

Rig Set-Up

Before starting setting up, make sure that the PMTs are in “safe” mode, then that your mouse is dark adapting.

Begin with turning on the four power strips (Figure 1). Turn on the monitors if they aren’t on automatically.

Perfusion Part 1

- Fill the large and small water baths with water halfway.
- Set the large water bath to 37° and the small water bath to 32°.
- Prior to setting up the perfusion lines, check the points below.
 - The backup vacuum is on.
 - The inflow and outflow tubes are pointed toward the middle of the dish.
 - The waste container is empty.
- Take out a 2 L Ames bottle from the fridge, place it on the lab bench, and unscrew the cap.
- Take an empty 250 mL bottle from one of the shelves, place it next to the 2 L Ames bottle, and unscrew the cap. Leave the caps in the sink.
- Wear gloves for the following steps. Pour 100 mL of Ames from the 2 L Ames bottle into the smaller bottle. Place a red weight on the large bottle and a blue weight on the small bottle.

Preparing 2 L Ames bottle

- From the 70° Incubation Fridge found opposite the rig rooms, take a bottle cap with 4 holes and a large bubbler. Each hole in the cap will be used for (1) oxygen, (2) inflow, (3) outflow, and (4) ventilation.
- Starting with (1) oxygen, the goal is to connect the bubbler inside the Ames bottle to the carbogen tank. Use the diagram below to assist you with the instructions.
 - Gather the parts for the *extended bubbler adapter* which are located around the lab bench (Figure 2a). Assemble the parts (Figure 2b).
 - Attach the extended adapter to the bubbler (Figure 2c).
 - String the extended adapter + bubbler the cap and tighten the cap (Figure 2d).



Figure 1: The locations of the power switches.

