USER GUIDE FOR THE VITROBOT

Bio-Imaging Center (Updated 11/10/2023)

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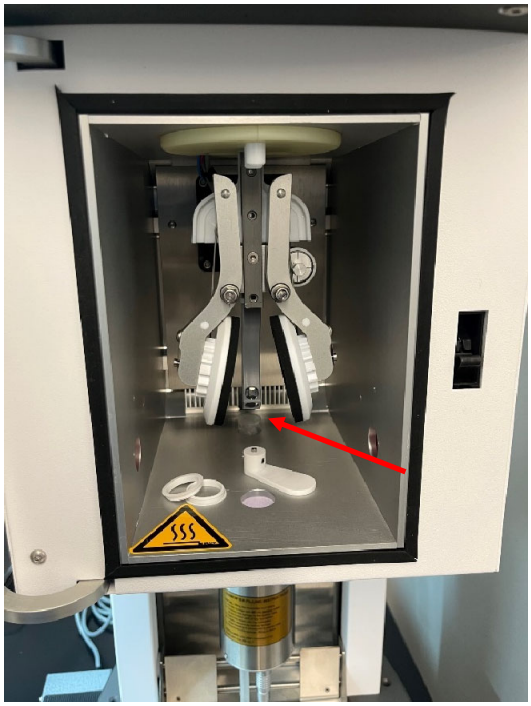
Vitrobot User Guide

Section 1: General Remarks

1. The maximum thickness of a sample for cryo-TEM is about 300nm.
2. Samples must be very concentrated for cryo-TEM.
    a. 5mg/ml is a good starting point for cryo-TEM.
    b. The range is generally 1-10mg/ml.
    c. For negative staining, you typically use 0.01-0.1 mg/ml as the negative staining procedure tends to concentrate the sample onto the carbon film of the grid.
3. Achievable resolution on the Talos L120C
    a. For single particle analysis applications on the Talos L120C with the Ceta-16M camera, the maximum attainable resolution is 10-12Å. For higher resolutions, a TEM with a field emission gun, higher accelerating voltage, and different camera would be required.
4. Throughput
    a. Be realistic!
    b. Don't expect to image more than 1-2 grids a day by cryo-TEM. It is a slow process.

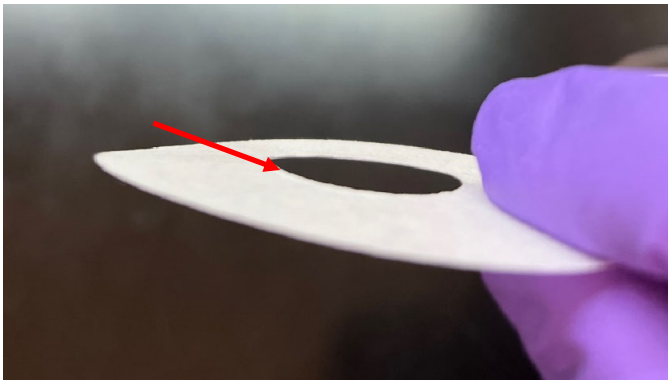
Section 2: Setting up the Vitrobot and Selecting Parameters

1. Things to check before starting:
    a. Make sure there are no forceps left in the Vitrobot before you turn on the instrument. If you power up the Vitrobot and it has forceps clamped in the arm, the machine will ram them into the shutter and ruin them. The Vitrobot forceps are expensive ($500!).

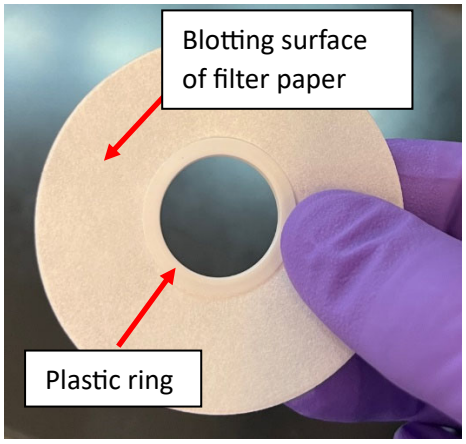


Before powering up the Vitrobot, check to make sure no forceps were left inside the machine!

