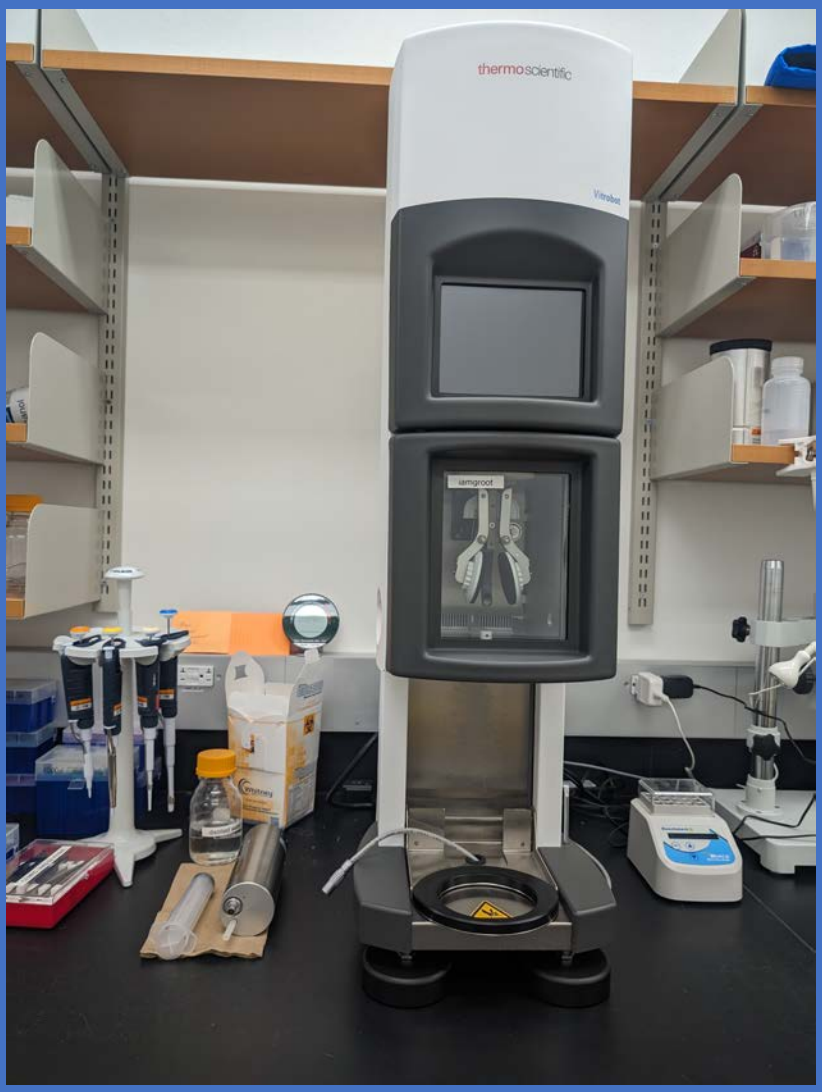
Cryo Electron Microscopy Research Center

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF WISCONSIN–MADISON

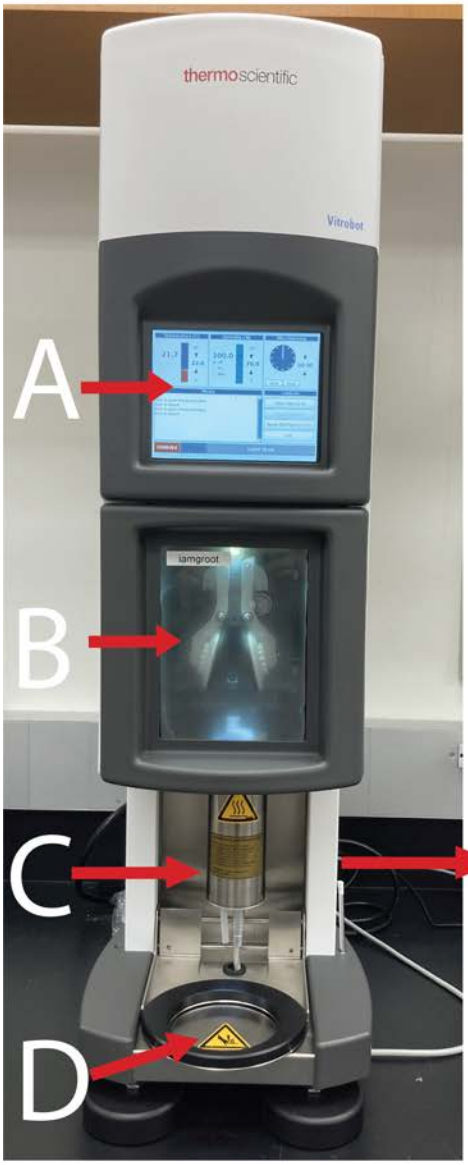


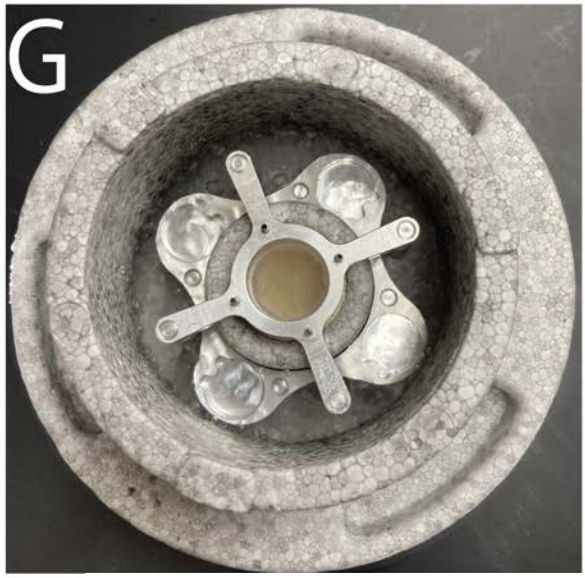
TFS Vitrobot Mark IV User Manual

Vitrobot Operations

IMPORTANT! DO NOT OPERATE THE VITROBOT ON YOUR OWN UNTIL YOU HAVE BEEN CERTIFIED BY CEMRC STAFF FOR INDEPENDENT OPERATION!

System Overview





F -Tweezers G -Ethane holder

E - Main switch (in the back)

A – Screen

B – Environmental chamber with blotting pads

C – Humidifier

D – Ethanelift

Short version

Caution! Safety and PPE:
Wear safety goggles and gloves when handling liquid nitrogen, gaseous and liquid ethane.

IMPORTANT! VITROBOT TWEEZERS ARE DEDICATED FOR PLUNGE FREEZING GRIDS ON VITROBOT ONLY. DO NOT USE THEM FOR OTHER GRID HANDLING PURPOSES.

1. PURPOSE OF THIS WORKFLOW:

The short version protocol for the TFS Vitrobot Mark IV is designed to serve as a rapid reference guide for experienced or trained users when freezing Cryo-EM grids.

2. ITEMS, REAGENTS, and MATERIALS:

Brought to the center by the users:

- ☐ Sample(s) to be frozen
- ☐ Dilution buffer (filtered water or buffer solution)
- ☐ Ice and ice bucket (samples should be put on ice before freezing)
- ☐ Grids (you can also purchase them from the center)
- ☐ Gold fiducials (for tomography samples; first time or forgot, ask the center)
- ☐ TetraSpeck beads (for CLEM samples; first time or forgot, ask the center)
- ☐ Tweezers

Provided by the center:

- ☐ Liquid Nitrogen (LN2)
- ☐ Ethane
- ☐ Cryo-gird box
- ☐ Tweezers (small, personal styles, longer forceps, Vitrobot tweezers)
- ☐ Pipetman and pipet tips
- ☐ Filter papers for blotting
- ☐ Ethane holder (Ethane cup, Spider, Grid box holder, Liquid nitrogen (LN2) container, Anti-contamination ring)
- ☐ CD cover (to avoid ice contamination)
- ☐ Heat block (for drying tools)
- ☐ Screw drivers and other tools for manipulating cryo-grid boxes
- ☐ Cryo-grid storage boxes and Puck
- ☐ Small LN2 Dewar (2-4x)
- ☐ Large LN2 Dewar (1x, 4L)
- ☐ Mini-centrifuge, vortex mixer, and storage tubes
- ☐ Distilled water (for Vitrobot humidifier reservoir fills)
- ☐ PPE (BSL1): Safety goggles, gloves, facemask and lab coat

3. GOOD PRACTICE IN GENERAL:

- ☐ Tweezers and other tools need to be pre-chilled with liquid nitrogen before handling frozen grids.

- ☐ To eliminate ice contamination, all tools that have touched liquid nitrogen need to be dried on the heat block before the next use.