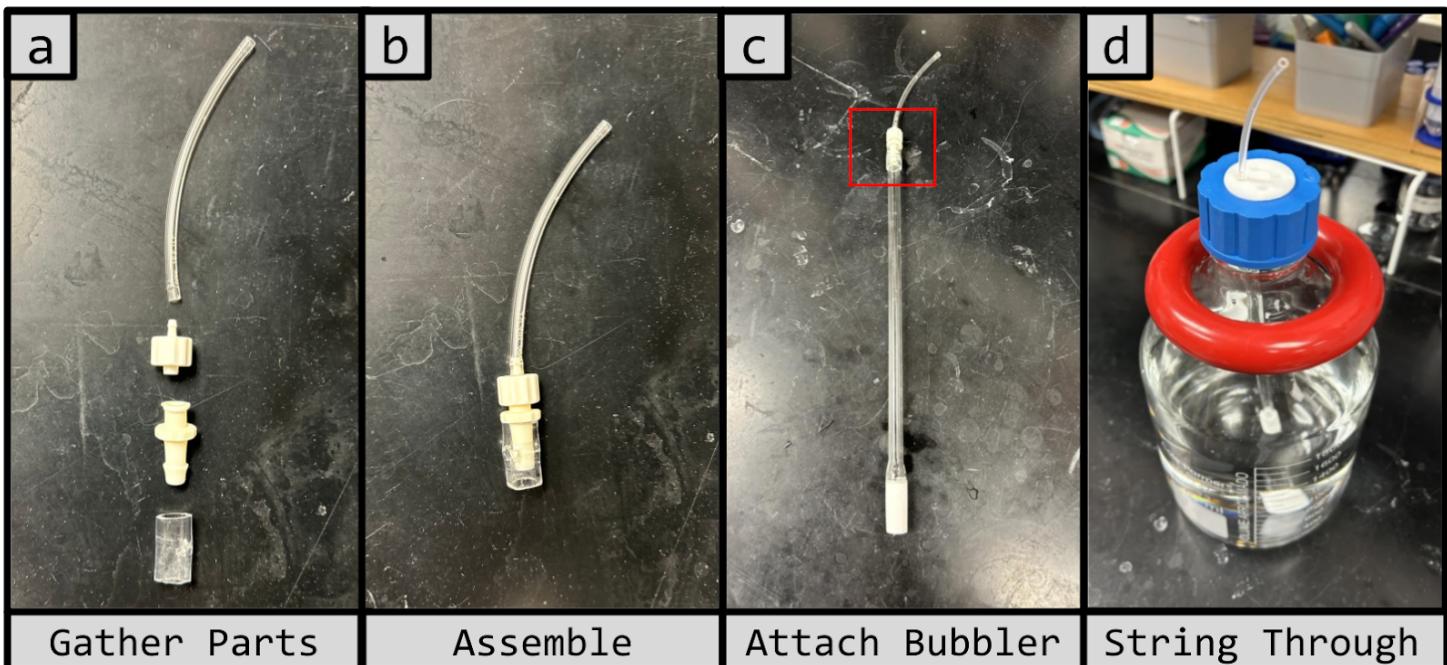


Figure 3: Visual instructions to make the extended bubbler adapter.

- Place the Ames in the water bath and connect the bubbler to the carbogen tank (Figure 3).

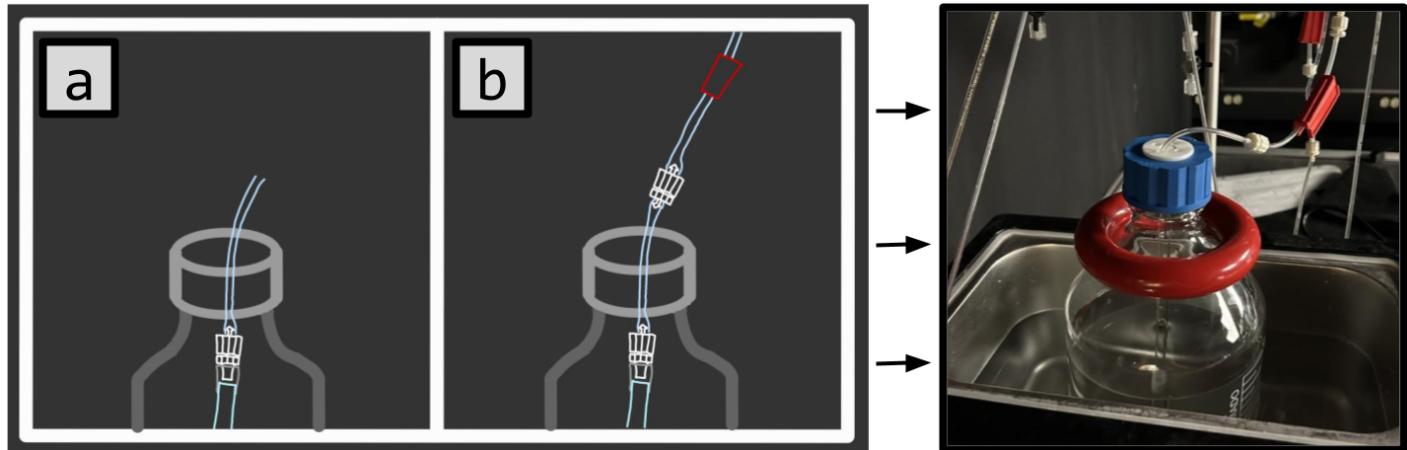


Figure 2: Visual instructions for attaching the extended bubbler adapter to the carbogen tank line. a) The extended bubbler adapter before being attached to the carbogen tank line. b) The extended bubbler adapter connected to the carbogen tank.

- After setting up the connection to the oxygen, find an L-tube on the lab bench to connect to the cap for (3) ventilation.
- String the (1) inflow and (2) outflow lines through the other two holes. The (1) inflow line should reach the bottom of the bottle. The (2) outflow line should dangle from the top, allowing the outflow to drip onto the surface of the Ames.
- The 2L Ames bottle should now look like the figure below.



Figure 4: Picture of a fully equipped 2L Ames bottle.