2. Load the Vitrobot filter paper.
    a. The filter paper rotates with each blot performed.
        i. 16 blots can be done before the filter paper needs to be changed (the machine keeps track of the blots).
        ii. After each use of the Vitrobot, new filter paper should be inserted. Once the machine has been power cycled, it loses count of the number of blots.
    b. The filter paper has two sides due to how the paper was punched out with a die. The edge where the paper is frayed upward (rough side) should not go against the grid. This side should go against the black blotting pads.
    c. Use the white plastic discs to clamp the filter paper evenly to the black surface. Run your finger over the plastic disc to ensure the filter paper is evenly secured.



The backside of the filter paper has its edges frayed upward. This side goes against the blotting pad.



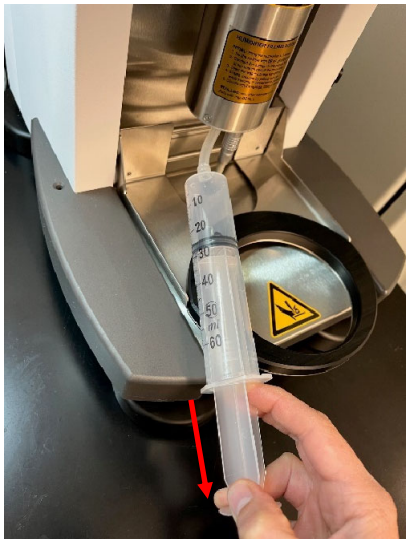
Insert the plastic ring

Clamp the filter paper against the blotting pad using the white plastic rings.

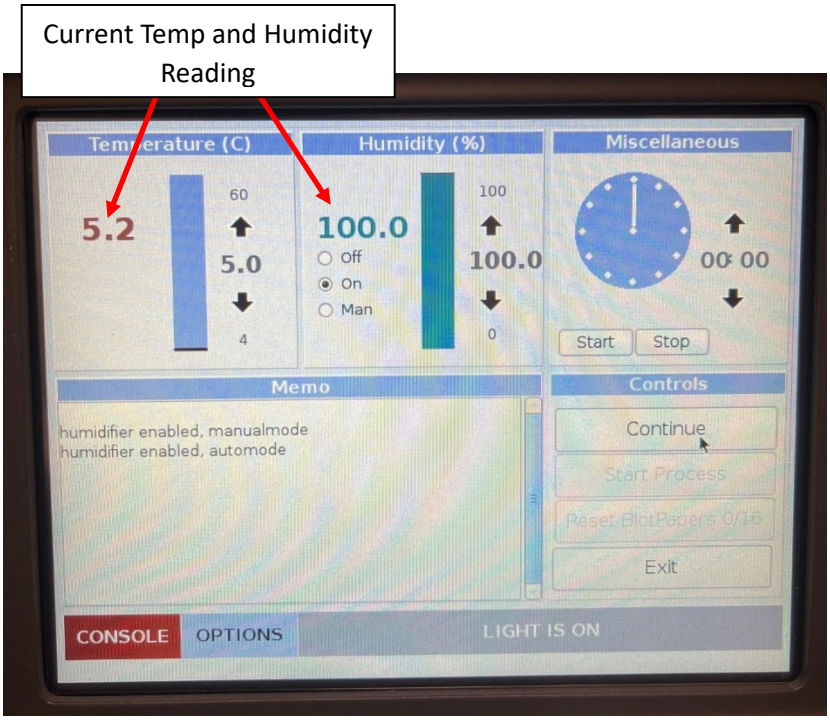
3. Set the temperature.
    a. The programmable temperature range is from 4-60˚C
    b. Usually a temperature range of 4-6˚C is used. 5˚C is a good starting point.
    c. If you use a sample that poses a biosafety risk, you can heat the Vitrobot to 60˚C to decontaminate the system.
4. Set the humidity.
    a. People generally use a humidity of 95-100%. 100% is a good starting point.
    b. Filling the humidifier:
        i. Fill the vessel with about 60ml distilled water (not the lab RO water!) using a 60ml syringe. Fill the vessel via the tubing at the bottom. When all the water has been pushed in, pull back the plunger of the syringe to make a vacuum before disconnecting.
        ii. You can test the humidity by going to 100% humidity and ticking Manual mode on the touch screen. You should see a mist enter the Vitrobot. Once you know it is working properly, set the humidity control back to Auto mode (On).
        iii. It takes 20-25 min for the temp and humidity to stabilize. It is best for the filter paper to equilibrate within the humidity before use.

