzBgdkg2k6z7wsHm8zzbVYAyCq8Tt5PzXtQk/edit>

⁶⁵ https://projects.iq.harvard.edu/files/weitzlab/files/131.5_weitzlab_guide_to_good_paper_writing_10-2012.pdf

⁶⁶ <https://journals.plos.org/ploscombiol/article?id=10.1371/journal.pcbi.1005619>

⁶⁷ <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 sentences⁶⁸
- Words to avoid from Arjun Raj⁶⁹
- Figure scripting from Raj lab⁷⁰

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...

⁶⁸ <https://www.americanscientist.org/blog/the-long-view/the-science-of-scientific-writing>

⁶⁹ https://docs.google.com/document/d/1r6nDcF43esu3xBjmk3ERAmaEHKEB75_HfISkk3zZhBk/edit

⁷⁰ <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

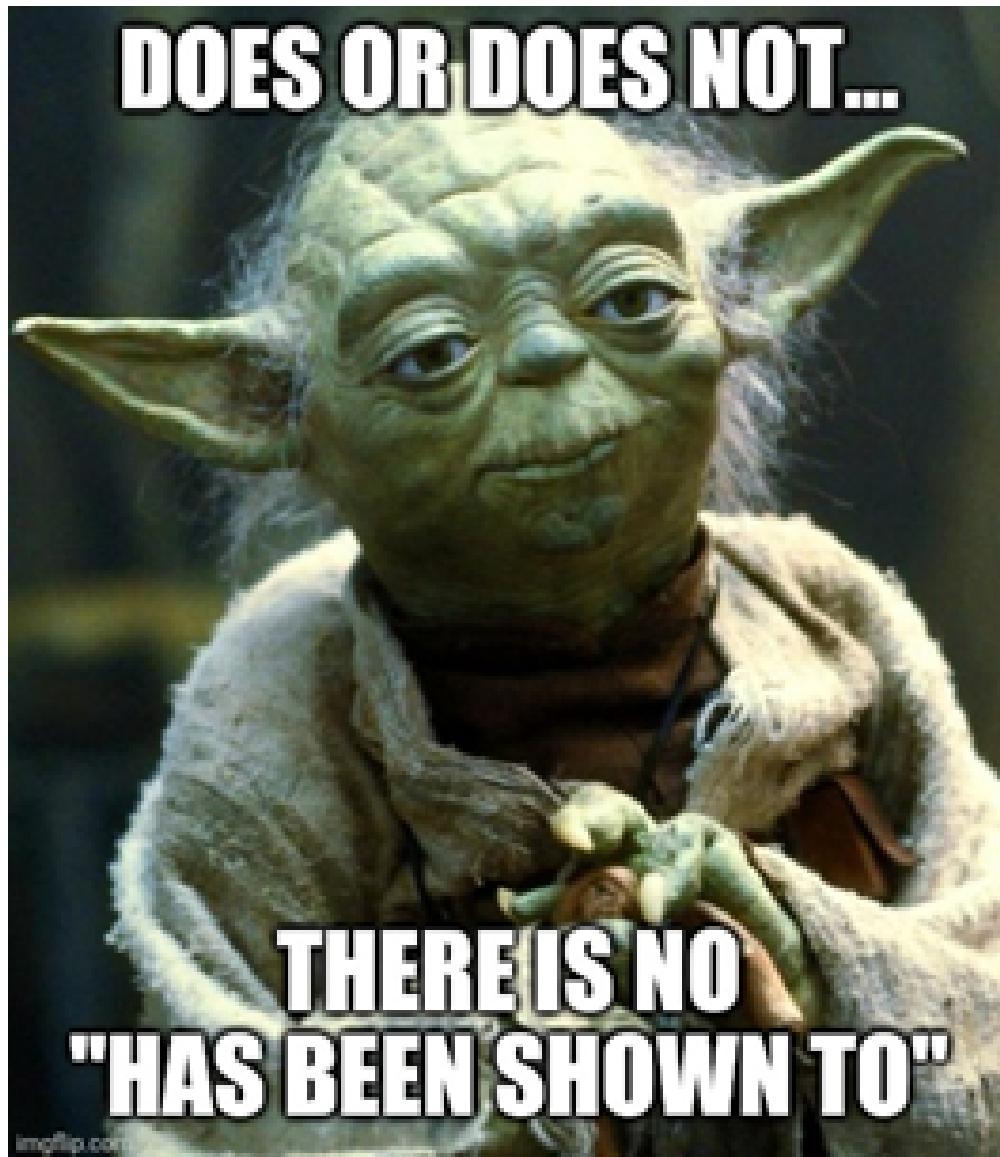
Always, always, always outline your work first. If the logical flow sucks, the words do not matter. The structure must be solid. Do not waste your time getting a flimsy structure dressed up with fancy words, syntax, etc. Get the structure correct and then polish a strong framing.

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.”

This sentence has five words. Here are five more words.

Five-word sentences are fine. But several together become

monotonous. Listen to what is happening. The writing is

getting boring. The sound of it drones. It's like a stuck record.

The ear demands some variety.

Now listen. I vary the sentence length, and I create music.

Music. The writing sings. It has a pleasant rhythm, a lilt, a

harmony. I use short sentences. And I use sentences of

medium length. And sometimes when I am certain the reader

is rested, I will engage him with a sentence of considerable

length, a sentence that burns with energy and builds with all

the impetus of a crescendo, the roll of the drums, the crash of

the cymbals—sounds that say listen to this, it is important.

So write with a combination of short, medium, and long

sentences. Create a sound that pleases the reader's ear. Don't

just write words. Write music.

-Gary Provost

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. Putatively, 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...

BOOTCAMP

7.1 IAP Bootcamp

7.1.1 Data analysis with Python

Why do data analysis with Python?

There's many GUI tools out there that do various types of data analysis: Excel, FlowJo, GraphPad Prism, and others. These are useful, but ultimately are only variably reproducible. "Reproducible" can mean a lot of things, but ideally scientific data analysis is:

1. Data reproducible: your raw data should be freely accessible and easy to load into analysis software.
2. Computer/OS reproducible: anyone on a reasonably modern computer should be able to do your analysis.
3. Tool reproducible: anyone can freely access the tools to do your analysis.
4. Minimally "artisanal": the final product of your analysis—graphs, tables, figures—should be as close to the analysis output as possible.

A related ideal is that of **transparency**, or how clear your data analysis is to your readers.

Introduction: the research problem

For the purposes of this, we are interested in a

- Introduce a research problem that will use both flow cytometry data.
- Generate some synthetic data for this

Good file layout practices

- All data in the OneDrive
- All analysis code in a git repo, setup reproducibly.

How do we deal with the fact that we are storing information in different locations? If we look carefully, there are really only three types of files here:

1. synced raw data in the OneDrive
 2. Code to do the analysis and generate output.
 3. Output, like figures, that you can regenerate by re-running the code.
- All notebooks in a *notebooks* folder.
 - All temporary/regeneratable output in an *output* folder that is *.gitignore*'d
 - Large cache data that takes a long time to regenerate may belong in the OneDrive.

The location of the OneDrive is going to change from computer to computer! We fix this by using a

Note: Ideally, at the end of the day, you should be able to take your laptop and throw it into the Charles River and lose no data and lose no progress. How do we practically make this the case?

If you store data in the OneDrive, it gets persisted. We also have a separate backup process that runs every day.

If you store your analysis code in a git repository (and sync with e.g. Github from time to time), then you will always retain a complete version history of your work, and also persist it.

There are some caveats that apply when dealing with extremely large datasets, e.g. next gen sequencing datasets that involves fun heavy boxes full of hard drives. If you are curious or need to do something with it, ask around.

To start, navigate to where you want your repository to be (with *cd* and *ls*). A common place create a new git repository, and create a Python virtual environment.

```
$ mkdir data_analysis_tutorial
$ cd data_analysis_tutorial
$ git init
$ python -m venv env
$ ./env/bin/activate # on MacOS, Linux
$ ./env/Scripts/activate # on Windows
```

We need to install the base packages we use, which are *pandas*, *seaborn*, and *rushd*. We will also be using Parquet files, which means we need to install *pyarrow*. Installing these packages also installs the other packages we use as dependencies (*matplotlib*, *numpy*, etc). You can also specify these explicitly if you want; it does not make a difference.

After we install packages, we snapshot the state into a *requirements.txt* file so that other people (or your future self) can exactly recreate your computational environment.

```
$ pip install pandas seaborn rushd pyarrow  
$ pip freeze > requirements.txt
```

The first time you open a notebook, it will want to pick a kernel and install ipykernel. Let it do this

Oh yaml, my yaml

- Let's start analyzing some flow data!
- Whoops, we have metadata we want to attach to these files.
- Time to write a yaml file!
- [learn YAML in Y minutes⁷¹](#)
- Example of special things,
- Example
- Using the YAML metadata visualizer in rushd.

Loading flow data

Introduction to Pandas

Tidy data

- Every data point / observation / cell is a `_row_`, with every measured variable in a column.

Indices

Certain columns can be made into an index/multi-index. The indices are special, because they allow for efficient (amortized constant time!) lookups into the dataframe, but are immutable and must be unique.

By default, none of the columns in your loaded data will be an index. Instead, each row gets a numeric index. This is still helpful! It means that even after a lot of dataframe slicing, you can still lookup that datapoint in the original dataframe!

For the purposes of analyzing flow data, we don't care to lookup specific columns in the dataframe, so we spend a lot of our time resetting the index.

⁷¹ <https://learnxinyminutes.com/docs/yaml/>

Saving cache files

- Cache file example

Downsampling data for kdeplots

-

Common data cleaning/manipulation approaches

Use the Pandas visual cheat sheet⁷²

Merging in extra metadata

(use this by defining a “plasmid” field to merge over in the fake data)

- The *join* function is only used for index joins
- Otherwise, you use the *merge* function.

Auto-selecting gates

Summarizing data

(e.g. group by)

Computing “condition means” to normalize by

(use of xs)

Plotting with Seaborn

7.1.2 Day 0: Software and training setup

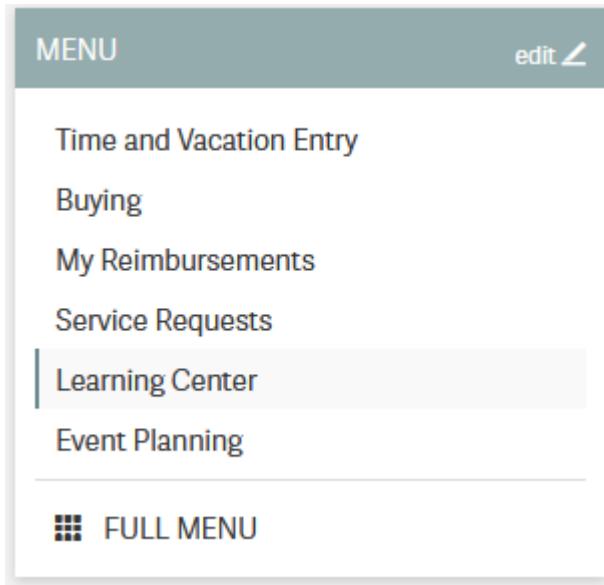
Our lab uses a lot of different software and various webservices. Before beginning IAP, you should install at least everything required on the list here, as well as completing all of the required EHS trainings.

⁷² https://pandas.pydata.org/Pandas_Cheat_Sheet.pdf

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. If you are an undergrad, do not select the BL2+ training group.

BIOSAFETY

- Use biological materials (including microorganisms, plants, animals, recombinant or synthetic DNA/RNA, etc.), requiring BL1 or BL2 containment.
- Supervise a laboratory that uses biological materials (including microorganisms, recombinant or synthetic DNA/RNA, etc.), requiring BL1 or BL2 containment.
- Perform research with human blood or body fluids, human cells or tissues, or human cell lines or using a material containing a bloodborne pathogen (e.g. HIV, HBV, HCV, malaria).
- Conduct research requiring BL2+ containment (e.g. viral vectors that can lead to oncogenesis, propagating HIV, HBV, HCV).

CHEMICAL SAFETY

- Use potentially hazardous chemicals in a laboratory (this includes even common chemicals such as oil, solvents, paints, alcohol, acetone, etc)

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 your 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, you will see many required trainings. Some have a required ‘classroom’ component, such as the *Lab Specific Chemical Hygiene training*, which will be completed with an in-lab walkaround.

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. If you are going to be helping with mouse work, still under the ‘My Profile’ tab, you should click **Join Another Group** under training groups and add the **68N: Mouse** training group.

Software and webservices

Core webservices

- Create a [Quartz account⁷³](#). Using your MIT email is recommended.
- Create a [Github account⁷⁴](#). 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 account⁷⁵](#). Using a personal email is recommended for permanence reasons.

⁷³ <https://www.quartzzy.com/>

⁷⁴ <https://github.com/>

⁷⁵ <https://www.zotero.org/user/register>

- Create an [ORCID](https://orcid.org/register)⁷⁶. Adding all of your active emails is recommended.

After creating these accounts, message your **Kerberos ID**, your **Github username**, your **Zotero username**, and the email you used to create your **Quartzy account** to the #iap slack channel. We'll get you added to all of the above. You will have to:

- Accept the Github invitation to the [gallowaylabmit organization](#)⁷⁷.
- Accept the Zotero invitation to the [gallowaylab group](#), checking that it appears in your [group list](#)⁷⁸.

Shared storage

We use **OneDrive** for shared lab storage. OneDrive's web interface is slightly clunky, and occasionally you have to wait a minute for syncing to occur, but it is the MIT option that offers us the largest amount of storage space, without having bandwidth limits (unlike MIT's Google Drive).

OneDrive also has an excellent implementation of “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.

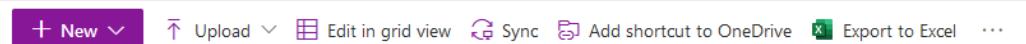
OneDrive also has tight integration with Office products, allowing Google Drive-esque live, multi-person editing of Office documents saved inside it.

We share presentations, software, primers, and plasmids with each other through the shared library, and all lab instruments will save data into OneDrive so everything is accessible.

When you see a file location like `instruments\data\attune` without an additional information, it is likely a path inside the OneDrive.

After being given access:

1. If not on a recent version of Windows 10 (e.g. Win 7, MacOSX, etc), download the [OneDrive client](#)⁷⁹. 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. After several minutes, it will show “OneDrive is up to date”, and all files should be accessible.

⁷⁶ <https://orcid.org/register>

⁷⁷ <https://github.com/gallowaylabmit>

⁷⁸ <https://www.zotero.org/groups/>

⁷⁹ <https://www.microsoft.com/en-us/microsoft-365/onedrive/download>

Experimental software

- **SnapGene:** We use SnapGene for our cloning analysis. Download it through IST [here⁸⁰](#), if asked for a registration code, get it [here⁸¹](#) (certificates 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.
- **FIJI:** For simple image analysis, Fiji (ImageJ) gives a nice GUI interface. Download it from <https://fiji.sc>
- **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 not, download the connector [here⁸²](#). We also have a shared Zotero group, to accumulate citations when writing manuscripts.

