

UNC at Chapel Hill Vitrobot Mark IV Protocol
Created by Joshua Strauss and Jared Peck

This Protocol is for Trained CryoEM Core Users.

The Vitrobot Mark IV is located in Glaxo Research Building room 011.

The Vitrobot Mark IV can be reserved on iLAB.

Where PPE and exercise caution when handling liquid nitrogen and liquid ethane.

If you need assistance please contact Jared Peck or Joshua Strauss.

If you noticed an error or damage to the equipment please tell Jared Peck or Joshua Strauss.

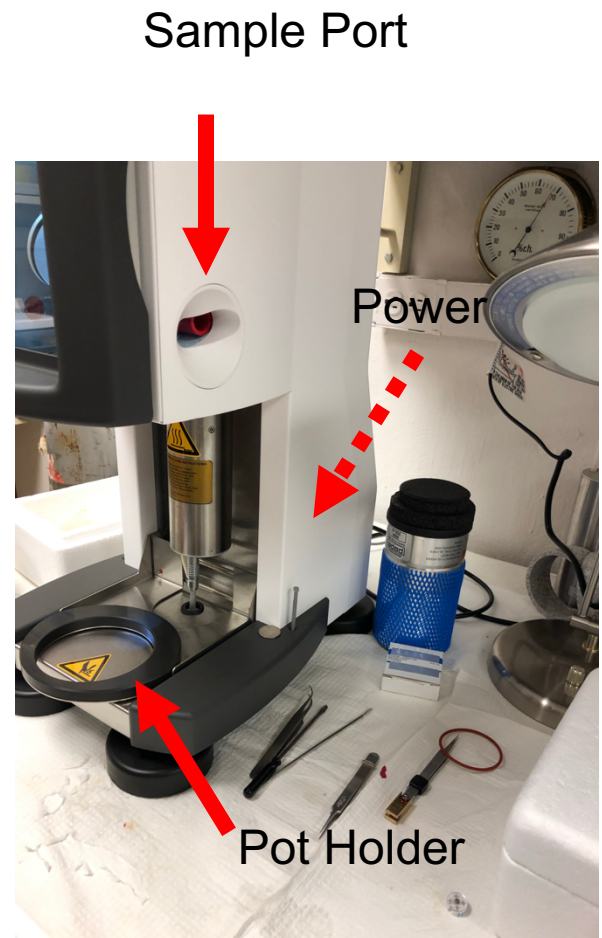
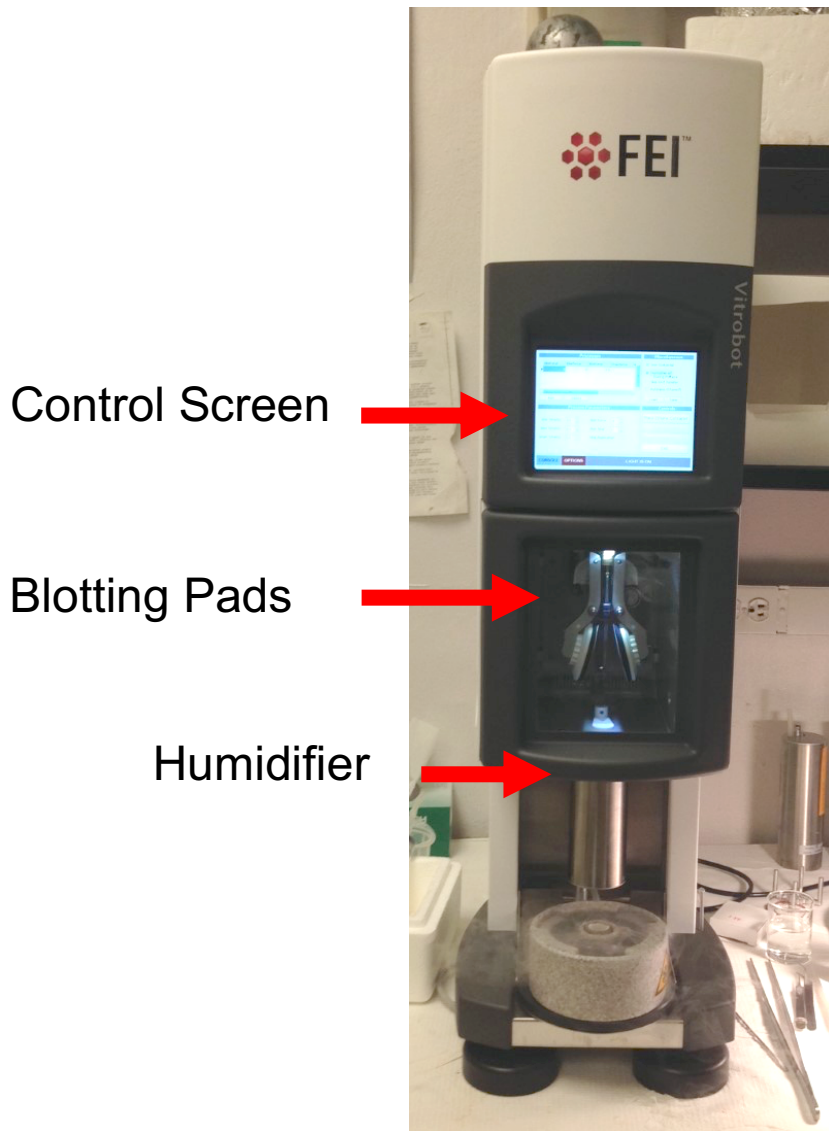
Make sure to fill out the Vitrobot Log Notebook (located on the Vitrobot bench). Enter your name, PI/Lab, institute, hours used, conditions used (if you choose), who to bill, and notes on the session. **This is critical!!!!**

Typical Vitrobot Session:

1. Setup Vitrobot
2. Assemble Vitrobot Pot and fill it with cryogen
3. Glow-discharge / plasma clean TEM grids
4. Prepare cryo-grids
5. Transfer cryo-grids to storage
6. Shutdown Vitrobot and clean-up after your self
7. Enter your information into the Vitrobot Log Notebook