Fill the syringe to 60ml with **distilled water.**

Connect the syringe to the tubing and inject the water into the humidifier reservoir.



Once all the water has been injected, pull back on the empty syringe to create a vacuum.

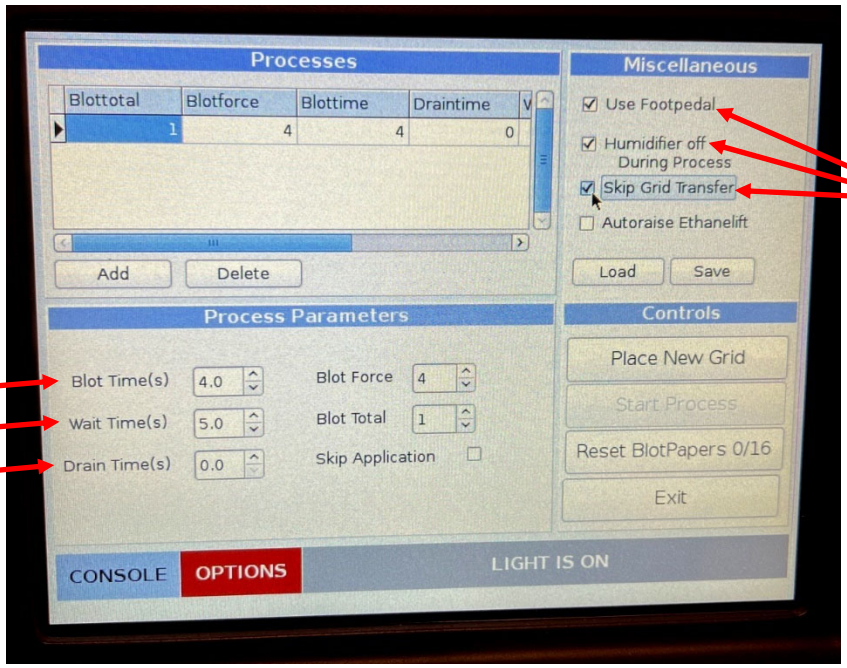


Wait for the temperature and humidity to reach the desired set points. It can take 20-30 min.

5. Options Tab
    a. Check these Boxes:
        i. Check “Use Foot Pedal” – Enables use of the foot pedal for the process.
        ii. Check “Humidifier Off During Process”
        iii. Check “Skip Grid Transfer”
        iv. “Skip Application” – Leave unchecked; this leaves the grid in a state where you can’t apply the sample to the grid.
    b. Set blotting conditions:
        i. Blot Time – How long the filter paper blots the grid.
            1. 4s is a good starting point.
            2. Generally, people use it in the range of 2-7s.
                a. If your ice is too thick, increase the blot time by 1-2s.
                b. If the grid is too dry, decrease the blot time by 1-2s.
        ii. Blot Force
            1. Range is +/- 25
            2. Generally, what is used is +/- 4
            3. This parameter is related to the instrument (not the sample) and should not be changed once set.
            4. For this instrument, use 4.
        iii. Wait Time – Time used to incubate the sample on the grid prior to blotting.
            1. 5s is a good starting point.
        iv. Blot Total – Number of blots
            1. 0: No auto blotting; blotting is done manually. This is used if cells are grown on a grid, and you would blot from the backside (non-cell side) to prevent cells from being ripped off the grid.

2. 1: One blot

v. Drain Time – How long the grid sits in the Vitrobot chamber after blotting before it is plunged into the cryogen.
    1. This is only useful for limited scenarios such as a viscous sample.
    2. Most samples have a drain time of zero to prevent drying.



