Gatan Plasma Cleaner - Solarus unit

Wear all necessary PPE and gloves for all steps.

The purpose of this step is to make the grid hydrophilic. The goal for cryo-TEM is to form vitreous ice (amorphous ice). Water needs to be attracted to the grids before the ice forms in liquid ethane when using the vitrobot.

Materials: Tweezers for TEM (ie. high precision tweezers), *Grid storage box (green) with lid, TEM grids for cryo-TEM (ie. Quantifoil holey grids or lacey carbon grids), Argon gas tank, oxygen gas tank.*

In the IMF turn on the Argon gas tank and oxygen gas tank.
Make sure the output is 40 psi for both tanks.
Turn the gas valves up to allow the gas to travel from the tanks to the solarus unit.

Open the solarus plasma chamber (pull up). Make sure the front side has the two rods with o-rings sealing up the two ports in front.

Place your TEM grids into a grid storage box (ie. 6 grids) using TEM tweezers.

Gently remove the lid from the grid box (too much motion may cause static and attract the grids).

Gently place the grid box into the plasma cleaner chamber and close the chamber.

Turn on the vacuum.

Select the hydrophilic recipe with Ar (Argon) and O (Oxygen) gas.
Set the time to 2 minutes (depends on the type of grids you use)

Click on start to plasma clean your sample. You may need to press the start icon several times before a successful start will begin. Plasma can be seen in the little viewing screen when plasma cleaning starts.

Vent the chamber and take out your sample. Protect your sample using the grid storage box lid (Use gentle slow motion to prevent static).

These grids should be good for the vitrobot.

Turn off the valves for the Ar and O tanks and close the tanks as well.

Vitrobot

I. Vitrobot Setup

Wear all necessary PPE and gloves for all steps.

The vitrobot needs to be set up to get 100% humidity, the ideal environment for the vitreous ice to form before it is plunged into the ethane bath. The vitrobot steps can be repeated as needed to make multiple samples and stored in LN2 for future viewing using cryo-TEM.

Materials:
Vitrobot related: Cryo-transfer tweezers for the vitrobot, vitrobot filter paper, vitrobot filter paper clips, Vitrobot stylist, Vitrobot styrofoam ethan container with ethane cup and spider.

Cryo-materials: cryo-grid box (hold 4 grids) with lid, lid removal tool for cryo-grid box (mechanical pencil), LN2 dewar (for LN2 transfer), LN2 dewar (for cryo grid box storage), Fresh LN2, ethane gas tank with pressure regulator and line for ethane.

Preparation materials: 5 ul Pipettor, Pipette tips, DI water, cryo-TEM sample in solution to apply on grid (need about 5 ul per sample), US Five cent coin (Nickel), Pliers, tweezers.

Turn on the vitrobot
Set the temperature to 5 degrees Celsius and humidity to 100% (turn humidifier on)
Use the plastic syringe to add 30 ml of DI water to the humidifier and retract about 30 ml of air before removing the syringe.

Use the plastic clips (white plastic rings) to add a vitrobot filter paper to the two vitrobot blotting arms.

Vitrobot setting:
Options -> Miscellaneous: Use the stylist to “check” on “Use foot pedal”, “Humidifier off during process”, and “Skip grid transfer”. Make sure that “Autoraise Ethane lift” is unchecked.

- ★ Use foot pedal
- ★ Humidifier off during process
- ★ Skip grid transfer
- ❏ Autoraise Ethane lift

Processing parameters

Blot time:	3 seconds	Blot force:	4
Wait time:	5 seconds	Blot total:	1
Drain time:	0 seconds		

It will take about 20 minutes for the vitrobot to reach 100% humidity (close the glass door and make sure the side ports (left and right for inserting the pipettors are closed).
In the meantime, prepare the liquid ethane using the vitrobot styrofoam container.

II. Liquid Ethane preparation:

Wear all necessary PPE and gloves for all steps.