Several helpful plugins can be downloaded; the recommended ones are:

Table 1: Recommended Zotero plugins

Addon name	Description
Zot-File ⁸³	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.
Zu-tilo ⁸⁴	Enables helpful tagging operations, such as the ability to copy/paste tags or easily add paper relationships.
Better Bib-tex ⁸⁵	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;

⁸⁰ <https://downloads.mit.edu/released/snapgene/vendor-registration.html>

⁸¹ http://downloads.mit.edu/released/snapgene/group-name_registration-code.txt

⁸² <https://www.zotero.org/download/connectors>

⁸³ <http://zotfile.com/>

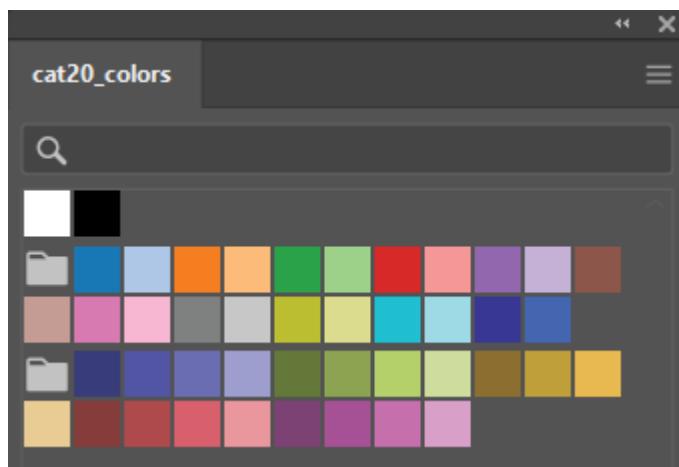
⁸⁴ <https://github.com/wshanks/Zutilo>

⁸⁵ <https://retorque.re/zotero-better-bibtex/>

left-clicking on download links will attempt to install the Zotero plugin as a Firefox plugin, which will fail.

- **Creative Cloud:** MIT has a site-license for students and staff. After installing the [Creative Cloud application](#)⁸⁶, login with your MIT credentials, after selecting “Work/School account”. You may have to wait 24 hours for activation after your first login. You should install **Acrobat** (for viewing PDFs) and **Illustrator** (for drawing vector art).
- **Inkscape:** (*Optional*) Inkscape is a free and open-source vector drawing program that can be downloaded [here](#)⁸⁷. Inkscape and Illustrator have many similar, but not completely overlapping features. If you have not learned to use either, pick one to start with to learn first (likely Illustrator). However, it’s likely eventually worth learning both if you don’t want to eventually pay for Creative Cloud. Inkscape’s (Cairo) PDF import also tends to be superior, if trying to import vector images from paper PDFs.
- **Color pallettes:** Having nice color-blind friendly, distinct colors to start drawings from is helpful. If making cartoon/stylized figures, the colors in-palette might be enough! If not, the palette can provide a good starting place.

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):



To use these palette files, see the [Illustrator documentation](#)⁸⁸ or the [Inkscape documentation](#)⁸⁹.

⁸⁶ <https://www.adobe.com/creativecloud/desktop-app.html>

⁸⁷ <https://inkscape.org/release/inkscape-1.0.1/>

⁸⁸ https://helpx.adobe.com/illustrator/using/using-creating-swatches.html#share_swatches_between_applications

⁸⁹ <https://inkscape-manuals.readthedocs.io/en/latest/palette.html>

Coding and collaboration

- **Slack** is how we communicate! After [downloading it⁹⁰](https://slack.com/downloads), 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.
- **Git**: For any code/code-like thing (LaTeX, other plain-text files) you write, Git is the standard way to share and collaborate with others in addition to tracking your entire version history.

You must install the base command-line tools from [here⁹¹](https://git-scm.com/downloads), make sure that you select your operating system and not the “Download source code” button!

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 (e.g. what name/email gets stored alongside the work you do).

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).

```
$ git config --global user.name "Full Name"  
$ git config --global user.email email_address@example.com
```

- **Github Desktop:** This program is a good basic GUI Git tool, in case the command line interface/built in editor interfaces aren’t for you. Download it [here⁹²](https://desktop.github.com/).
- **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 from [here⁹³](https://www.python.org/downloads/). 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.

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.

⁹⁰ <https://slack.com/downloads>

⁹¹ <https://git-scm.com/downloads>

⁹² <https://desktop.github.com/>

⁹³ <https://www.python.org/downloads/>

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.

After installing, to make sure it worked, open a new terminal and type the following, checking that the output looks similar to the following.

```
$ 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.
```

Exit the Python prompt by typing `exit()`

After exiting Python, you can install the normal “semi-base” packages needed for most data analysis:

```
$ pip install numpy scipy pandas matplotlib jupyter jupyterlab
```

- **R:** Many bioinformatic tools are written in R, so we also use R. From [here⁹⁴](#), download the main package (MacOS) or both the base entry and the Rtools entry (Windows).
- **VSCode:** (*Optional*) Having a good *plain-text editor* (e.g., not Word) is important for coding, and is ultimately up to personal taste. If you have your own favorite, feel free to not install VS Code. If you are used to language-specific IDE's 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 how you want, you can use it for all of your coding.

The recommended editor is VS Code, downloadable [here⁹⁵](#).

After installing, you should click the extensions button:



and search and install the following extensions (type in the name, click the install button).

⁹⁴ <http://lib.stat.cmu.edu/R/CRAN/>

⁹⁵ <https://code.visualstudio.com/>

Table 2: Recommended VS Code extensions

Addon name	Image	Description
Python		Enables Python debugging, running, and IntelliSense (in-line help while typing).
Pylance		Faster ‘language server’ for Python, which means the IntelliSense is faster and more accurate.
Snakemake Language		Snakemake syntax highlighting for editing computational pipelines.
R		Base language support for R.
R LSP Client		The VS Code side of the R language server. Before installing this, run <code>install.packages("languageserver")</code> 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.
Jupyter		Inline Jupyter notebook support. No more need to launch Jupyter in a web browser, just do it inside VS Code!
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.

- **RStudio:** (*Optional*) If you don’t feel like using VS Code for your R work, the excellent, well-polished standard IDE is RStudio Desktop, downloadable [here⁹⁶](https://rstudio.com/products/rstudio/download/#download).

⁹⁶ <https://rstudio.com/products/rstudio/download/#download>

7.1.3 Computational environment check

Many of the tools, including this protocols website, depend on Python. Contributing to analysis code and to protocols also requires that your git install is working as expected.

There is a repository at <https://github.com/GallowayLabMIT/environment-check> that contains a Python script that checks for installation problems by checking that simple commands can succeed.

This page does not give specific, copy-and-pastable instructions, as these will slightly differ. See *Day 0: Software and training setup* (page 314) for instructions on installing both Python and Git, and *An introduction to Git* (page 326) for git help.

Initial environment check

Note: This section of the setup is meant to be a minimal test of basic functionality. It mimics some of the steps you may want to do when working with a computational repository. See the *Startup checklist when working with repositories* (page 370) for common “startup tasks” that normally need to be done when starting a new repository / downloading someone else’s.

When working with the protocol repository, you will have to repeat the virtual environment step, for example!

If you run into errors, ask someone who has their environment fully setup! All of the following steps should be done *inside* a local copy of the environment-check repository.

1. Clone the **environment-check** repository at <https://github.com/GallowayLabMIT/environment-check>. You can put this repository wherever you like. 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.

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 effort , git tracks lots of small files internally which means a lot of syncing effort.

2. Create a **virtual environment** within the environment-check repository; see *Python/Sphinx setup* (page 408) for help creating this environment. Remember to add your virtual environment folder to the `.gitignore` so it is not tracked by git! [KEG: This just involves adding the file ‘env’ to the “`.gitignore`” file]
3. Activate the virtual environment, and install the listed dependencies with `pip install -r requirements.txt`. [Note: “`requirements.txt`” can be found in the protocol repo]
4. Create and switch to another branch (for example, `cjohnsto`). [KEG: I found the easiest way to do this is via Github Desktop. Branch > New branch]

5. 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.
6. Create a `datadir.txt` file that contains the path to your locally-synced OneDrive. [KEG: Just make a text file and copy the path to your OneDrive (aka SharePoint) folder for the Galloway lab so you can link directly to your data using “rushd”.] For example, the `datadir.txt` file might look like this (on MacOS):

```
/Users/username/Library/CloudStorage/OneDrive-SharedLibraries-  
↳MassachusettsInstituteofTechnology/GallowayLab - Documents
```

You don't need any quotes or other characters around the path.

Note: The environment-check script expects that you point the data directory at the *root* of the OneDrive, e.g. the folder with subdirectories like `projects`, `instruments`, etc.

In your own code, you can point the `datadir` wherever you want, but the root is a typical choice because it lets you access both the raw data in `instruments`, project folders, and more. You can also always create a “local folder” variable in Python, e.g:

```
flow_datadir = rd.datadir/'instruments'/'attune'/'chris'  
# later in your code  
# the following argument resolves to  
# OneDrive/instruments/attune/chris/2022.06.21  
some_flow_function(flow_datadir/'2022.06.21')
```

-
7. Commit your changed files and push your new branch to Github. [KEG: Stage changes by clicking on the ‘+’ in the ‘Changes’ tab. Then types some message on what you will commit into the space for ‘Source Control’. By clicking on the check mark, you will commit changes.]
 8. Run `python check.py` or `python3 check.py` (MacOS) to see if you get all green checks! [KEG: This only works if you are in the ‘environment-check’ folder]

Note:

Some tips from Katie on getting going.

1. Your virtual environment is basically a container for projects that will help you track packages, versions, ectera. So you need one for spaces you'll be running python (for instance in `/protocols`)
2. Don't have permissions to run scripts, see here: https://docs.microsoft.com/en-us/powershell/module/microsoft.powershell.core/about/about_execution_policies?view=powershell-7.2
3. If something isn't working, try restarting VS Code. Sometimes it just needs to restart.
4. To install reStructuredText, click here: <https://marketplace.visualstudio.com/items?itemName=lextudio.restructuredtext>

5. Are you sure you installed Sphinx? <https://pypi.org/project/Sphinx/>
 6. Visually confirming the protocols' preview is working is encouraging. Try 'Ctrl+Shift+P' or 'Ctrl+Shift+R'
 7. If you get stuck, ask for help!
-

Protocols check

After getting all green checks, clone the protocols repo (<https://github.com/GallowayLabMIT/protocols>) and edit this file (docs/bootcamp/iap/environment_check.rst), adding your name to the completion list. Commit and push those changes and you are done! [KEG: To save, "Ctrl+S"].

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)

7.1.4 An introduction to Git

Acknowledgements

This bootcamp session is heavily inspired by the Software Carpentry Git lesson⁹⁷ (CC-BY-4.0).

The initial motivation section is inspired by [The Git Parable](#)⁹⁸.

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](#)⁹⁹

Say you are trying to do version control for files related to a paper manuscript. You have some figures, and a main .docx

⁹⁷ <https://swcarpentry.github.io/git-novice>

⁹⁸ <https://tom.preston-werner.com/2009/05/19/the-git-parable.html>

⁹⁹ <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
```

(continues on next page)

(continued from previous page)

```

  └── 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 its correct 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.

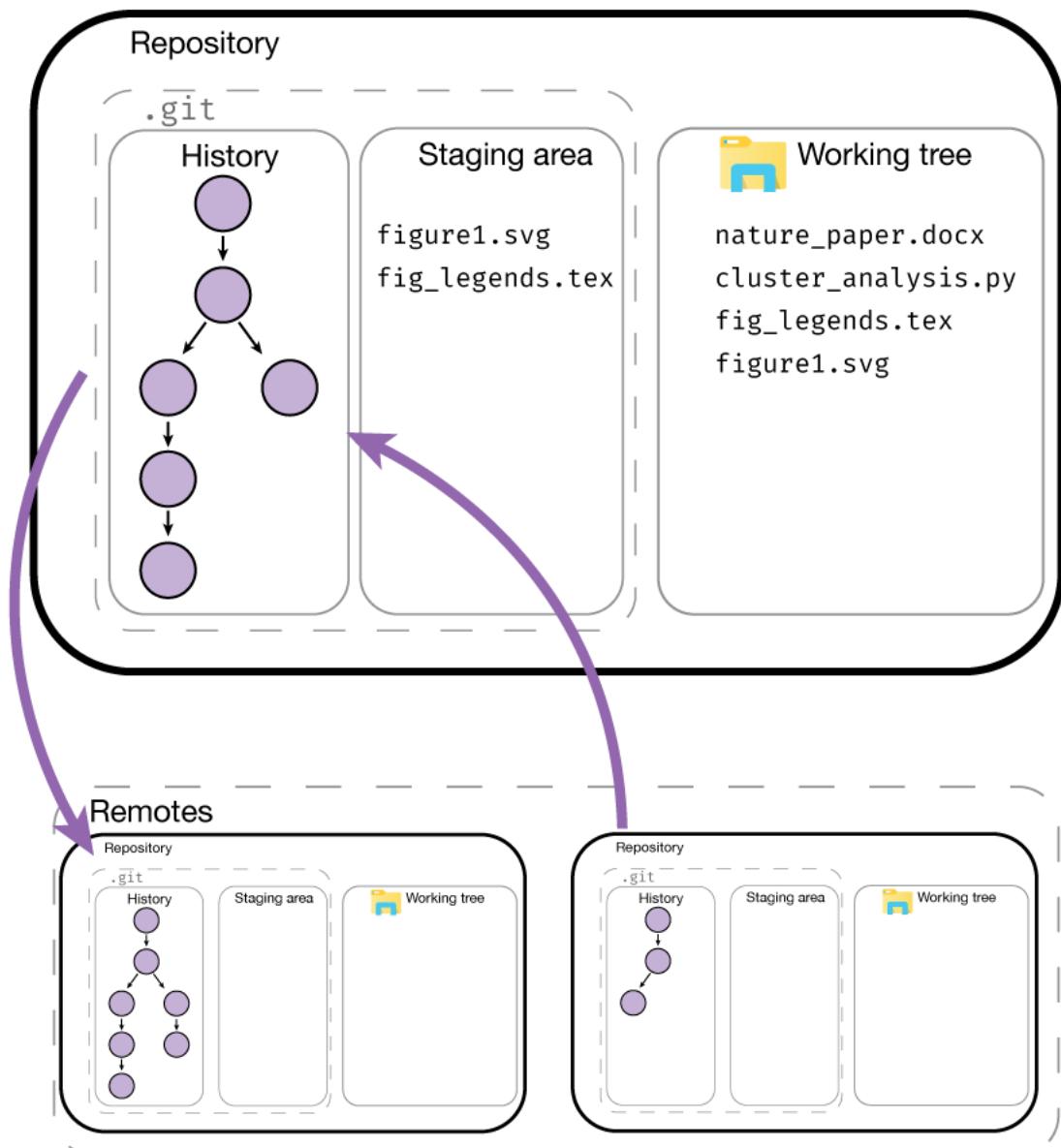
In Git, we call the entire set of files and folders that we are interested in a **repository**, or **repo** for short. Typically, repositories are going to include everything related to a single project. This could mean a set of analysis scripts for the same project, an entire R package that is being 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 inefficient 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**.