Overview of the Vitrobot

Vitrobot Mark VI



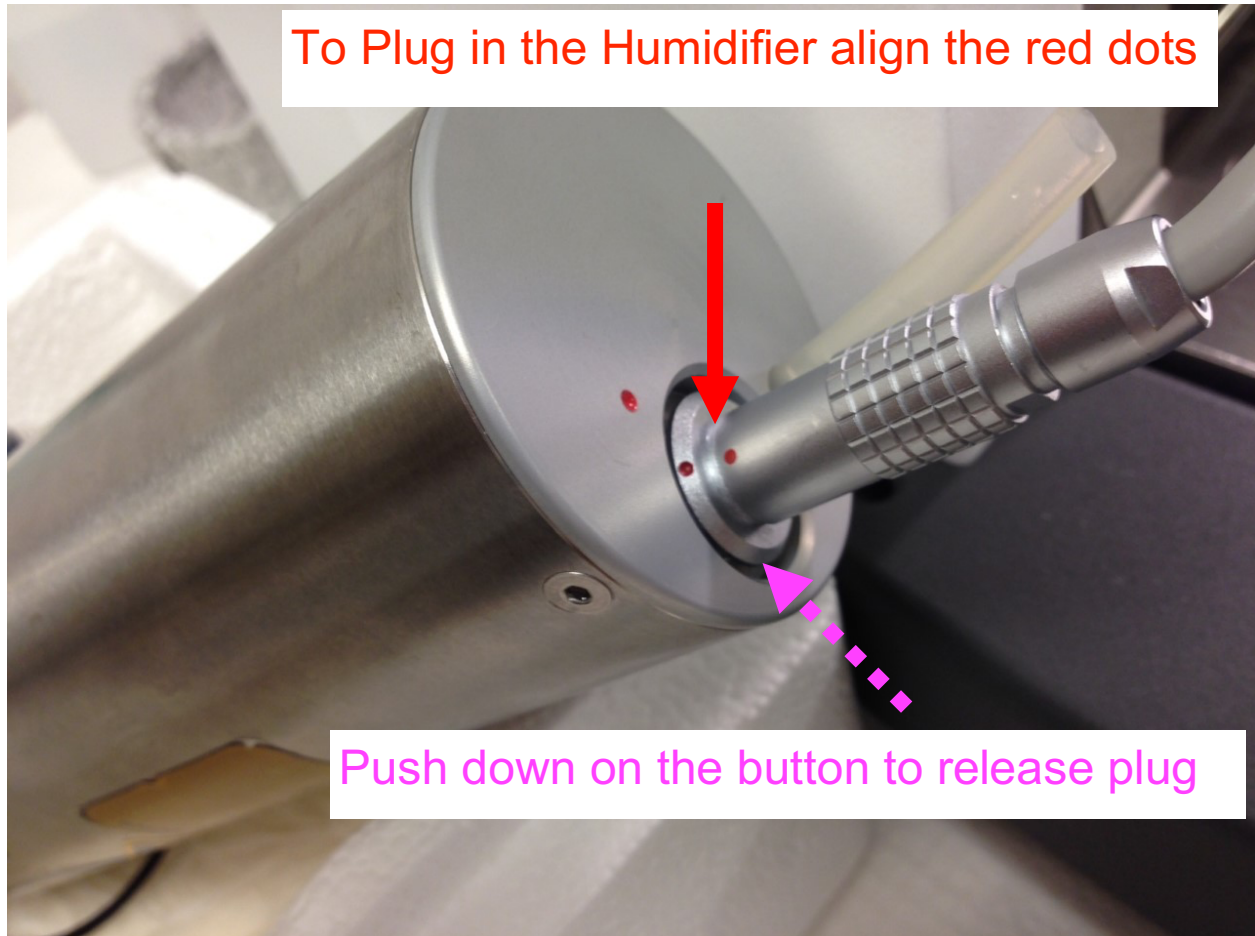
Before preparing cryo-grids:

1. Organize the tools and materials for the experiment
2. Check the LN2 tank and propane/ethane tanks
3. Check LN2 dewar make sure it does not contain ice or water
4. Check the Vitrobot
5. Check the Pelco EasiGlow or Tergeo-EM
6. Make sure you have enough sample for your experiment

NOTE: It is recommended to let the filter paper incubate in the Vitrobot chamber at high humidity for 30-60 mins before preparing grids.

Setting up the Vitrobot:

Normally the Humidifier is plugged into the Vitrobot, if not please follow these directions. Plug in the cable into the bottom of the Vitrobot Humidifier. Align the red dot on the cable with the red dot on the bottom of the Vitrobot Humidifier. To remove the plug push down on the button (indicated in purple), as you pull the cable away from the humidifier. failure to do so will damage the Humidifier. **This part is critical! As the Core for Help on this step!!!**



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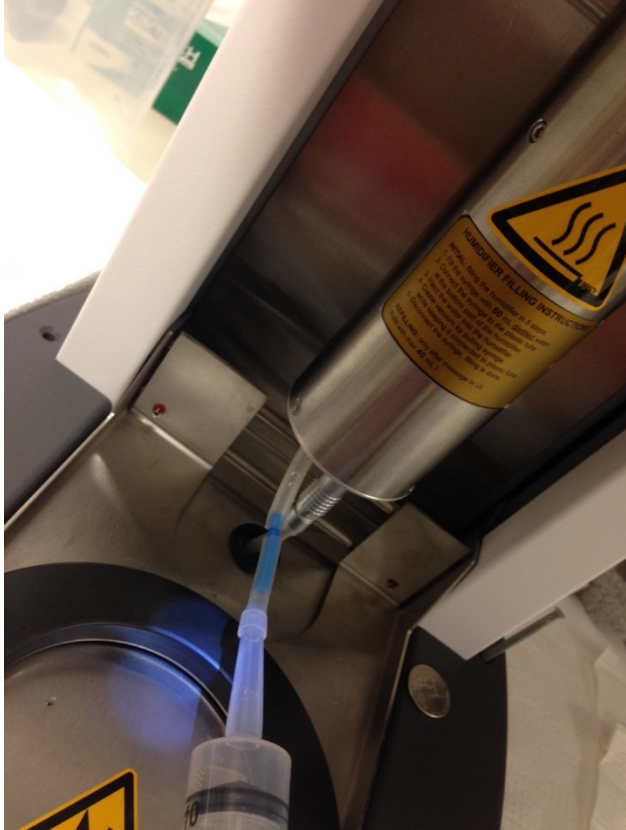
Make sure that the orange **rubber O-ring** is mounted on the top of the Vitrobot Humidifier before sliding it into the Vitrobot. Place the Vitrobot Humidifier into the Vitrobot so that the sticker faces you (as shown in the picture).