Materials: Vitrobot Ethane container with spider, nickel, cryo-grid box, pliers, dewar of fresh LN2 (to prevent contamination), ethane gas tank with regular and pipette tip line, vitrobot stylist or metal rod to break up solid ethane

The Ethane container is the vitrobot styrofoam container with the ethane brass cup in the center with the styrofoam pieces. Essentially it is a styrofoam container that has a LN2 bath surrounding an ethane brass cup. In the LN2 bath area surrounding the cup, there are ports to add the TEM cryo-grid boxes (4 slots). You can add a cryo-grid box to ports now before starting. Uncap the cryo-grid boxes. The “spider” (metal piece with 4 legs) is used to transfer the heat from the brass cup to the LN2 bath. This allows the LN2 to make the condense the ethane gas into liquid ethane in the brass cup. Prepare the ethane container after setting up the vitrobot and have your TEM grids plasma cleaned beforehand (you do not want to plasma clean while preparing ethane because we do not want sparks while working with ethane gas).

CAUTION: While using ethane gas, do not have any sparks inside the room.

Setup the Ethane container with the spider. Make sure it is completely dry and free of ice contamination.

Add LN2 directly to the brass container and fill it up with LN2 once (the LN2 will evaporate but will help cool down the brass cup). Fill up the LN2 bath region around the ethane brass cup and do not pour anymore LN2 directly into the brass cup. Allow the spider to cool down the brass cup as you fill up the side of the container with LN2. (Once the LN2 bubbles, then the metals in the ethane container should have reached equilibrium temperature with the LN2.)

CAUTION: While using ethane gas, do not have any sparks inside the room.

Open the ethane gas tank and allow the ethane gas line to release. The flow should be slow and feel like a gentle fan blowing. Adjust the gas flow as needed.

At the end of our ethane gas line is a pipette tip to release the flow of ethane gas.

Use the ethane line to add ethane gas to the brass cup (there should not be any LN2 in the brass cup). The pipette tip should be close to the side walls of the brass cup. Liquid ethane should condense and you will hear a hissing sound.

Adjust the flow of ethane as needed as the brass cup is filled with liquid ethane. Keep the pipette tip below the liquid ethane level so you do not splash liquid ethane out of the brass cup. As the liquid ethane level rises, you should also rise the level of the pipette along the side walls of the brass cup.

Decrease the gas flow to a halt once the brass cup is full of liquid ethane and then remove the pipette tip.

Add LN2 to the LN2 bath region on the outside as needed to keep the brass cup cold.

The liquid ethane will solidify into a white crystal. Use the backend of the vitrobot stylist and tap on the solid ethane to break up the solids at the bottom of the brass cup. The ethane should return as a liquid ethane and have a mirror-like appearance.

Grab a nickel and place it on the spider (the warm of the nickel is enough to warm up the spider). As you see it thaw, use the pliers to pick up the spider and remove it from the ethane container.

Now the ethane container is ready for the vitrobot.

III. Preparing a sample with the Vitrobot:

Before this step:
Remember to remove the spider from the Ethane container and have a cryo-grid box ready in the ethane container in the LN2 bath region on the container.
The vitrobot should be at 100% by the time the ethane container is ready and nearby the vitrobot.
Have your plasma treated TEM grids ready.

Materials: All previous materials, especially need sample ready, plasma treated TEM grids, pipette tips

Add a plasma treated TEM grid onto the vitrobot cryo-tweezers. Have the carbon side of the gird face the direction you plan to add the sample to via the pipetters ports (left or right on the vitrobot).

The cryo-tweezer will hold the grid in place when you use the black plastic band and move it to the grooves on the tweezers (use up to the first groove).

Attach the tweezers to the vitrobot tweezer arm (there should be a click when it's in position).

[*(Click) - Either use the foot pedal or use the stylist to click on the next process on the vitrobot.]

(Click) - Move cryo-tweezers into the humidifying chamber.
(Click) - Lower cryo-tweezers to allow sample to be added via the side ports.

Open the side port of the vitrobox (either left or right of the vitrobot humidifier chamber)
Use the 5 ul Pipettor to add 5ul of sample to the cryo-TEM grid. You can use both hands to balance the pipettor. Move the tip as close as possible to the grid without touching and then add the drop to the grid.

Make sure the "spider" is removed from the ethane container and the LN2 level is full for the LN2 bath that is surrounding the ethane brass cup in the ethane container. There should still be liquid ethane in the center brass cup. Add LN2 as needed to the side.

