

Vitrobot and Glow Discharge Protocol

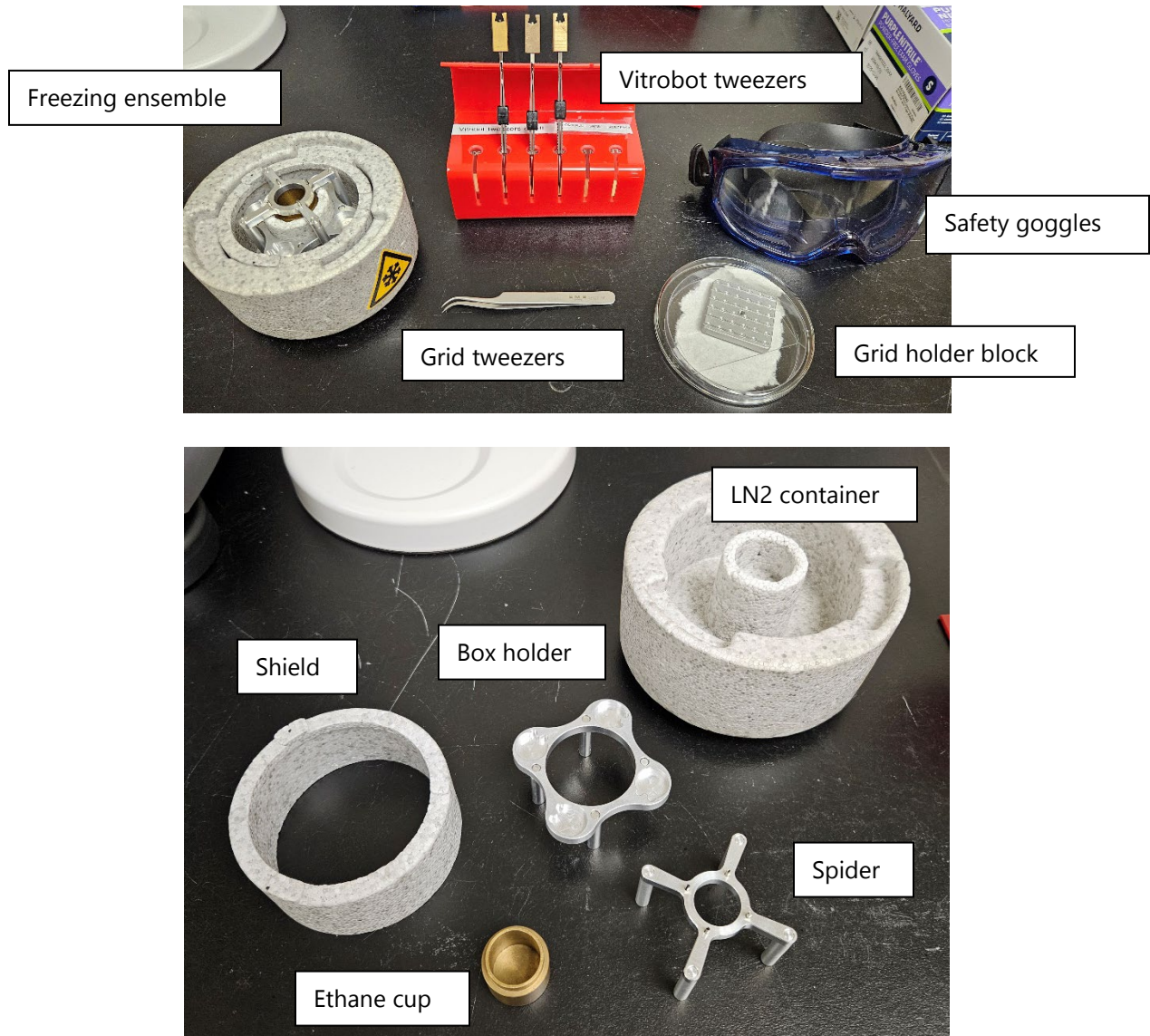
CEMC

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Materials



General precautions

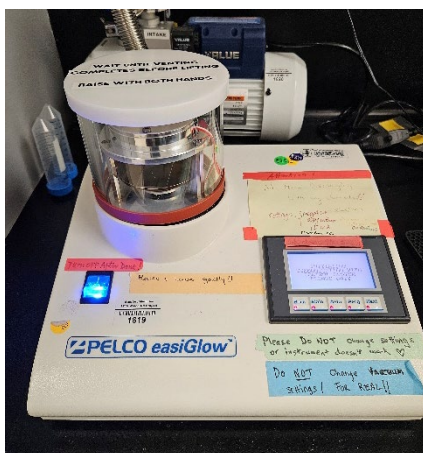
1. Make sure all equipment is completely dry before use, otherwise large ice contamination will form.
2. Liquid nitrogen (LN2) is a cryogen. Prolonged contact with your body will cause cold injuries. Long pants and close-toed shoes are necessary when using LN2. In-person training is required before handling.
3. Liquid ethane is also a cryogen. However, liquid ethane will very easily penetrate bare skin and **will burn below the skin** immediately upon contact. Use extreme caution when handling liquid ethane, and long sleeves and safety goggles are required for protection. In-person training is required before handling.

About Grids

Grids have a topside and bottom. The box that the grids come in usually says which way the tops face. For Quantifoil grids there is a carbon coating on the topside, which gives it a darker appearance. You can use this as a guide. Always place grids topside up.



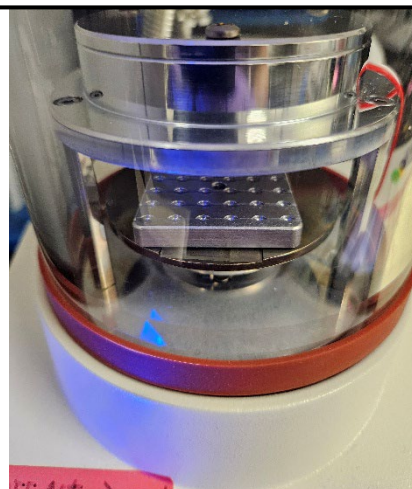
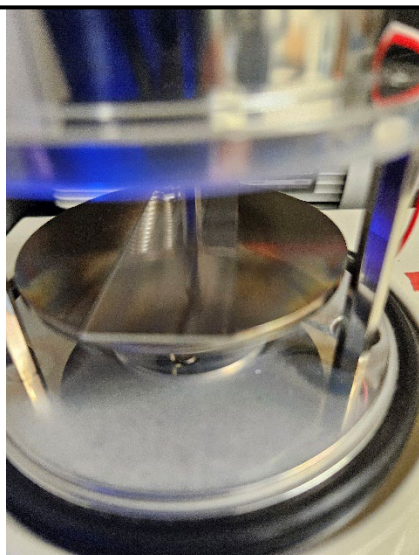
Glow Discharge



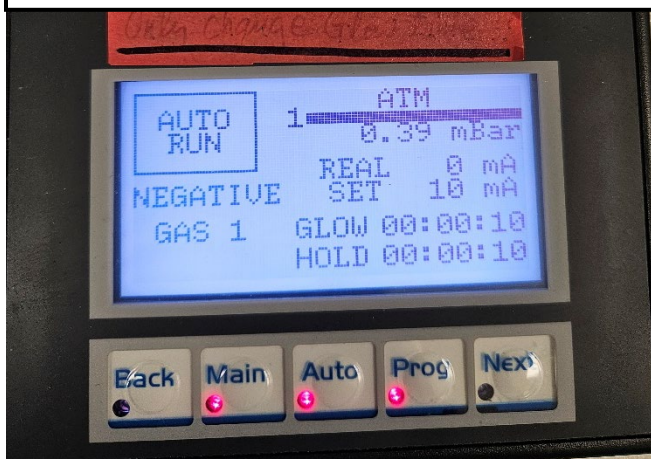
The glow discharger is located right of the fume hood in Fairchild and on the bench in ChemH. Use both hands when removing or replacing the glass jar, using a careful, straight, vertical motion.



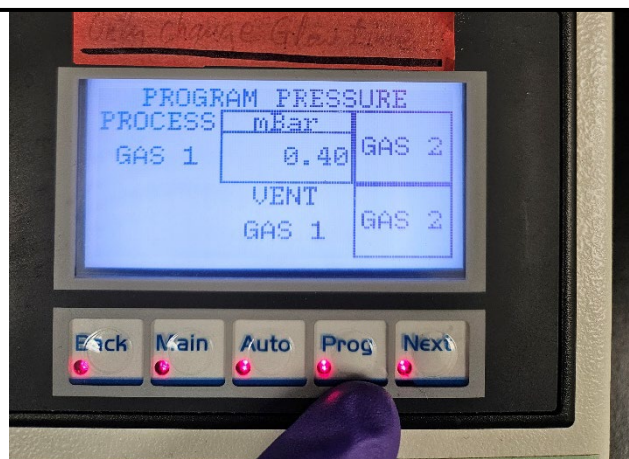
1. Place the grid(s) within the divots on the grid holder block.
2. With the glow discharger turned on and the glass jar removed, place the grid holder block on top of the stage. Note that there should be a glass cover slip between the stage and holder block to help dissipate charge. Replace the jar.