4. PROCEDURES

Step 1: Vitrobot set up

- ☐ Install the humidifier. Fill the humidifier with 60 ml of distilled water.
- ☐ Turn on Vitrobot (main switch from back).
- ☐ Set to desired temperature and humidity parameters on the CONSOLE tab and select “On”.
- ☐ Set up desired blotting parameters on the OPTIONS tab.
- ☐ On the Miscellaneous window (under the OPTIONS tab), select the top three items: “Use Foot pedal”, “Humidifier off During Process”, and “Skip Grid Transfer”. Do not select the “Autoraise Ethanelift”.
- ☐ Turn on the heat block.

Step 2: Do a few practice runs without inserting the Vitrobot tweezers. Perform at least one dry run through the cycle to verify the process, ensuring that everything is functioning correctly and that you remember all the steps during the freezing cycle.

Step 3: Determine normalized blot force zero point. This is the minimum blot force that positions the bare blotting pads close enough to hold an index card.

- ☐ The blotting pads will not stabilize to their blotting positions until they’ve been cycled a few times. Before you start calibrating the blot force, always do at least 1 run without the tweezers after setting up parameters.

Step 4: Prepare liquid nitrogen.

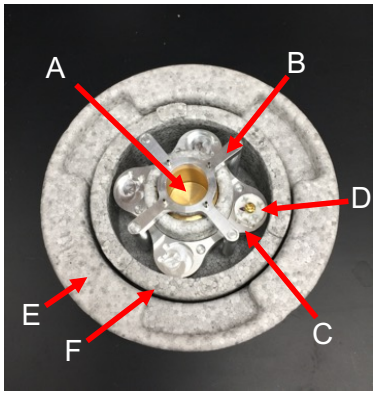
- ☐ Make sure the empty 4L LN2 Dewar is dry.
- ☐ Fill liquid nitrogen in the 4L LN2 Dewar.

Step 5: Glow discharge your grids (sample application side facing up).

Step 6: Cooling ethane holder and condensing ethane.
Caution! Wear PPE for eye protection! This is dangerous and you’ll need staff support unless you have already been certified for independent operation. (See the Ethane tank operation sheet on the wall next to the tank)

- ☐ Assemble the ethane holder.
- ☐ Cool the ethane holder and cup with liquid nitrogen.
- ☐ Condense ethane.
- ☐ Wait until a very thin layer of solid ethane coats the inner walls of the ethane cup to ensure that the liquid ethane is cool enough for proper vitrification.
- ☐ Remove the spider before starting the freezing cycle.

Assembled ethane holder



A, Ethane cup; B, Spider; C, Grid box holder; D, Grid Box; E, Liquid nitrogen container; F, Anti-contamination ring

Step 7: Freeze your grids.

- ☐ Insert the blotting paper. Place the blotting paper with the protruding (slightly curved) side facing the blotting pads. (For consistency, use filter paper of the same brand/type).
- ☐ Fill two small Dewars with liquid nitrogen.
- ☐ Pick up a grid using the Vitrobot tweezers.

- ☐ Gently tap tweezers to make sure the grid is stable and does not fall off the tweezers.
- ☐ Attach and center the Vitrobot tweezers on the plunge rod.
- ☐ Check the grid sample application side (e.g., carbon foil) is facing the side that you want to apply your sample from.
- ☐ Check the liquid nitrogen level before you put the ethane holder on the ethanelift.
- ☐ Apply 3-4 µl of sample using a pipet to the sample application side of the grid.
- ☐ Plunge freezing grids.

Step 8: Remove the Vitrobot tweezers and place your grid into the gird box. Ensure that both the grid and grid box remain submerged in the cryogenic agent throughout.

- ☐ Quickly check if the lid opening is on top of the slot that you want to put your grid in. if not, use the pre-chilled personal tweezers to adjust the lid positioning.
- ☐ Remove the Vitrobot tweezers from the plunge rod.
- ☐ Move the ethane holder off the ethanelift.
- ☐ Lift the black tweezer slide clip up to the open position while keeping the tweezers closed to prevent the grid from falling off while it is still submerged in liquid ethane.
- ☐ Swiftly transfer the grid to the outer liquid nitrogen pool with the tweezers and place the grid into the corresponding slot of the submerged grid box.

Step 9: Align the grid box opening to the next empty slot. **IMPORTANT!** Do not use the Vitrobot tweezers. User pre-chilled personal tweezers to do this step.

Step 10: Dry the Vitrobot tweezers. Get the Vitrobot tweezers ready for the next round of grid freezing cycle. You can dry the tweezers with hair dryer or wipe it gently with Kimwipes in 70% ethanol. **IMPORTANT!** Do not dry Vitrobot tweezers on the heat block. It will damage the tip.

Step 11: Completion of one grid box. After filling one grid box, close it by tightening the screw on the lid with pre-chilled screwdriver. Quickly transfer it to a small Dewar filled with liquid nitrogen. Proceed to fill the next grid box if necessary.

Step 12: End of session checklist:

- ☐ Close your grid box and transfer it to a small Dewar filled with liquid nitrogen.
- ☐ Place ethane holder under snorkel to warm/dry.
- ☐ Remove, clean and store the Vitrobot tweezers.
- ☐ Exit the Vitrobot program on the screen. Read and follow messages on the screen. Please make sure the tweezers are removed before you click “Exit”. Wait until the screen goes dark before proceeding to the next steps.
- ☐ Switch off Vitrobot (switch on the back right-hand side).
- ☐ Remove the blotting paper. Leave the white plastic rings inside of the chamber.
- ☐ Take down and empty the humidifier.
- ☐ Turn off the heat block and return all dry tools to their designated containers.
- ☐ Leave the chamber door ajar to allow excess humidity to evaporate.
- ☐ Store your frozen grid box(es). (ask center staff for assistance)
- ☐ Fill out the logbook.
- ☐ Close the main valve of the ethane tank, vent the lines, and close all valves (after venting the lines, both gauges should be zero).
- ☐ Update your time on iLab as needed.

Detailed version

Caution! Safety and PPE:
Wear safety goggles and gloves when handling liquid nitrogen and gaseous and liquid ethane.

IMPORTANT! VITROBOT TWEEZERS ARE DEDICATED FOR PLUNGE FREEZING GRIDS ON VITROBOT ONLY. DO NOT USE IT FOR OTHER GRID HANDLING PURPOSES.

1. PURPOSE OF THIS WORKFLOW:

The detailed version protocol for the TFS Vitrobot Mark IV serves as a comprehensive manual, providing thorough explanations, tips, and cautions for each step involved in freezing Cryo-EM grids. It's designed to help both fresh and experienced users fully understand and master the vitrification method, ensuring optimal results and efficient operation of the Vitrobot Mark IV.

2. REVISION CONTROL

Revised by:	Approved by:	Date	Changes Made
Kai Cai	CEMRC microscopy staff (KT, BS, AK, JY, EW)	6/3/2021	First draft released
Kai Cai	CEMRC microscopy staff (KT, BS, AK, JY, EW)	2/27/2022	Reviewed, updated, re-released
Yumeng Liu	CEMRC microscopy staff (BS, AK, JY, PM, AB, SG, EW)	3/9/2024	Reviewed, updated, re-released

3. REFERENCES

1) Iancu, C.V. et.al., Electron cryotomography sample preparation using Electron cryotomography sample preparation using the Vitrobot, Nature Protocol, 2006;1(6):2813-9. doi:10.1038/nprot.2006.432
2) How-to videos for operating the Vitrobot Mark IV System, ThermoFisher Scientific, Website link: https://www.thermofisher.com/us/en/home/electron-microscopy/products/sample-preparation-equipment-em/vitrobot/instruments/vitrobot-mark-iv.html?cid=msd_ls_xbu_xmkt_acc-vitrobot_285811_na_pso_gaw_wwhv8r&gad_source=1&gclid=CjwKCAiAopuvBhBCEiwAm8jaMXv9fG81ax
3) Cryo EM 101, Cryo-EM Grid Preparation: Plunge Freezing by Blotting, Website link: https://cryoem101.org/chapter-2/#part2
4) Vitrobot Standard Operating Procedure, NIH Cryo-EM Centers Merit Badge Program, Website link: https://www.cryoemcenters.org/merit-badge/tfs-vitrobot-mark-iv/tfs-vitrobot-mark-iv-sop/

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ITEMS, REAGENTS, and MATERIALS

Brought by the users:

- ☐ Sample(s) to be frozen
- ☐ Dilution buffer (filtered water or buffer solution)
- ☐ Ice and ice bucket (samples should be put on ice before freezing)
- ☐ Grids (you can also purchase them from the center)
- ☐ Gold fiducials (for tomography samples; first time or forgot, ask the center)
- ☐ TetraSpeck beads (for CLEM samples; first time or forgot, ask the center)
- ☐ Tweezers

Provided by the center:

- ☐ Liquid Nitrogen (LN2)
- ☐ Ethane
- ☐ Cryo-gird box
- ☐ Tweezers (small, personal styles, longer forceps, Vitrobot tweezers)
- ☐ Pipetman and pipet tips
- ☐ Filter papers for blotting
- ☐ Ethane holder (Ethane cup, Spider, Grid box holder, Liquid nitrogen (LN2) container, Anti-contamination ring)
- ☐ CD cover (to avoid ice contamination)
- ☐ Heat block (for drying tools)
- ☐ Screw drivers and other tools for manipulating cryo-grid boxes
- ☐ Cryo-grid storage boxes and Puck
- ☐ Small LN2 Dewar (2-4x)
- ☐ Large LN2 Dewar (1x, 4L)
- ☐ Mini-centrifuge, vortex mixer, and storage tubes
- ☐ Distilled water (for Vitrobot humidifier reservoir fills)
- ☐ PPE (BSL1): Safety goggles, gloves, facemask and lab coat

Good practice in general:

- ☐ Tweezers/other tools need to be pre-chilled with liquid nitrogen before handling frozen grids.
- ☐ To eliminate ice contamination, all tools, after touching liquid nitrogen, need to be dried on the heat block before the next use.