Add the Ethane Container onto the Vitrobot lift. The vitrobot menu process should say "Place Ethane Container"

(Click) - The Ethane container will rise up. Some LN2 will spill. The TEM grid will be blotted and then plunged into the liquid ethane in the center of the ethane container. Then, the ethane lift will lower down.

Add LN2 to have the ethane container to have the free floating styrofoam cylinder rise up and protect the grid in a layer of LN2 mist.

Looking at your TEM grid, remove the cryo-tweezers from the vitrobot using both hands, while keeping the TEM grid (the one that's on the end of those tweezers) in the liquid ethane.

Hold the cryo-tweezers in one hand while the TEM grid is still dipped in liquid ethane. Use your other hand to carry the ethane container out of the lift and on to the side of the vitrobot while still keeping the TEM grid (on the cryo-tweezers) submerged in liquid ethane.
With the ethane container down on a flat surface and the cryo-tweezers still holding on to the grid submerged in liquid ethane, use one hand to keep the tweezers closed, while using the other to move the black plastic band out of the groove on the cryo-tweezers (keep the tweezers closed with the other hand so you don't drop your grid).

Quickly transfer the grid from the liquid ethane to the LN2 bath outside of the ethane cup but still in the styrofoam container.

Add the grid to the grid box located in the LN2 bath.

Refill the ethane container with LN2 by pouring LN2 on the side of the container (region outside of the free floating styrofoam cylinder) into the LN2 bath region. (Do not pour LN2 into the liquid ethane)

Repeat the above process with the vitrobot until your cryo-grid box is filled (4 slots). Before capping your cryo-grid box, cool down the cryo-grid box lid and the tool for it in the LN2 bath, then cap your cryo-grid box when you're done. If you run low on liquid ethane, you may need to prepare more again with the spider. Remember to take out the spider before using the vitrobot again. The blotting paper can only be used to blot 16 times before you need to change the filter paper again.

Store the cryo-grid box in the LN2 dewar for storage (ie. customized falcon tube). When doing so, make sure the transfer is quick. Use the string to keep the sample afloat.

<u>Clean up for vitrobot:</u>
Leave the ethane container in the fume hood to allow the LN2 and liquid ethane to evaporate.
Remove the filter paper and open the glass window for the humidifier to dry.
Leave the white rings (plastic clips for the filter paper) inside the humidifier chamber for drying and storage.
Take out the water unit for the humidifier and pour out the water (you can shake the water unit to remove the water. Leave the unit down to dry.

On the vitrobot menu screen, turn off the vitrobot program. Then, turn off the power to the vitrobot using the switch at the back right corner of the vitrobot.

Cryo-holder bake out - Gatan Pump station

*Ideally, the bake out on the Elza cryo-transfer holder is done the day before your TEM session.

Materials: Elza holder, Gatan pumping station, Elza holder pumping tube, Gatan temperature controller with cable for Elza holder

Insert the Elza cryo-transfer holder into the pumping tube (black long container with vacuum hose attached between itself and Gatan Pumping Station.

Attach the vacuum hose on the Pump station to the Elza cryo-transfer holder (this is located on the dewar of the Elza holder. The connection is a metallic pipe. Above the pipe is a black knob for closing and opening the vacuum port in the dewar of the Elza holder. The black know should be closed clockwise.

Turn on the pumping station.

Once the pumping station starts pumping, you can unlock the valves on the pumping station (V1 and V2) and the valves leading to the Elza holder dewar and cryo-transfer holder tip. 4 black valves total on the Gatan pumping station.

On the cryo-transfer holder, open the black valve on the dewar of Elza holder to maintain a vacuum around the dewar.

If the Gatan temperature controller is turned off, press and hold onto the G button on the temperature controller.

Attach the temperature controller cable from the Gatan temperature controller to the bottom of the Elza holder (align the red dot on the cable to the red dot on the dewar side of the Elza holder to connect).

Setup Bake out with the temperature at 100 degrees Celsius and bakeout time to 99 hours. Press start to begin the bakeout.

The temperature will slowly ramp up to 100 degrees Celsisu and as the vacuum improves, the current will drop below 200 mA. Generally, this will take 2-3 hours for the bakeout to complete. To make sure the bake out is completed, we can set this up the day before our TEM session and turn off the bakeout.

Before using the Cryo-transfer station, close all the valves on the Gatan pumping station and close the black valve on the Elza holder.