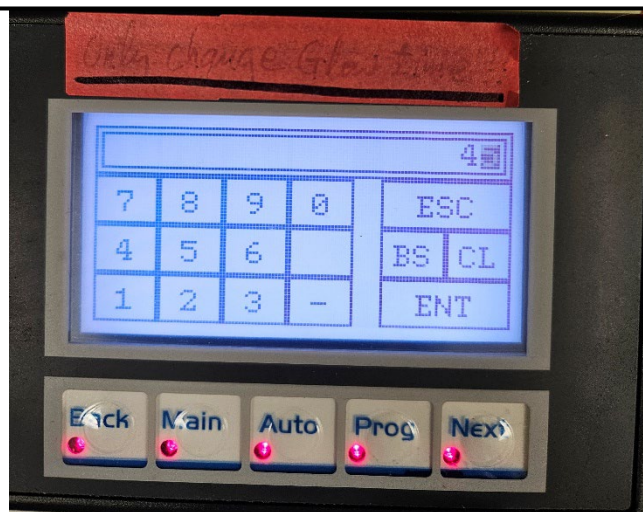
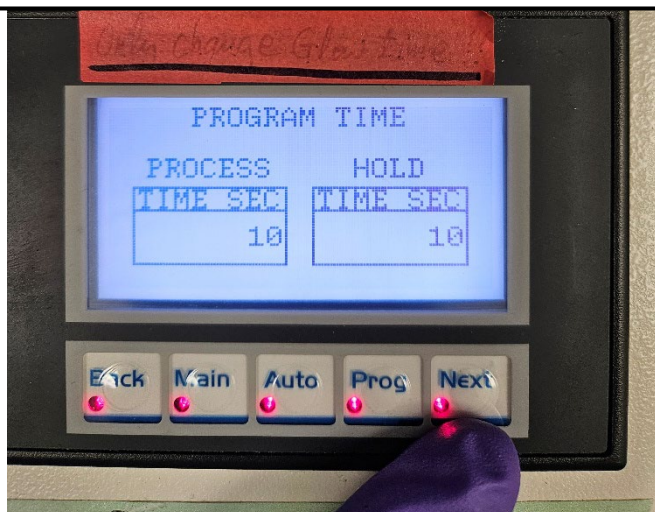
- Operating the glow discharger is a combination of physical buttons and a touch screen. Always check the settings to see if they match what you need. For common grids, the following settings are good: 15 mA, glow time 45 s, hold time 15 s. The image below shows a different setting that we will change.



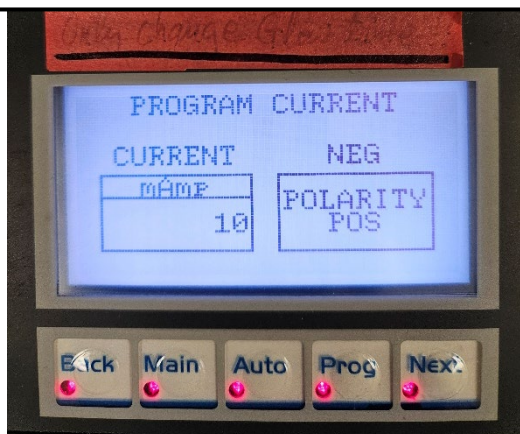
- To change settings, first press the "Prog" button. DO NOT change the vacuum settings (mBar). Not only is it dangerous to do so, you are unlikely to need to. Pressure should be 0.40 mBar and will display 0.39 mBar on the home screen.



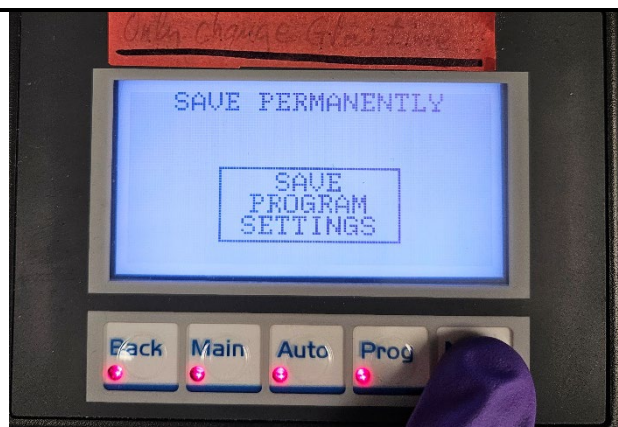
- Press the "Next" button (it can be unresponsive so you may need to press hard). Here, you can change the time for Glow (Process) and Hold. Glow is how long the plasma will last; hold is how long the vacuum will be held without glow. Touch the screen to change the settings, which brings up a numerical touchpad. Press "Enter" to save the setting. Press "Next" to advance to the next option screen.



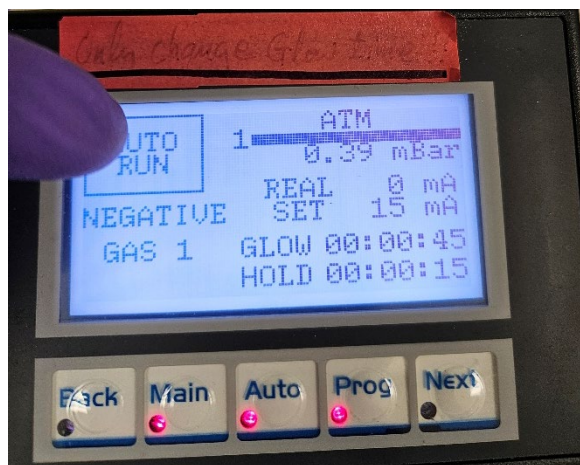
6. Press the Current box to change the current. Make sure it says NEG above "Polarity POS". Press "Next".



7. Press "Next" on the following screen. You do not need to save the setting.



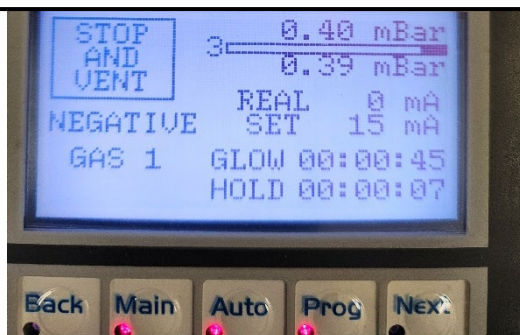
8. With the desired settings now in place, start the process by pressing "Auto Run" on the home screen.



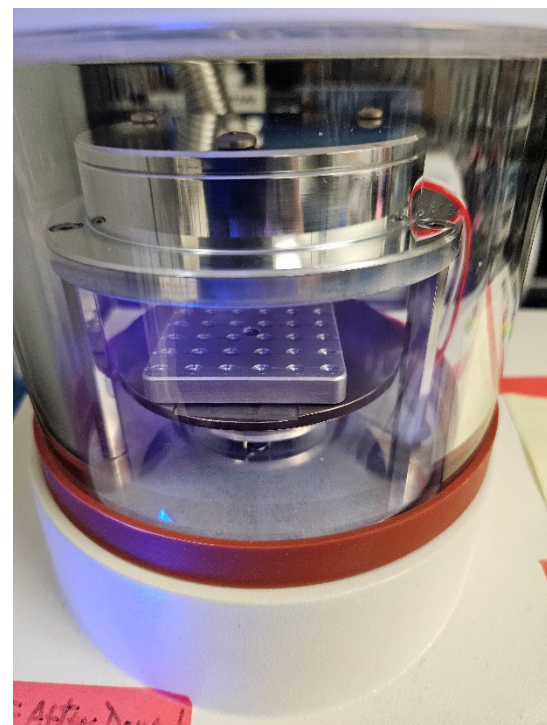
9. You should hear the vacuum starting. The vacuum status is displayed in the top right. If you ever need to interrupt the process, tap "Stop and Vent".



10. Once the vacuum is reached, the Hold will begin and count down.

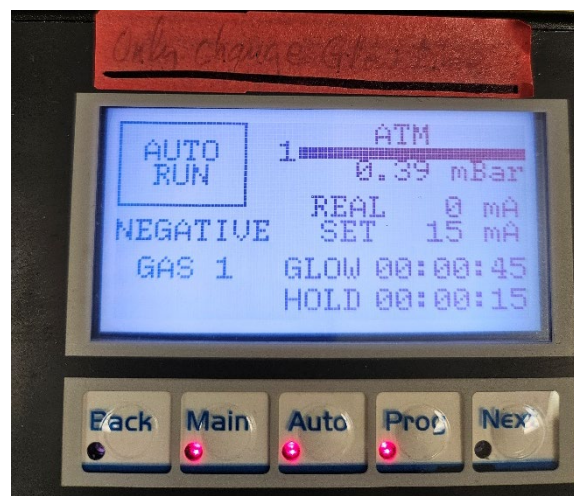


11. After the Hold, the machine will automatically proceed to Glow. During this phase, the machine will use plasmized air to charge the grid, which also helps to remove some volatile contaminants if present. You will see the plasma as a purple cloud between the two large discs inside the chamber. PAY ATTENTION for any signs of electric arcing, or "lightning", as this is a sign that there is too much junk on the sample or the stage and your grid could be compromised. If this happens, clean the stage and block with alcohol and start again. You may want fresh grids, too. You may also clean with alcohol beforehand to prevent issues.