START THE VITROBOT

Purpose of this section: Get the Vitrobot prepared for freezing grids.

Step 1: Set up humidifier. Angle the humidifier and insert it into the Vitrobot. The humidifier should be installed with the label facing the front and in a locked, steady position.

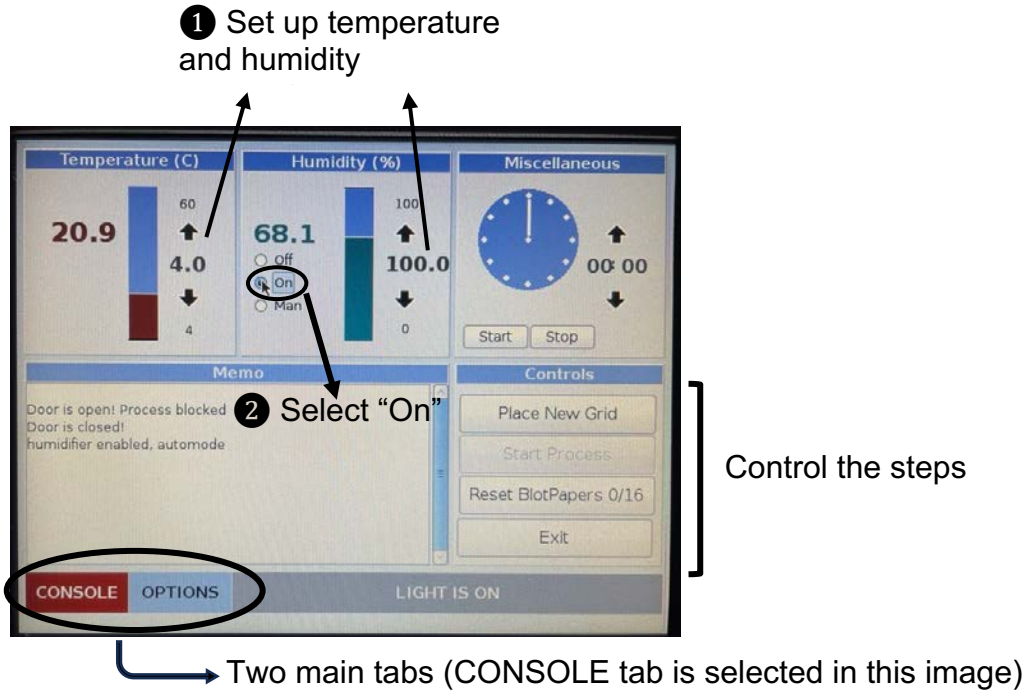
❶ Plug in the bottom of the humidifier. Align the red dots and gently push the plug in.
❷ Insert the top of the humidifier. Alight the screw and the mark on the top of the humidifier, then gently push the humidifier in along the mark and turn counterclockwise until you reach the stop.
❸ Fill the humidifier with 60 ml of distilled water (provided by the center, not regular tap water or de-ionized water) using the provided syringe. Gently push the water through the rubber tube at the bottom of the humidifier. After all the water is dispensed, remove air bubbles by slightly pulling back on syringe (<15 ml).

Step 2: Turn on the Vitrobot. The on/off switch is at the back right side. You should turn on the heat block now so it can reach to the desired temperature (55˚C) for drying the tools during the plunge freezing cycle.

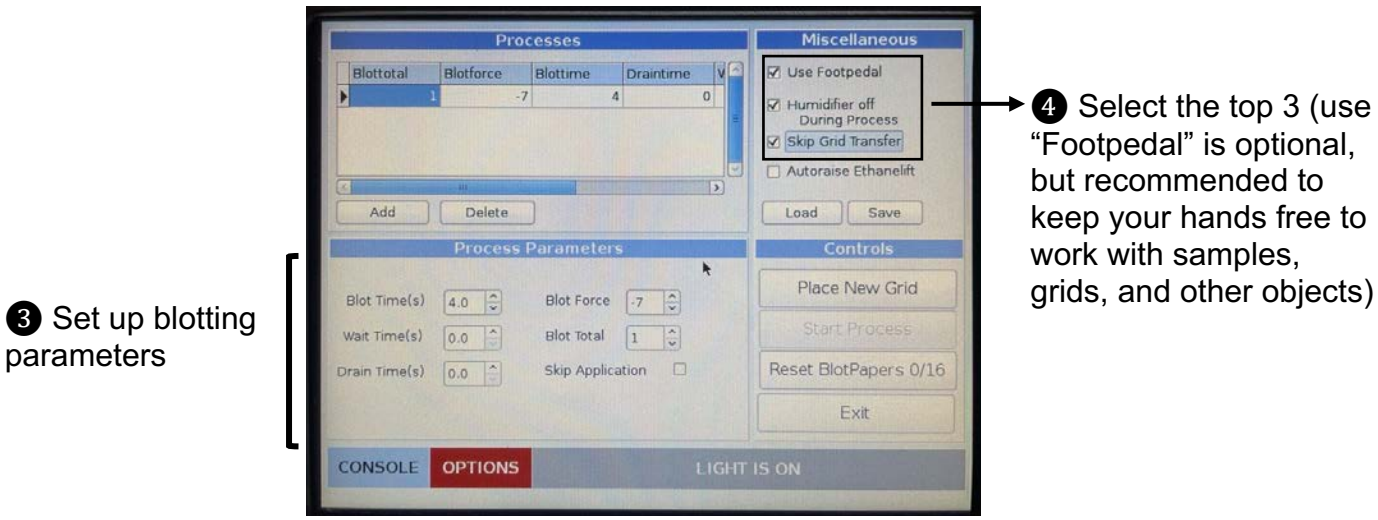
Step 3: Set to desired parameters for vitrification. The humidifier will not start until the temperature gets close to the setting you want. Set the desired temperature first and set humidity to 100% and select “On”. When 100% humidity is reached, you may lower it to ~95% humidity (or less) so that the chamber is not constantly being humidified (misted).

There are two main tabs shown at the bottom left of the screen: CONSOLE and OPTIONS.

Under the CONSOLE tab:



Under the OPTIONS tab:



Make sure that on the Miscellaneous window (under the OPTIONS tab), select the top three items: "Use Footpedal", "Humidifier off During Process", and "Skip Grid Transfer". Do not select the "Autoraise Ethanelift".

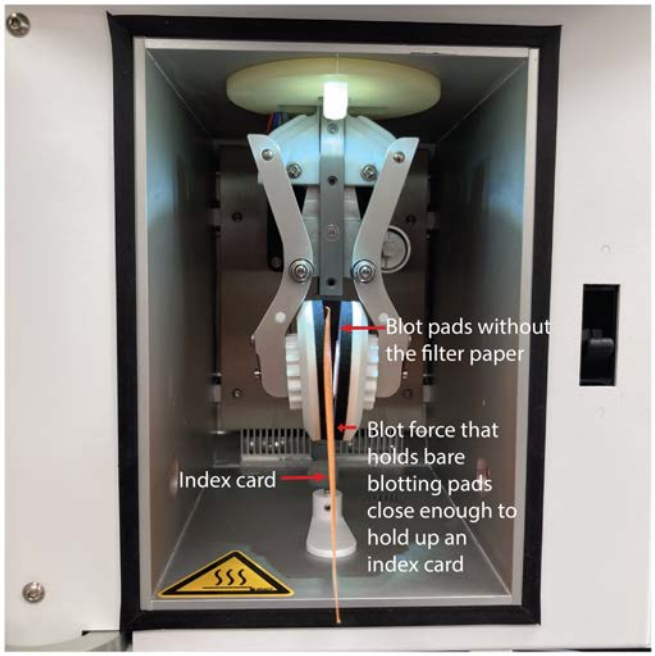
Note: Make sure to change the blotting parameter before you start a new run. Any changing of parameters in the middle of the freezing cycle might not be saved and applied.