Cryo-transfer station:

For this step, you need to have:
1. Your samples should have been pre-prepared using the vitrobot and stored in LN2 (dewar).
2. The Elza cryo-transfer holder needs to be baked out in vacuum about 2-3 hours before use (Ideal bakeout the day before at 100 degree Celsius)
3. The Talos F200C G2 needs the cryo-box at least 26% filled with LN2.
4. Fresh dewar of LN2.

Materials: Elza holder, Cryo-transfer station, Gatan Temperature Controller with cable, Clipping tool for Elza holder (removes clipping ring that holds grid in place at the tip of the Elza holder), precision tweezer, cryo-grid box with samples in LN2 dewar storage

I. <u>Sample grid holder to Elza holder (cryo-holder)</u>

Make sure the black valve on the Elza holder is closed.
Make sure the valves on the Gatan pump station are all closed and the pump is off.
Unplug the vacuum hose that attaches the Gatan pump to the Elza holder dewar.
Keep the temperature controller cable attached and the bake out sequence is off (We'll use the cable to check the temperature of the Elza holder tip: location of the sample grid slot for our samples)

Make sure the Cryo-transfer station is completely dry and the loading dock is aligned to allow the tip of the Elza holder to dock. (The tip of the Elza holder is where the TEM grid will be loaded. The gray knob at the back of the Elza holder near the dewar is used to open or close the shutter for the sample in one smooth motion to prevent the shutter from being stuck. For now, keep the shutter closed.)

Take out the black plug on the Cryo-transfer station and put it aside.
Move the Elza holder into the Cryo-transfer station. Make sure it's completely in (the loading dock needs to be aligned to the pins at the bottom of the station)

Remove the cap of the dewar on the Elza holder and add the small plastic funnel.
Pour LN2 into the funnel (you can lift the funnel to have the LN2 drain into the Elza dewar faster) while also pouring LN2 into the Cryo-transfer station loading dock. Cover the loading dock with the glass lid when not pouring liquid nitrogen to keep the loading lock cold.

Continue filling up LN2 on the Elza dewar until it is full and fill up the loading dock to a level above the tip of the Elza holder until the temperature reaches -177 degrees Celsius (~15-20 minutes). Remove the funnel and recap the Elza dewar.

Once the temperature reaches -177 degrees Celsius, wait for about 1-2 minutes for the temperature to stay cold.

Open the shutter (using the gray knob) for the Elza holder, place the clipping tool (metal threaded side) and tweezers into the LN2 bath in the Cryo-transfer station loading dock to cool down the tools.

Use the clipping tool to remove the sample ring by mounting the tool on the sample ring and turning clockwise (½ turn) slightly (DO NOT PUSH DOWN) and then lifting the tool up (stay within the LN2 mist). The sample ring should now be attached to the end of the clipping tool.

Place the clipping tool with the sample ring attached into the LN2 bath of the loading dock.

Use the tweezers to remove the old TEM grid from the Elza holder tip sample slot position (previous covered by the sample ring). If you take out the tweezers outside of the LN2 mist to remove the old grid, then heat up the tweezers with a blow dryer to remove ice contamination and dry it to room temperature before placing the tweezers back into the LN2 bath in the loading dock. (Add more LN2 to the loading dock) You want to make sure the tweezers are at LN2 temperature when you use it on your grids.

Have a styrofoam box ready and partially fill the box with LN2 nearby the cryo-transfer station.

Transfer your cryo-grid box with lid from the storage LN2 dewar to the styrofoam box. Then use the lid removal tool to attach to the lid of the cryo-grid box. Quickly transfer the cryo-grid box from the styrofoam box to the cryo-transfer station lock. There is a grid box docking location in the LN2 bath.

Use the lid removal tool to open the cryo-grid box. Move the lid to the LN2 bath just in case a TEM grid is stuck at the bottom of it when you open the cryo-grid box lid. If it does get stuck, it can be washed into the LN2 bath and picked up by the tweezers to be set back up in the cryo-grid box. If there is nothing stuck, transfer the lid back into the LN2 in the styrofoam box with the removal tool attached (saving it for later when we need to seal the cryo-grid box after successfully transferring a grid to the Elza holder sample slot position).