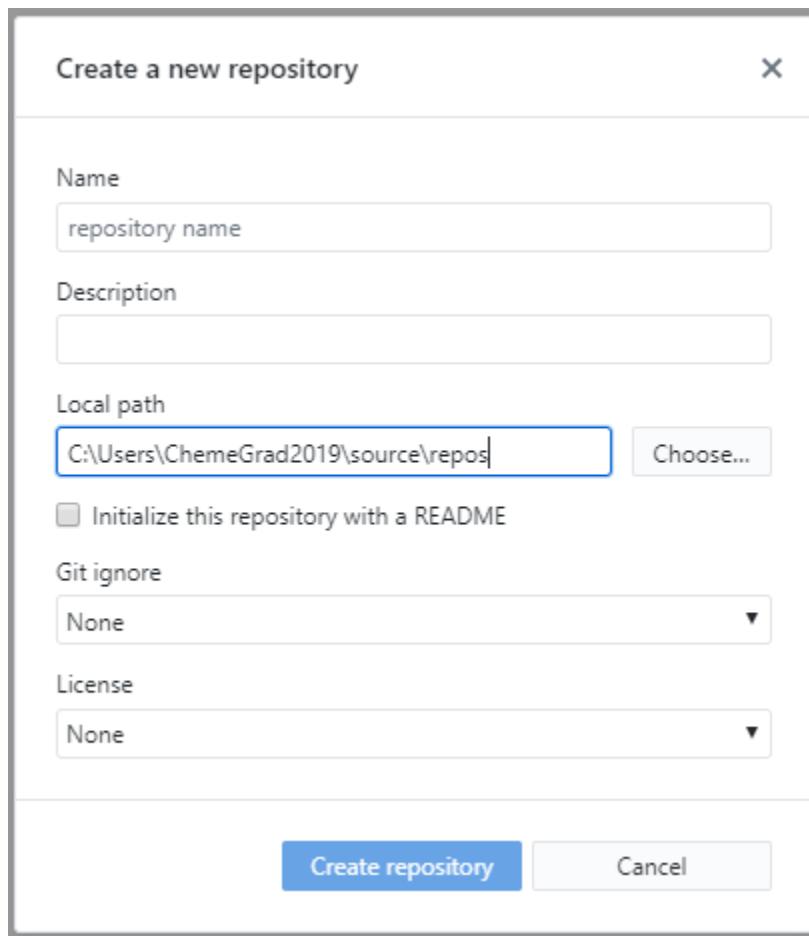
Downloading or starting a repository

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.

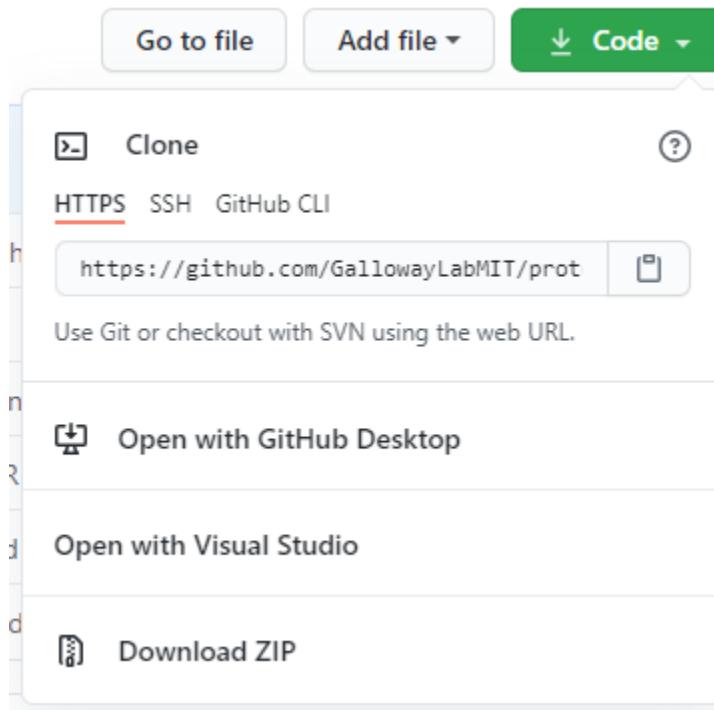


The graphical interface gives the additional option to start with some content pre-generated, in particular a **copyright license** and a **.gitignore** file. We will cover the ignore file later. If you choose one of these options, you can immediately push to a remote, because there is content to push!

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.
```

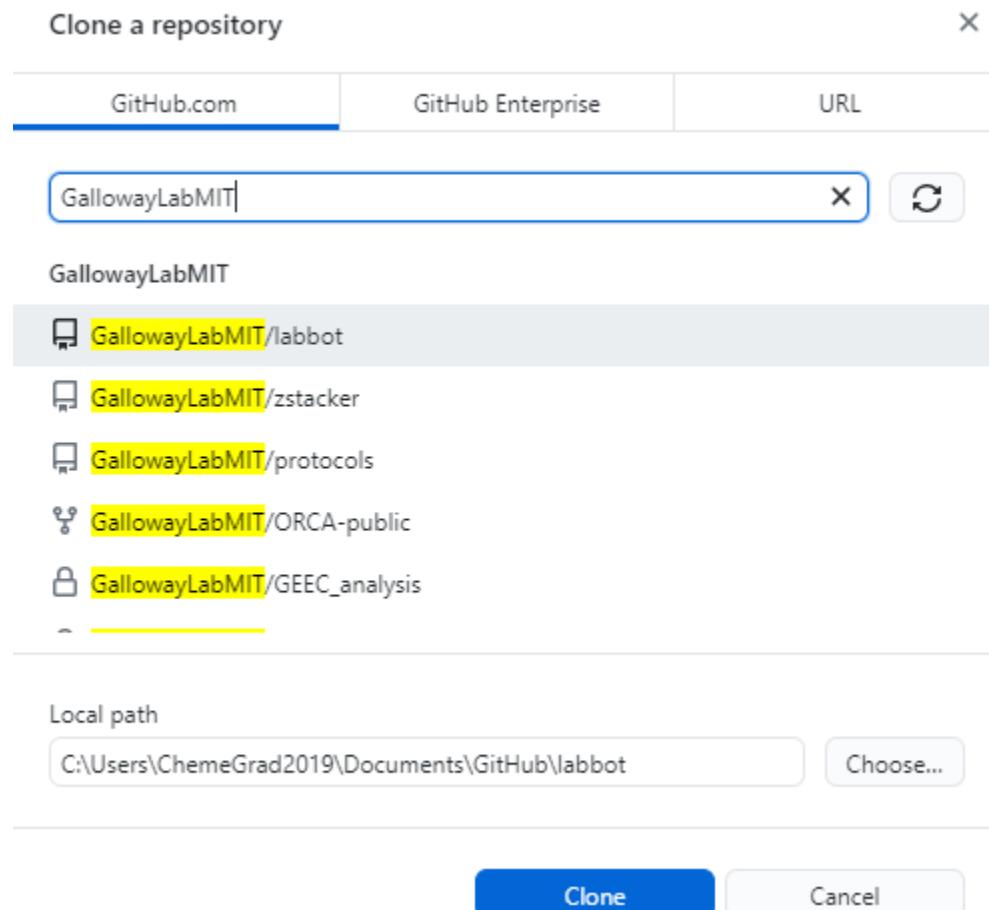
If you haven't added an SSH key, you can use the https URL:

```
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.
Receiving 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 deletion 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.
Receiving 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
```

(continues on next page)

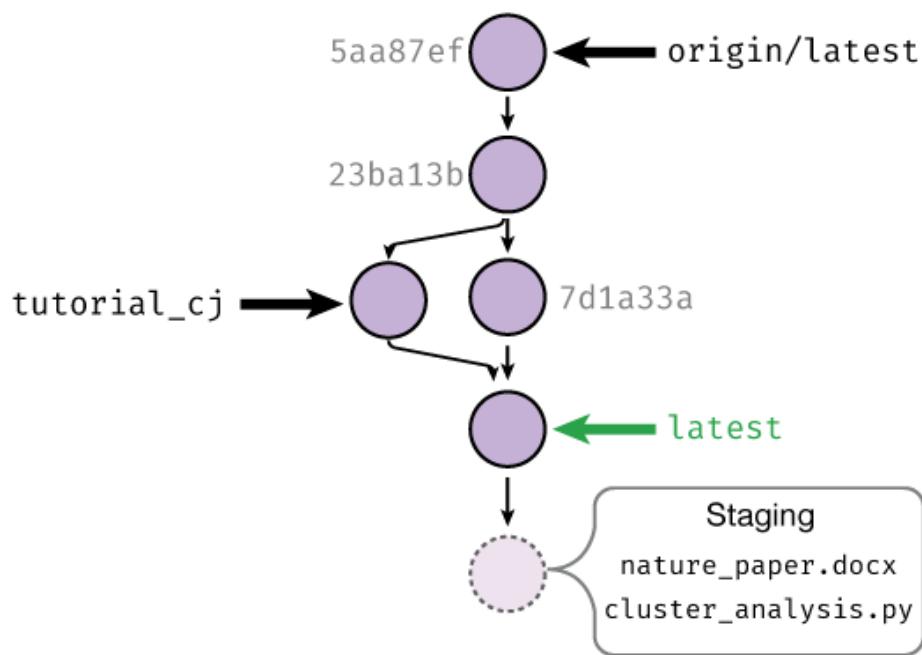


Fig. 1: An example history is shown. Each commit has a random name (in gray) that is assigned by Git. Shown with arrows are the various **branches**, which can be thought of as movable commit names. Shown in green is the currently checked out branch; staged changes will be committed on top of this branch.

(continued from previous page)

```
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
```

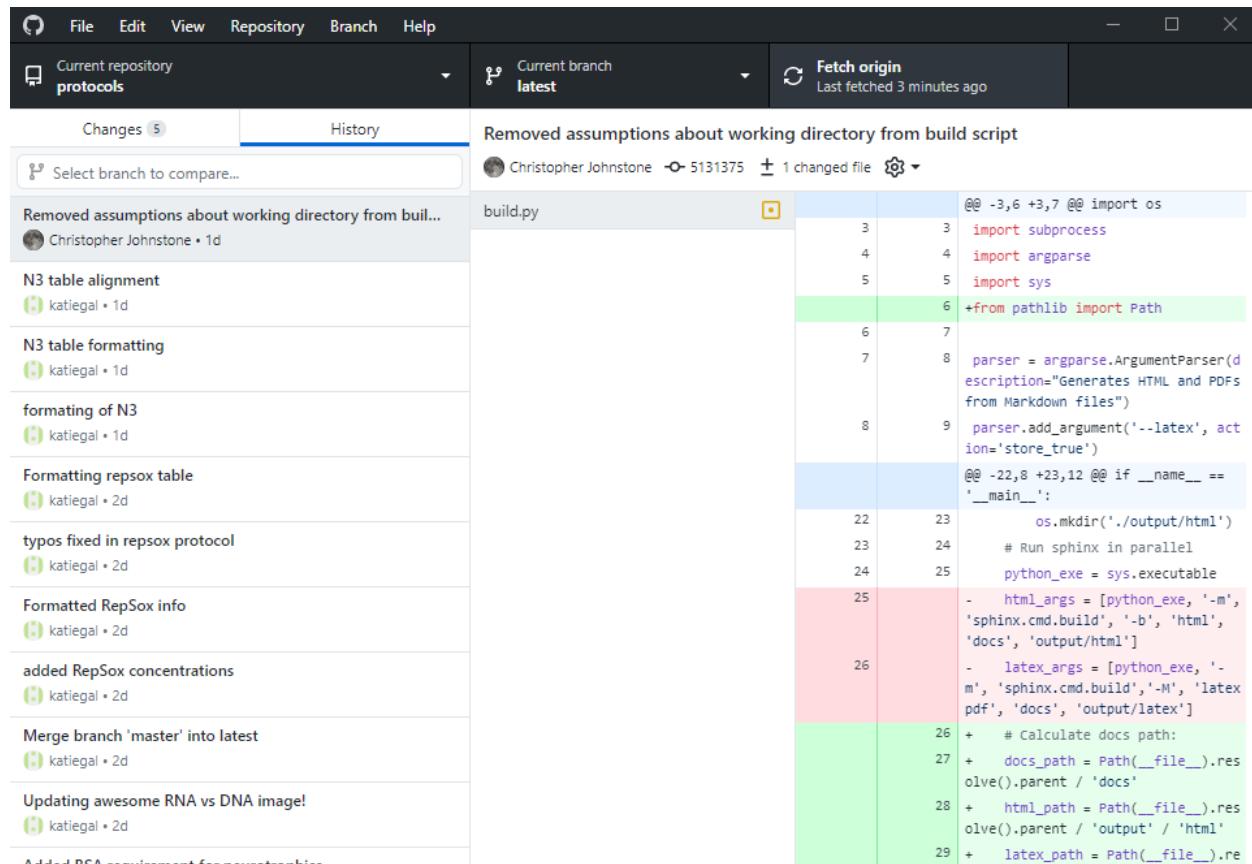
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```
* | 667a652 (origin/tech_wip) Added details on no-spaces rationale
* | 9a3dda0 Switched ISO date to explicitly link to xkcd
* | 55bb3eb Updated shell and software tips and tricks with shell extras
* | f65c1e5 Added helper script run info
```

In this example graph view, we see that two branches came together at commit e0a749e.

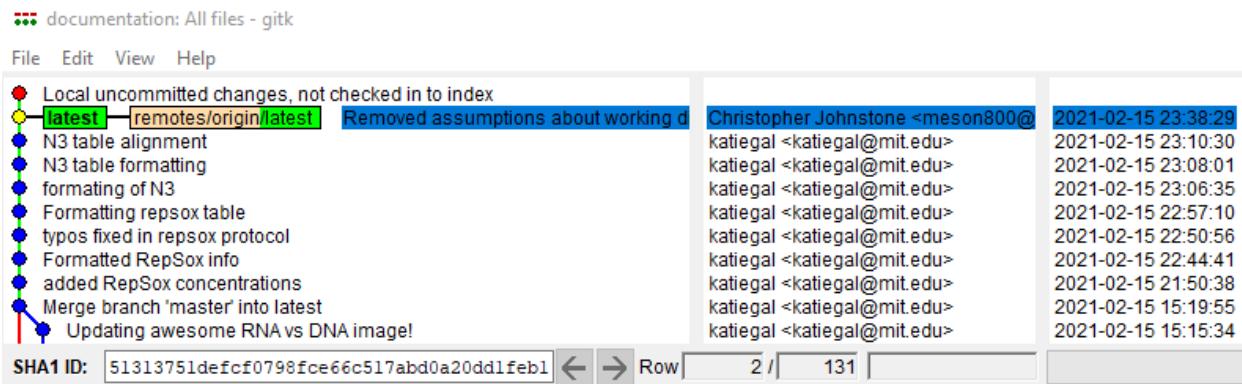
We can use various GUI programs to get the same result! In Github Desktop, you can switch to the history tab to see a linearized history (e.g. like the default git log):



The screenshot shows the Github Desktop application interface. The top navigation bar includes File, Edit, View, Repository, Branch, and Help. The main window has tabs for Changes (5) and History, with History selected. A sidebar on the left lists commit messages from different users. The central area displays a diff view of the file 'build.py' between two specific commits. The diff highlights code changes, with additions in green and deletions in red. The commit message above the diff reads 'Removed assumptions about working directory from build script'.

```
Removed assumptions about working directory from build script
Christopher Johnstone -O- 5131375 + 1 changed file ⚙️
build.py
@@ -3,6 +3,7 @@ import os
import subprocess
import argparse
import sys
+from pathlib import Path
@@ -22,8 +23,12 @@ if __name__ == '__main__':
    os.mkdir('./output/html')
# Run sphinx in parallel
python_exe = sys.executable
- html_args = [python_exe, '-m', 'sphinx.cmd.build', '-b', 'html', 'docs', 'output/html']
- latex_args = [python_exe, '-m', 'sphinx.cmd.build', '-M', 'latex pdf', 'docs', 'output/latex']
+ # Calculate docs path:
+ docs_path = Path(__file__).resolve().parent / 'docs'
+ html_path = Path(__file__).resolve().parent / 'output' / 'html'
+ latex_path = Path(__file__).re
```

You can also run gitk in a terminal to bring up a different graphical view which shows the entire tree:



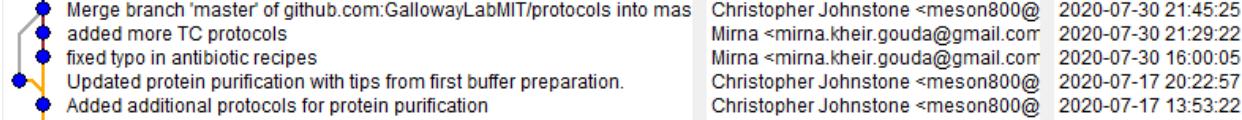
In summary, `git log` and `git status` and their GUI equivalents should be your go-to tool to find out what is happening in history. If you are ever feeling confused, take a look at the log!