Step 4: Activate foot pedal (optional). Using the foot pedal will allow you to proceed to the next step of the freezing cycle while both hands are occupied.

Step 5: Do a few practice runs without placing the Vitrobot tweezers. Perform at least one dry run through the cycle to verify the process, ensuring that everything is functioning correctly and that you remember all the steps during the freezing cycle.

Step 6: Determine normalized blot force zero point. This is the minimum blot force that positions the bare blotting pads close enough to hold an index card. This should be done without the filter paper on the blotting pads. Every user should record their determined zero blot force number in the Vitrobot logbook. The blot force number that was used by the previous user is a good start for your testing. Adjusting the blot force setting to a more negative value will decrease the force. For consistency, blot force should be normalized for every session. The blot force for every Vitrobot is also different (what you determine for the CEMRC Vitrobot will not be the same for a Vitrobot located in another laboratory).

Determine the normalized blot force zero point



❶ Set blot force to the normalized zero blot force determined by the previous user (you can find it on the logbook)
❷ Set the blot number to 2, blot time 5s. (5s allows you enough time to pause the blotting, as described in the next step)
❸ Start one dry run. Pause the cycle when the blotting pads touch each other by opening the chamber door.
❹ Insert the index card between the blotting pads to check if the card is held up.
❺ If the card is held up, repeat steps 1-4 with a 1 blot force decrease.

⇒ If the card is not held up, then the blot force set in the previous round is the determined zero blot force.

⇒ If the card is still held up, continue decreasing the blot force until you find the force that doesn't hold up the card. The blot force one level higher than this is the determined zero blot force.

Note: The blotting pads will not stabilize on their blotting points until they've been cycled a few times. Always do at least 1 practice run without tweezers after setting up parameters.

PREPARE LIQUID NITROGEN

Purpose of this section: Fill the 4L large LN2 Dewar

Step 1: Make sure the empty 4L LN2 Dewar is dry (use the one that is resting upside down by the wall for drying). Ensure there is no ice frost or water present at the opening and bottom of the Dewar.

Step 2: Fill liquid nitrogen in the 4L LN2 Dewar. The LN2 Dewar should be standing in the plastic container to avoid the LN2 spilling on the floor. The funnel should be tilted slightly to allow nitrogen vapor to be released during the filling process.

Note: Always fill liquid nitrogen in the Dewar slowly to reduce splashing cryogenic hazard and waste. **Caution! Wear safety goggles and cryogenic gloves when handling liquid nitrogen.**

GLOW DISCHARGE THE GRIDS

Purpose of this section: Improving grid hydrophilicity (wetting) to improve sample dispersion during sample application.

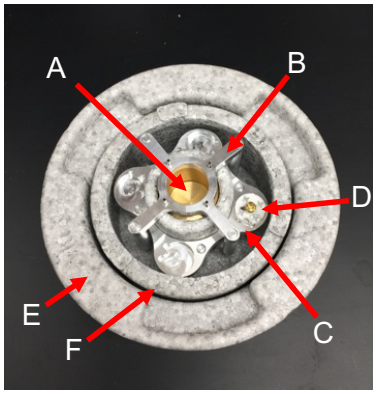
Follow the separate protocol for this step, depending on the instrument (GloQube, easiGlow, or the others) you use.

Note: Glow discharged grids undergo changes in surface hydrophilicity over time following the glow-discharging step. Typically, they are good for use for approximately 30-60 mins after glow discharging. It's important to note that the time elapsed between the glow-discharging step and sample application may introduce variability, affecting the consistency of frozen cryo grids.

COOL THE ETHANE HOLDER

Purpose of this section: Get the ethane holder and cup ready for condensing ethane.

Assembled ethane holder



A, Ethane cup; B, Spider; C, Grid box holder; D, Grid box; E, Liquid nitrogen container; F, Anti-contamination ring

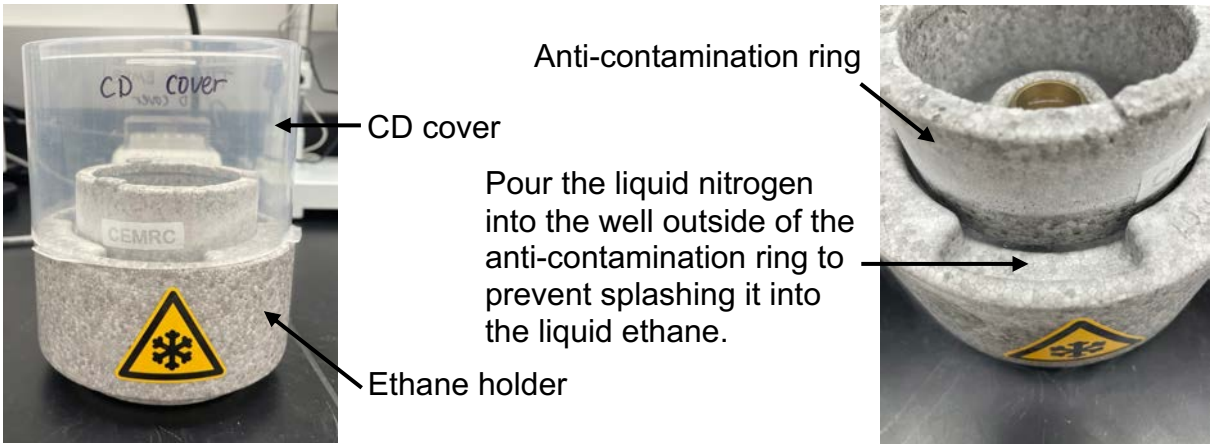
Caution! This step should be done in a well-ventilated area. Large amount of nitrogen vapor and some ethane gas will be released. Liquid nitrogen, liquid ethane, and cryogen-cooled metallic tools (such as forceps or tweezers) are extremely cold: be sure to wear appropriate personal protective equipment (gloves, goggles, lab coat, and closed-toe shoes) and be careful when handling these reagents and tools.

Step 1: Assemble the ethane holder. Place the ethane cup, metal frame (spider), metal grid box holder and the grid boxes in the holder (see image to the right). Alternatively, grid boxes can be placed in outer pool of liquid nitrogen (or liquid nitrogen filled small Dewar) to cool more effectively prior to being placed in the designated wells.

Grid box notes: Loosen the top screw with the screwdriver, slide the top opening to the grid box slot you want to put grid into. You want to use a grid box that is labeled on top and bottom and has no cracks and the plastic top slides easy. **IMPORTANT!** Only use non-Vitrobot tweezers to slide top of grid box to new slot, NOT the Vitrobot tweezers! It can warp or break the Vitrobot tweezers over time. Check grid box for extra (old) grids before cooling with liquid nitrogen. Dispose the old grids in the 10% bleach bottle on the bench.

Step 2: Start cooling the ethane holder and cup. First, fill both the central ethane cup and outer well of the container with liquid nitrogen until they are both completely full. This will allow both parts cool quickly. The subsequent pours of liquid nitrogen should be directly into the liquid nitrogen container (outer well) and not the ethane cup. Make sure there is no liquid nitrogen in the ethane cup before you start condensing ethane.

Note: To refill the liquid nitrogen levels while doing the vitrification, pour liquid nitrogen into the wells on the very outer edge of the cup (outside of the anti-contamination ring), so you do not accidently get any liquid nitrogen into the ethane. This will contaminate the ethane. Or you can add carefully from the inside of the anti-contamination ring without getting LN2 into the ethane. Place CD cover over the cup when not actively working to keep frost out (eliminate ice contamination).

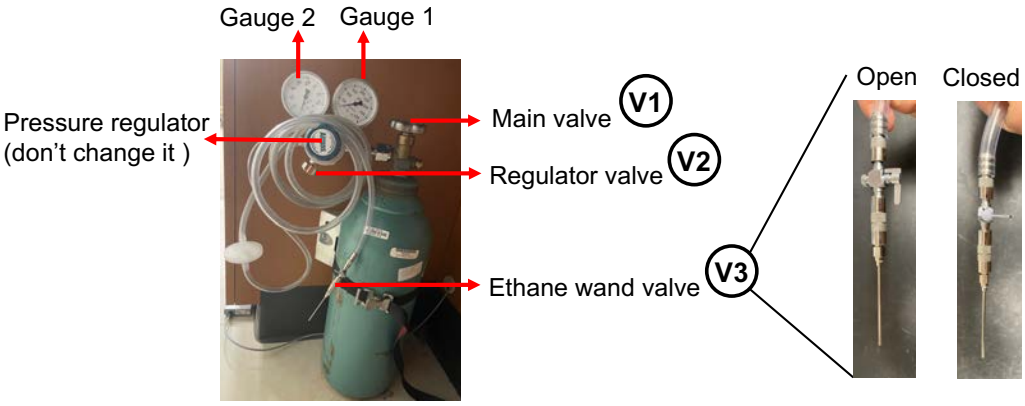


Step 3: Keep replenishing the liquid nitrogen in the container so that the grid box is kept completely submerged.