Wait for the LN2 level to be under the sample position slot. Use the tweezers to quickly transfer a cryo-TEM (the one with samples prepared via vitrobot) grid from the cryo-grid box to the sample slot position. Make sure you stay within the LN2 mist and center the grid with your tweezers quickly (put the tweezers back into the LN2 bath).

Grab the clipping tool and quickly tap the end of it 3 times to remove any contamination on it's tip before using it to seal the cryo-TEM grid in place **(Center the tool and push down (hear a small click) and turn counterclockwise and then lift the tool**). The sample ring should be now on the sample slot and securing the cryo-TEm grid in place. Add LN2 to the loading dock (avoid splashing, since your cryo-grid box is open) and this can help wash the grid of trace

contamination if you have enough time. Otherwise, quickly close the shutter (gray knob) in one smooth motion until the shutter is completely closed (spring resistance felt), then add LN2.

Use the lid removal tool to grab the cryo-grid box lid to reseal the cryo-grid box. Transfer this back to the styrofoam box with LN2 and then restore the cryo-grid box into the LN2 dewar.

Remove the tweezers and clipping tool and blow dry as needed.

Now one sample is ready on the Elza holder while your other samples are safely stored in the LN2 dewar.

Fill the docking station with LN2 and cap the docking station with the glass lid. Setup the microscope and cryo-transfer station to get ready for sample insertion into the microscope.

II. <u>Sample insertion into the microscope</u>

Setup the microscope using the TEM touchscreen:

Cryo-instructions - 3 minute (180 seconds) pump down time setup and stage reset.

Have the stage tilted -55 degrees.
Have it check for cryo-holder/outgassing holder

Fill up the LN2 in the Cryo-transfer station and cap the docking station with the glass lid.
Remove the temperature controller cable attached to the Elza holder.

Move the Cryo-transfer station as close to the TEM as possible with a good height (where you easily move the holder out of the station and quickly into the microscope.)

Review the following instructions before you insert the holder:

Have the styrofoam box ready under the TEM to catch LN2. Remove the dewar cap of the Elza holder and set it aside.

NOTE: The dewar opening on the Elza holder points in the same direction as the small pin that needs to be aligned with the TEM when inserting the holder into the TEM. You can use the dewar opening as your guide. During the holder insertion, you will spill LN2 so be careful.

Quickly transfer the Elza holder into the TEM - dewar opening on the Elza holder will be facing horizontally to your right to align with the pin on stage (3 o'clock position) when the stage is tilted -55 degrees. Push the Elza holder all the way in to secure the o-ring and initiate the pump down (Do not force it and make sure the pin/dewar opening is in the correct position and the holder is completely in). Half of the LN2 will spill down (hopefully you have a styrofoam box to

catch the flow.)

Select the ST Cryoholder during the pump down. (During this time, you can replug the Cryotransfer station with the black plug and add LN2 to the loading dock to keep the station cold. This will allow you to quickly be able to replace your sample if your current sample is not good for cryo-TEM. You can periodically add LN2 until you know that your current sample is good.)

After the pump down sequence (3 minutes), turn the holder counterclockwise until resistance is felt (the dewar should face about 45 degrees counterclockwise from the 12 o'clock position.), then guide the holder straight into the microscope.

Tilt the stage back to 0 degrees (the dewar opening should now face the 12 o'clock position - upright)

Use the funnel to add LN2 to the dewar on the cryo-holder until it is full. Recap the dewar on the Elza holder.

<u>TEM setup for preview</u>

On the TEM software, insert the cryo-box (Setup expansion tab)

Wait for the column vacuum to be below 20. (Again, add LN2 to the Cryotransfer station dock as needed just in case this is a bad sample for cryo)

Open the shutter on the Elza cryo-holder (gray knob) in one smooth motion (counterclockwise) until it is fully open.

Change the magnification to LM 210x and spot size to 7 (this is to protect your sample before you open the column valves).

Open the column valves and spread out the beam. (If you do not see anything, did you remember to open the shutter on the holder? Make sure the beam is spread)

Preview your sample (check all over the grid with the search function) and determine if it's appropriate for imaging. Vitreous ice should be clear (Ideally your sample will be inside this for viewing). Vitreous ice (amorphous ice) lacks the crystal structure of hexagonal and cubic ice and will appear transparent to the electron beam. It will appear lighter than carbon film but not as light as a hole. Contamination will appear black, such as hexagonal/cubic ice and solid ethane.