Exercise

Do the following with the `git log` command or one of the graphical tools.

- 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?
- What was the second commit in the protocols repository?
- Find the embedded emails of two contributors to the protocols repo.

1. One example is this one, shown in graph form with Gitk:



- 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

- 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 recommended¹⁰⁰ 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
```

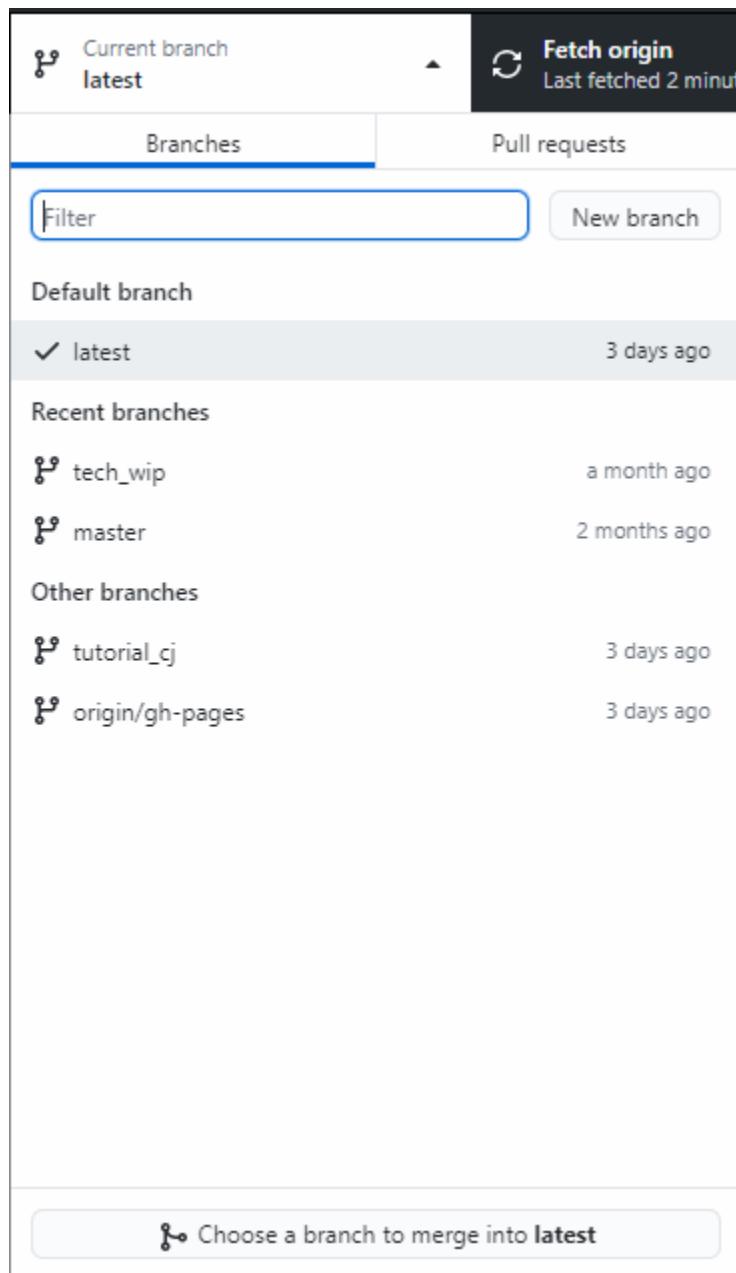
Note that creating a new branch **does not switch to it**. To actually switch branches, we need to tell Git to update the directory we actually see (the **working tree**). We do this with the **checkout**

¹⁰⁰ <https://sfconservancy.org/news/2020/jun/23/gitbranchname/>

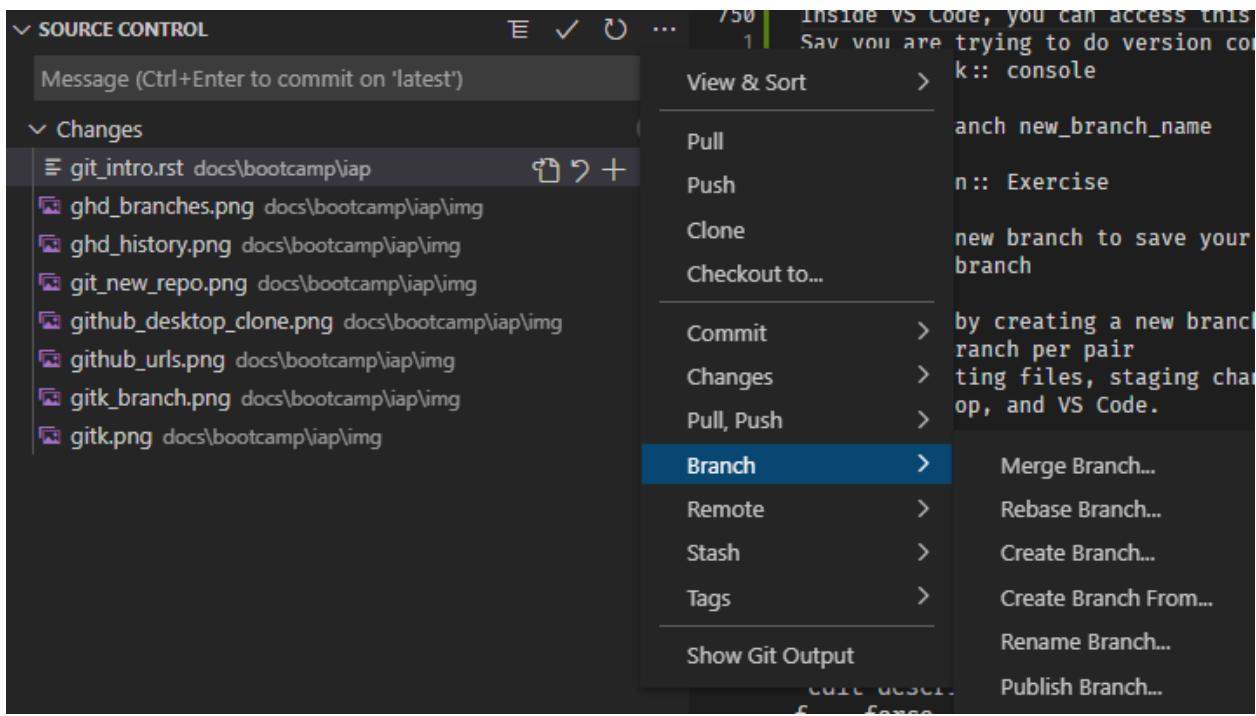
command:

```
$ 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 people.

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'.
```

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-
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
```

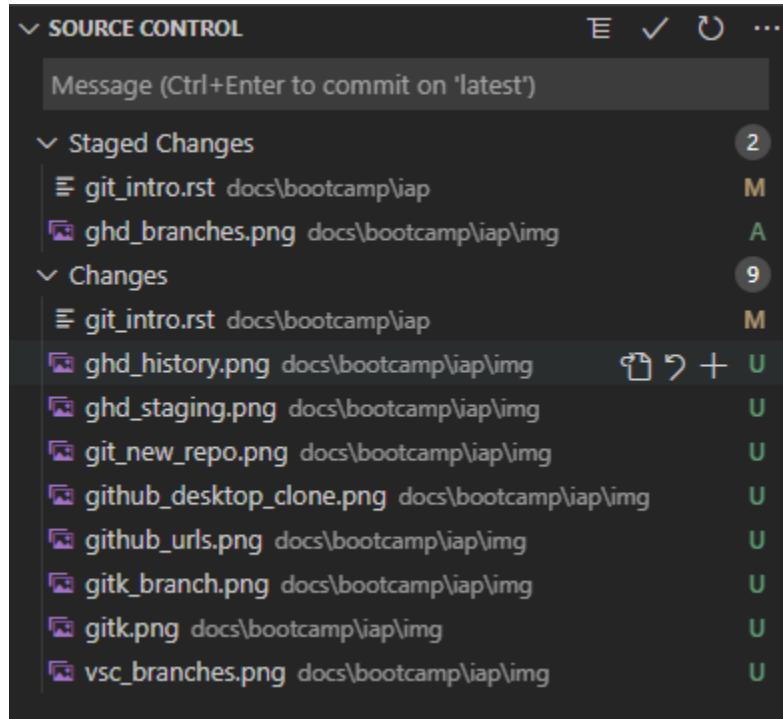
We can do the same thing in the GUI tools. In Github Desktop, changes are staged automatically:

The screenshot shows the GitHub interface for a repository named "protocols". The top navigation bar includes a "Current repository" icon and the repository name "protocols". Below the navigation bar, there are two tabs: "Changes" (which is selected) and "History". Under the "Changes" tab, it says "9 changed files". A list of files is shown, each with a checked checkbox and a plus sign icon. The first file listed is "docs\bootcamp\iap\git_intro.rst".

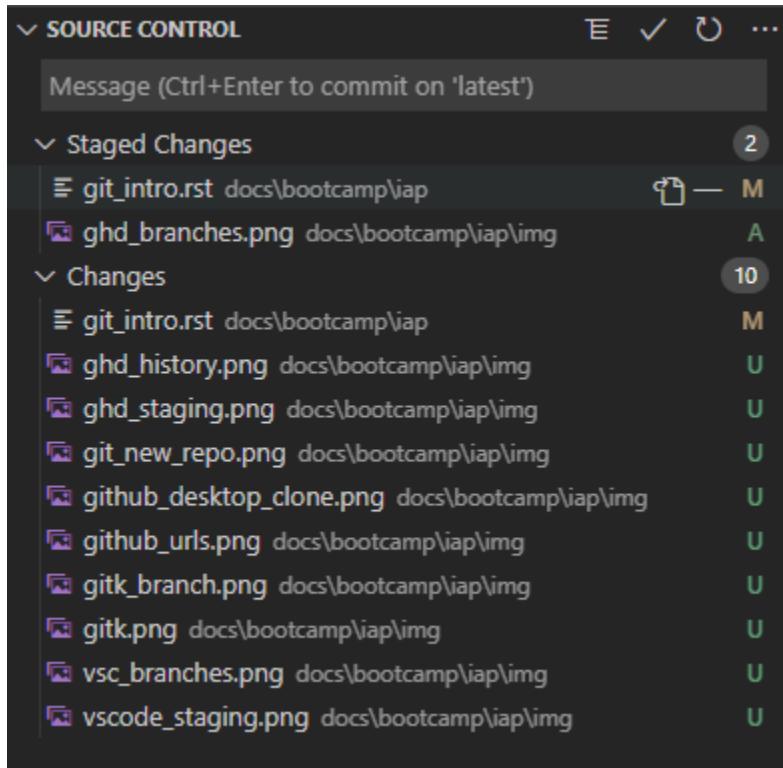
File Path	Action
docs\bootcamp\iap\git_intro.rst	+
docs\bootcamp\iap\img\ghd_branches.png	+
docs\bootcamp\iap\img\ghd_history.png	+
docs\bootcamp\iap\img\git_new_repo.png	+
docs\bootcamp\iap\img\github_desktop_clone.png	+
docs\bootcamp\iap\img\github_urls.png	+
docs\bootcamp\iap\img\gitk.png	+
docs\bootcamp\iap\img\gitk_branch.png	+
docs\bootcamp\iap\img\vsc_branches.png	+

The screenshot shows the VS Code interface for committing changes. It features a "Summary (required)" input field with a placeholder "Summary (required)". Below it is a "Description" input field with a placeholder "Description". At the bottom, there is a large blue button labeled "Commit to latest".

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 unstaged 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/bootcamp/iap/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.
-

Snapshutting 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 committing  
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 committing 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 up 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
```

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```
$ git revert 57e1f4e
[tutorial_cj a0afdee] Revert "Just testing"
 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

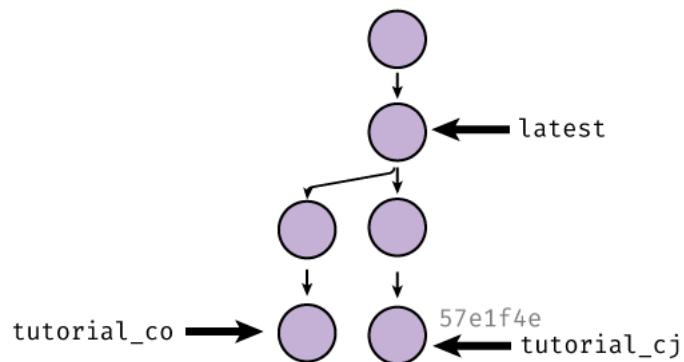
  Revert "Just testing"

  This reverts commit 57e1f4ea06f25a7c05c6d03fcc297efa458bc8e4.

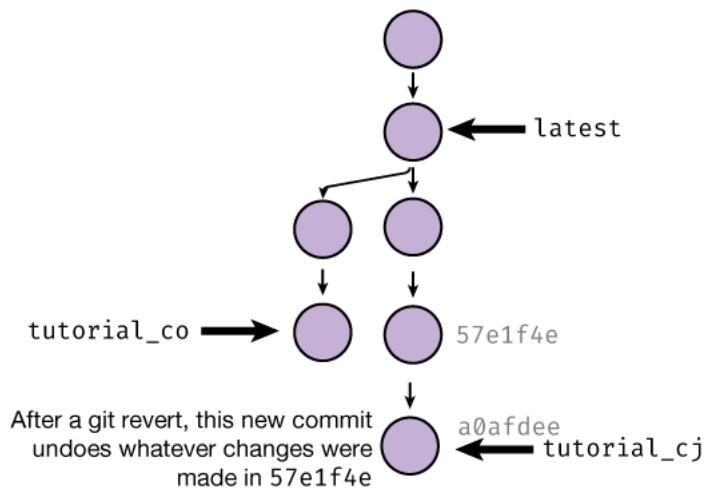
commit 57e1f4ea06f25a7c05c6d03fcc297efa458bc8e4
Author: Christopher Johnstone <meson800@gmail.com>
Date:   Thu Feb 18 22:24:21 2021 -0500

  Just testing
```