Note: Be careful with the floating anti-contamination ring. Its purpose is to keep the cooled liquid nitrogen vapor above the grids and grid boxes to avoid ice contamination. If the anti-contamination ring is broken, contact a facility member.

Step 4: Condense ethane. After all the liquid nitrogen has evaporated from the ethane cup, after vigorous liquid nitrogen bubbling has ceased (especially around the spider), wait for at least an additional 5 minutes. At this time, one is able to begin condensing the ethane gas to the liquid-phase in the central ethane cup.

Operate the ethane tank: To do this, open the ethane tank slightly to produce a modest flow rate. To minimize any ethane contamination in the LN2 container, turn on the ethane gas flow (by controlling the ethane wand valve V3) when moving the tip to the bottom of the ethane cup. The tip should be directed toward the bottom or side of the cold cup wall. (Please see **Appendix II** for detailed information on using the ethane tank) **Caution! Wear PPE for eye protection!**

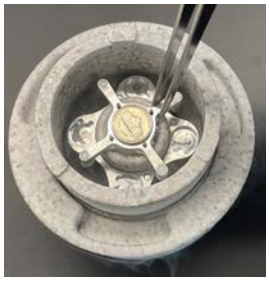


Note: If the flow rate is too low, the ethane will solidify in the tip of the ethane wand. If the flow rate is too high, it will not liquefy and will blow out of the ethane cup. You can test the flow rate by feeling it on your skin (e.g., inner wrist). Holding the tip of the ethane wand against the inner wall of the ethane cup facilitates condensation. You can test the flow rate by listening to the sound of the ethane condensing. The proper flow rate should be like a gentle hissing sound. Move the ethane wand slowly in circles while condensing.

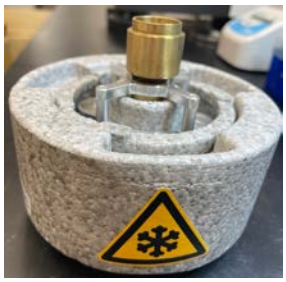
Step 5: Stop condensing ethane. Continue filling the ethane cup until it is full, typically right below the rim of the spider. If you fill the ethane cup too full, frozen ethane tendrils will be formed and contaminate the liquid nitrogen.

Step 6: Remove metal frame (spider). Wait until a very thin layer of solid ethane coats the inner walls of the ethane cup to ensure that the liquid ethane is cool enough. Remove the metal frame (spider) to prevent the ethane from rapidly solidifying. You will need to use 2 pairs of tweezers to hold the ethane cup in place while removing the spider. Place spider under snorkel to dry, if the spider is frozen to the ethane cup (e.g., stuck) you can warm it with an extra room temperature ethane cup because it is the right size. Alternatively, place a nickel on the top of the spider (right size) to warm the edge and remove the spider with the nickel on top. Please ask a center staff for help if you are struggling with this step.

Thin layer of solid ethane and the ethane level is right below the spider.



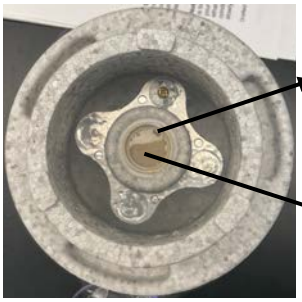
Place a nickel on top of the spider to warm its edges and remove the spider with the nickel on top.



Place a new ethane cup on top of the spider to warm the edge. Remove the cup first before removing the spider.

Step 7: Generate ethane slurry that is perfect for vitrification. If the liquid ethane looks clear, this means it is too warm for proper vitrification. You can cool the ethane by placing the spider (remember to pre-chill the spider in liquid nitrogen before use) back to the ethane cup. The ethane suitable for vitrification should appear as a milky slurry.

Note: If too much of the ethane becomes solid inside of the ethane cup, you can gently put room temperature forceps in contact with the ethane cup to melt the solidified ethane. A thin layer of solid ethane along the wall of the ethane cup is ideal. **IMPORTANT!** A thick solid ethane layer may come in contact with and bend grids or damage the Vitrobot tweezers. Keep an eye on this.



Ethane slurry, perfect for vitrification

Step 8: When necessary, refill the ethane cup to the brim. The liquid ethane level decreases during the vitrification process. You can condense more liquid ethane and refill the cup if it is needed. To avoid ice contamination, make sure that the tip of the ethane supply tube is dried completely each time you introduce it into the ethane cup. If not, wipe it dry with Kimwipes. **Caution! Wear PPE for eye protection!** First, place the pre-chilled spider (make sure the spider is dry) back on top of the ethane cup. Wait until a thin layer of solid ethane is formed along the inner wall of the ethane cup. Second, open the ethane tank and ensure the flow rate is low to prevent splashing of the liquid ethane when the tip of the ethane wand is moved close to the surface. Lastly, place the tip of the ethane wand slightly below the surface of the liquid ethane, to start condensing more

ethane. Shut off the ethane tank after you are done. (Please see **Appendix II** for detailed information on using the ethane tank)

Note: If ethane is completely solidified or the ethane level has dropped significantly you may want to consider condensing ethane in a new holder and cup instead of attempting to revitalize the current cup. Ice contamination builds up during the vitrification process. Typically, you can freeze at least 8 grids in one ethane cup during standard run. Re-condense ethane in a dry new cup if you are going to freeze more grids.

USE THE VITROBOT

Purpose of this section: Freezing cycle/plunge freezing

IMPORTANT! VITROBOT TWEEZERS ARE DEDICATED FOR PLUNGE FREEZING GRIDS ON THE VITROBOT ONLY. DO NOT USE IT FOR OTHER GRID HANDLING PURPOSES.

Step 1: Place new blotting paper. Place the fresh blotting paper onto the white plastic rings. For consistency, use filter paper of the same brand/type for all your experiments. If that type is not present by the Vitrobot, contact a staff member for help.

Note: Notice (feel) the difference between the two sides of the paper depending on the cut direction when they were manufactured. The side with the protruding (curved) edge should face the blotting pad (in other words, insert the plastic ring along the protruding direction of the filter paper). If the blotting papers start to warp due to the humidity in the chamber, they should be replaced (approximately every 8-12 grids).

Step 2: Fill two small Dewars with liquid nitrogen. Make sure the Dewars are dry. One Dewar is for pre-chilling tools. The second Dewar will be for storing your grid boxes containing frozen grids temporarily. Later, these grids can be transferred to the long-term storage Dewar at the end of the freezing session.

Step 3: Gently pick up a glow-discharged grid with Vitrobot tweezers. Pick up the grid using the Vitrobot tweezers with the screw-side facing up (the foil side of your grid should also be facing up). Close the tweezers by moving the black tweezer slide clip up to the first ridged notch and until the tweezer is closed. You should use the tweezers to hold the very outer edge of the grid (not in the mesh-filled center). Typically, on an EM grid there is a thicker outer ring (edge) and this is the region to secure the tweezer to, however, you do want to make sure the grid is securely clamped and does not fall off (see the next step). If you are unsure of grid placement, use the stereomicroscope to check positioning and/or ask a staff member.

Step 4: Gently tap tweezers to make sure the grid is stable and does not fall off the tweezers

Step 5: Place the Vitrobot tweezers on the plunge rod. Be sure to place the Vitrobot tweezers so the screw-side (e.g., at top of the tweezer block that 'clicks' onto the plunge rod) is always facing right. If you are applying your sample from either the right or left side, the screw-side should always face to the right. **IMPORTANT!** Make sure the tweezers are placed on the center of the rod and you feel the slight click. If the Vitrobot tweezer is off center on the plunge rod, the side of the tweezers

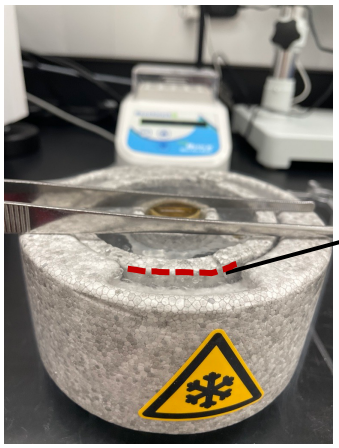
may come in contact with the bottom of the Vitrobot, will cause a loud noise when raised, and jam the machine's mechanism. If this happens, contact a staff member for help.