Prepare the small Ames bottle

- Refer to Figure 5 for visual instructions.
- Take a small bubbler from the 70° Incubation Fridge and place it into the 250 mL bottle. Attach a large bubbler adapter to it.
- Place it into the small water bath and connect it to the oxygen line.
- Adjust the oxygen so that it is lightly bubbling.

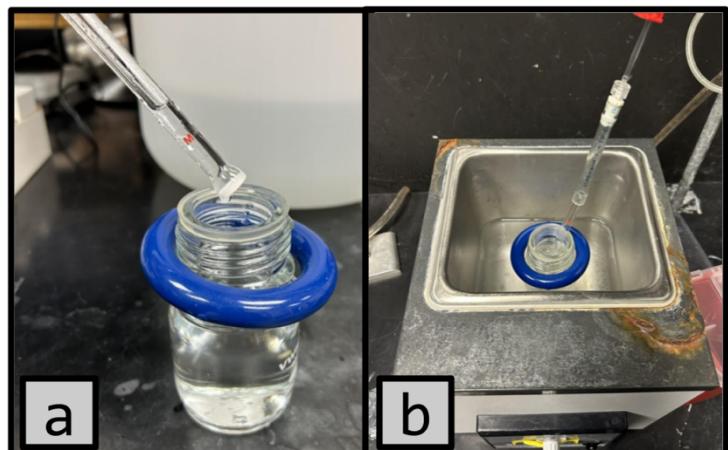


Figure 5: The small Ames bottle. a/ The filled bottle with the small bubbler adapter and blue weight. b/ The bottle in the small water bath attached to the carbogen line.

Perfusion Part 2

- Take two drip tubes from the lab bench and place them into the perfusion pump
- Connect the closed system perfusion lines. Use the diagrams below to guide you.
 - **Blue**: Inflow, Ames bottle → Pump.
 - **Red**: Inflow, Pump → Dish.
 - **Orange**: Outflow, Dish → Pump.
 - **Yellow**: Outflow, Pump → Ames bottle.

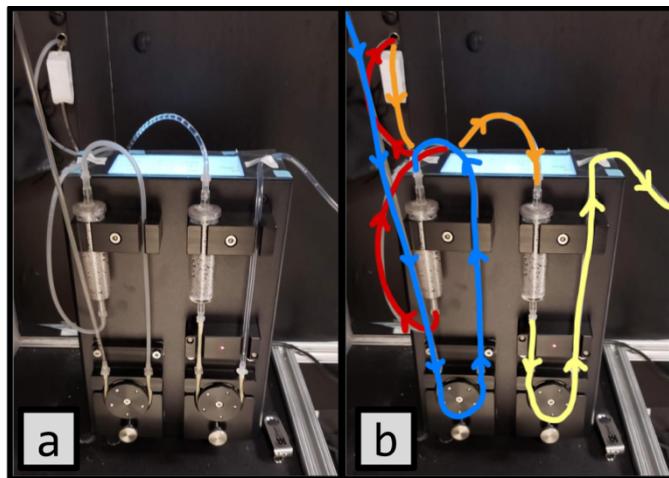


Figure 6: The fully set up perfusion pump. a/ Without the flow color scheme. b/ With the flow color scheme.

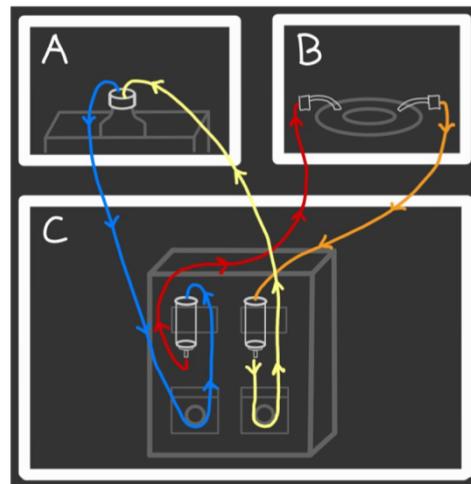


Figure 7: Graphic visualization of flow. It is color coded with the scheme above.