Check these

Recommended starting parameters for blotting:

Parameters in the Options Tab

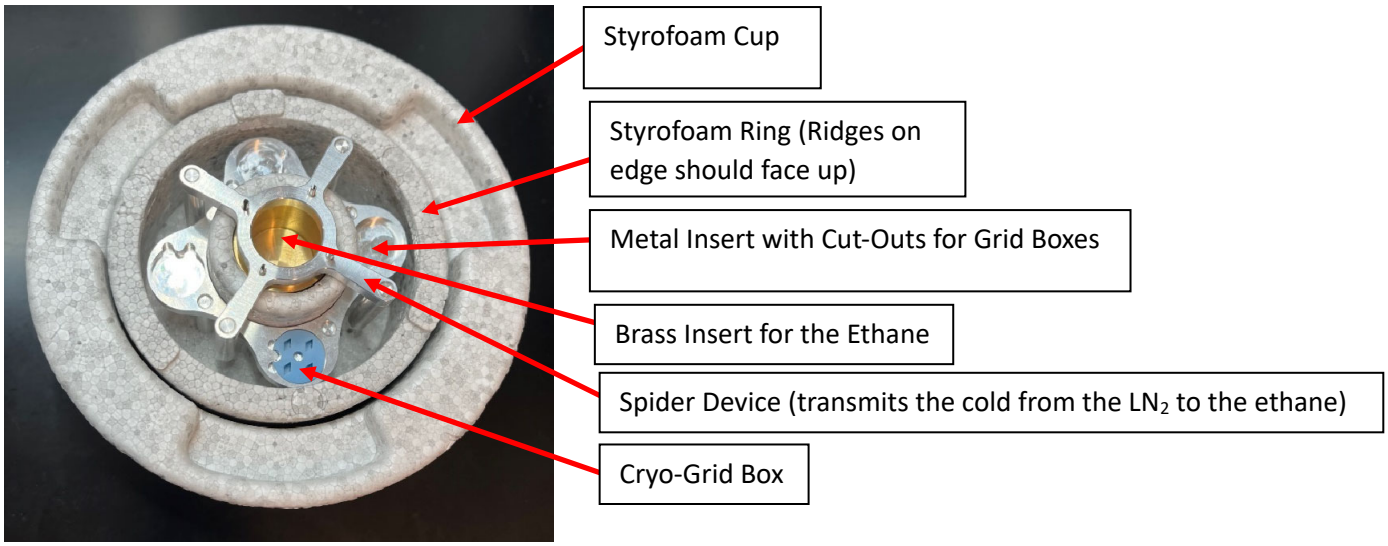
Section 3: Glow Discharge Parameters

1. Glow Discharge Current
    a. For cryo-TEM, a current of 10-20mA works well.
        i. The lower the current, the gentler the glow discharge
        ii. Higher currents are more aggressive to the grid film.
2. Glow Discharge Time
    a. For cryo-TEM, a glow discharge time of 20-90s works well.
        i. If you use a low current (10mA), use a longer glow discharge time (90s).
        ii. If you use a high current (20mA), use a shorter glow discharge time (30s).
3. Grids for Cryo-TEM
    a. Quantifoil R1.2/1.3 300 mesh copper are a good starting point.
        i. These generally yield very good results.
        ii. These grids have a carbon film containing 1.2 micron diameter holes separated by 1.3 microns
    b. Quantifoil grids with gold bars or a gold continuous film are typically only used for a more difficult sample where nothing else works.
        i. Gold grids yield less beam-induced motion in theory because the gold shrinks less than copper when cooled.
        ii. But these grids are more difficult to handle because they are very fragile and auto-routines don't always work well due to the Bragg reflections produced by the gold.

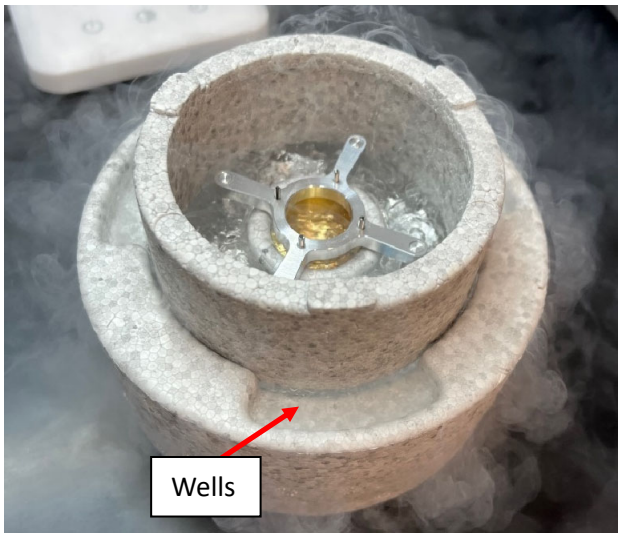
c. Quantifoil R2/2 300 mesh copper
    i. The advantage of these grids is the holes are bigger and you may be able to get multiple images per hole to increase throughput.
d. Lacey Carbon Grids
    i. Can be a good starting point to see if the sample prefers the carbon film over the ice.