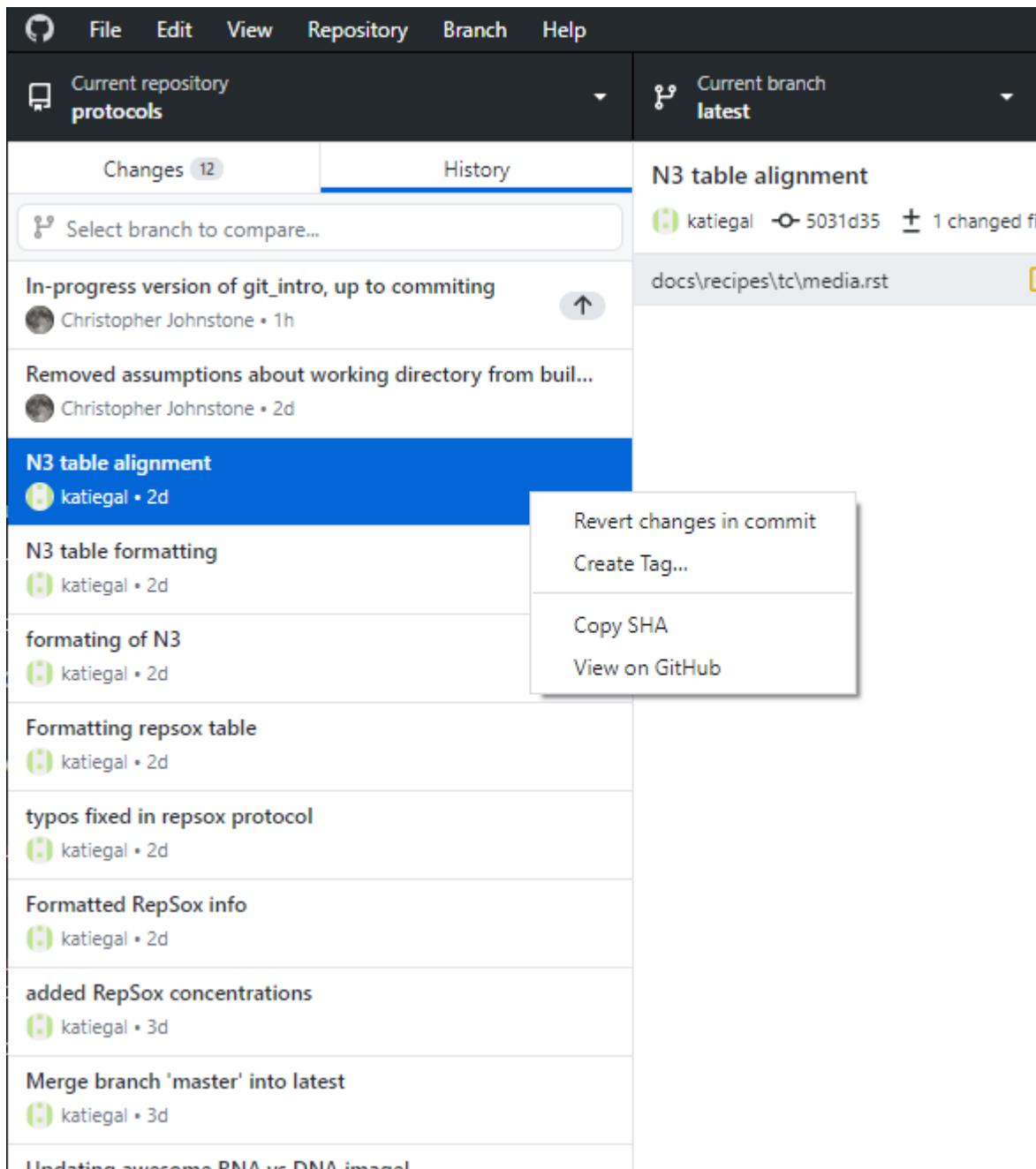
Visually, what has happened is that we have gone from this tree:



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 initialize 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

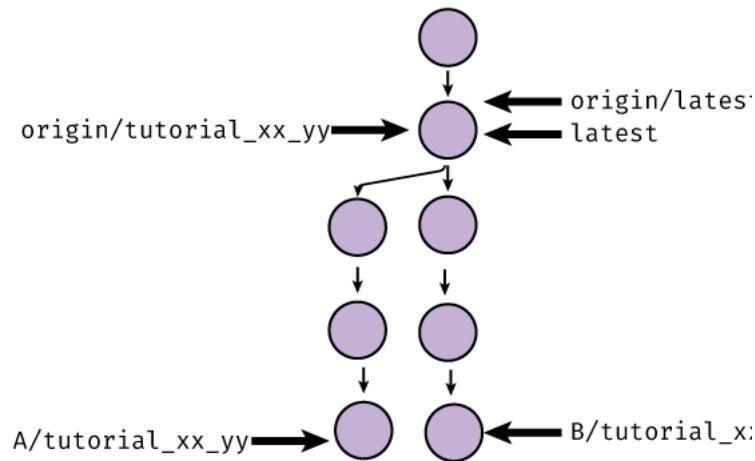
One partner should push the shared branch first:

```
$ 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
```

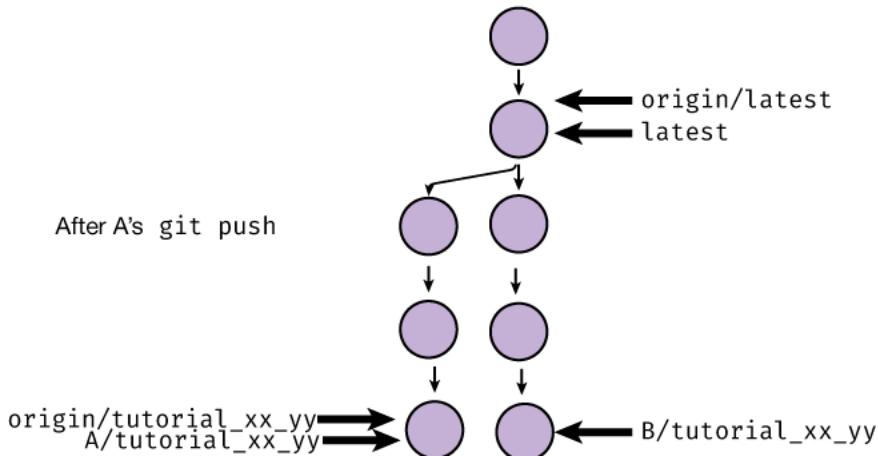
What happens when the other person now tries to push?

```
$ 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 initialized at the beginning, with each person having local commits:

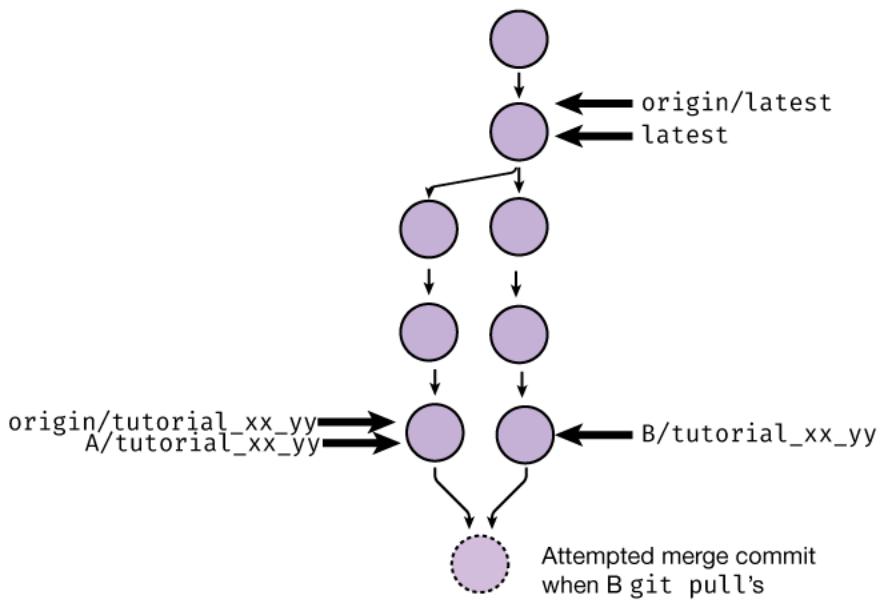


The first person's push (A) set the remote branch `origin/tutorial_xx_yy` equal to **their** version of `tutorial_xx_yy`:



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/bootcamp/iap/git_intro.rst
CONFLICT (content): Merge conflict in docs/bootcamp/iap/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/bootcamp/iap/git_intro.rst
```

(continues on next page)

(continued from previous page)

```
no changes added to commit (use "git add" and/or "git commit -a")
```

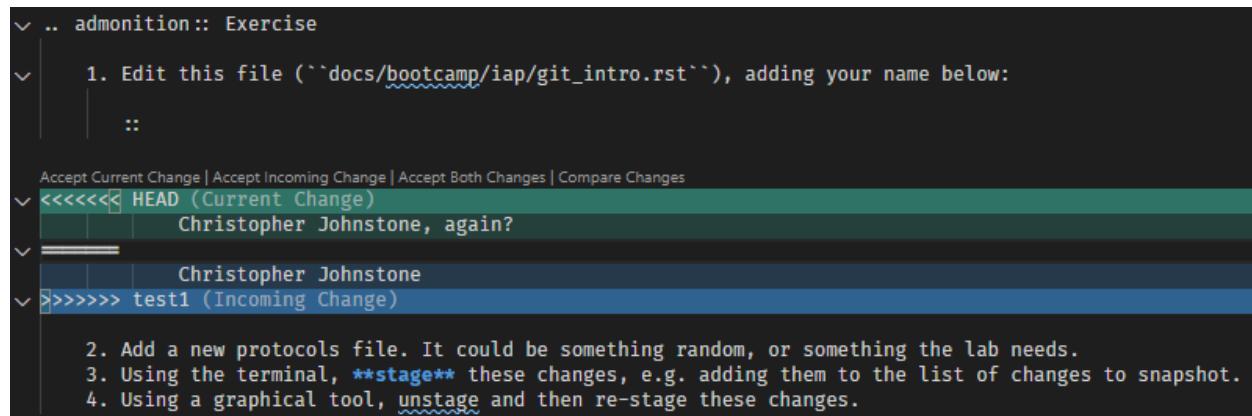
It tells us that we have to 1) fix the merge conflicts, 2), use `git add` to tell Git that we fixed the conflicts, and 3), run `git commit` to finish the merge.

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/bootcamp/iap/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:



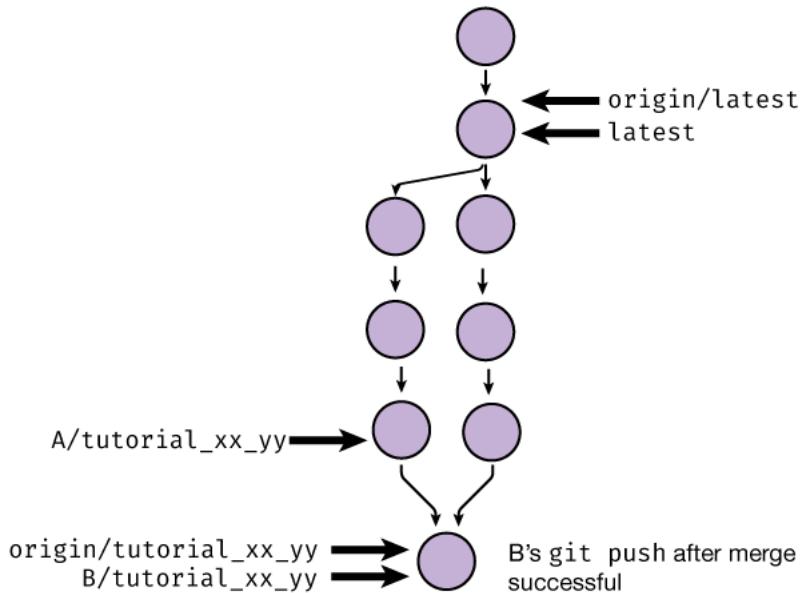
```
.. admonition:: Exercise  
1. Edit this file (``docs/bootcamp/iap/git_intro.rst``), adding your name below:  
:::  
  
Accept Current Change | Accept Incoming Change | Accept Both Changes | Compare Changes  
<<<< HEAD (Current Change)  
      Christopher Johnstone, again?  
=====  
      Christopher Johnstone  
>>>> test1 (Incoming Change)  
  
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.
```

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/bootcamp/iap/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 :)

7.1.5 Software Tips and Tricks

One date format to rule them all/naming



101

Fig. 2: Courtesy of xkcd¹⁰²

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.

This date format is both unambiguous and also sorts well (e.g. a lexicographic/alphabetical sort sorts in the correct time order).

¹⁰¹ https://imgs.xkcd.com/comics/iso_8601.png

¹⁰² <https://xkcd.com/1179/>

Finally, avoid using spaces in file and directory names! It's not a problem for most code, but it's just a good best practice that makes typing these paths in easier in a terminal.

Paper RSS feeds

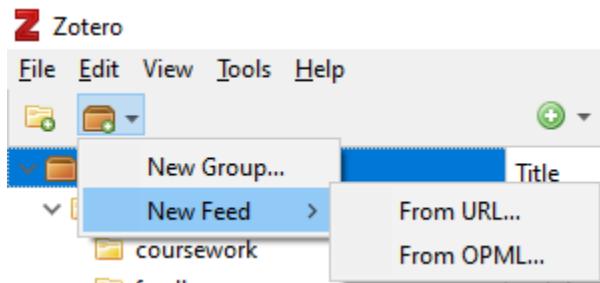
Staying on top of the firehose of papers can be difficult. Using a RSS (Really Simple Syndication) aggregator can be helpful. RSS is a relatively old technology that allows for websites to push lists of content over time; the first podcasts were actually syndicated/released using RSS.

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 [Feedly¹⁰³](#). 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!

¹⁰³ <https://www.feedly.com/>

The screenshot shows the PubMed.gov search interface. At the top, the search bar contains the term "supercoiling". Below the search bar are links for "Advanced", "Create alert", and "Create RSS". To the right are "Search" and "User Guide" buttons. Below the search bar are buttons for "Save", "Email", and "Send to". To the right are "Sorted by: Best match" and "Display options" buttons. The main area is titled "Your RSS Feed". It shows a field "Name of RSS Feed:" with "supercoiling" entered, a dropdown for "Number of items displayed: 100", and buttons for "Create RSS" and "Cancel". Below these are links for the RSS feed URL (<https://pubmed.ncbi.nlm.nih.gov/rss/s>) and a "Copy" button.

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:

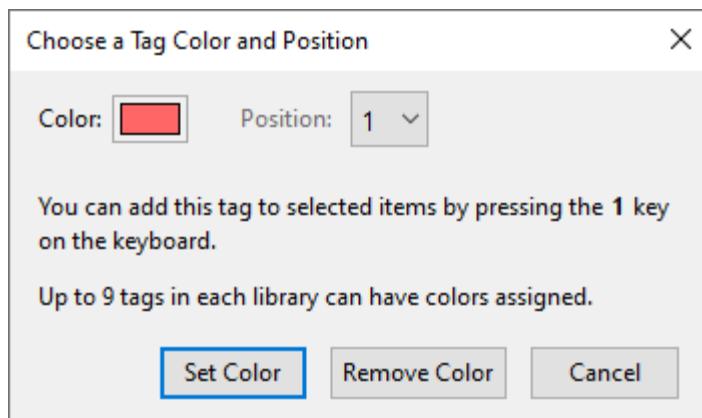
The screenshot shows the Proquest search interface. At the top, there are tabs for "Modify search", "Recent searches", and "Save search/alert ▲". A dropdown menu is open over the "Save search/alert" tab, listing four options: "Save search", "Create alert", "Create RSS feed", and "Get search link".