- Before starting the perfusion, which loops outflow into inflow, run the perfusion into the waste flask to wash out contaminants. Use Figure 8 below as a reference. Alternatively, you can run the perfusion in the large water bath.
- After washing the perfusion lines, connect outflow back into the loop and start the perfusion.

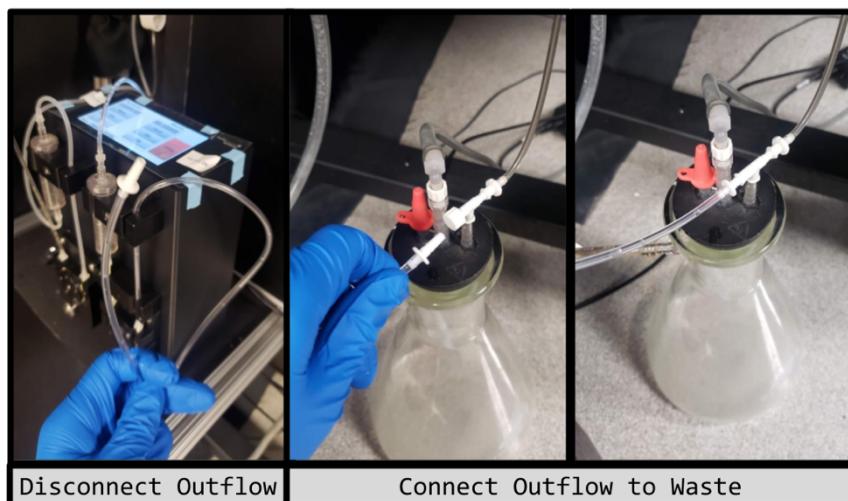


Figure 8: Visual instructions for connecting the outflow to waste.

Objectives Part 1

- Turn on the infrared lamp (found on the same table as the stage and electrode controls).
- Turn on the Stage Computer (found on the lowest compartment under the keyboard; the power button is found underneath a flap on the right side) and open the following software.
 - LinLab2 (Stage Control)
 - Multiclamp (Electrode)
 - Symphony (Projection)
- With the 10x move the objective approximately to the center of the dish. Then, zero x and y of the objective on LinLab2.
- Move the 10x down to focus on the bottom of the dish.
 - While moving down in z, move x and y back and forth until you see debris moving. Adjust the light using the wheel on the infrared lamp as necessary.
 - When you can focus on a piece of debris, you've focused on the bottom of the dish. Avoid accidentally focusing on the debris on the bottom of the dish
- Switch to the 60x and focus on the bottom of the dish.
 - Same procedure as focusing the 10x, essentially find a debris to focus on. This time, open a perfusion line because the 60x objective should be submerged in the solution.
- Zero z. Now xyz should be relative to the position of the 60x focused on the dish.
- Focus the condenser.
 - On the objective control panel, flip the switch to go from objective to condenser mode.
 - Using the knob on the Field Stop Aperture (Figure 9), partially close the aperture enough so that the edge is visible on the screen.
 - Move the condenser in z to have the edge as sharp as possible. Zero the condenser, then open the aperture again.
- Turn off the LED, then project the alignment cross on Symphony. Center the alignment cross using knobs on the condenser (Figure 10). The knobs move the condenser diagonally.
- Choose a different light stimulus to remove the alignment cross, then turn the infrared LED back on.

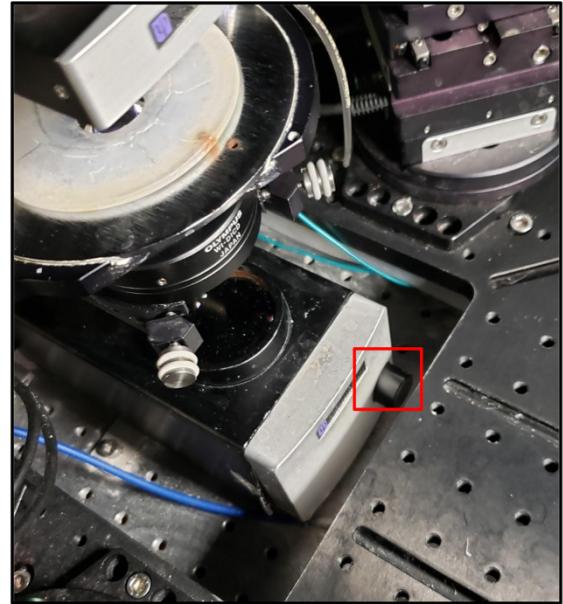


Figure 9: The knob which controls the field stop aperture.