Rubber O-ring



Sticker Faces You

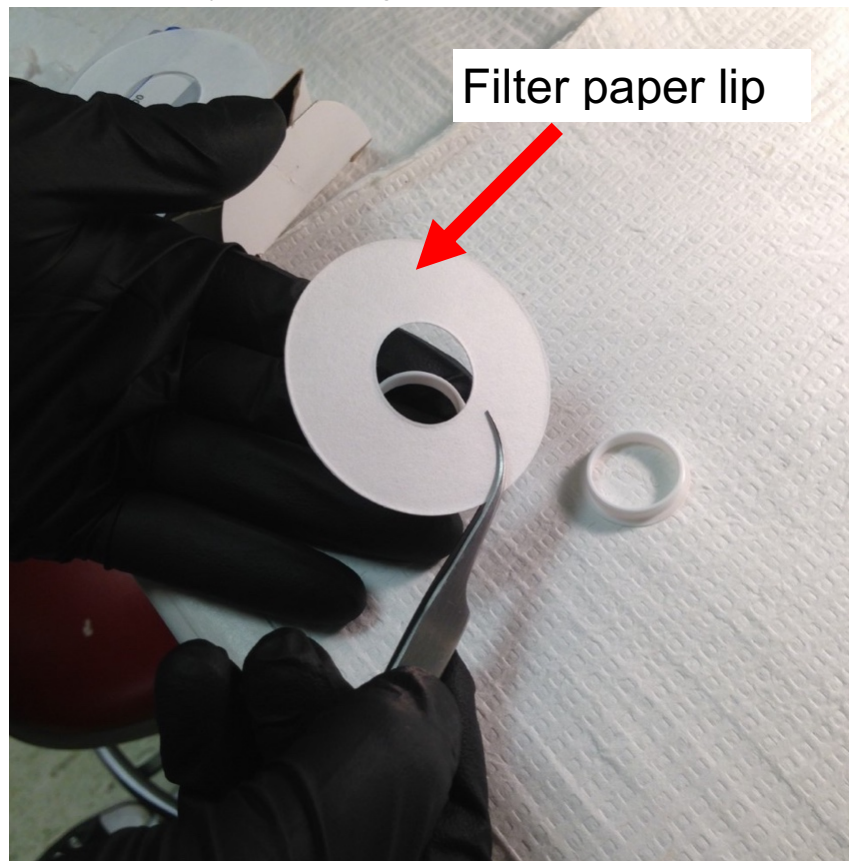


Fill the **humidifier with 30 ml of dH₂O** with the 60 ml Syringe. After injecting the water into the humidifier, pull back on the syringe to make a vacuum in the humidifier.

Turn on the Vitrobot main power switch, located in the back of the unit. Wait for the computer to boot and the main control screen to appear before starting the experiment.

Put filter paper on Vitrobot Blotting pads

Load Vitrobot Filter Paper (Whatman 595) onto the Plastic Ring. Don't touch the filter paper with your bare hands, wear gloves, and use forceps. Orientate the filter paper with the lip of the filter paper away from the ring.



Mount the Plastic Rings with Filter Paper on to the Vitrobot Blotting Pads. Make sure that the rod is in the upright position inside the chamber. Make sure the filter paper and plastic rings are flush with the blotting pads. **Wear gloves** when handling the Filter Paper and/or use forceps, don't touch the filter paper or inside of the Vitrobot Chamber with dirty hands.



Cooling the Vitrobot Pot

Assemble the Vitrobot Pot: Ethane Cup, Pedestal, Spider, Styrofoam Ring, and Styrofoam Cup. Make sure all of the components are clean and not damaged.

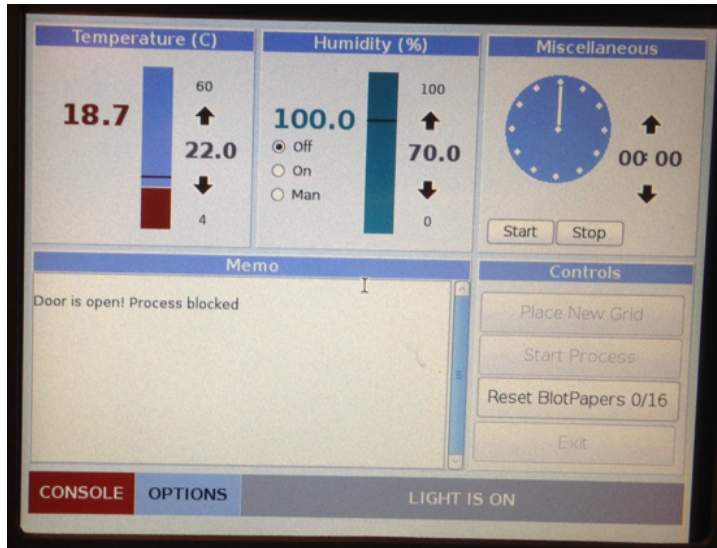


Pour clean LN2 into the Vitrobot Pot and Ethane Pot.



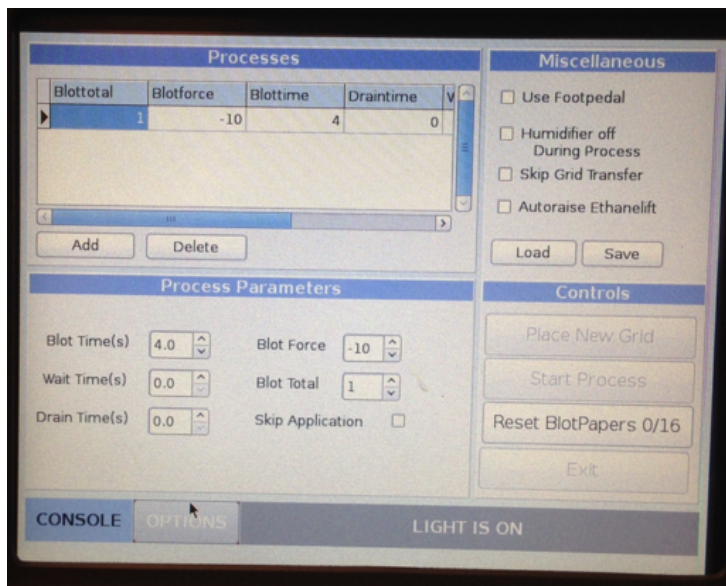
It should take ~15 mins to cool the ethane cup, during this time add more LN2, and cover the top of the Vitrobot Pot with a plastic beaker or styrofoam box to prevent frost from building up in it. Alternatively, pre-cool the ethane pot and spider with LN2 in a separate container then assemble using pliers to handle the metal components. Once the liquid nitrogen stops boiling it is ready to dispense the ethane or ethane propane into the ethane cup.