12. After the Glow, the vacuum will be slowly released. During this time, the box at the top left of the screen will still display "Stop and Vent". DO NOT press this button or attempt to remove the glass jar while this button is displayed. Be patient and let the vacuum slowly release. Once it is safe to remove the jar, the button will change back to display "Auto Run". At this point, carefully remove the jar and take out the grids. Replace the jar and shut off the machine.

Glow discharging lasts about 30-60 min, but sometimes up to 2 hours.



Preparing Liquid Ethane

CAUTION: liquid ethane easily burns skin. Wear goggles and long sleeves while handling

For this tutorial, water is being used for clarity. Expect cold vapor emanating from the cryogenics in the real procedure.

Always cool down your tools in LN2 before touching to your grid/sample to avoid warming up. You can tell that the tool is cool when the boiling around it stops.

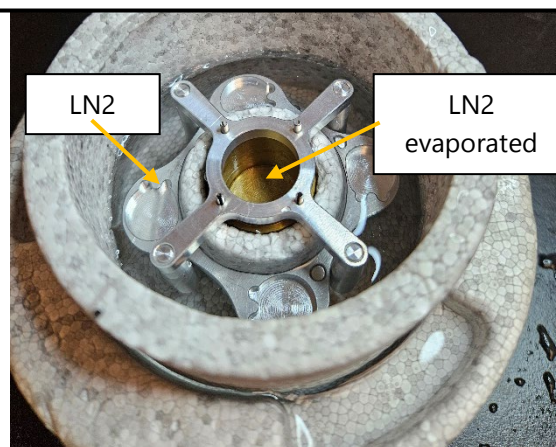
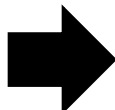
To help you pour LN2 from the manual Dewar into the ensemble, you can use a small thermos or a Styrofoam coffee cup with a triangular slit.

1. Start with a dry and fully assembled freezing ensemble as shown to the right: LN2 container, box holder, ethane reservoir, spider, and shield. Cups are stored in a drawer beside the fume hood in Fairchild and by the snorkel in ChemH. Note: Place the shield with the little bumps facing up. The shield will float on the LN2. It is designed to help keep a curtain of cold nitrogen vapor over the cup which keeps atmospheric air out. You can use the 3 cutouts in the base to pour more LN2.



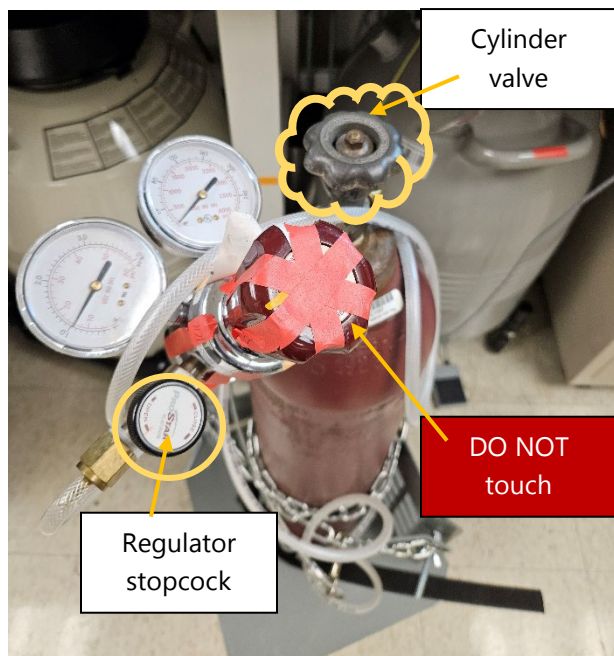
2. Dispense LN2 into a manual Dewar. You will need training on handling and dispensing LN2 from a tank before being able to do it yourself. Use general precautions (long pants, closed shoes, proper safety equipment.)

3. Pour LN2 into the ethane cup to cool it. Afterwards, continue pouring directly into the bulk reservoir of the LN2 container. Let the LN2 in the ethane cup fully evaporate before proceeding.



The ethane cylinder is dark red and chained to the leg of the table holding the Vitrobot. The only valves you need to touch are the Cylinder Valve and the Regulator Stopcock. DO NOT touch the regulator adjustment valve (big red valve with tape on it).

4. Always make sure that the regulator stopcock is closed before opening the cylinder. Open the cylinder valve on the ethane cylinder. You only need to open it slightly, about $1/8^{\text{th}}$ to $1/4^{\text{th}}$ turn.



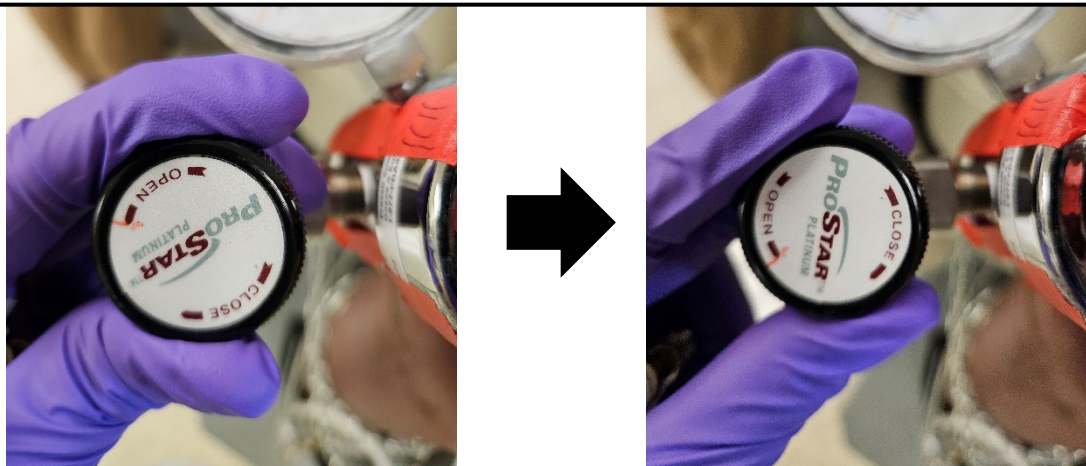
5. With your dominant hand, hold the ethane lineout by the top of the pipette tip and place the tip into the ethane cup. With your left hand, hold onto and manipulate the regulator stopcock.

Always keep your left hand on the regulator stopcock in case you need to quickly close it during the procedure.

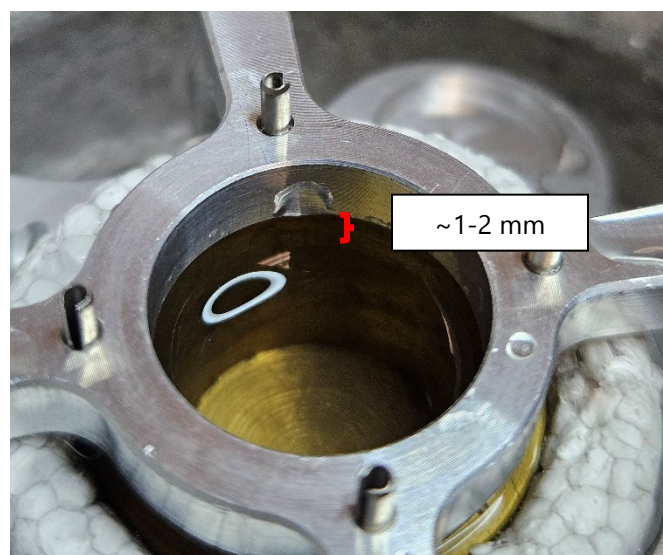
Ethane freezes easily. Try to avoid touching the tip to the cup, as the ethane can freeze and block the tip. If this happens, close the stopcock and let the tip thaw. You can then proceed.



6. Begin the flow of ethane by slightly opening the stopcock. It only takes about a $1/8^{\text{th}}$ turn to get a good flow rate of ethane. Too little and it won't dispense; too much and you will blast away the ethane before it liquefies. There is a red line marked on the stopcock as a guide. The line is parallel to the regulator line when closed.



7. A whitish plume will emit from the pipette tip accompanied by hissing/gurgling sounds. These are normal. Monitor the level of liquid ethane. You may need to occasionally shut off the flow and let the white plume dissipate to see it. Fill the cup until the liquid is a few millimeters from the top of the brass, and then close the stopcock. Do not let the liquid touch the spider, or the ethane may freeze the spider to the cup.
8. Let the liquid ethane sit until it begins to look slightly cloudy. Remove the spider with tweezers and place on slide warmer.



If the ethane freezes (turns solid white), take a warm metal rod (such as a screwdriver or blunt tweezer) and stick it into the cup/liquid to thaw it. You can also invert a warm ethane cup. This is also a good technique if the spider freezes to the cup.