Note: For normal processes, the foil (carbon, gold, other) side of the grid should face the side to which the sample will be applied. There are two times during the cycle that you can attach the tweezers, when the plunge rod is fully extended (after plunging, not preferred cycle time), or the step after when it is slightly retracted (after clicking "Place new grid", preferred cycle time).

Step 6: Retract tweezers into the humidified chamber. After the tweezers are attached to the plunge rod, if you are at the "Place new grid" step, press the foot pedal (or click "Continue" on the screen), to retract the tweezers into the humidified chamber.

Step 7: Place ethane holder to the center of the stage. Check the level of liquid nitrogen in the outer pool of the ethane holder, to make sure the grid box is submerged.

Optimal liquid nitrogen level for one plunge freezing cycle: It is important to check the liquid nitrogen level before you put the ethane holder on the Vitrobot. To check liquid nitrogen levels, push down the anti-contamination ring slowly using the large forceps. The liquid nitrogen should be touching the top edges of the liquid nitrogen container, but not over-flowing. You will want the level to be high, because the liquid nitrogen will be evaporating during the freezing process. If it is too high, when the Vitrobot raises the ethane holder, liquid nitrogen can overflow into the ethane or freeze the top of the ethane cup to the Vitrobot.



Liquid nitrogen levels the top of the well

Step 8: Raise the ethanelift. Press the foot pedal (or click "Place ethane container" on the screen).

Note: **Caution!** If you forget to put the ethane holder on, DO NOT place the holder on the ethanelift as the ethanelift is being raised. The speed of the ethanelift raising seems slow at the beginning but speeds up during the cycle. Just wait and do a dry run.

Step 9: Apply your sample with a pipetman from the opening on the side of the chamber. Continue the process with the foot pedal (or click "Process" on the screen). The plunge rod will lower the tweezers to the sample application position. There is a red door that slides so that you can see an opening. Use 3-4 µl of sample. Try to release the drop so it is sitting on the edge of the tip and then transfer the drop to grid without touching the grid. Close the red dorr after sample is added to minimize changes to the humidity and temperature within the Vitrobot chamber.

Note: You can apply the sample multiple times using the multi-sample application set up on Vitrobot. (See **Appendix I** for more detailed information)

Step 10: Blotting and plunge freezing. Continue the process with the foot pedal (or click "Continue" on the screen).

Note: After plunging, the ethanelift lowers with the grid submerged in the ethane. Keep filling up outer pool with the liquid nitrogen. **IMPORTANT!** Please be careful with pouring the liquid nitrogen, try to eliminate the splashing during the process. Always keep grid boxes under the liquid nitrogen level!

Step 11: Verify the position of the grid box lids opening. Quickly check if the lid opening is on top of the slot that you want to put your grid in. If it is not at the correct spot, use a set of pre-chilled personal tweezers to adjust the lid position.

Step 12: Carefully detach the Vitrobot tweezers from plunge rod while keeping the grid submerged in liquid ethane. Detach tweezers from the plunge rod very carefully, do not touch the sides of the ethane cup with the grid, or let the grid come out of the ethane.

Step 13: Carefully move the ethane holder down while keeping the grid submerged in liquid ethane. Use both hands to move the ethanelift if it is needed.

Step 14: Quickly transfer the grid to the liquid nitrogen and then into the submerged grid box.

Note: You should lift the black tweezer slide clip up to the open position while keeping the tweezers closed (otherwise, the grid will fall off), while the tweezers and grid are still submerged in the ethane cup. Then, quickly transfer the grid to the outer liquid nitrogen pool using the tweezers and place the grid into the appropriate slot of the submerged grid box.

Step 15: Align the grid box opening to the next empty slot. **IMPORTANT!** Do not use the Vitrobot tweezers. User pre-chilled personal tweezers to do this step.

Step 16: Dry the Vitrobot tweezers. Get the Vitrobot tweezers ready for the next round of the grid freezing cycle. You can dry the tweezers with a hair dryer or wipe them gently with Kimwipes in 70% ethanol. Click “Place New Grid” on the screen (or use the Footpedal) to start the next freezing cycle.

Step 17: Transfer the filled grid box into the small liquid nitrogen Dewar. After filling a grid box, align the lid opening with the notch and lock the lid in place by tightening the screw with screwdriver. Ensure that all the tools are pre-chilled in liquid nitrogen before use. Then quickly transfer the grid box into the small liquid nitrogen Dewar.

SHUT DOWN THE VITROBOT

Purpose of this section: Use this as a check list to clean up the bench and the used instruments. The section is very important to maintain the instrument in good condition for all the users in the center.

Step 1: Close your grid box and transfer it to a small Dewar filled with liquid nitrogen.

Step 2: Place the ethane holder under the snorkel. Allow the nitrogen and ethane evaporate.

Step 3: Put back the Vitrobot tweezers. Remove the Vitrobot tweezers, clean them with 70% ethanol, put back the tip protector, and put the tweezers back to their designated containers.

Step 4: Tap the "Exit" button on the Vitrobot screen.

Note: The Vitrobot can only exit the program when the chamber door is closed. When you click the "Exit" button, there will be a message on the screen asking you if you remove the tweezers. Please make sure the tweezers are removed and you can click "yes" to continue exiting the program. Wait until the screen goes dark before you proceed to the next steps.

Step 5: Turn off the Vitrobot. (main switch in the back, right-hand side)

Step 6: Remove the blotting papers. Clean the white plastic rings with ethanol. Place rings back to the Vitrobot chamber.

Step 7: Carefully remove the humidifier.

Note: To unplug the humidifier, press the little ring at the base of the plug and detach the chamber from the bottom first. Then, rotate the chamber clockwise to detach it from the top. You can the dump the excess water out in a beaker or cup. You will need to invert and dump humidifier at least 5 times to clear the reservoir. Place the humidifier on its side on paper towel next to the Vitrobot to dry.

Step 8: Turn off the heat block and return all dry tools to their designated containers.

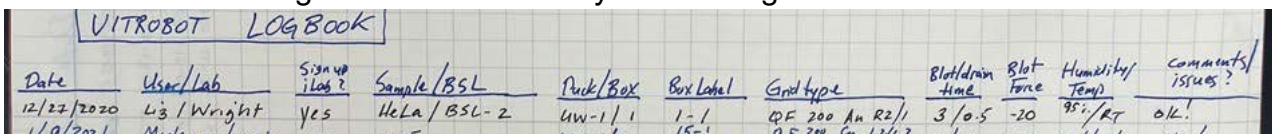
Step 9: Leave the chamber door ajar to allow excess humidity to evaporate (after instrument is fully shutdown).

Step 10: Store your frozen grids boxes in the appropriate storage Dewar.

Please request the center staff to store your grids. If you are permitted to store your grids in the center storage Dewar yourself, ensure to check the liquid nitrogen level in the Dewar before placing your grids inside. If the level is low, please notify the center staff to refill the Dewar. The liquid nitrogen level should be just below the edge of the chamber where the lid is secured.

Step 11: Other items to complete after your session:

1) Fill out the Vitrobot logbook with details of your freezing run.


VITROBOT LOGBOOK

Date	User/Lab	Sign up + Log?	Sample/BSL	Puck/Box	Box Label	Grid type	Blot/drain time	Blot Force	Humidity/ Temp	comments/ issues?
12/27/2020	Li / Wright	yes	HeLa / BSL-2	UW-1 / 1	1-1	QF 200 Au R2/1	3 / 0.5	-20	95% / RT	OK!

2) Close the main valve on the ethane tank, fully open the regulator valve to vent the residual ethane in the line (you will hear a clear hissing sound, and the pressure on both gauges should turn to 0). Make sure to close all valves again after you are done.

More tips on optimal freezing conditions:

1) Longer blot time means thinner ice (See **Appendix III** for more detailed information on freezing conditions).
2) For consistent results, maintain the same interval between glow discharge and freezing of your grids.
3) A different set of Vitrobot tweezers may change the ice thickness, gradients across grids, etc. Different settings may be required when using different pairs of tweezers.
4) Do not use the Vitrobot tweezers to slide grid box lids to a new slot as this will slowly bend the tweezers over time. Use your own personal tweezers to slide grid box lids to new slot.

Appendix

I: Multi-sample application set up.

You can apply samples to one grid more than once with either the same or different blotting conditions. This approach provides flexibility in experimental design and allows for multiple iterations or variations in sample application.