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; I use the following note template:

Top line summary:

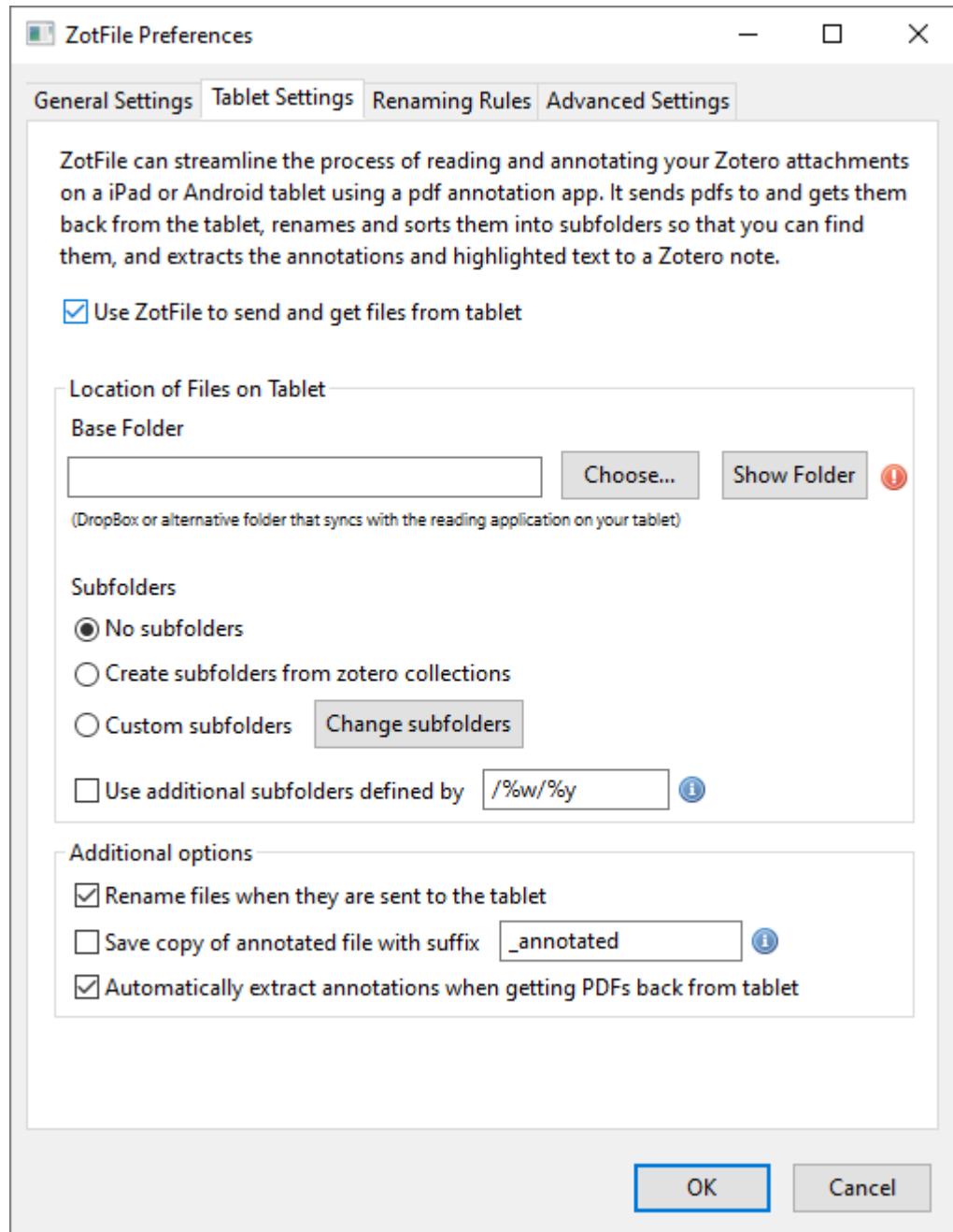
What they did/highlights:

Why do we care:

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.



Better Quartzy

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 drag the CAS # field to the second position:

<input type="checkbox"/> ITEM NAME ▾	CAS #	VENDOR	AMOUNT	LOCATION	TYPE	OWNER	ADDED	UPDATED	
<input type="checkbox"/> pKG521	pENTR...			Bacteri...	Adam Bel...	Jan 5, 2021	Jan 5, 2021	Request	
<input type="checkbox"/> pKG520	pMXs...			Bacteri...	Adam Bel...	Dec 15, 2020	Dec 15, 2020	Request	
<input type="checkbox"/> pKG519	pCW5...			Bacteri...	Adam Bel...	Dec 15, 2020	Dec 15, 2020	Request	
<input type="checkbox"/> pKG518	phage...			Bacteri...	Adam Bel...	Dec 15, 2020	Dec 15, 2020	Request	
<input type="checkbox"/> pKG517	pENTR...			Bacteri...	Adam Bel...	Dec 15, 2020	Dec 15, 2020	Request	

To fix this, there is a Quartzy enhancer userscript that makes it look like the following!

<input type="checkbox"/> ITEM NAME ▾	CAS #	VENDOR	AMOU...	LOCATI...	TYPE	OWNER	ADDED	UPDAT...	
<input type="checkbox"/> pKG521 	pENTR-MCP-mCherry-NLS				Bacteri...	Adam B...	Jan 5, 2021	Jan 5, 2021	
<input type="checkbox"/> pKG520	pMXs-ERKKTR-Clover 				Bacteri...	Adam B...	Dec 15, 2020	Dec 15, 2020	
<input type="checkbox"/> pKG519	pCW57-mCherry-MCP 				Bacteri...	Adam B...	Dec 15, 2020	Dec 15, 2020	
<input type="checkbox"/> pKG518	phage-mCherry-MCP 				Bacteri...	Adam B...	Dec 15, 2020	Dec 15, 2020	
<input type="checkbox"/> pKG517 	pENTR-IsI1-Halo				Bacteri...	Adam B...	Dec 15, 2020	Dec 15, 2020	

The plasmid field is larger, and the plasmid resistance (Amp/Kan/Chlor) is listed directly 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.

First, you should install a userscript manager like [Tampermonkey](#)¹⁰⁴.

Then, you should be able to 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:

The screenshot shows the Tampermonkey Utilities tab interface. At the top, there's a header with the Tampermonkey logo, version v4.11, and links for 'Installed userscripts', 'Settings', and 'Utilities'. Below the header, there are four sections: 'Cloud' (with 'Type:' dropdown set to 'Google Drive', 'Export', 'Show backups', and 'Revoke Access Token' buttons), 'Zip' (with 'Export' button and 'Import' field containing 'Choose File No file chosen'), 'File' (with 'Export' button and 'Import' field containing 'Choose File No file chosen'), and 'TextArea' (with 'Export' and 'Import' buttons and a large text area). At the bottom, there's an 'Install from URL' section with a text input field and an 'Install' button.

Regex help

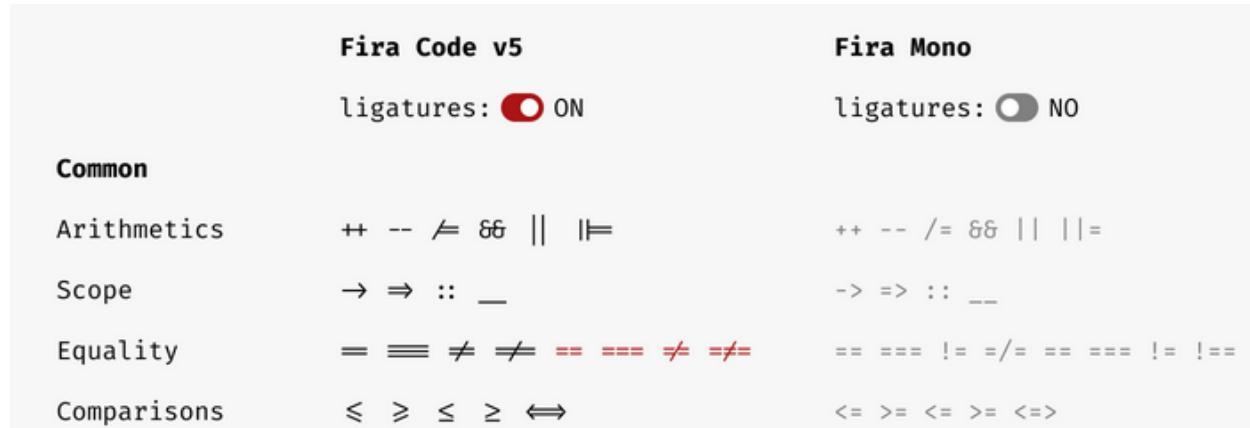
If you ever need help debugging or designing a regular expression, try using <https://regex101.com/>

¹⁰⁴ <https://www.tampermonkey.net/>

Fonts

Helvetica Neue is a good sans-serif font that is based on everyone's favorite font, Helvetica. Download it [here](#). Windows needs a slightly different version of the fonts, available [here](#)

For a good monospaced/code/terminal font, [Fira Code](#)¹⁰⁵ is excellent. 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.



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`

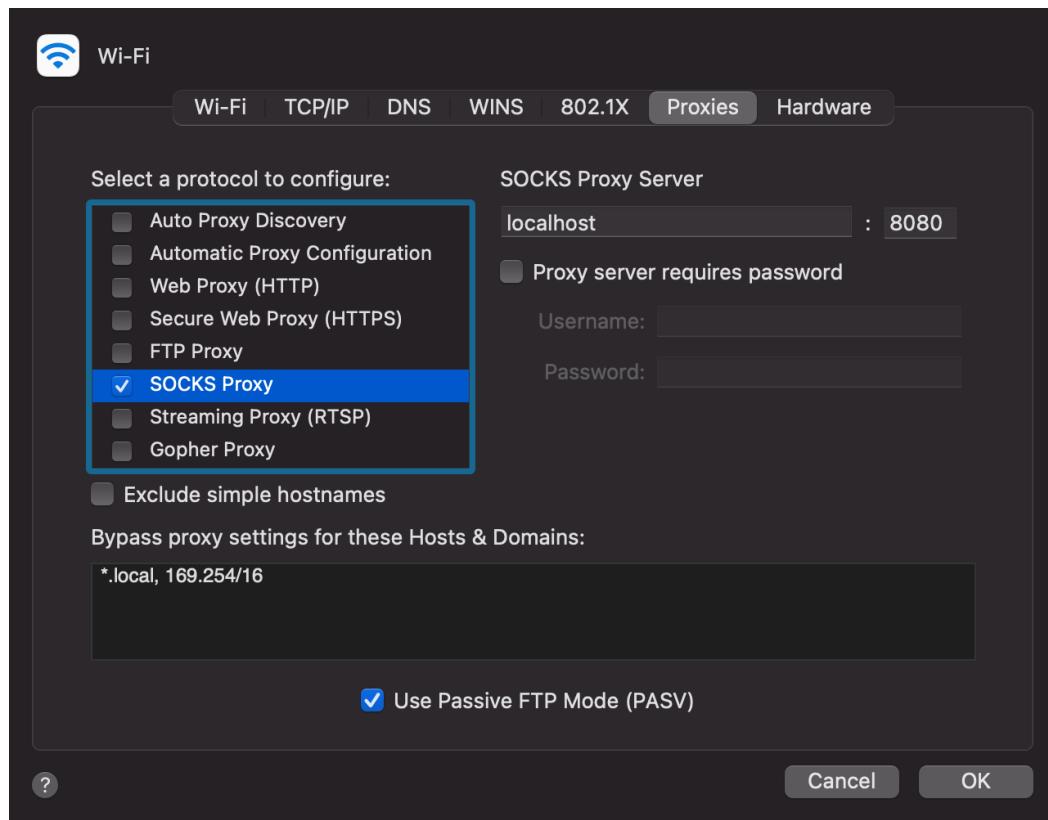
```
Nathan — zsh — 80x24
Last login: Wed Dec 29 13:26:35 on ttys000
Nathan@Nathans-Air ~ % ssh -D8080 nbwang@athena.dialup.mit.edu
```

2. Login with your MIT password and do a Duo push in bash/powershell/etc. **Leave this window open.**

```
Duo two-factor login for nbwang
Enter a passcode or select one of the following options:
1. Duo Push to XXX-XXX-3372
2. Phone call to XXX-XXX-3372
3. SMS passcodes to XXX-XXX-3372
Passcode or option (1-3): 1
```

3. Set your system proxy settings to use a SOCKS v5 proxy to localhost , port 8080

¹⁰⁵ <https://github.com/tonsky/FiraCode/releases>



4. Check that the system proxy works by typing “what is my ip address” into Google. You should get an IP starting with 18

A screenshot of a web browser showing a Google search result. The search query "what is my ip address" is typed into the search bar. Below the search bar, the navigation bar shows "All" selected, along with links for Books, News, Shopping, Videos, More, and Tools. The search results page displays the message "About 1,540,000,000 results (0.46 seconds)". The first result is a card with the heading "What's my IP" and the IP address "18.9.64.28" prominently displayed. Below the IP address, it says "Your public IP address". At the bottom of the card, there's a link "→ Learn more about IP addresses".

7.1.6 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 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** and 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.

Owner * Repository name *

 GallowayLabMIT /

Great repository names are short and memorable. Need inspiration? How about [fictional-engine](#)?

Description (optional)

 Public
Anyone on the internet can see this repository. You choose who can commit.

 Private
You choose who can see and commit to this repository.

Initialize this repository with:
Skip this step if you're importing an existing repository.

Add a README file
This is where you can write a long description for your project. [Learn more](#).

Add .gitignore
Choose which files not to track from a list of templates. [Learn more](#).

.gitignore template: [Python](#) ▾

Choose a license
A license tells others what they can and can't do with your code. [Learn more](#).

License: [None](#) ▾

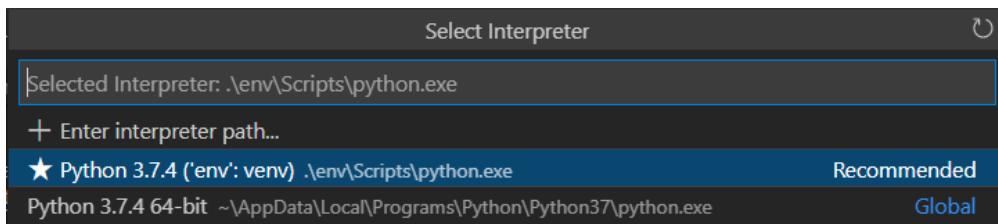
This will set  main as the default branch. Change the default name in Galloway Lab's [settings](#).

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, and open a terminal (or VS Code instance) into the repository folder.
3. Create a virtual environment (`python -m venv env`) for this project.
4. If in a terminal, activate the virtual environment (see *Python/Sphinx setup* (page 408) for help activating)

Note: If you are working inside VSCode, 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 should use that environment by default.

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`, type in `select interpreter`, select `Python: Select Interpreter` and click the Python installation in your newly created virtual environment.