9. You have two options on how to proceed from here. You can keep the shield on or you can take it off. The shield will help to prevent ice contamination during freezing. However, some find it cumbersome to work around during the next steps. The shield will also become compressed during the Vitrobot steps, which may cause splashing. For less contamination, choose to keep the shield on. For easier handling, take the shield off. If using the shield, you can keep swapping it out to avoid ice buildup as you freeze.

Shield

Pro: Less ice contamination

Con: Harder to handle



No Shield

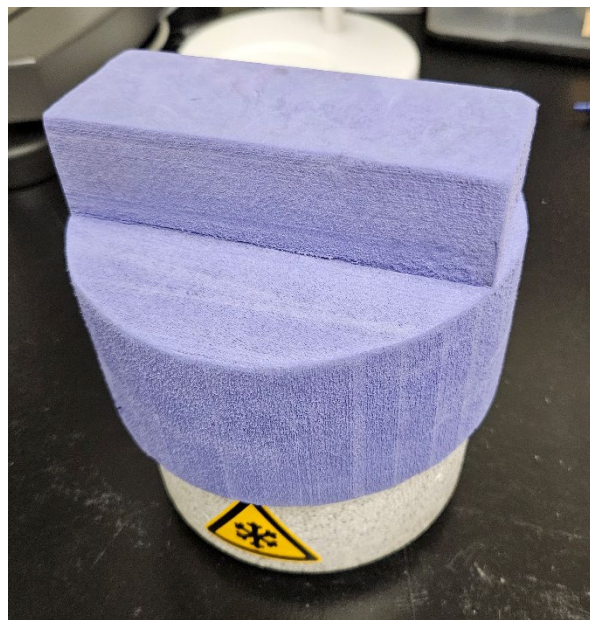
Pro: Easier to handle

Con: Higher risk of ice contamination

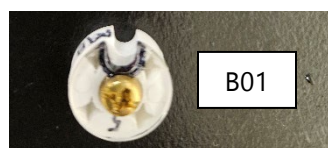


Whichever method you chose, covering the ensemble when not in use, especially for long intervals, will help prevent ice contamination (Image to the right).

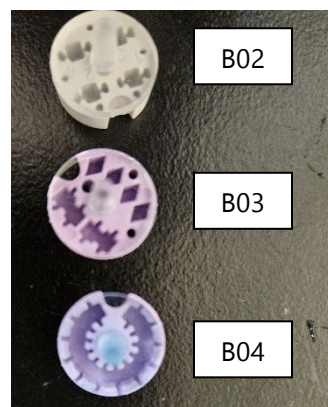
10. Place your grid box(es) onto the box holder to cool them and prepare for grid placement. See "About Grid Boxes" below regarding general information and how to handle them.



About Grid Boxes



B01



B02



B03



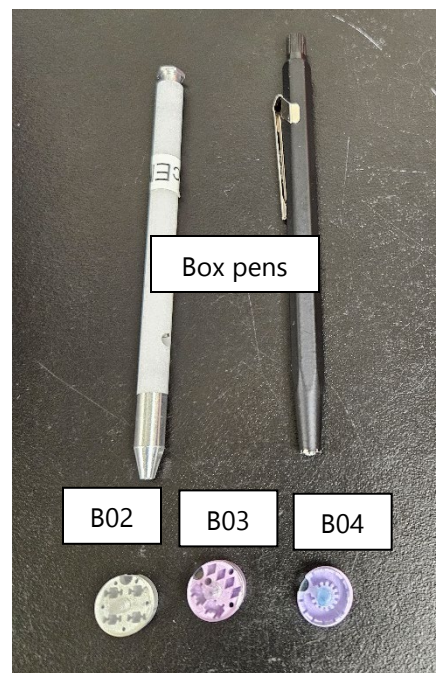
B04

- B01 – Regular grid box (unclipped)
- B02 – Autogrid box (clipped)
- B03 – Hybrid box (clipped and unclipped)
- B04 – 12-grid box (12 clipped grids)



Box tweezer

B01



Box pens

B02

B03

B04

There are 4 main styles of boxes, 2 styles of pens, and 1 type of box tweezer. The two pens are used to manipulate B02, B03, and B04. The tweezer is used for B01 (grab it by the screw).

Make sure that the pens are bone-dry before cooling in LN₂. Any water can freeze their moving parts, rendering them unusable.

For B01: You will need the appropriate screwdriver to open/close the screw (lefty-loosey, righty-tighty). When closing the box, it helps to place a tweezer at the cutout to prevent the lid from turning with the screw.

For B02-B04: The lid for B02, B03, and B04 is separate from the box. To open the box, you need to press down with the pen attached to the lid and then screw left. Screw right to close.

- White pen: Press the spike of the lid into the pen. The button on the top of the pen releases the box (a lot of force is required.)
- Black pen: The button on the black pen pushes out a set of claws that grabs and releases the lid spike.

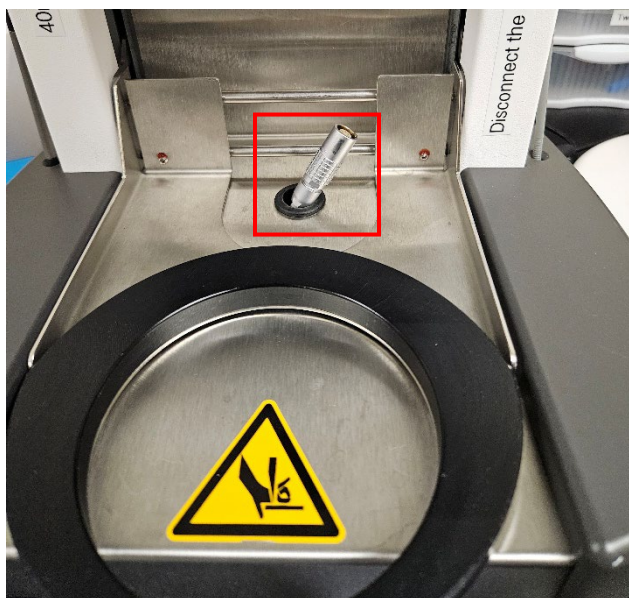
Vitrobot Preparation

The Vitrobot preparation steps can be done before glow discharging and preparing the liquid ethane.

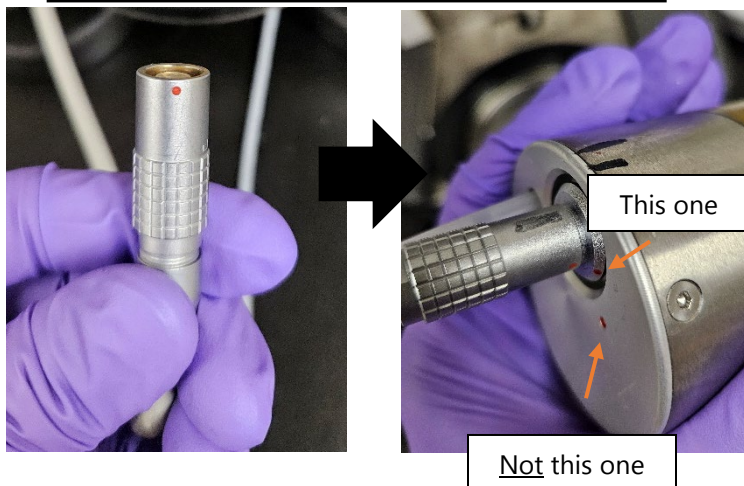
1. Beside the Vitrobot is a plastic cup containing the humidifying chamber and several large syringes. The humidifying chamber is always stored inverted to allow water to drain out. Pick up the chamber.



2. At the bottom of the Vitrobot is a cord. Pull this out.



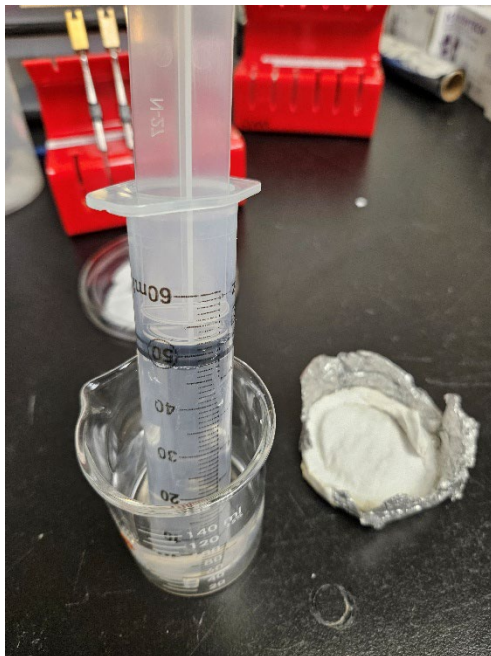
3. Connect the cord to the bottom of the chamber lining up the red dots on the cord and the chamber. (Align it to the red dot on the collar; ignore the one on the chamber casing. The black pen lines will also line up.)