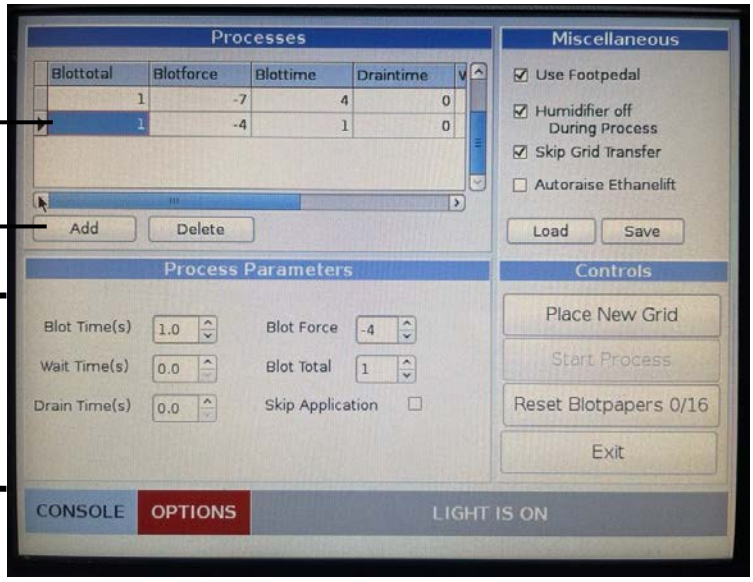
To achieve this, you can simply add another set of blotting parameters

Go to the OPTIONS tab:

This is the second blotting condition you added.

❶ Click “add”. There will be a new set of blotting parameters displayed underneath.

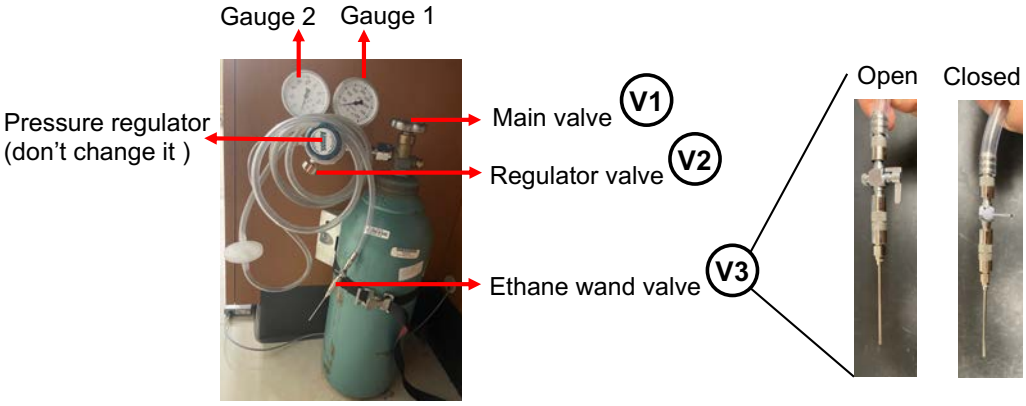
❷ Set up the parameters for the set of blotting conditions that was selected (the second set of blotting was selected and shown in this image).



In the above example, the setup is for two sample applications per freezing cycle:
1). After the first sample application, click “Continue” in the Controls panel. The grid will be blotted with the following conditions: blot time 4s; wait time 0s; drain time 0s; blot force -7; blot total 1.
2). Then the tweezers will return to the sample application position, allowing you to apply sample again.
3). After the second sample application, click “Continue” in the Controls panel. The grid will be blotted with the following conditions: blot time 1s; wait time 0s; drain time 0s; blot force -4; blot total 1.

II: Ethane tank operation.

Ethane tank operation sheet



Before you turn on the ethane tank

1) Gauge 1 and 2 should both be zero.
2) All the valves should be closed.

Please notify the center staff right away if you see the gauges are not zero, or valves are open.

Turn on the ethane tank

1) Open the Main valve (V1) , Gauge 1 should read approximately 500 psi.
2) Open the Regulator valve (V2) , turn the knob until Gauge 2 read approximately 10 psi
3) Open the Ethane wand valve (V3) , test flow rate by feeling on your skin (e.g., inner wrist) . Turn off once you feel the air flow on your skin.
4) Hold the tip of the ethane wand against the inner wall of the cold ethane cup and open (V3) to start condensing ethane. Tune (V3) to adjust the flowrate to a proper speed.

Turn off the ethane tank

1) Close (V3) .
2) Close (V2) .
3) Close (V1) .

After (V1) is closed, there is still residual ethane gas trapped in the lines, both gauge 1 and 2 still detect the pressure and read the values. It is important to vent the lines after use.

Vent the lines (make sure (V1) is closed)

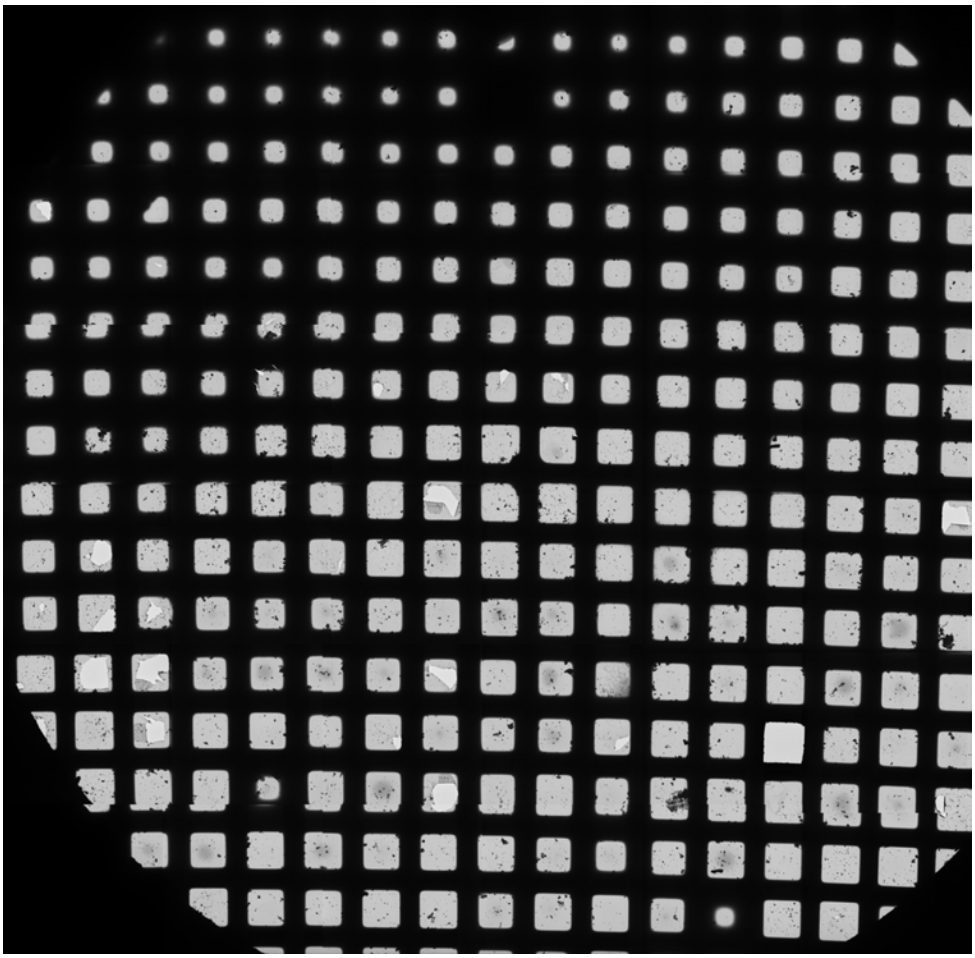
Open both (V2) and (V3) , you will hear a venting sound. !Important! After the venting sound stops and both the gauges display zero, **CLOSE ALL THE VALVES**.

III Examples of Vitrobot settings have been used.