Set parameters in the Vitrobot Control Screen (Console and Options Tab)



The Console tab:

- Temperature
- Humidity
- Controls
- Light
- Miscellaneous



Options Tab

- Processes
- Process Parameters
- Blot time**
- Wait Time**
- Drain Time**
- Blot Force**
- Blot Total**
- Controls
- Miscellaneous
- Use Footpedal
- Humidifier off during the process

What does this mean?

Blot Time: how long the filter paper makes contact with the grid

Wait Time: time in-between applying sample and blotting

Drain Time: time in-between blotting and plunging

Blot Force: how strong the blot is

Blot Total: number of blots

Typical parameters that we use are...

Blot time (rule of thumb, 1 second per microliter) 2-5 seconds

Single blot

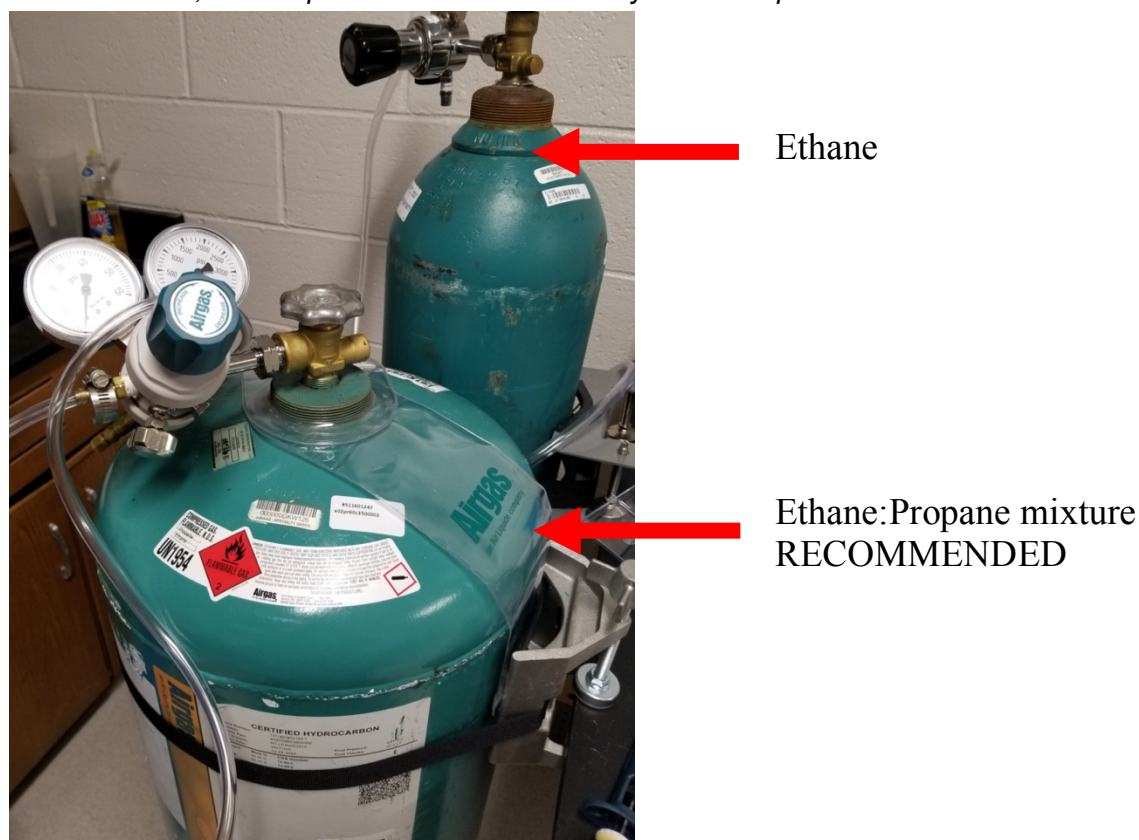
Drain time 0

Apply 3-4 ul of sample

Set Temperature to 22 degrees
Set Humidity to 95%
Check "Use Footpedal"
Check "Humidifier off during process"
Blot force +20

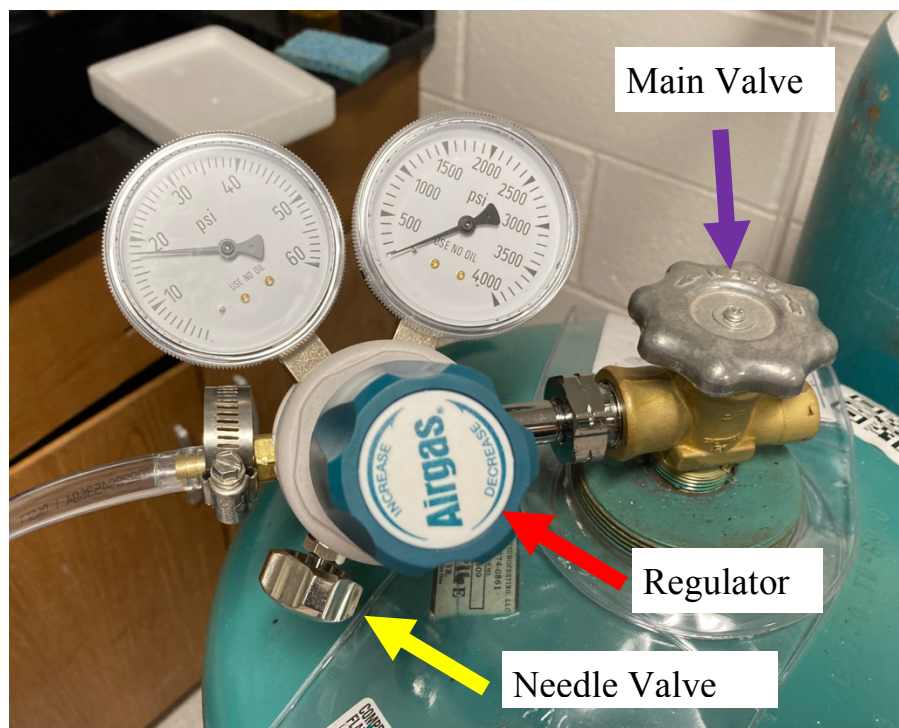
Once the Vitrobot Cup is cooled, will take ~10-15 mins, fill the ethane cup with ethane propane.

Note The ethane propane mixture is recommended for use because it will not solidify at liquid nitrogen temperatures, which reduces the chance of damaging grids with an ethane ice layer. Check the temperature of the ethane/propane mixture with a thermometer before preparing grids. After dispensing the ethane propane let it cool for a few mins using the spider, It should cool to be between -186 to -190 C°. Handel the ethane propane mixture using the protocol outlined below, but keep in mind it will not solidify like the liquid ethane.