Section 4: Liquefying the Ethane and Freezing Grids

1. Wear a mask when working with the ethane and liquid nitrogen to minimize contamination. A user's warm breath can introduce ice contaminates.
2. Assemble the unit for liquefying ethane (Styrofoam cup, brass insert for the ethane, metal holder to the brass cup, metal spider device, and the Styrofoam ring).
    a. Pour liquid nitrogen directly into the brass cup until it has cooled.
    b. Subsequently, pour liquid nitrogen into the wells of the Styrofoam cup.
    c. Wait until all the components have stopped boiling and have reached temperature. The liquid nitrogen level should be at the rim of the Styrofoam cup.
    d. The ridges on the Styrofoam ring should face up.
        i. If the Styrofoam ring develops frost, you can swap it with a new one (there are 3 available).
        ii. The Styrofoam ring creates a liquid nitrogen vapor layer that helps maintain temperature and minimize ice contaminants.



Assembly for liquefying the ethane



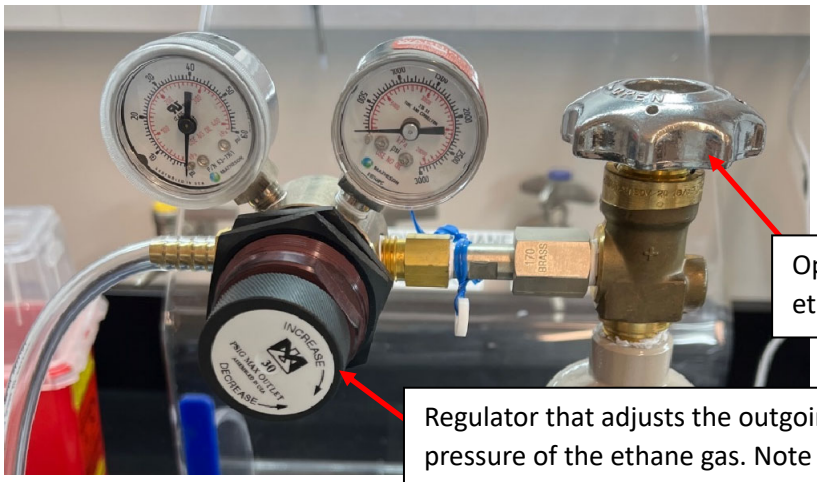
Start cooling the assembly by first pouring LN_2 directly into the brass cup. As you pour the liquid nitrogen, the Styrofoam ring will rise. Once the violent bubbling has subsided it has reached LN_2 temperature. Maintain the LN_2 level so that the LN_2 fills the wells of the Styrofoam cup by pouring LN_2 into the wells of the Styrofoam cup (not into the brass cup).

3. Liquefy the ethane.

CAUTION: Ethane is very flammable! Do not use if there is an open flame in the prep lab!

Liquid ethane can cause severe burns!

a. Fill the brass cup with ethane. You want to use a high-purity ethane (99.999%).
b. The pipette tip on the plastic tubing from the ethane bottle should touch the bottom of the brass cup.
c. Open the ethane tank and adjust the ethane regulator until you get an adequate flow of ethane gas into the brass cup. Don't have the pressure set too high as you don't want ethane splashing out of the cup. CAUTION: Liquid ethane will cause severe burns!!! Exercise extreme caution!!
d. Move the pipette tip up and down as you fill it to prevent the tip from clogging.
e. Fill the brass cup to the top with ethane. When you are finished with the ethane, adjust the regulator so there is no flow, close the tank, and return the ethane tank to the gas cabinet.
f. Wait until you see the ethane begin to freeze. Once you see the ethane start to solidify, you know that it is at the right temperature to properly vitrify samples.
g. Remove the spider device with forceps.
    i. The spider device will be frozen to the top of the brass ethane container.
    ii. Place a nickel on top of the spider device. This will warm it just enough so that the spider device can be removed with forceps.
h. If you see a thin film floating on top of the liquefied ethane, blot it away with a filter paper wedge.