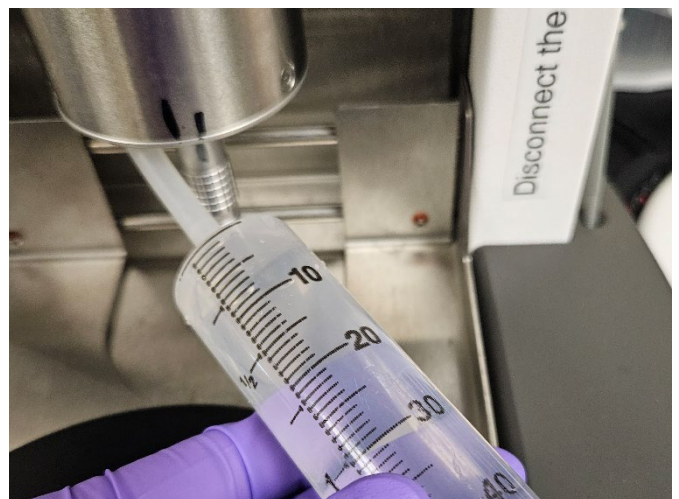
4. Notice at the top of the chamber there are 3 backwards "L" grooves. (There should also be a red O-ring; make sure it is there). To attach the chamber to the Vitrobot, carefully bring the chamber to the Vitrobot while threading the cord back into the hole. Be careful not to bend the cord too much, as it can damage it. Align the screws on the side of the collar underneath the Vitrobot to the tops of the grooves. Push the chamber up, letting the screw follow the groove. At the end of travel, twist the chamber counterclockwise until you feel a snap. The chamber is now locked into place. Note that the Heat Warning Triangle and Black Dot should be facing you.



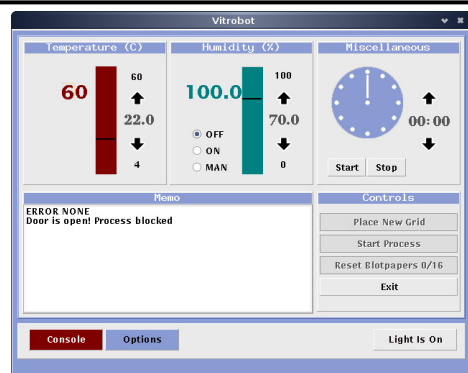
5. Fill a large syringe with 40-50 mL of water using a beaker as shown below.



6. Attach the syringe to the flexible tube at the bottom of the chamber. Draw back to suck out any trapped air. Disconnect the syringe, push the water to the tip of the syringe, and then reattach and inject the water into the chamber. Without detaching, pull back the piston to vacuum seal.

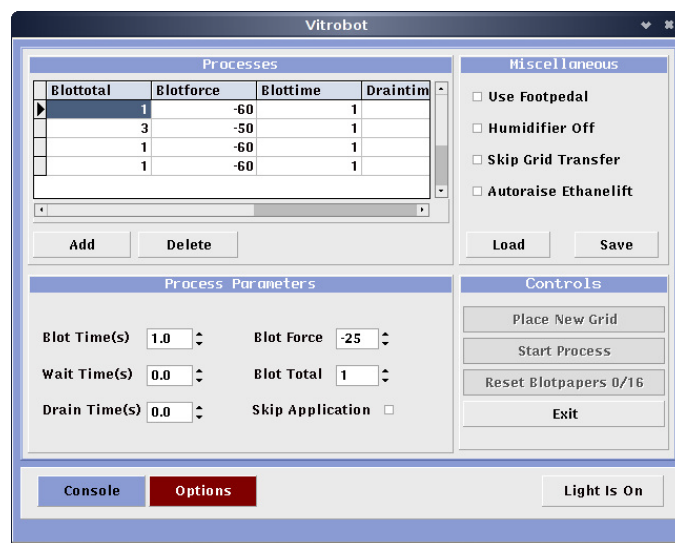
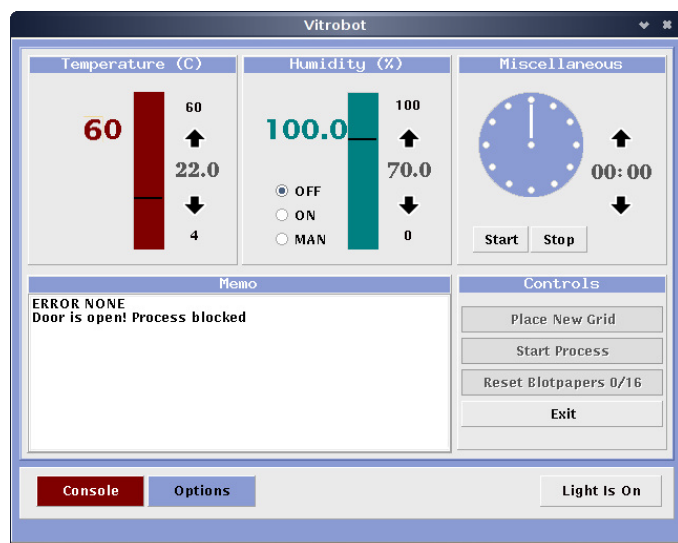


7. Now the Vitrobot is ready to be turned on. The switch is located in the back. Flip it on. The system will boot up and eventually you will see the home screen (below). You can use the touchscreen, a mouse, or a foot pedal to interact.



8. Select "Humidity on" and use the arrows to adjust humidity level. 100% works for most. Adjust temperature as needed. Let these equilibrate before freezing.

9. Hit "Options" in the bottom left. Select "Humidifier off During Process" in the upper right. Select your settings (orange box below), then hit console.



Freezing settings are something you will have to determine per sample. Generally, longer blot times and higher blot force will produce thinner ice but also wick away your sample. Here are some general guidelines.

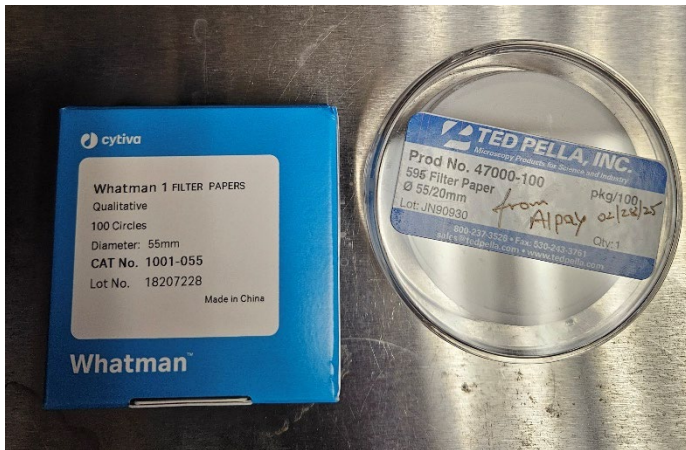
Blot time: Blot time has the most effect on ice. Around 3 seconds is a good place to start.

Blot force: Range +/- 25. Usually 3 or 4 is good. Less effect on blotting and some users always stick to 3.

Wait time: How long it will wait before blotting. Good for sample adherence or for adding ligands *in situ*.

Drain time: How long after blotting and before plunging into ethane. Will thin the ice more. Usually not used.

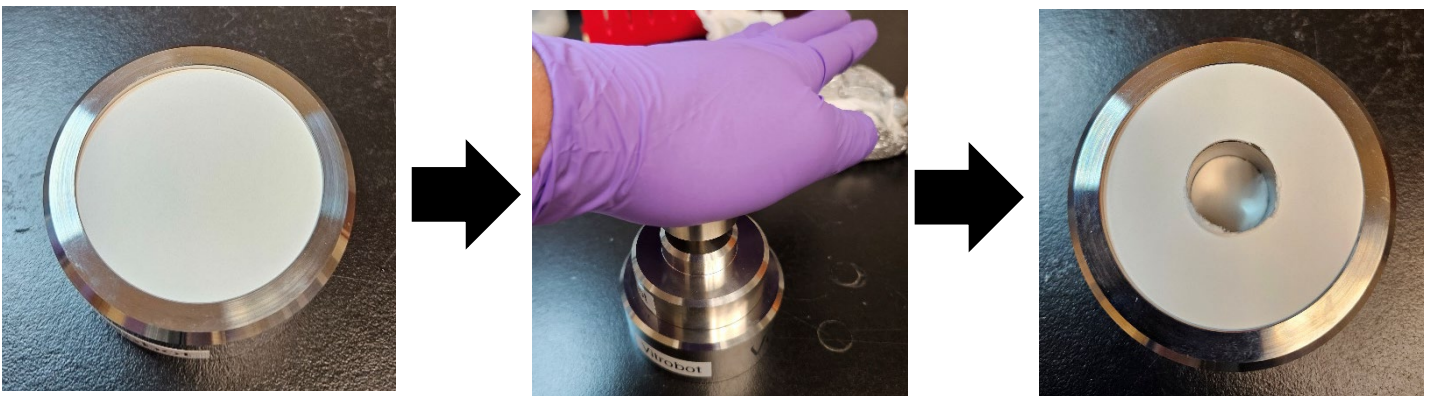
10. Grab some filter paper. At Fairchild, filter paper is stored in the top drawer of the cabinet below the Vitrobot. At ChemH, it is by the Vitrobot. There is some filter paper made by Ted Pella that is specifically made for the Vitrobot. You can also choose to use Whatman filter paper (works just as good and you can choose different types for your needs).