-
5. Install packages you need. For data-analysis projects, this is likely `pip install numpy scipy matplotlib seaborn rshd`
 - a. If you are planning on using Jupyter notebooks, it is a best-practice to additionally install `nb-clean` via `pip install nb-clean`. Then, run `nb-clean add-filter`. From then on, `nb-clean` will automatically run alongside Git as a filter, making sure that extraneous notebook metadata does not get committed to version control.
 6. 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`.
 7. If using data in the OneDrive/Sharepoint, create a `datadir.txt` file in the top-level folder of the repository that contains one line with the full, absolute path.
 - a. Add `datadir.txt` to your `.gitignore` file. This means editing the `.gitignore` and adding a line anywhere, typically at the top, that contains just `datadir.txt`.

New repository (Julia)

1. Create a new repository 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** and you should select **Julia** as the **.gitignore** template. Setting the **.gitignore** means that Git will start off by ignoring all Julia-related temporary files. You can update and modify the ignore list later.

Owner * Repository name *

 GallowayLabMIT /

Great repository names are short and memorable. Need inspiration? How about [fictional-engine?](#)

Description (optional)

 Public
Anyone on the internet can see this repository. You choose who can commit.

 Private
You choose who can see and commit to this repository.

Initialize this repository with:
Skip this step if you're importing an existing repository.

Add a README file
This is where you can write a long description for your project. [Learn more.](#)

Add .gitignore
Choose which files not to track from a list of templates. [Learn more.](#)

.gitignore template: [Julia](#) ▾

Choose a license
A license tells others what they can and can't do with your code. [Learn more.](#)

License: [None](#) ▾

This will set  main as the default branch. Change the default name in Galloway Lab's [settings](#).

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, and open a terminal (or VS Code instance) into the repository folder.
3. 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.
4. Inside the Julia prompt, press `]`; the prompt should change to `(folder_name) pkg>`. Type `add pkg1 pkg2` to install packages into the virtual environment.

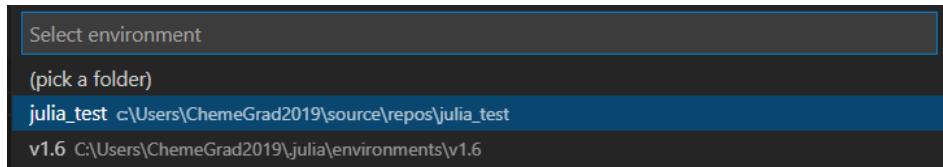
```

~\source\repos\julia_test $ julia --project=.
  Documentation: https://docs.julialang.org
  Type "?" for help, "]?" for Pkg help.
  Version 1.6.1 (2021-04-23)
  Official https://julialang.org/ release

(julia_test) pkg> add Plots
  Updating registry at `C:\Users\ChemeGrad2019\.julia\registries\General`
  Updating git-repo `https://github.com/JuliaRegistries/General.git`
  Resolving package versions...

```

- a. When you add or update packages later, be sure to commit the `Manifest.toml` and `Project.toml` files! These encode how others can reproduce your set of packages.
5. Once you have `.jl` files, VSCode 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 virtual environment.



New repository (R)

Warning: TBD. R's virtual environment system is non-built-in and is also bad compared to other systems. 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) currently under beta testing.

Existing, non-data-driven repository (e.g. protocols)

1. Clone the repository to some local folder.
2. Start a terminal/VSCode instance in the folder.
3. If using Python:
 - a. Create a virtual environment using `python -m venv env`.
 - b. Activate the environment (see *New repository (Python)* (page 370) for details on activating in VSCode/terminal).
 - c. Install the current package versions for this project using `pip install -r requirements.txt`.
4. If using Julia:

- a. Start Julia within a local virtual environment with `julia --project=..`.
 - b. Enter package mode by pressing `]`.
 - c. 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.

Existing, data-driven repository (e.g. `tangles_model`)

1. Perform the steps in the above section, *Existing, non-data-driven repository (e.g. protocols)* (page 373).
2. If using Jupyter notebooks, run `nb-clean add-filter` to register the Jupyter notebook cleaning filter with Git.
3. If using `rushd`, add a `datadir.txt` file to the root folder of the repository, containing the absolute path to the OneDrive/Sharepoint folder (e.g. the folder that *directly* contains the folders such as `projects`, `manuscripts`, etc).

7.1.7 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
```

¹⁰⁷ MacOS image from <https://ohmyz.sh/>

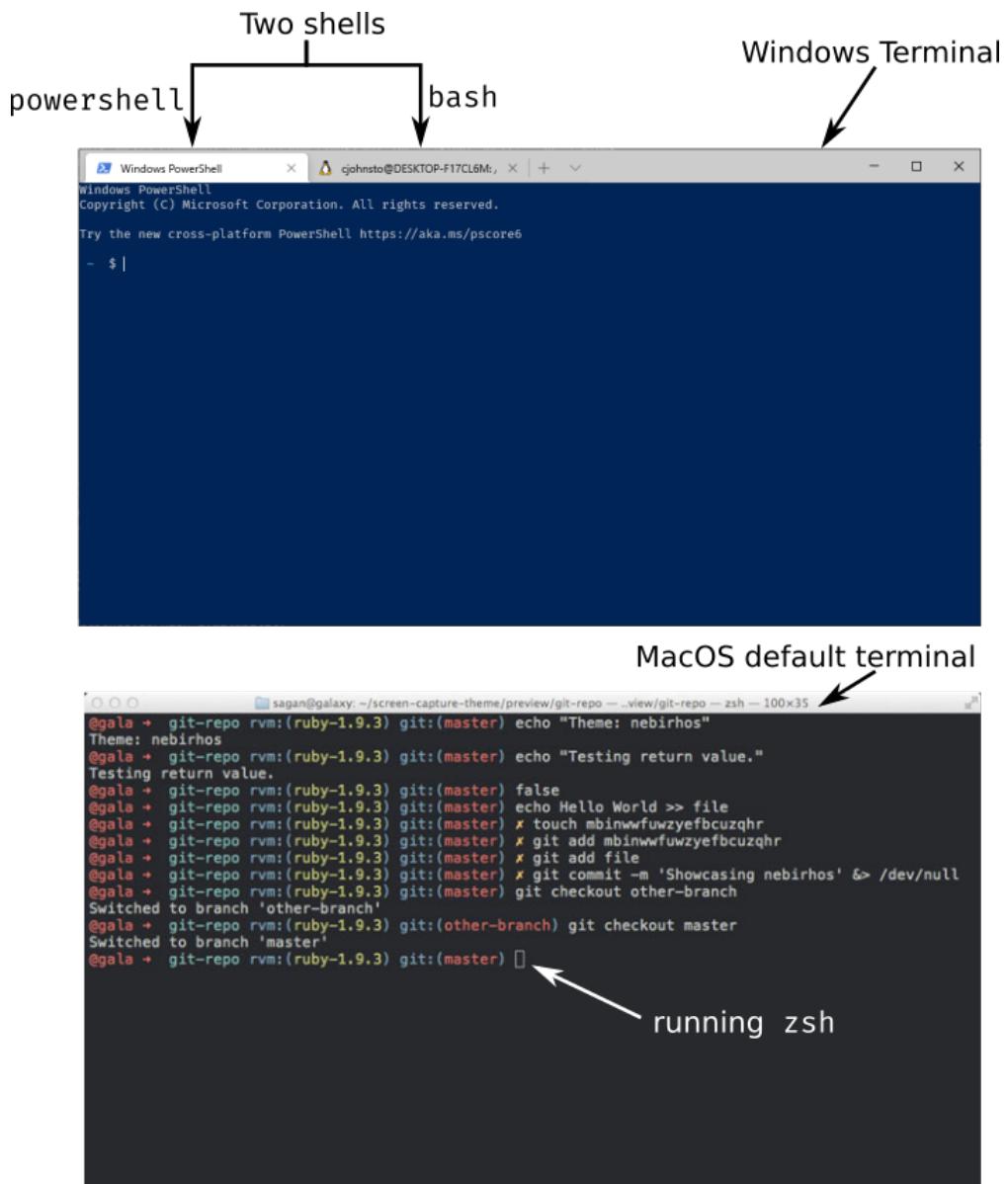


Fig. 3: An example of the difference between terminals and shells Page 375, 107

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 its **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 “short-hand” 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 foreground).
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
```

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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
```

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```
$ cd ~          # return to 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.¹⁰⁸

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
```

¹⁰⁸ Inspired by the Software Carpentry examples^{Page 382, 109}, license CC-BY-4.0

¹⁰⁹ <https://swcarpentry.github.io/shell-novice/02-filedir/index.html>

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.

Now that we can create directories/folders, how do we actually move files around? Using `mv`!

Command: `mv`

`mv` stands for **m**ove, 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 though 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
```

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```
$ 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 concatenate. 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) ]
```

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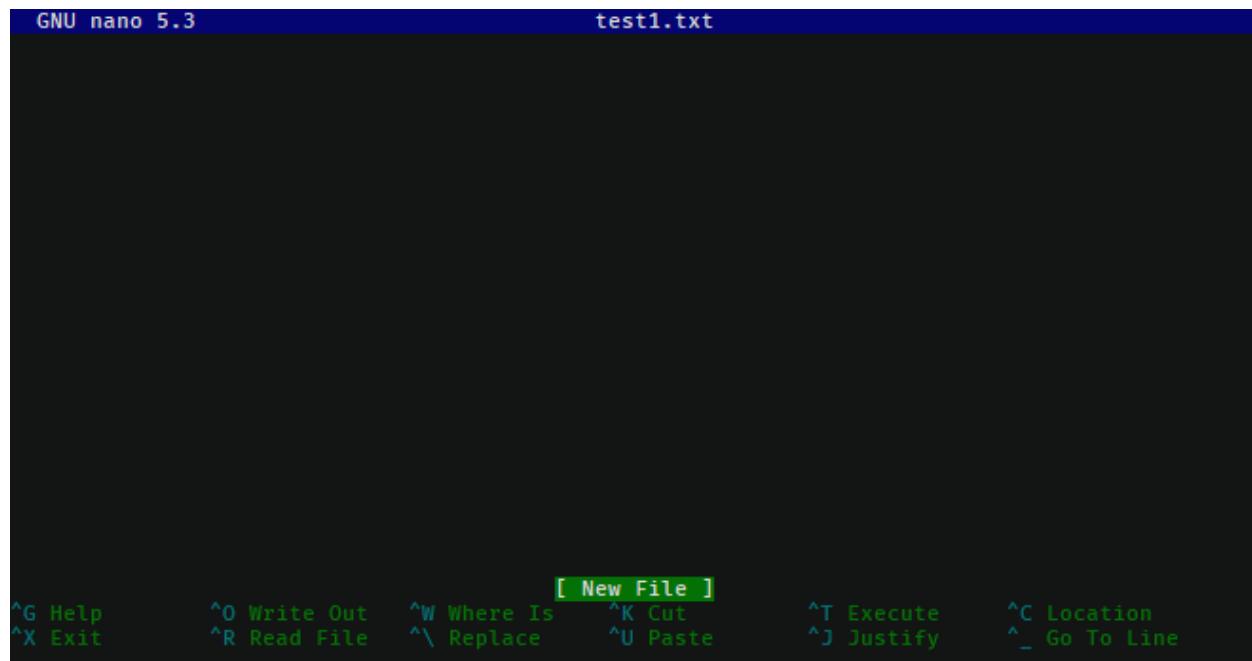
<code>^G Help</code>	<code>^O Write Out</code>	<code>^W Where Is</code>	<code>^K Cut</code>	<code>^T Execute</code>	<code>^C ↵</code>
<code>↖ Location</code>					
<code>^X Exit</code>	<code>^R Read File</code>	<code>^\\ Replace</code>	<code>^U Paste</code>	<code>^J Justify</code>	<code>^_ Go ↵</code>
<code>↖ To Line</code>					

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.

The screenshot shows a terminal window with the title bar "GNU nano 5.3" and the file name "test1.txt". The main area of the window is completely blank, indicating an empty file. At the bottom of the window, there is a status bar with several options:

- File Name to Write: test1.txt
- "G Help
- "C Cancel
- M-D DOS Format
- M-M Mac Format
- M-A Append
- M-P Prepend
- M-B Backup File
- ^T Browse

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 more 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 'global/regular expression/print'.

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 regular expression syntax is supported in this mode.

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Following the example in the Software Carpentries example¹⁰⁶, 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
```

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¹⁰⁶ <https://swcarpentry.github.io/shell-novice/07-find/index.html>

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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
        ├── .git
        └── env

```

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 agtccagtgtCATCAACAAGTTGTACAAAAAAG
nbw_primers.txt:GG_lenti_bb_fwd ACAGCGTCTCATcctTAAAAGAAAAGGGGGGAC
nbw_primers.txt:GG_WPRL_fwd ACAGCGTCTCATgcCGATAATCACACCTCTGGATTACAAAATT
nbw_primers.txt:link-NLS-VPR_rev
→gaaagctgggtctagatatcTAAACAGAGATGTGTCGAAGATGG

```

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```

nbw_primers.txt:"mCherry-MCP-VP16_rev      "
  ↵gaaagctgggtctagatatcCTACAGCATATCCAGATCAAATCGTC
nbw_primers.txt:Rsites_UbC_MCS_fwd      "
  ↵tccagttggcatgcgctagcctcgagGCATCAACAAGTTGTACAAAAAAG
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](#)¹¹⁰.

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](#)¹¹¹. 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](#)¹¹² 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": [
    {
```

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¹¹⁰ <https://ohmyposh.dev/>

¹¹¹ <https://ohmyposh.dev/docs/installation>

¹¹² <https://ohmyposh.dev/docs/installation/#4-replace-your-existing-prompt>

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```
"type": "prompt",
"alignment": "left",
"segments": [
    {
        "type": "session",
        "style": "plain",
        "foreground": "#ffffff",
        "properties": {
            "postfix": ":",
            "user_color": "#ffd93d",
            "ssh_icon": "\u260e"
        }
    },
    {
        "type": "path",
        "style": "plain",
        "foreground": "#44B4CC",
        "properties": {
            "style": "agnoster_full"
        }
    },
    {
        "type": "git",
        "style": "powerline",
        "powerline_symbol": "\u260e",
        "foreground": "#193549",
        "background": "#a1c60b",
        "properties": {
            "local_working_icon": " W",
            "local_staged_icon": " S"
        }
    },
    {
        "type": "python",
        "style": "powerline",
        "background": "#00897b",
        "foreground": "#193549",
        "powerline_symbol": "\u260e",
        "properties": {
            "display_version": false,
            "display_virtual_env": true,
            "display_mode": "context"
        }
    },
    {
}
```

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```
"type": "text",
"style": "plain",
"properties": {
    "text": "$"
}
}
]
}
```

TODO

Add IAP bootcamp info

7.2 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.

7.2.1 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.

7.2.2 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 α_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 α_m .
2. How does increasing α_p affect the steady state mRNA and protein levels? Increasing β_m ? Increasing β_p ? Choose one of these parameters (α_p , β_m , or β_p) and plot mRNA and protein levels over time for several different values of that parameter.
3. Optional: How does increasing β_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.

7.2.3 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 k_{cat} 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?

7.2.4 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?