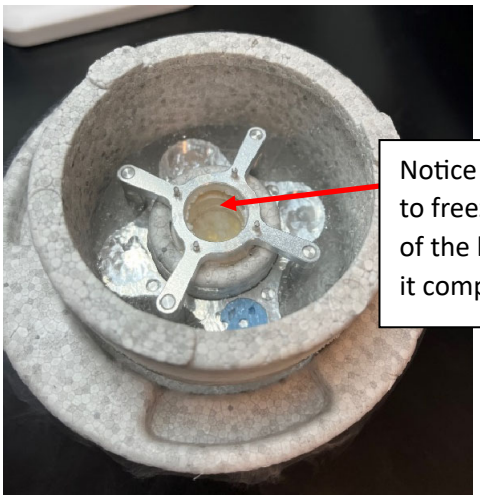


Opens/closes the ethane tank

Regulator that adjusts the outgoing pressure of the ethane gas. Note the direction to increase or decrease the flow.

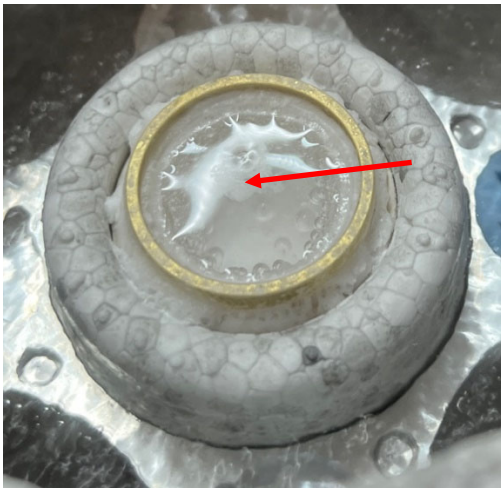
When you dispense the ethane, the gas can make it hard to see. Make sure your fingers are a safe distance away from the ethane as liquid ethane can cause severe burns!

Newly liquefied ethane is clear. It is not yet cold enough to properly vitrify grids.



Notice the ethane starting to freeze around the edges of the brass cup. Don't let it completely solidify!

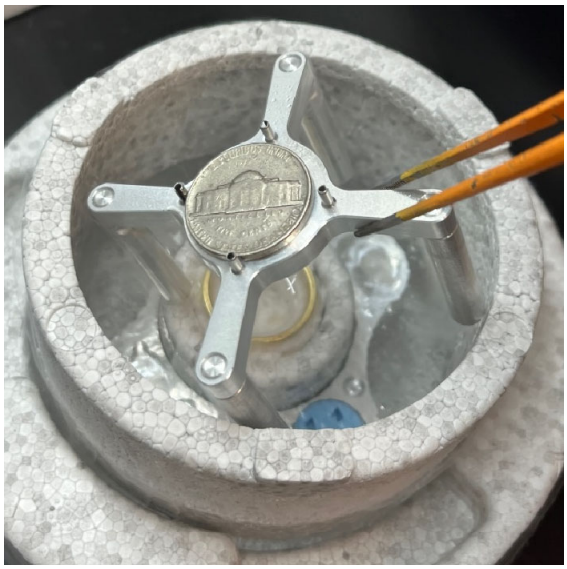
Allow the ethane to cool via the spider device until it just begins to freeze. It is now the proper temperature for plunge freezing.



If there is a film floating on the surface of the ethane, blot it away with filter paper.

The spider device will be frozen to the brass cup containing the ethane. To safely remove it, use forceps to place a nickel on top of the spider device.

The nickel will transfer enough heat to the spider device so that it can be removed.