For single particle:

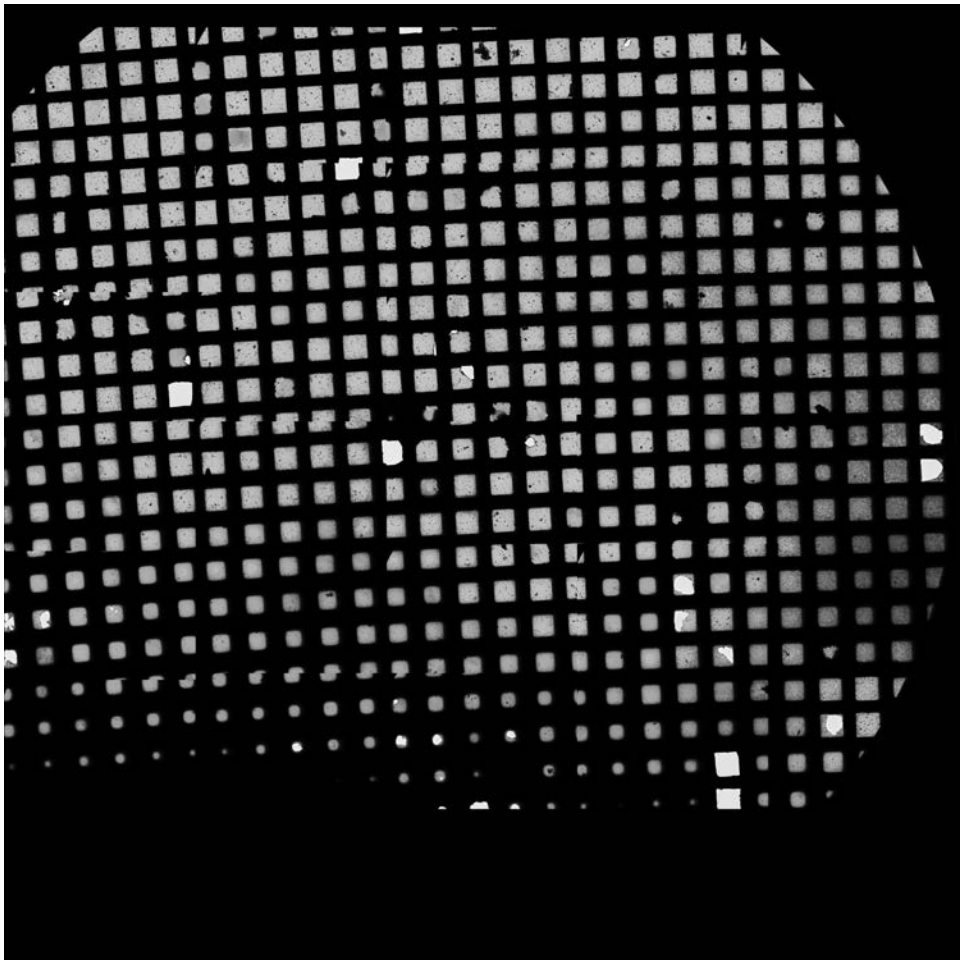
Blotting time: 1s, 2s, 3s, 4s, 5s
Blot force: -8 (calibrated zero blot force)
Wait time: 0s, 45s
Drain time: 0.5s
Grids type: QF Cu200 R1.2/1.3, QF Au200 R1.2/1.3, QF Au300 R1.2/1.3, GO QF Au300 R1.2/1.3, QF Cu300 R2/2, AuF Au300 R1.2/1.3, AuF Au200 R1.2/1.3, C-Flat Cu300 R2/1.

Representative Atlas image for single particle 1:



Freezing condition:
Grid: Quantifoil Cu200 R1.2/1.3
Sample: Apoferritin 5.5 mg/mL
Glow discharge: 60s at 10mA
Blot time: 3s
Blot force: -8 (calibrated zero blot force)
Wait time: 0s
Drain time: 0.5s
Temperature: 4℃

Representative Atlas image for single particle 2:

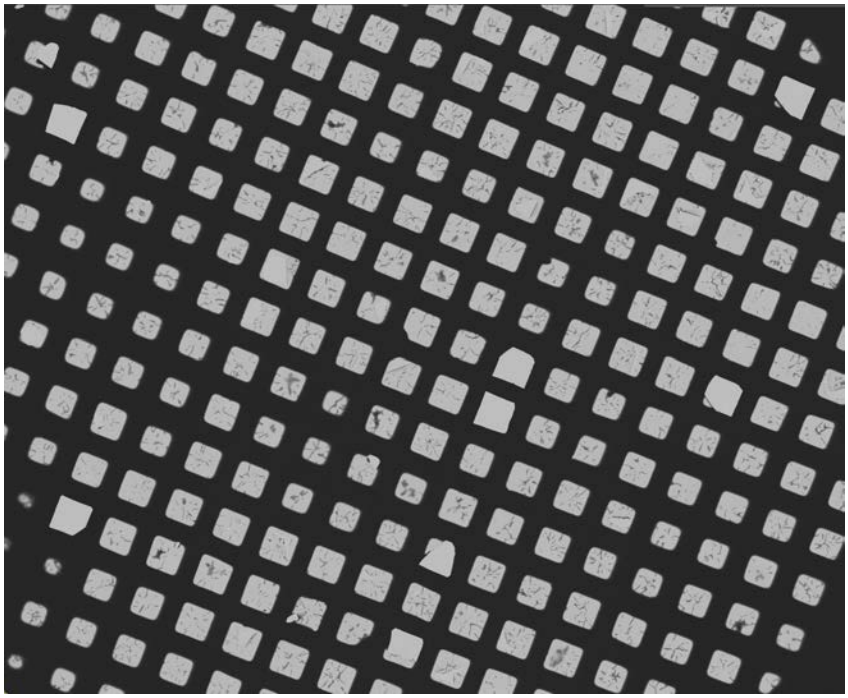


Freezing condition:
Grid: Quantifoil Cu300 C-Flat R2/1
Sample: Apoferritin 5.5 mg/mL
Glow discharge :20s at 20mA
Blot time: 3s
Blot force: -8 (calibrated zero blot force)
Wait time: 0s
Drain time: 0.5s
Temperature: 4℃

For Bacteria:

Blotting time: 2s
Blot offset: -8 (calibrated zero blot force)
Wait time: 60s
Drain time: 0.5s
Grids type: Cu200 R2/1, Cu200 R3.5/1

Representative Atlas image for bacteria:



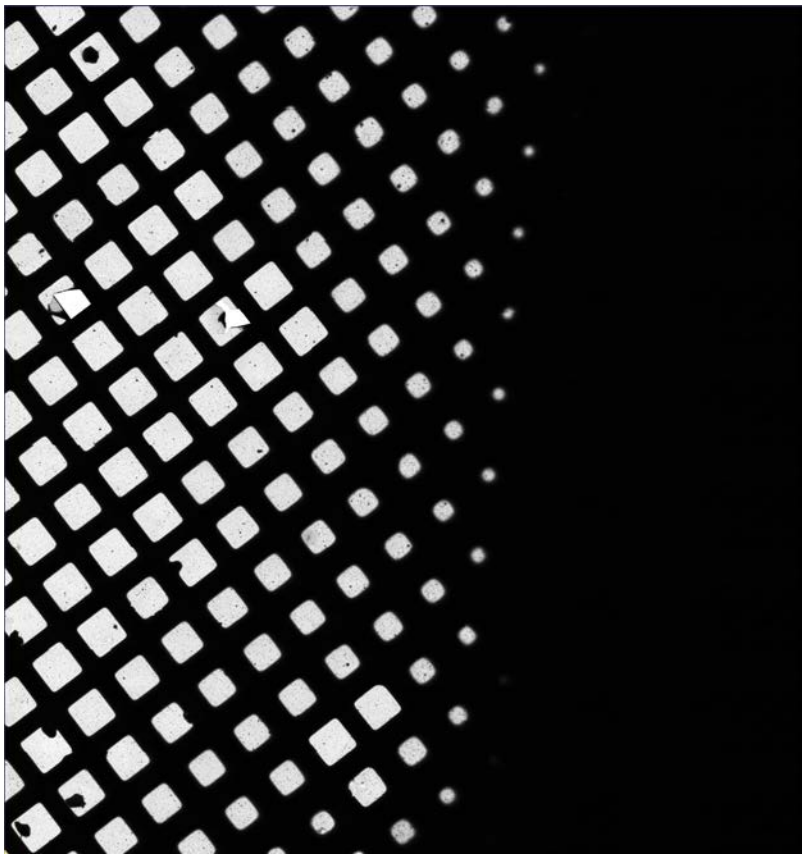
Freezing condition:
Grid: Quantifoil Cu200 R2/1
Sample: *Rhodobacter sphaeroides*
Glow discharge: 30s high Harrick Glow discharger
Blot time: 2s
Blot force: -15 (-8 is the calibrated zero blot force)
Wait time: 60s
Drain time: 0.5s
Temperature: 25 ℃

Additional information: There were 5 μL of sample and 4μL of 10 nm gold fiducials on the grid during blotting.

For Virus:

Blotting time: 2s, 4s
Blot offset: -8 (calibrated zero blot force) or lower
Wait time: 45s
Drain time: 0.5s
Grids type: Quantifoil Cu200 R2/1, Quantifoil Cu300 R1.2/1.3+2nm_Carbon

Representative Atlas image for virus:



Freezing condition:
Grid: Quantifoil Cu200 R1.2/1.3
Sample: Rhinovirus
Glow discharge: 60s at 10mA
Blot time: 4s
Blot force: -15 (-8 is the calibrated zero blot force)
Wait time: 45s
Drain time: 0.5s
Temperature: 22 ℃