7.2.5 Tutorial 5: Hill functions

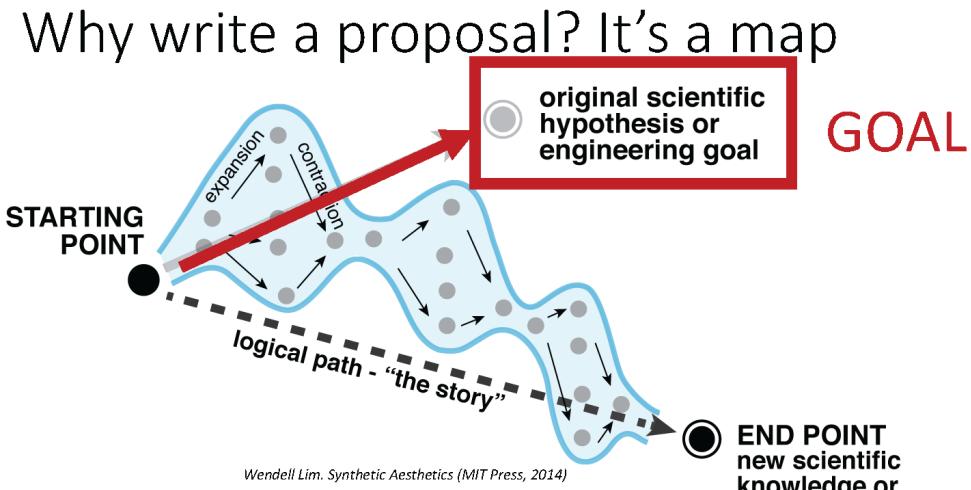
- Lesson PDF: [Lesson5_HillFunctions.pdf](#)
- Scripts: [L5_repressor_script.m](#), [L5_repressilator_script.m](#)

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 ($\alpha_0 > 0$)?

7.3 “How to” Training Series

7.3.1 Tutorial 1: How to Write a Proposal



Value:

1. You have outlined the journey and where you expect to reach
2. You can share that goal and plans with others!
3. They might even give you money for your map
4. You can reflect on where deviations occur

- 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!**



The task:

Read paper A

EXPECTATION:

Read paper A

REALITY:

Read paper A

Read paper B mentioned in paper A

Read paper C mentioned in paper A

Read paper D mentioned in paper A

Read paper E mentioned in paper A

:: Read paper F mentioned in paper A

- View these slides.

7.3.3 Tutorial 3: How to Review a Paper

A suggested recipe for journal club

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

General guideline of when to start deciding on details

Big picture

1st Level

- Size
- Layout
- Color palette
- Font

2nd Level

- White space
- Level of physical/scientific accuracy
- Color separations/overlays

3rd Level

- White space (yes again!)
- Alignment
- Embellishments (outlines, shadows, effects, etc.)

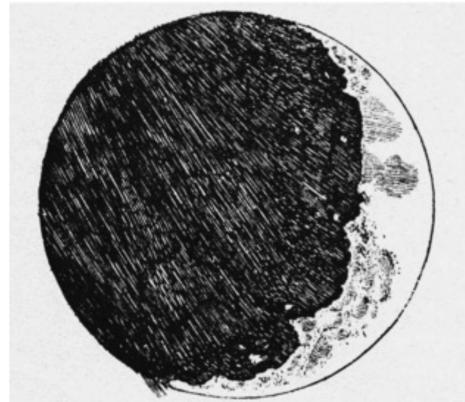
Small details

- 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.

TECH DOCUMENTATION

8.1 Repository setup

This repository is automatically built and pushed to <https://gallowaylabmit.github.io/protocols>

See the *Repository setup guide* (page 403) if you are interested in how the continuous build system works, or you'd like to fork this repository to make it your own.

TODO

Add info about labbot, repo setup, git, a more detailed RST primer, etc.

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 website¹¹³](#), in addition to a printable PDF version of all of the protocols and recipes, available [here¹¹⁴](#).

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 412).

If you already are familiar with text editors and have an editing/programming environment already setup, you can go directly to [Local building](#) (page 410).

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:

¹¹³ <https://gallowaylabmit.github.io/protocols>

¹¹⁴ https://gallowaylabmit.github.io/protocols/galloway_lab_protocols.pdf

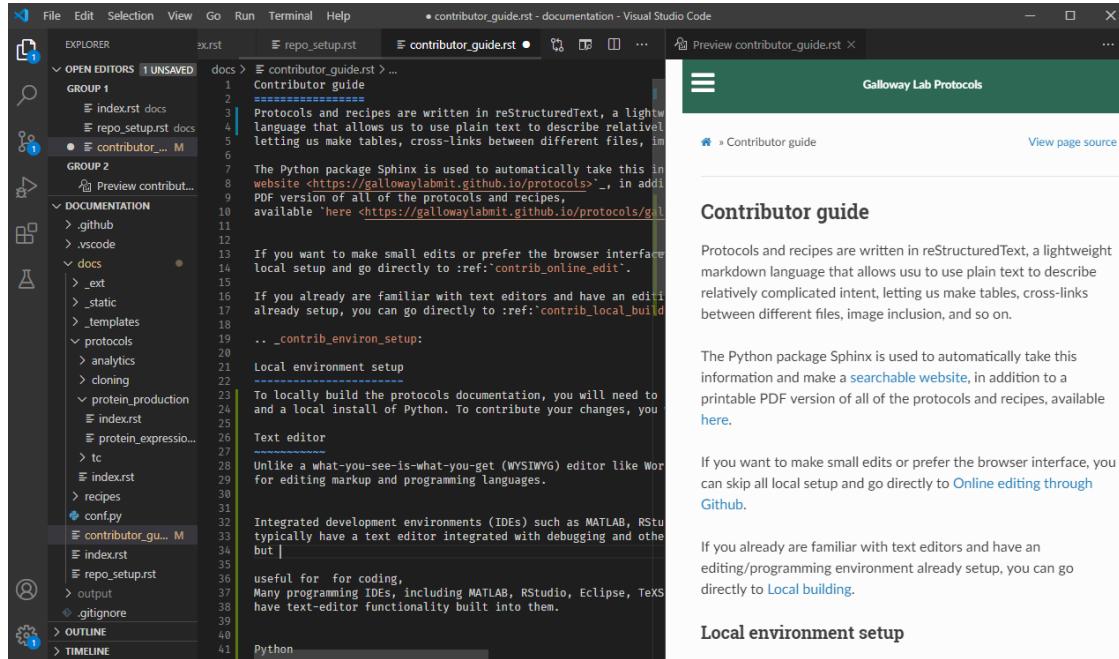
- **Built-in editors:** Every major OS comes pre-installed with a plain text editor. Windows has *notepad*, Mac OSX has *TextEdit* (while this defaults to a rich-text editor, it can also be used as a plain-text editor), and most Linux distros have *vi* or *GEdit*.
- **Terminal-based editors:** *nano* 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++*¹¹⁵. More recently, there are the commonly used editors *Sublime Text*¹¹⁶, *Atom*¹¹⁷, and *VS Code*¹¹⁸.

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:



¹¹⁵ <https://notepad-plus-plus.org/>

¹¹⁶ <https://www.sublimetext.com/>

¹¹⁷ <https://atom.io/>

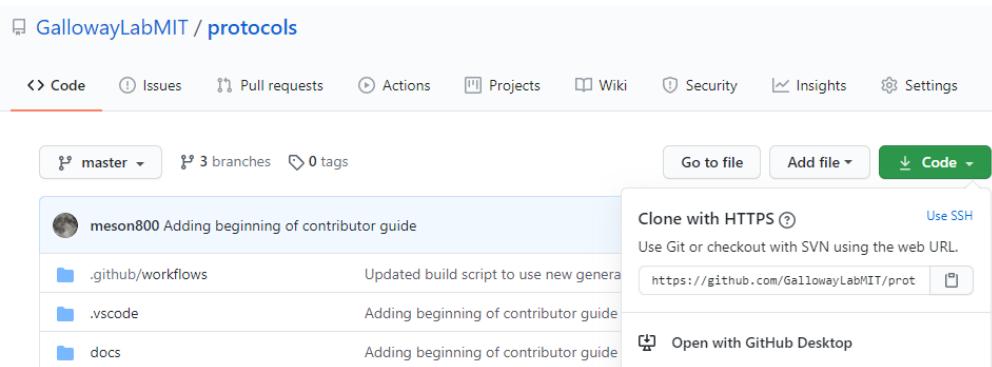
¹¹⁸ <https://code.visualstudio.com/>

9.1.2 git

We use git to manage version history, simultaneous editing, and other features. There are several¹¹⁹ excellent¹²⁰ tutorials¹²¹ elsewhere that explain how to use git.

If you don't have git, you can [install it from here](#)¹²², and install a GUI tool if you wish (such as the standalone [Github Desktop](#)¹²³, 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.

¹¹⁹ <https://git-scm.com/book/en/v2>

¹²⁰ <https://try.github.io/>

¹²¹ <https://gitimmersion.com/>

¹²² <https://git-scm.com/downloads>

¹²³ <https://desktop.github.com/>

9.1.3 Python/Sphinx setup

Sphinx¹²⁴ 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 installer¹²⁵. 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.

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 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 408) section.

¹²⁴ <https://www.sphinx-doc.org/en/master/>

¹²⁵ <https://www.python.org/>

4. When there are no build errors, add the 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](#)¹²⁶.

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.
```

¹²⁶ <https://github.com/GallowayLabMIT/protocols/actions>

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 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 `reStructuredText: Open Preview to the Side`.

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:

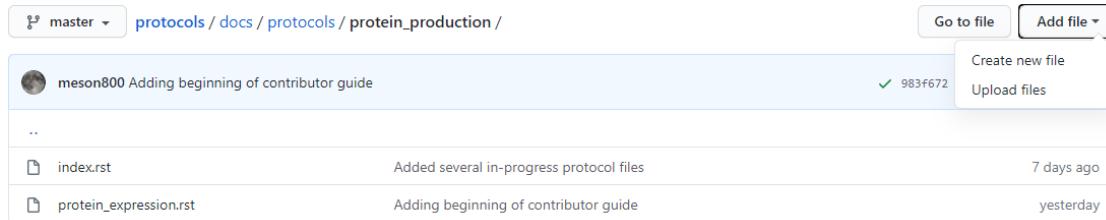
```
python build.py --force-rebuild
```

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 410) 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:

The screenshot shows a GitHub repository interface. At the top, there's a navigation bar with a dropdown for 'master', the path 'protocols / docs / protocols / protein_production / protein_expression.rst', and buttons for 'Go to file' and '...'. Below this is a commit history card for a user named 'meson800' with the message 'Adding beginning of contributor guide'. It shows '1 contributor' and a 'Latest commit' timestamp. Further down, there's a stats summary: '44 lines (32 sloc) | 1.95 KB' and a set of file operations buttons ('Raw', 'Blame', 'Copy', 'Edit'). The main content area starts with a section titled 'Protein expression'. Inside, there's a note block containing the text '... time::: 2 days'. Below this is a 'Protocol' section. Under 'Protocol', there's a 'Note' heading followed by text about E. coli strains and a bulleted list of strains. Then, there's a numbered list of four steps for protein expression.

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, inoculate 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.

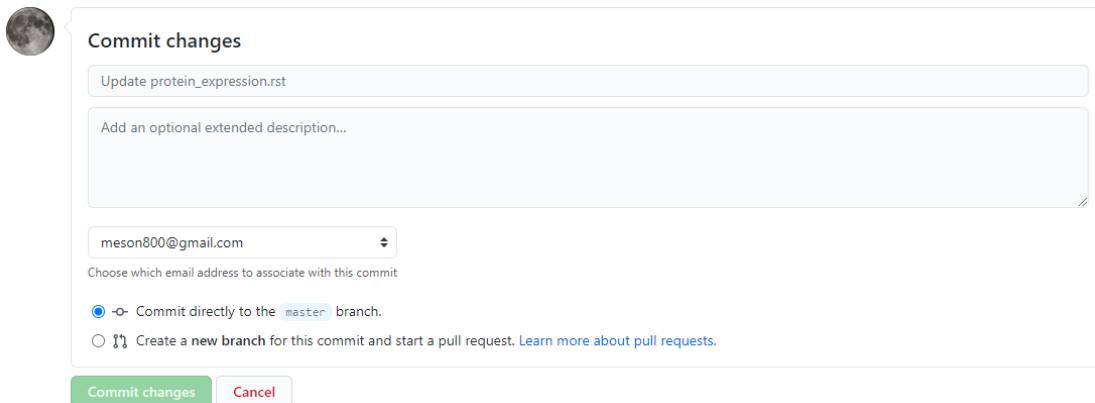
This will open an editor window:

protocols / docs / protocols / protein_production / protein_expression.rst Cancel

<> Edit file Preview changes Spaces 4 Soft wrap

```
1 =====
2 Protein expression
3 =====
4
5 .. time:: 2 days
6
7
8 Protocol
9 =====
10 .. note::
11     The BL21 strain of E. coli is best for protein production. It has several proteases knocked
12     out, among other modifications. There are several sub-strains that can be relevant:
13
14     - **BL21**: The classic, useful if your promoter is a standard E. coli promoter
15       such as lac, tac, trc, ParaBAD, PrhαBAD, or T5.
16     - **BL21(DE3)** : A strain with integrated T7 polymerase. Can be used in place of **BL21**
17       but is required if using the T7 or T7-Lac promoters.
18     - **BL21-RIL** : A strain with extra copies of certain tRNAs, in order to correct for the
19       rarity of some codons in E. coli. This can be helpful in upping protein titers,
20       especially when expressing some mammalian proteins.
21
22 1. In the morning, start a **5 mL** culture of your strain, in LB/antibiotic media.
23 2. Prepare :ref:`TB media <tb_media>` in Erlenmeyer flasks. For proper aeration,
24   flasks should be filled to at most 30% the listed volume.
25 3. Before leaving for the day, inoculate **20 mL** LB/antibiotic cultures with **400 uL** of the 5 mL culture.
26 4. The following morning, use the NanoDrop to calculate the OD of the overnight cultures. Inoculate large
27   flasks to an OD600 of 0.075 (e.g. three doublings to get to 0.6).
```

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 protocol, 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 here¹²⁷ 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

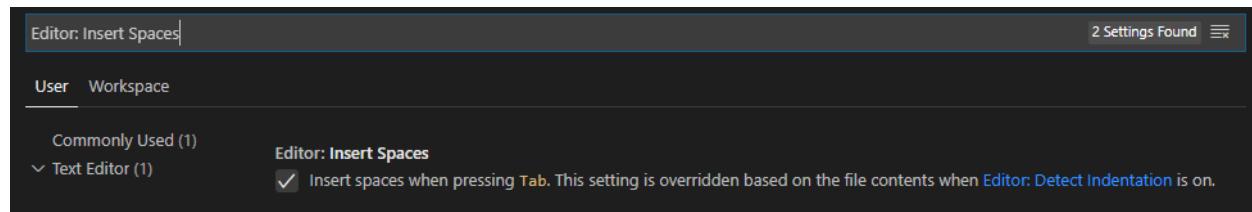
¹²⁷ <https://www.sphinx-doc.org/en/master/usage/restructuredtext/basics.html>

if you are programming in whitespace-dependent languages like Python).

Without wading too deeply into the [holy war¹²⁸](#), 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:



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`.

¹²⁸ <https://softwareengineering.stackexchange.com/questions/57/tabs-versus-spaces-what-is-the-proper-indentation-character-for-everything>

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
  * ??????
* 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](#)¹²⁹ 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

¹²⁹ <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](#)¹³⁰

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 405) 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](#)¹³¹, noting that labels that you create are global and shared across the entire repository!

¹³⁰ <https://example.org>

¹³¹ <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

$$E = mc^2$$

CONTRIBUTING

See the [Repository setup guide](#) (page 403) 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 405) for a more detailed walkthrough of how to contribute to this protocols repository.

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