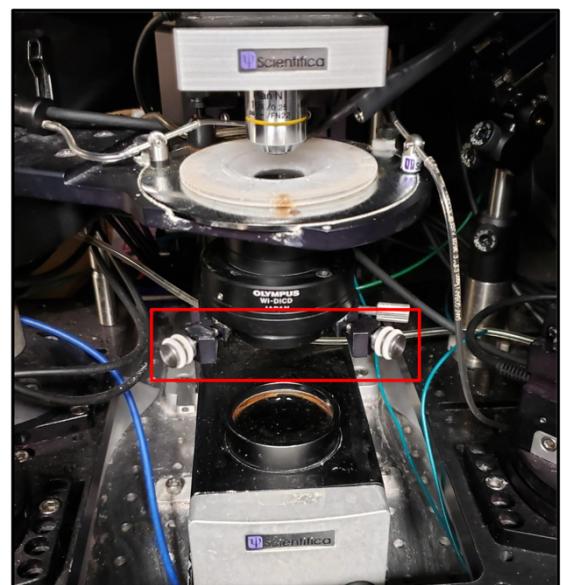


Figure 10: The knobs that center the light stimuli.

- Switch to the 10x and move the objective to 7000um in y.

Electronics Part 1 (Applicable for Two Electrodes)

- Carefully unscrew the electrode holder from the electrode arm on the rig, then go to the metal plating station in the main lab space.
- Sand off the old chloride from the tip of the wire.
- Perform metal plating on the wire (Figure 11).
 - Place the red clamp onto the back of the electrode holder.
 - Uncap the 3M sodium chloride solution.
 - Turn on the pulse generator.
 - Place the tip of the electrode and the black wire into the sodium chloride for about 15 seconds or until the wire tip is sufficiently black. Do not let the electrode and wire touch while in the solution.
- Firmly screw back the electrode holder back into the electrode arm (Figure 11c).