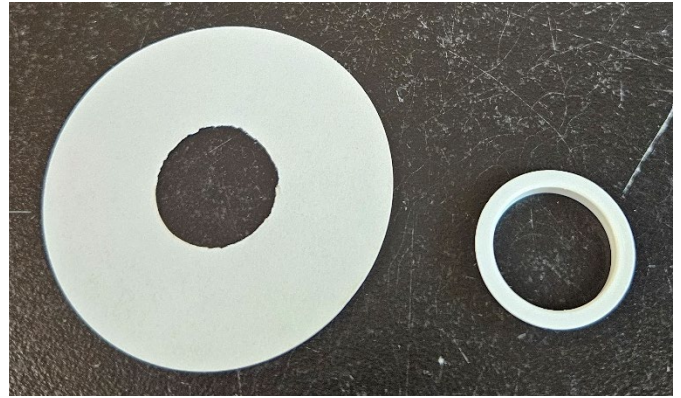
11. To use Whatman filter paper, you will need to punch a hole to fit the Vitrobot paddles. The hole punch is in the bottom drawer of the same cabinet at Fairchild. Make sure to use the one labelled "Vitrobot," as there is one for the Leica that has a different hole size.



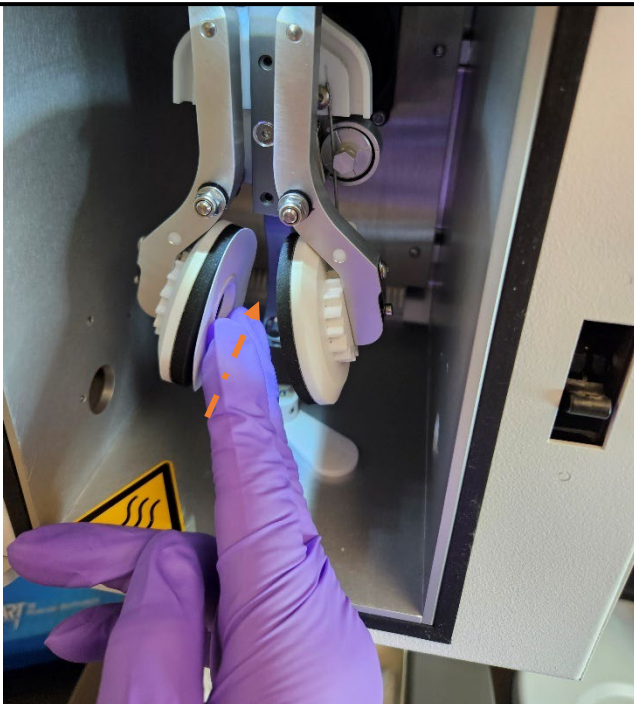
12. The hole punch is in two parts. Remove the top part (with the plunger). Next, put 2-6 pieces of filter paper in the impression of the bottom part. Replace the top part and press down on the plunger. Put any excess punched papers back in the box; you will only need 2.



13. The paper is secured to the Vitrobot paddles using the white plastic ring as shown to the right. These rings are stored in the main Vitrobot chamber. Place the paper onto the ring as shown below. Repeat for the second piece of paper.

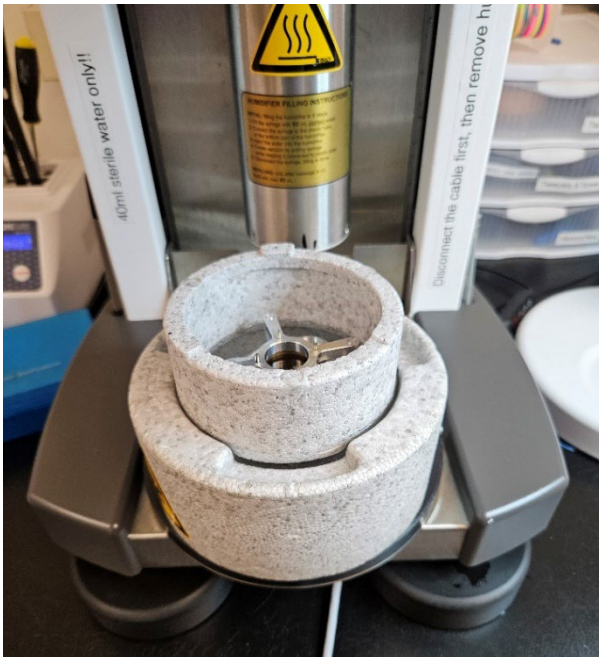


14. To place the filter papers onto the paddles, first gently slide them into position. Next, gently pinch the ring into the hole. Be careful not to press too hard or bump the paddles too hard, or you might de-calibrate the paddle positioning.

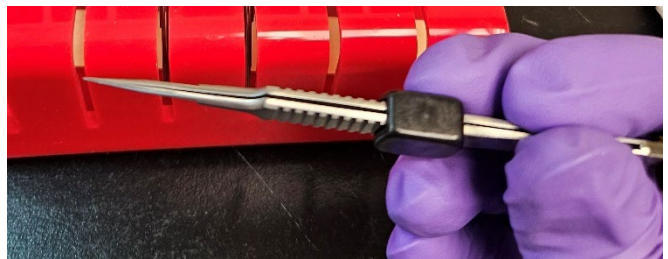
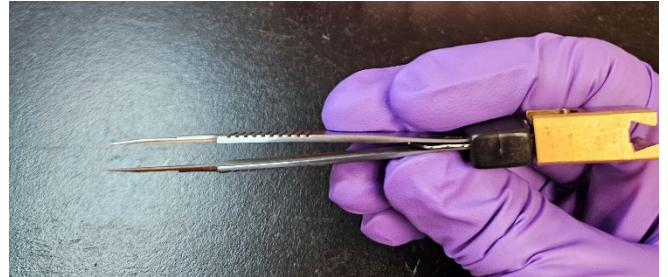


Vitrobot Operation

1. Place the freezing ensemble onto the stage (black ring at the bottom) of the Vitrobot. Have the grid box and the pour cutout positioned closest to you. The Vitrobot will raise and lower the stage during the processes.



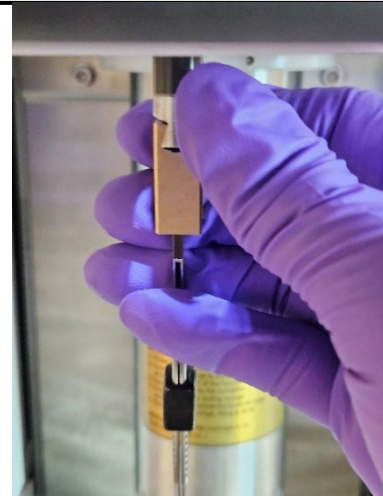
2. The Vitrobot tweezers have a black piece of plastic that is used to lock the tweezers shut. There is a piece of metal at the top to hold the black piece in the open position. Sliding this black piece down to the first groove near the pointed end locks the tweezers shut, maintaining a good grip on the grid.



3. Pick up a grid with the Vitrobot tweezers as close to the edge of the grid as possible while maintaining a secure grip. You can test if the grid is secure by tapping the edge of the broad part of the tweezers against the edge of your finger. Place a Kimwipe underneath to catch the grid if it falls.

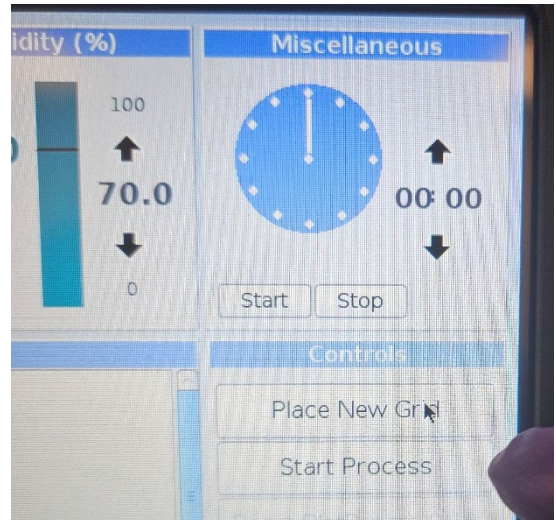
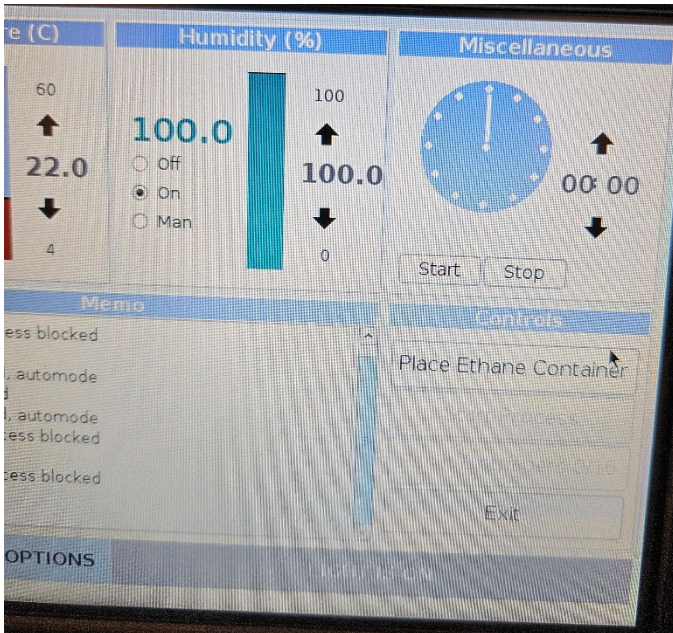


4. To put the tweezers on the Vitrobot arm, slide it onto the dovetail from behind and pinch between your fingers. It will snap into place. Make sure to orient the top of the grid towards your dominant hand.