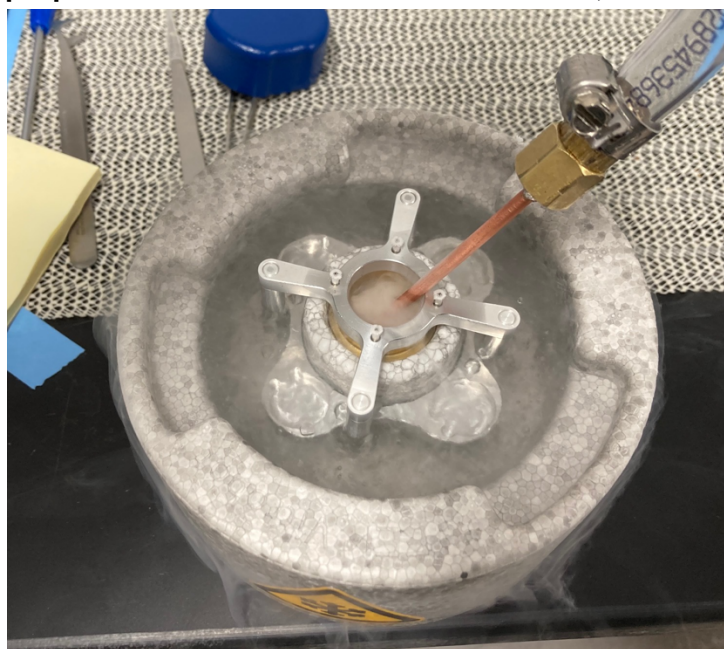


Fill ethane pot with ethane propane

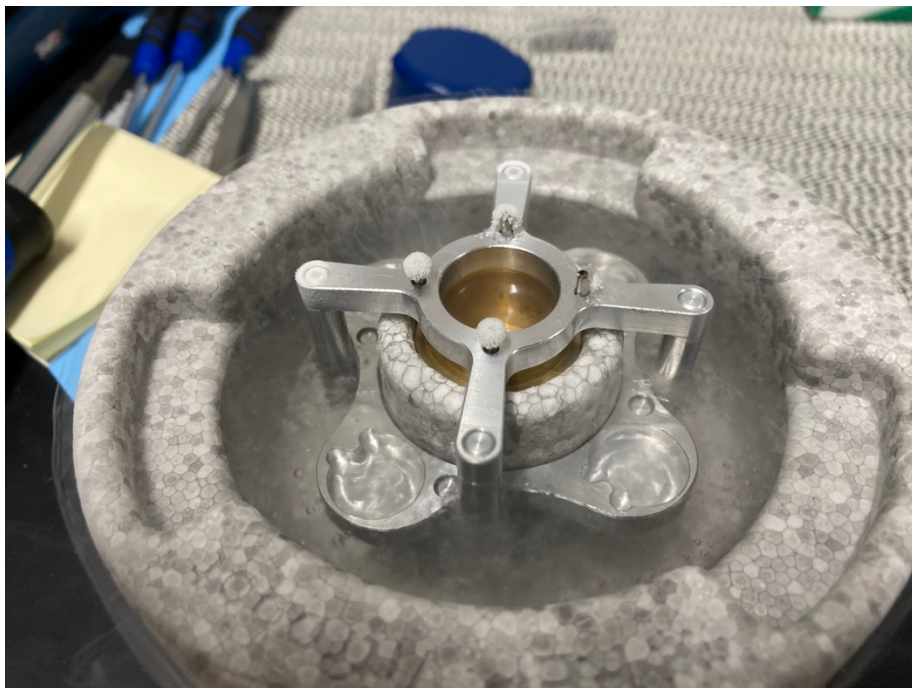
Open the main valve on the ethane propane tank. **Adjust the regulator so that the PSI is ~15.** To dispense the ethane propane slowly open the needle valve, the PIS on the regular will drop, and gas will come out of the tube.



Before dispensing the ethane propane mixture, place the copper tube inside the ethane pot, orientate the tube at an angle with the bottom of the tube touching the bottom of the ethane pot. Then open the needle valve to dispense the ethane: propane gas. The gas should flow out slowly and will condense inside the ethane pot. **When you are finished using the ethane propain make sure to close the main valve, and needle valve.**

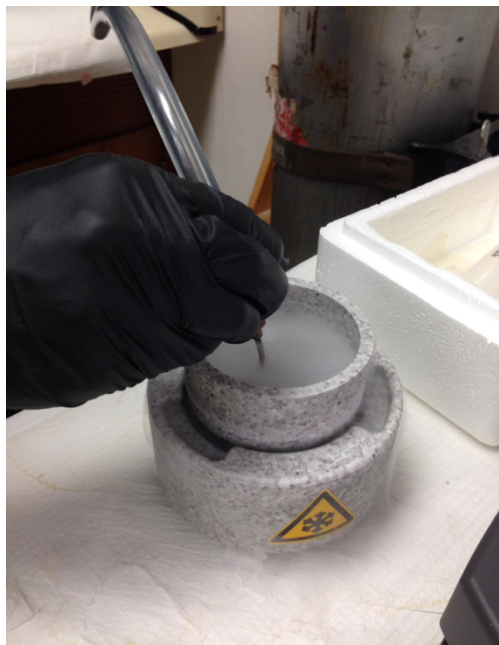


Continue to fill Ethane Pot until the liquid fills the very top of the cup, a few mm below the Spider.

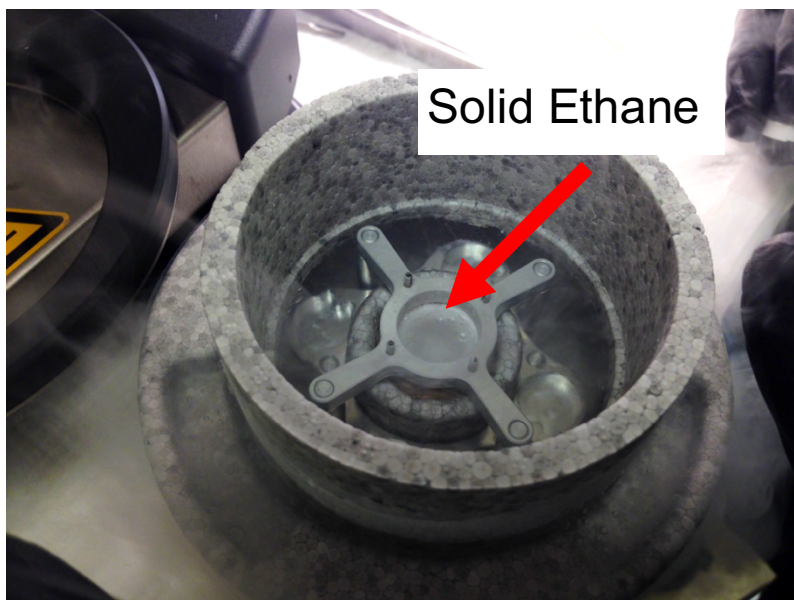


Wait a few mins for the ethane propane mixture to cool down to 180-190 C°. Check the temperature of the ethane propane mixture using a thermometer. Once the ethane propane mixture is cooled you can remove the Spider, and place it on the hot plate.