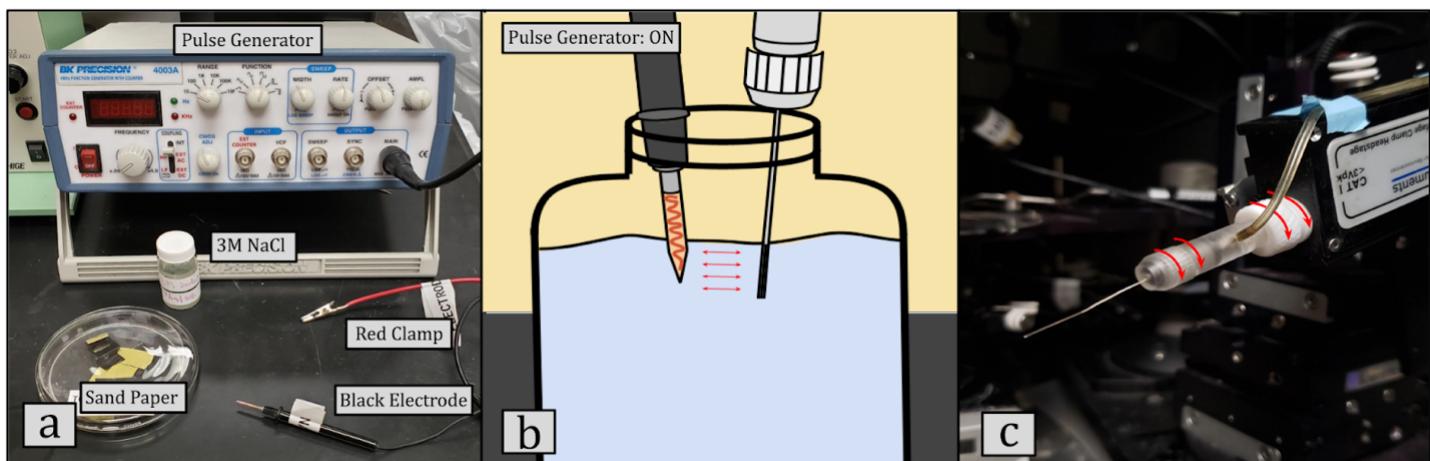


Figure 11: Preparing the wire for the electrode. a/ An overview of the metal plating station. b/ The electrode and wire (not touching) inside the 3 M salt solution. c/ Tightening the electrode holder into the electrode arm.

Pipettes

Pulling Pipettes

- If electrode pipette tips are not already available, make them in the Pipette Pulling Machine (Figure 12).
 - Press the orange button called “MAINS” to start the machine.
 - Open the pipette chamber. Take a glass rod by its end and set it in the chamber (Figure 12a). Use a rubber-tipped tweezer to press the rod against the screw on the right side.
 - Close the chamber, then input the desired pipette program (Figure 12b).

- Press START, which begins the pulling process (Figure 12c). When the machine pauses press START again to receive one pipette, then START again to receive another.



Figure 12: Visual procedure of pulling a glass pipette. a/ Placing the glass rod into the holder, tightly against the screw on the right. b/ Inputting a program. c/ Pressing START the first time to begin the pulling process.

- Inspect at least the first pipette to check if the machine is working properly. The filament might not heat up properly right after starting the machine.
- Place pipettes into a foam pipette holder and bring them with you to the rig.
- Carefully put the glass pipette into the electrode holder, encasing the wire.
 - You should feel the pipette go through the 2 O-rings inside.

Mouth Pipette

- Cut a 1 mL pipette (plastic) at the tip with a razor blade then attach it to the mouth pipette tube. Refer to Figure 13.
- Test if the pipette holds air pressure.
 - Breath into the mouth pipette with positive or negative pressure, then close the pressure valve for a moment. If the pressure is moving, try retightening the screws on the electrode holder (Figure 11c).

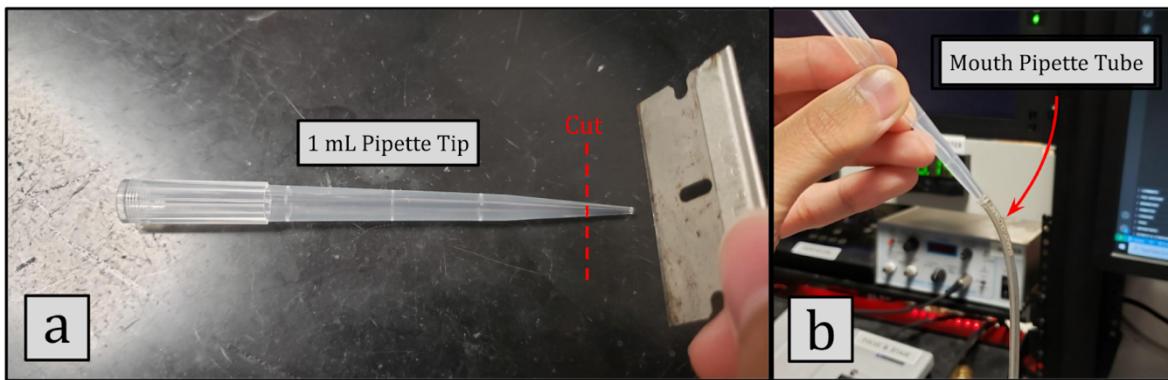


Figure 13: Setting up the mouth pipette. a/ The approximate cut location on the 1 mL Pipette tip. b/ The mouth pipette connected to the breathing tube.