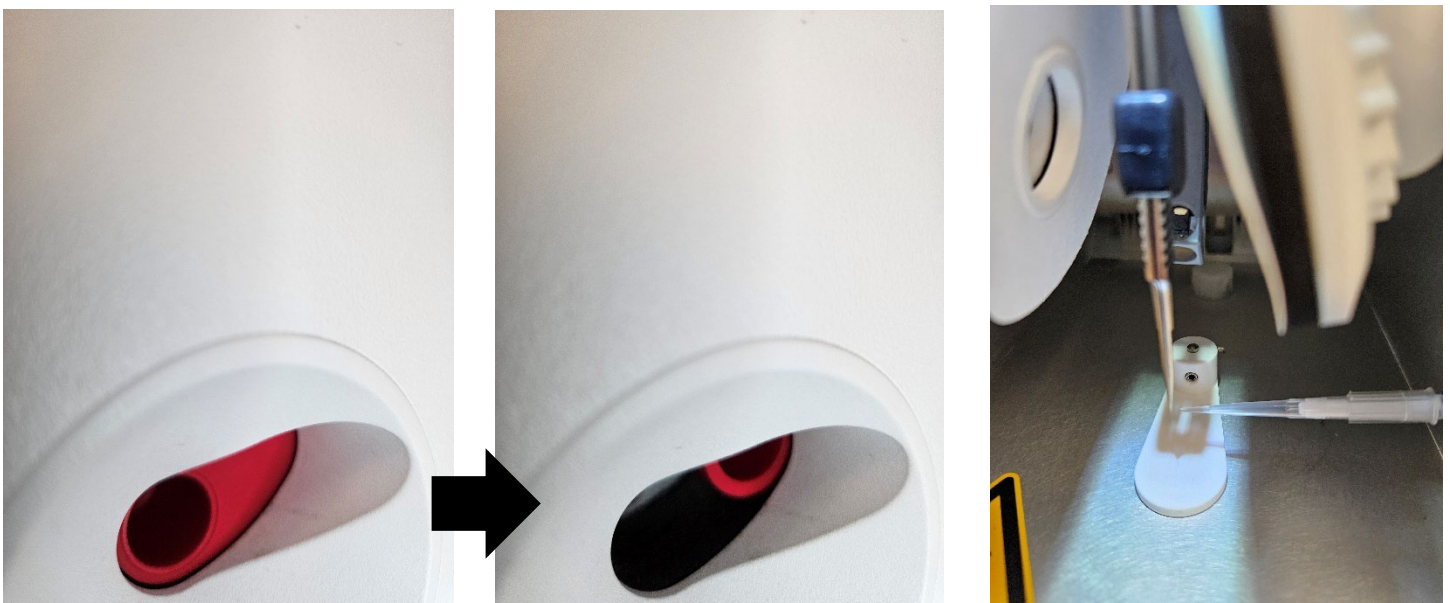


5. Hit "Place Ethane Container". The arm will retract and the "ethane container" (freezing ensemble) will be brought to the top.

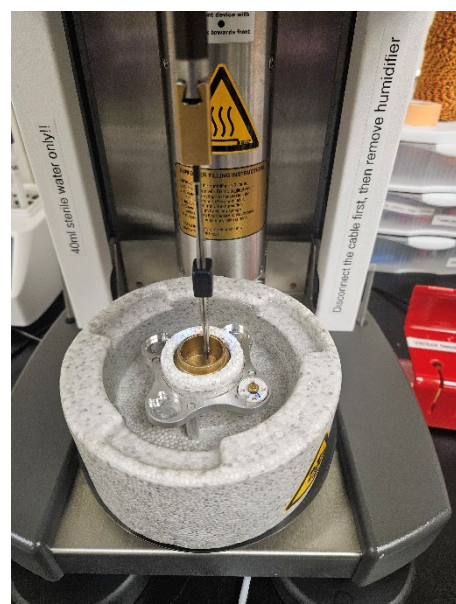
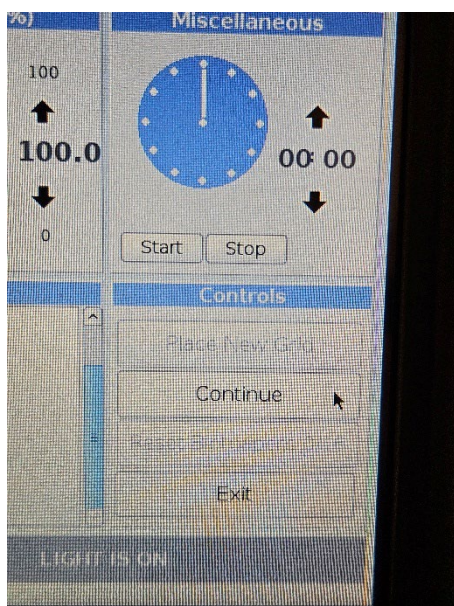
6. Once the motion has stopped, hit "Start Process." The arm will lower just a bit to allow you to inject your sample. Hitting "Place New Grid" will instead reset the positions (in case you need to place a new grid). If you do this by accident, there is no harm. Just hit "Place Ethane Container" again.



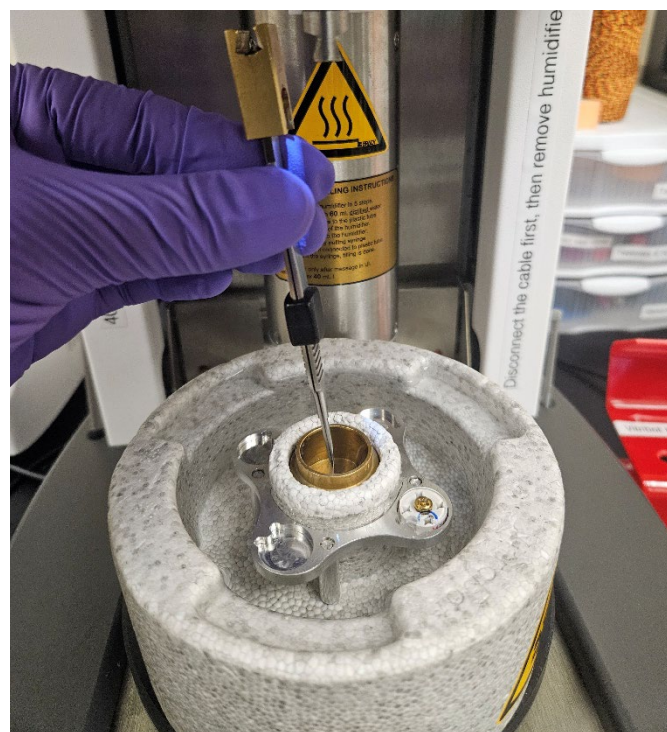
7. To inject your sample, there is port with a red swinging door on either side of the VitroBot. This is to help keep the chamber conditions in equilibrium. Push this door to the side with your finger. Using a P10 or P20 pipette, dispense 3 μ L of sample onto the grid (topside/carbon side) through the side entry port. (This is why you wanted to orient the grid towards your dominant hand in Step 4.)



8. Hit "Continue". The Vitrobot will blot according to your conditions and then plunge the sample into the ethane cup. The stage will lower and then pause.



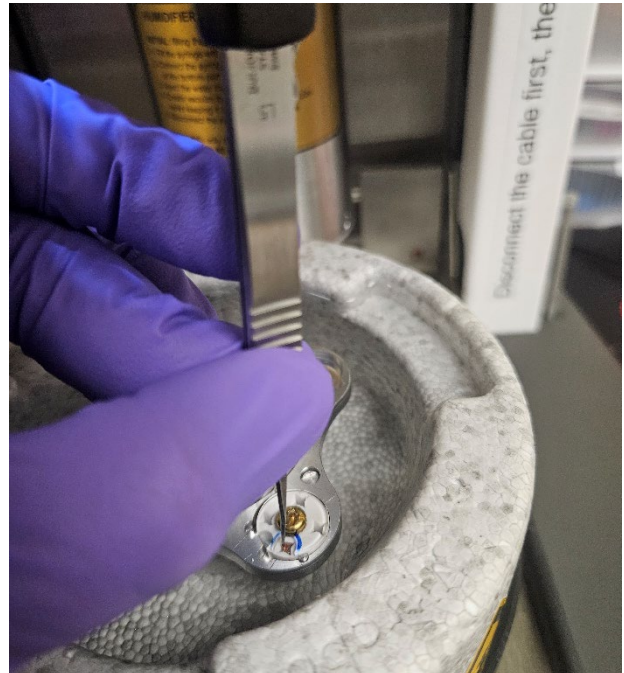
9. The next step is to take the tweezers off the arm and transfer to the nitrogen. First, refill the LN2 reservoir in the ensemble. Next, carefully slide the tweezers off the holder while keeping the grid submerged in the liquid ethane. BE CAREFUL NOT TO BEND THE GRID ON THE SIDE OF THE CUP. At this point, you can hit "Continue" on the console and the stage will move to give you more room for the next steps. You will, however, have to follow the tweezers with the movement of the stage/cup so as to avoid exposing the grid to air. If you do not need the extra room, and you would rather not risk the grid, you can also do the next step without hitting continue.