Follow the same procedure when using liquid ethane. The main difference is that the ethane will solidify at liquid nitrogen temperatures. Dispense liquid ethane into an ethane cup, keep the spider on it to cool the ethane cup. Be very careful when handling liquid ethane propane mixture. Both are flammable, and unlike liquid nitrogen, if you spill ethane on you it will burn your skin.



Wait for the ethane to solidify around the bottom and sides of the ethane cup. If too much of the ethane solidifies it will damage the TEM grid when plunging. Melt the excess solid ethane with forceps or add more liquid ethane to the pot. Check the temperature of the ethane with a thermometer.



You may use the PELCO EasieGlow or the Tergeo EM to render your grids hydrophilic, see below for instructions on using both.

Note: *plasma clean or glow discharge TEM grids right before preparing cryo-grids, within one hour. Typically, we do this after dispensing the ethane.*

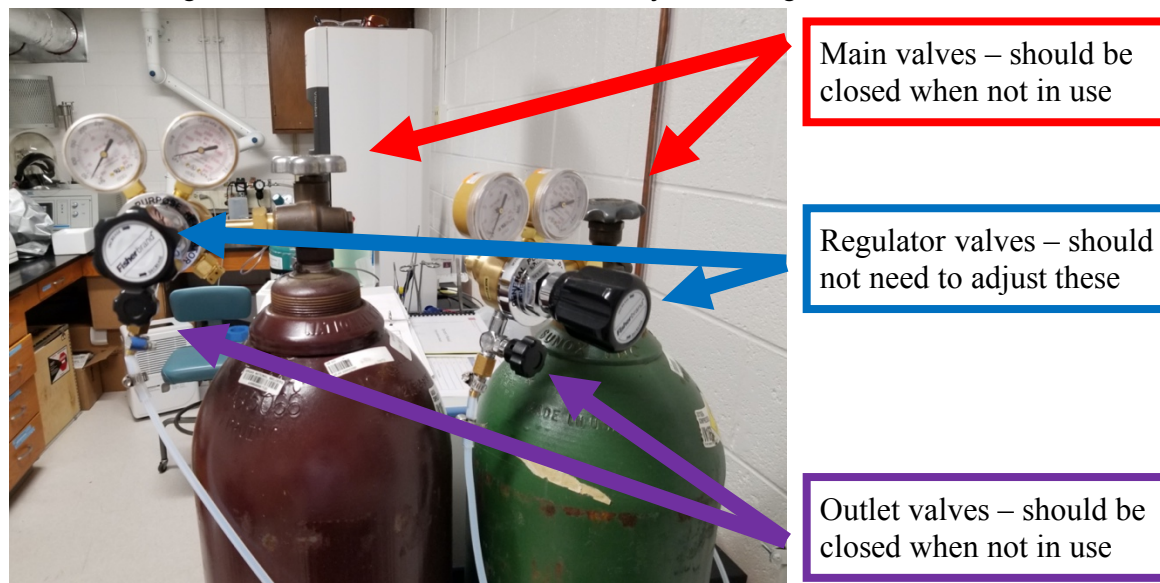
Tergeo EM Plasma Cleaner

Typical Settings:

Quantifoil TEM Grids: Recipe 1: Indirect plasma 25% Oxygen and 75% Argon

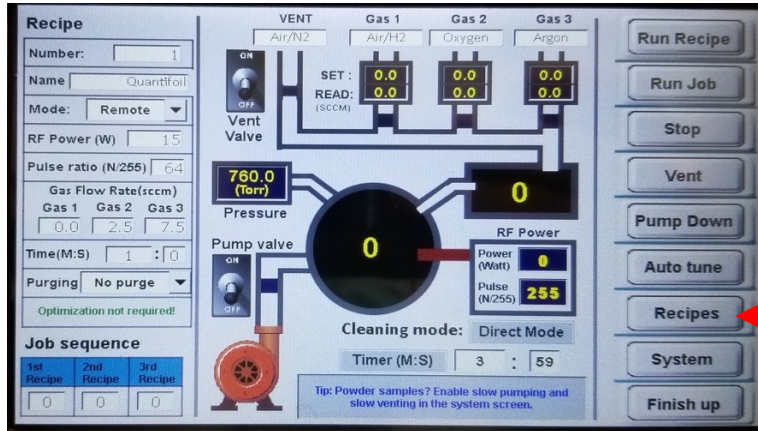
Ultrafoil TEM Grids: Recipe 2: Direct plasma 25% Oxygen and 75% Argon

Turn on the plasma cleaner using the power switch on the back, right side of the machine. Open the **main valves** on the Oxygen and Argon tanks next to the plasma cleaner, ensuring that the **outlet valves** are also open. You should not normally need to adjust the regulator valves, the regulators should read ~150. If not adjust the regulator valves.

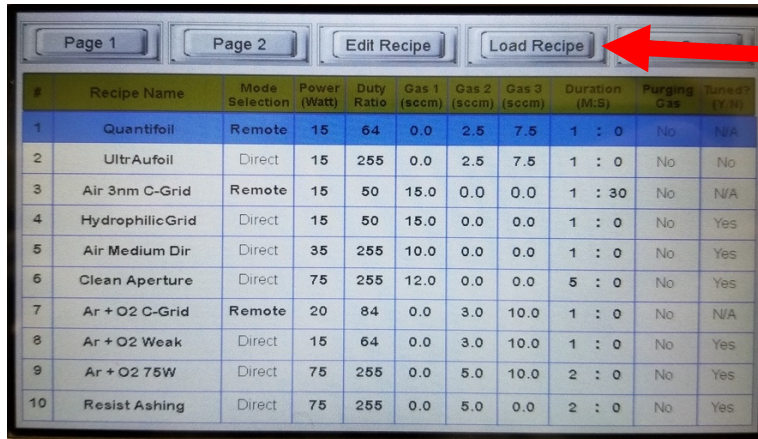


Open the Tergeo-EM chamber door by turning it counterclockwise. Place the grids to be plasma cleaned on a glass slide and **set the glass slide or metal pedestal inside the chamber** on top of the clear glass holder, pushing the slide a couple of inches back into the chamber. Place the chamber door back on the plasma cleaner and turn clockwise until it stops. The door will feel loose until the vacuum is applied, this is normal. On the touchscreen, select **“Recipes”** and find the recipe that is suitable for the type of grids you are using.