Objectives Part 2

- Carefully swivel the electrode arm under the objective. You may need to adjust the electrode height beforehand.
- Find the electrode under the 10x.
 - You may need to move the electrode in x and y significantly if it is not immediately seen under the objective.
- Center the electrode on the screen, then zero the xyz of the electrode (the z of the objective should be 7000um).
- With the 10x, put the electrode in focus at 3000um (in reference to the objective)
 - This requires moving both the electrode and objective down in z, but always moving the objective down before the electrode.
- Switch to 60x, then put the electrode in focus at 3000um (3000um at 10x is different from 3000um at 60x).
- Move the electrode tip to the upper left corner of the screen, then hold “Home In” on the electrode manipulator for about 3-5 seconds.
 - You can test whether the Home In position is saved by moving the electrode a bit and then clicking the button. The electrode should snap back to the upper left of the screen.
- Flip the “Approach” button on the electrode manipulator and begin extracting the electrode using the x-knob. You want to extract it until the z position of the electrode is positive, since a positive z is safe to swing out.
 - If the electrode is “stuck” using the x-knob, you may need to flip the Approach and raise the electrode with the z knob to achieve a positive z position.
- Hold the “Home Out” on the electrode manipulator for about 3-5 seconds. Test Home In and Home Out to ensure the electrode is moving to the correct places (under the objective for Home In and out into a swingable position for Home Out).
- Switch the approach mode to off.
- Make sure the electrode arm is swung in and Home In.

Electronics Part 2

- Move both the ground wire and electrode so that they are submerged in the solution.
 - The electrode should be submerged when focused at 3000um with the 60x.
- Turn on the Oscilloscope.
- Prepare MultiClamp (Figure 8).
 1. Click the reset button.
 2. Click open file then open "StandardConfig.mcc" when the file explorer pops up.
 3. Under Pipette Offset, click the lock then Auto.
 4. Check the box for Irms. The noise should be less than 10, ideally around 5. If all is well, uncheck the box and continue.
- Click Home Out and swivel the electrode arm out.
- Dispose of the glass pipette from the electrode holder.
- Withdraw the ground wire and perfusion tubes, allowing easy access to the dish.
- Turn off the monitors and lower the black sheets to prepare for dissection.

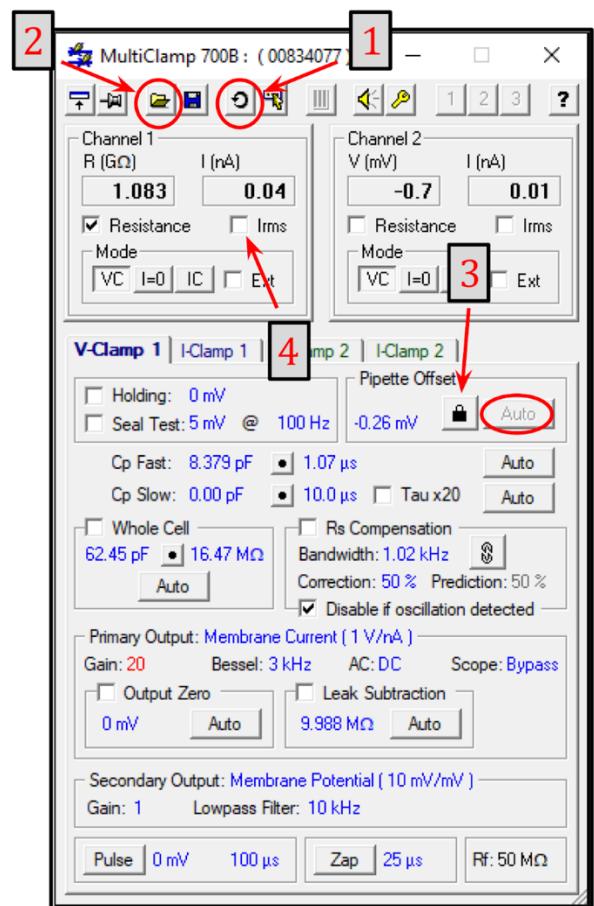


Figure 14: The highlighted locations of where to click on the MultiClamp GUI