10. Carefully transfer the grid, while still held by the tweezers, to the bulk liquid nitrogen. You will want to lift the tweezers from the ethane and plunge into the nitrogen as quickly but as confidently as possible, reducing the time that the grid is exposed to air but at the same time making sure not to drop or damage the grid in the process. Note that this one of those steps that are easier if you had removed the shield already.



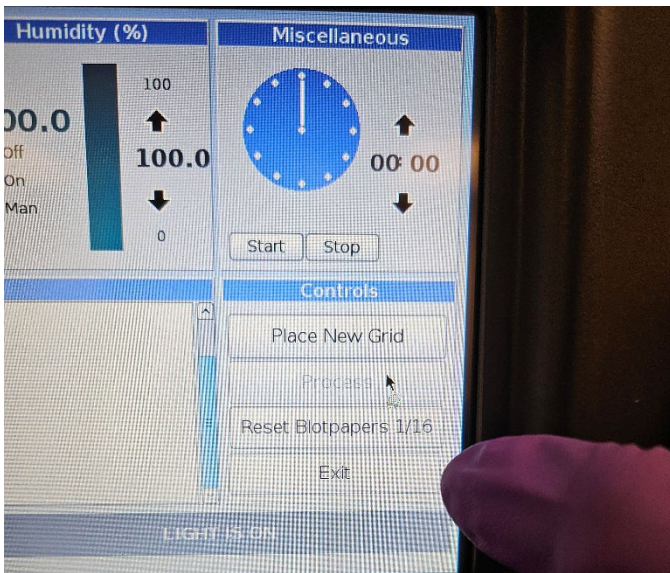
11. Next, squeeze the tweezers with your fingers where the ridges are to keep them shut. With your other hand, lift up the black piece of plastic to the top of the tweezers and let it lock into place. While still holding the tweezers shut, navigate the tweezers to an open slot in the grid box. Carefully open the tweezers and let the grid fall into the slot. Rotate the lid to the next open spot if needed.



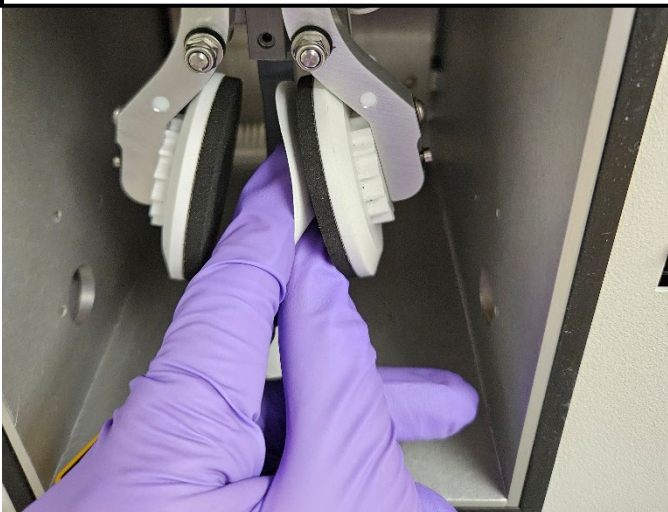
12. To freeze more grids, place the Vitrobot tweezers on the slide warmer and grab a fresh, dry set of tweezers. Repeat from Step 5 as needed.

Ending the Session

1. When you are done freezing your grids, carefully remove the freezing ensemble from the stage (avoid spilling or splashing the cryogen). Using the correct tool, secure the lid onto the grid box, then pick up the box and transfer it to a container of liquid nitrogen. Store the grids in a puck (See Puck Storage Manual). Alternatively, you can use a Falcon tube, but pucks are recommended.
2. Hit "Exit" on the console. The arm will retract and the screen will shut down, but the Vitrobot will not shut off.
3. To completely shut off the Vitrobot, flip the power switch in the back.



4. Carefully remove the blot paper by sliding your fingers between the paper and the pad and letting the white plastic piece pop out.



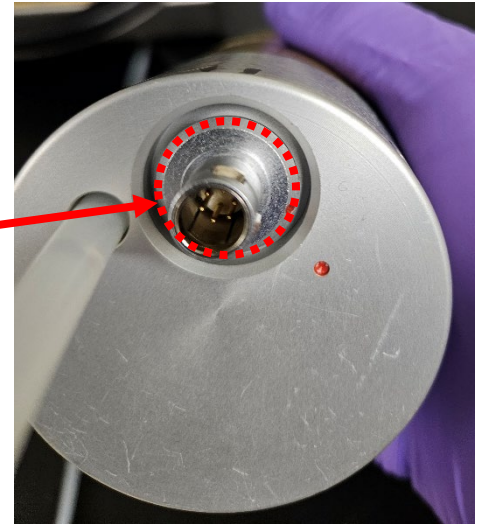
5. Place the plastic pieces back in the Vitrobot. Leave the door ajar to air out.



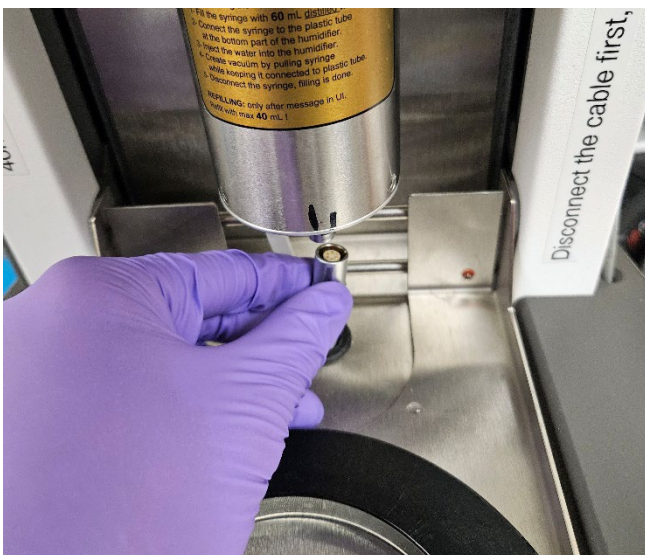
6. Press up onto the collar around the cable connection of the humidifier. Use your finger tip. Simultaneously, gently pull down on the cable head until it releases. This is a difficult process and may require a few attempts before it releases.



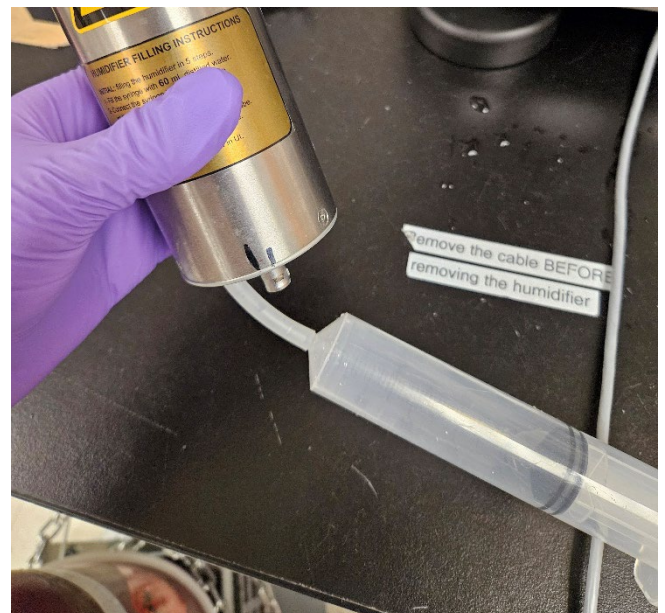
This is the part you are pressing. It requires a lot of force to press it. **Note: The chamber shown on the right is detached for clarity. Keep the humidifier on the Vitrobot as shown to the left while you press and detach the cable.**



7. Once the cable releases, tuck it slightly into the hole while keeping the head within access.



8. Remove the humidifier by twisting it clockwise and pulling it free from the screws. Empty the excess water into the sink. Use an empty syringe and suck out the remaining water on an inverted humidifier.



- Place the inverted humidifier back in the beaker. Take the freezing ensemble and **carefully** carry it to the fume hood to let the gases evaporate. Clean up your materials, place cold items on the slide warmer, and check that the **ethane valves are shut** on the cylinder before leaving.