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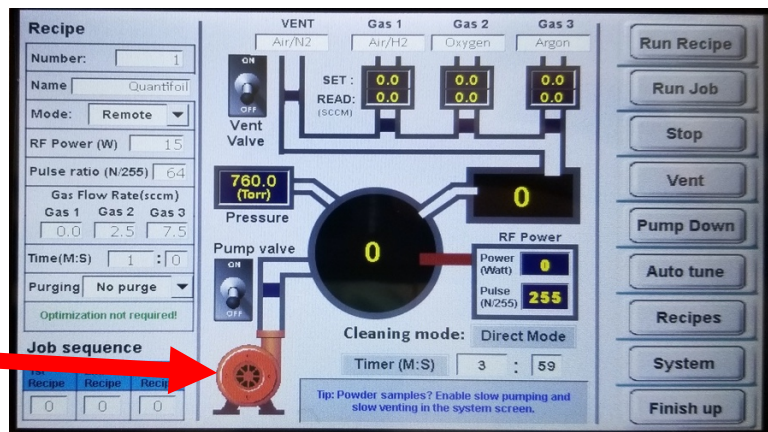
Select "Recipes"



Choose the appropriate recipe and select "Load Recipe"

Select the recipe by clicking on it and selecting "Load" at the top of the screen. At the home screen, once the grids are inside the chamber and the door is on, select "Run Recipe" and monitor the screen until complete. The vacuum pump will turn red to indicate that it is on and the pressure will start to drop as indicated on the screen. Once the vacuum is at the appropriate pressure, the gas will begin to flow and the plasma will ignite. At this point, the timer will start to count down. After the timer reaches zero, the system will be in a standby state with the vacuum pump on. Tap the pump icon on the screen (shown in red) to turn off the pump; the icon should turn gray.

Click on the pump icon after plasma cleaning to turn pump off. Icon will turn gray.



Press the "Vent" icon on the screen and wait until the chamber reaches atmospheric pressure, then press "Stop". You can now remove the chamber door and retrieve your grids.

Note: *Occasionally there will still be a slight vacuum in the chamber after venting and the door will be tight and difficult to remove. If this happens, do not attempt to force the door, you may damage the seal. Instead, repeat the vent procedure and attempt to remove the door again; it should come off easily. When you are finished, replace the door, turning clockwise until seated.*

Then **turn off the plasma cleaner**, turn off the switch on the back of the machine. **Close the Oxygen and Argon gas valves after shutdown.**

Glow discharge TEM grids with the Pelco EasieGlow

Typical Settings:

Quantifoil TEM Grids: 30 seconds at 15 mA

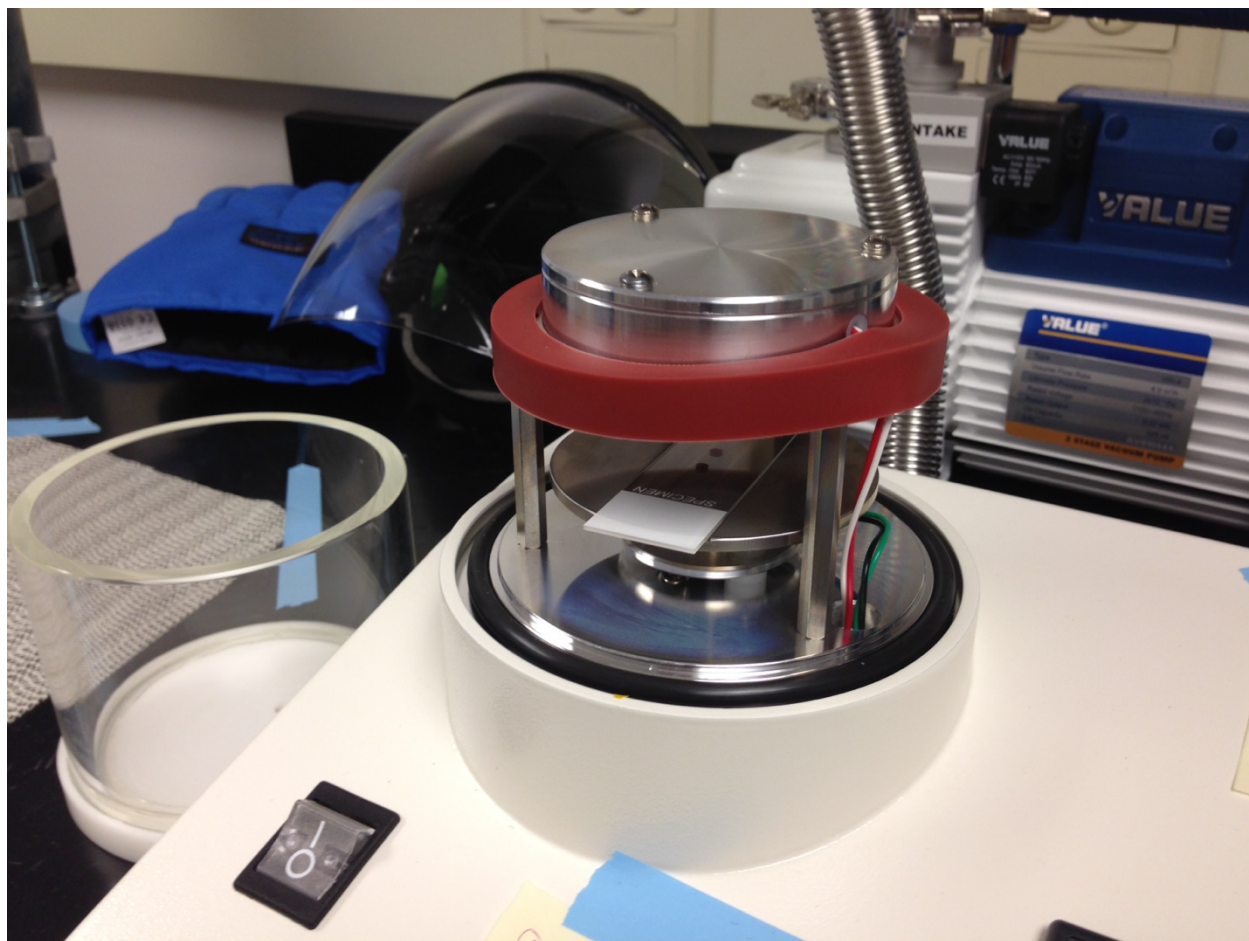
Quantifoil TEM Grids with 2nm Carbon: 30 seconds at 10 mA

UltraAufoil TEM Grids: 45 seconds at 15 mA

Check Settings on the Viewing Screen. You can change the settings by pressing the “Prog” button. Refer to the manual for details on operating it. To glow-discharge, the TEM grids click “AUTORUN” on the touch screen.