Use "High Contrast" mode if needed. In addition, the defocus can be set to -200um if needed to see your sample.

If the sample is good for cryo-TEM imaging, you can ignore the cryo-transfer station for now and continue to TEM alignment for Cryo-TEM.

If your sample is not good. Close the column valves.

Make sure the cryo-transfer station is still cold with no ice contamination and has LN2.
Following the instructions below to remove the sample.

Removing the sample -
If you plan to add another sample, make sure the cryo-transfer station still has liquid nitrogen. If not, use the blow dryer to cool heat up the station and remove all ice contamination. Make sure the dock is aligned. Then, add liquid nitrogen to cool down the cryostation dock again. (The black plug to the cryostation can be kept there to keep the station cold until you're ready to reload the cryo-holder.)

Before removing the sample:
Retract the cryo-box and close the column valves

Close the shutter on the Elza holder (closing the sample) using the gray knob at the end of the holder (turn it clockwise in one full motion until it stops). This will protect the sample from falling into the column.

Unplug the black plug from the cryo-transfer station if you plan to transfer the cryo-transfer holder back into the cryo-transfer station to replace the sample.

Press the "Remove Sample" icon on the TEM display screen.
This should reset the stage.
Tilt the stage -55 degrees (minimizes spilling of the LN2)
Have the styrofoam container ready to catch LN2 under the holder.
Uncap the dewar on the Elza holder and set it aside.

Use your right hand to hold the cryo holder and pull out enough so you can use your left hand to push against the white panel on the TEM. Keep pushing with the left as you pull with the right hand until the holder stops naturally (resistance). IMPORTANT: you need to maintain the push and pull so the column vacuum does not crash.

Turn the holder clockwise until resistance is felt and then pull out smoothly *and quickly reposition the dewar upright and re-insert into the cryo-transfer station if you plan to transfer the cryo-transfer holder back into cryo-transfer station.*

If you've successfully returned the Elza holder back into the cryo-transfer station, then you can repeat the steps to cool the cryo-transfer station and add a new sample. You would need to re-attach the temperature controller cable and check to make sure the Elza holder tip is still at the right temperature.

If there is ice contamination on the Elza holder, then you will need to remove the LN2 from the Elza dewar and then start another bakeout using the Gatan pumping station and Gatan temperature controller again for about 2-3 hours. Warm up the cryo-transfer station with a blow dryer to remove all water. Then, you can restart the cryo-transfer steps again from the very beginning if you plan to look at another sample.

TEM - Alignment

Make sure the column vacuum is below 20.

Start at a small spot size (ie. 6 or 7) to make sure the sample won't be damaged by teh beam.

Open the column valves.

Setup C2 aperture (ie. 150 or 75), spot size (ie. 5 or 3), gun lens (ie. 5 or 3) to get proper exposure

For contrast: Add the objective aperture (ie. 100) and make sure it's aligned in diffraction space.

Normal alignment protocol - Align most parameters at SA 11000X, align rotation center at a high magnification (ie. SA 92000X). Check the objective stigmation and coma free alignment on the BM-Ceta camera.

Exposure - high magnification, dose around 20-40 $e^-/(A_o^2.s)$ Image near focus for better details (can increase exposure time 1 to 2 seconds) to get more lighting.

Low Dose:
Search:Make sure dose is below 1.0 $e^-/(A_o^2.s)$ when searching to prevent damage to the sample. Can have the defocus at -200 um to see more contrast.
Focus: Set Focus distance from sample center and "x" degrees away: High magnification will sacrifice this location to get a good focus on sample location. (Can setup Focus 1 and Focus 2 for two potential locations to sacrifice)

Exposure: Setup illumination for camera, center the beam and spread the beam to provide enough dose to the camera. Target 20-40 $e^-/(A_o^2.s)$.

Use low dose Search to look for the sample (either using the BM-Ceta camera or flucamera)
Click on low dose Focus to setup the defocus for the image
When ready to take an image, in low dose, click on the "Expose" in the "Expose" tab in the low dose options. The integration time should be set for 1 or 2 seconds.

Images can also be taken in Search mode, but making sure the screen is lifted and click on acquire on the BM-Ceta Camera controls.