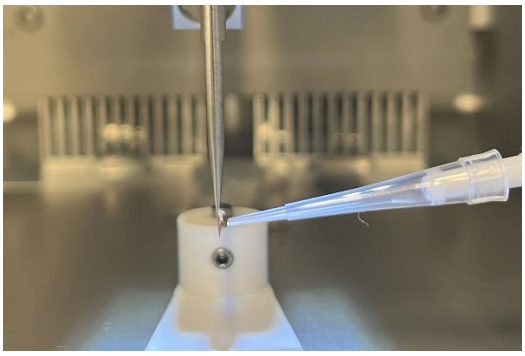
Remove the spider device with forceps. Caution! The metal spider device will be very cold and can cause severe burns if it contacts your skin.

4. Freezing a Sample
    a. Follow the on-screen prompts of the Vitrobot under “Controls”. You can proceed from one step to the next using the foot pedal.
    b. Briefly, the entire process for freezing the grids is as follows:
        i. You will load the forceps holding a grid into the arm of the Vitrobot.
        ii. You will be prompted to place the ethane assembly on the lift. The ethane assembly will be lifted, and the Styrofoam ring will compress against the underside of the temp/humidity chamber. Some LN_2 will spill.
        iii. You will be prompted to pipette sample onto the grid through one of the side ports of the temp/humidity chamber.
        iv. The grid will be blotted with the filter paper based on the user-defined parameters.
        v. The grid will be plunged into the liquid ethane.
        vi. The ethane assembly along with the grid and forceps will be lowered simultaneously, and then the user will have to disengage the frozen grid and forceps from the Vitrobot arm and store the vitrified grid in a cryo-grid box that is kept under LN_2.
    c. Holding the grid with the Vitrobot forceps.
        i. The sample will be applied to the carbon side of the grid. If you are right-handed, the carbon side should face the right. If you are left-handed, it should face the left.
        ii. Ensure the forceps are properly loaded onto the Vitrobot arm. You should hear a click.
            1. If the forceps are not loaded correctly, they can get jammed in the instrument.
            2. If a jam occurs:
                a. Use a sturdy pair of forceps to manually pull down the arm until you can disengage the forceps from the arm.
                b. Then power cycle the instrument to reset the positions.

Ensure the Vitrobot forceps are properly engage with the arm to avoid a jam.

d. Apply the sample.
    i. Introduce the sample through one of the side ports.
    ii. Pipette 3.5 µl onto the carbon side of the grid.
    iii. Once the grid has plunged into the ethane, refill the Styrofoam cup from the wells. You want to ensure the grid box is kept under liquid nitrogen.

Apply sample through one of the side ports of the temp/humidity chamber.



Pipette 3.5 µl onto the carbon-side of the grid.

e. Storing the frozen grid
    i. Once the grid is blotted and plunged into the ethane, carefully disengage the forceps from the Vitrobot arm while keeping the grid submersed in the ethane.
    ii. Quickly transfer the grid from the ethane to the surrounding liquid nitrogen reservoir.
    iii. With the grid above a slot of the grid box, open the forceps to allow any chunks of frozen ethane to fall off, and then insert the grid into one of the slots of the grid box.
    iv. From here, cap the grid box and quickly move it to a small liquid nitrogen dewar for temporary storage until it can be loaded into the TEM.

Section 5: Shutting Down the Vitrobot

1. When you are finished using the liquid ethane assembly, carefully move it to a fume hood to allow it to reach room temperature. The ethane will evaporate into the fume hood. Place the plexiglass shield in front of the ethane assembly to ensure no one accidentally touches it while it is still cold.
2. Ensure there are no forceps left on the Vitrobot arm.
3. Remove the filter paper and discard it. Leave the rings that clamp the filter paper in the chamber so they don't get lost.
4. If there is any liquid in the bottom of the temperature/humidity chamber sop it up with a paper towel.
5. Hit the Exit button.
6. Turn off the power using the switch on the back of the Vitrobot.
7. Empty the water vessel.
    a. Disconnect the water vessel by twisting it clockwise and lowering it.
    b. Pour the water into a beaker. Swirl to release more water and pour again. Continue until no more water can be removed.
    c. Leave the water vessel on the side of the instrument to let it air dry.

Remove water from the humidifier and leave the empty humidifier beside the Vitrobot to air dry.

8. Open the front door and leave it open so the temp/humidity chamber of the unit can air dry.