Place the TEM grids carbon side facing up on a glass slide. Carefully remove the bell-jar, place it on the bench with the plastic top-down, to avoid damaging the glass bell-jar.



Check the O-ring for dust before placing the bell-jar back on the unit.

When handling the TEM grids use a fine tip forceps, and grab the edge of the TEM grid at the 6 o'clock position.

Note: do not use plastic petri dish, the TEM grids will adhere to it due to static. Use a glass petri dish with a piece of filter paper inside it to store the TEM grids and glass slide. Some people will coat the glass slide with parafilm, you don't need to do this, the grids will not jump off the glass slide.

Note: examine the TEM grids before and after glow discharging with the light microscope to verify that the TEM grids do not have any defects and that the film does not have any cracks in it.

During the experiment watch the sample adhere to the surface of the TEM grid. If the surface of the TEM grid is hydrophobic then the liquid will ball up on the TEM grid. If the surface of the TEM grid is hydrophilic then the liquid will spread out over the surface.

Remove the spider, use pliers or large forceps, put a nickel on top of the spider if it is stuck (caused by ethane solidifying the spider and ethane cup).

Note: If there is too much solid ethane then melt it by adding more liquid ethane to the cup or stick a metal forceps inside the ethane cup.

Preparing cryo-grids:

1. Place cryo-grid boxes on the pedestal. Make sure that the screw/top is loose.
2. Keep the cryo-boxes and grids under LN2 during the experiment. **Label the cryo-boxes with a unique identifier**, we recommend your initials + date + alpha-numeric character.
3. **Pickup a TEM grid with the Vitrobot Forceps**. Slide the clip down one-two notches down to secure the TEM grid, then gently tap the forceps to verify that the TEM grid is secure. **Note: keep track of the orientation of the TEM grid relative to the forceps**. As I am right-handed and I prefer to pick-up the TEM carbon side facing the Right Sample Port of the Vitrobot. Make sure to pick up the TEM grid from the edge and don't bend the TEM grid. The forceps should grip the outer ring of the TEM grid. Make sure the grid is not bent or damaged. On the Vitrobot Control Screen select "**place new grid**", this should raise the rod. Or use the Foot-pedal.
4. Place the **Vitrobot Forceps on the rod**, keep track of the orientation of the forceps and TEM grid. **Make sure that the clamp is flush with the rod...this This is critical!!! Or you might damage the Vitrobot.**
5. Place the Ethane Dewar on the Vitrobot, then select "**place ethane**" or use the foot pedal.
6. Select "**Continue**" or use the foot pedal to raise the rod into the chamber.
7. Select "**Continue**" to lower the rod and forceps to the level of the blotting pads and filter paper.
8. Apply the sample onto the surface of the TEM grid. Avoid having the pipette make direct contact with the TEM grid.
9. Select "**Continue**" or foot pedal to blot the grid.
10. After the Vitrobot plunges the TEM grid into liquid ethane **transfer the cryo-grid into the grid box**. **Note: when transferring the cryo-grid from the Ethane into the cryo-grid box make sure to work quickly and keep the grid under liquid ethane or LN2.**
11. **Continue this process** (steps 4-13) until you have multiple cryo-grids, typically 4-8 for each sample.

Note: Use the syfoam ring, it will help keep a layer of cold nitrogen vapor in the Vitrobot Cup.



Note: Also keep the LN2 level at above the cryo-grid boxes.



Note: You store the cryo-grid boxes at the bottom of the Vitrobot Cup after you finished preparing your grids. This way you don't have to worry about the LN2 level dropping below the pedestal or the grid box. Just make sure that the top is closed to prevent grids from popping out of the box.



Shutdown procedure

Transfer cryo-grids to storage

Tighten the screw on the cryo-grid boxes.

Transfer the cryo-grid boxes to storage in LN2.

Ask Jared Peck or Joshua Strauss to transfer your grid boxes into a puck and Biochain 34 LN2 dewar for long-term storage.

*If you are working afterhours you can put your grids into a falcon tube and place the falcon tube in the Dewar. Make sure that your falcon tube is connected to a string and has drain holes on the top to let LN2 into the tube. **If you transfer your cryo-grids into a Falcon tube contact Jared Peck or Joshua Strauss and tell them where your grids are. This is critical!!!!!!***

Note: *only CryoEM Core Staff Personal are permitted to handle the pucks.*

Remove filter paper form the blotting pads

Remove blotting paper and plastic rings, clean rings with 70% ETHO, leave rings in the chamber.

Clean Vitrobot tweezers with 70% ETOH

Clean the tips of Vitrobot tweezers then place tweezers back into storage.

Drain Vitrobot Humidifier

Unmount the Humidifier from the Vitrobot, poor the water from it into a plastic cup. Use the syringe to remove any remaining water. Place the humidifier inside the foam box to dry. You don't have to unplug the humidifier.

Do not unplug the humidifier..... this is critical!!!! To unplug it correctly hold down the button located on the bottom of the Vitrobot Humidifier with one finger to release the plug, then gently pull the plug out. **Critical!!!! if you don't push down on the button you will damage the plug.**

Ethane Tank

Close Ethane tank valves, this is critical! First, close the main valve, then turn the middle valve (used to set the flow rate), open the needle valve to purge the tubing of any remaining ethane gas, then close the needle valve.

Vitrobot shutdown

On the Vitrobot Control Screen select "shutdown Vitrobot", this is critical do not skip this step!!!!. After the computer shuts off turn the main power switch off located in the back of the unit.

Clean up

Put all the tools and equipment back where you found them.

Put the Vitrobot dewar under the snorkel to evaporate.

Wipe the Vitrobot Table with 70% ETHO

Vitrobot Note Book

Enter your information into the Vitrobot Log Notebook. Include your name, who to charge to (ie Lab), Institute, number of hours you used the Vitrobot, Vitrobot settings you used, and notes. **If you notice an error with the Vitrobot make sure to write it down in this notebook and tell Joshua Strauss.**