Using the Vitrobot

The Vitrobot Mk 3 (aka Mark III, Mark 3, *etc.*) can control both the local temperature and the local humidity during the process of plunge-freezing a grid for cryoEM. It does require a significant amount of time to equilibrate after the temperature and/or humidity have been set, so please remember to allow time for this to happen. The following pages list the steps involved in using this device for a routine grid freezing session. First time users should watch an experienced staff member freeze grids a few times before attempting this alone. Also, if you are trying to do anything even slightly exotic, please talk to the staff of the EMC before starting.

Setting up the Vitrobot

1) Attach the metal humidity reservoir to the main device. This metal tube has a bayonet-style mount which is designed to work when the large yellow triangle (or the small black dot) on the side of the cylinder is facing the front: With the cylinder in that position, insert it into the mount and lock it into place by rotating counter-clockwise as one would look down onto the top of the cylinder. There is also a cord that must be attached to the bottom of the metal cylinder. Connect this cord before or after attaching the cylinder to the main device, depending upon which you find easier.
2) Fill the cylinder with 60 ml of fresh, distilled/filtered water using a 60 ml plastic syringe. The syringe connects to a piece of clear plastic tubing that pokes out from the bottom of the cylinder (beside where the cord attaches). After filling the cylinder, pull back on the syringe's plunger to remove any air from the system.
3) Open the main valve on the cylinder of air. The cylinder is currently located behind and to the left of the Vitrobot. Please do not adjust any

of the settings of the gas regulator: merely open (and later close) the main valve.

4) Turn on the Vitrobot. The power switch is on the back (near the bottom and close to the monitor) of the main device. It is a regular toggle switch that you can locate by touch and flip into the on position. The monitor is normally powered on, and in most circumstances, you should simply see the computer and then the Vitrobot program start.
5) Make certain that the blotting paper will suffice for your needs. Each pair of blotting papers can be used 16 times, and we currently try to mark the number of times that a set of blotting papers has been used by leaving a note on the front of the Vitrobot. If there is no filter paper, or if the remaining blots are not sufficient for your freezing session, change the filter paper now (instead of wasting time in the middle of a freezing session). Gloves are recommended for handling the filter papers.
6) Set the temperature and humidity. Most of our freezing is done at 22° C, but we have successfully used the Vitrobot at temperatures as low as 4° C and as high as 55° C. The humidity is normally either set to 100% (which means that when the system is at equilibrium, any evaporation of water from the grid is immediately replaced by condensation from the surrounding air) or not set at all (which means that the humidity within the blotting chamber will be whatever combination of ambient humidity and the Vitrobot's temperature setting allows). Allow about an hour for the temperature and humidity to equilibrate.
7) Set the plunge freezing parameters. Click the "Options" button at the lower left corner of the Vitrobot display to setup the blotting conditions. Specimens will differ in the amount of time needed to blot properly, but values on the order of 3 to 4 s are generally a good starting point. The user can also control what are called "Wait time" (the time between initiating an actual plunge freeze cycle and when the blotting occurs) and "Drain time" (the time after the blotting has happened but before the grid is plunged into the cryogen). Few people here have found a use for Wait time but some of us routinely use a Drain time of 1 to 2 s. In addition, the "Offset" setting makes slight adjustments to the vertical

position of the tweezers during the blotting process. It is often better to fine tune the thickness of an ice layer by changing this offset value instead of trying to adjust the actual blotting time, but this requires that the user be exceedingly consistent in how a grid is grasped by the tweezers.

8) Customize other features of the Vitrobot. For example, you may want to use the foot pedal to control the device or you may want to turn on the LED lights that illuminate the blotting chamber. Such things are all settings on one of the pages of the Vitrobot controls.

Cooling the Cryogen

9) Cool the cryogen. Make certain that the parts (liquid nitrogen reservoir, float, brass cryogen cup, metal storage box holder and metal "spider legs") are clean and dry. Some people put the entire assembly in a Styrofoam box so that the atmosphere around it all is cold, dry nitrogen. This eliminates (or at least minimizes) contamination by water vapor in the atmosphere during the cryogen preparation procedure. Make certain there is good thermal contact between the cryogen cup and the metal spider legs. Fill the reservoir and the brass cryogen cup with liquid nitrogen. Keep refilling the reservoir as the nitrogen boils off, but do not fill the brass cryogen cup more than one or two times. Allow the temperature of the system to stabilize such that the reservoir can sit for 5 or 10 minutes without needing to be refilled. If using ethane as the cryogen, open the main valve on the small ethane cylinder (currently under the table that holds the Vitrobot) and adjust the ethane flow using the needle valve on the gas regulator. Do not adjust any of the other settings of the regulator. Slowly bleed ethane into the cryogen cup by putting the pipette tip end of the ethane line in the corner of the cryogen cup (*i.e.*, where the bottom and the wall come together) and opening or adjusting the needle valve on the ethane cylinder. The

ethane should start to liquefy and the cup should slowly fill. You may need to refill the reservoir with liquid nitrogen during this process. Fill the cryogen cup completely, reduce the flow from the ethane cylinder and rapidly pull the tip out of the liquefied ethane. These steps can be performed without having the float in position, especially if all this is done inside a Styrofoam box. Be sure to close the main ethane valve either at this point or at the end of your freezing session. The ethane in the cryogen cup will remain liquid for ~1 hour under normal operating conditions, though repeated freezing may force the user to replace ethane that is lost during plunge-freezing. Some people like for the ethane to be cold enough to solidify along the sides of the cryogen cup, but care must be taken to prevent the ethane ice from getting too thick and solid, in which case the tweezers can be damaged when they plunge into the (no longer) liquid ethane. Excess ethane ice can be melted by plunging a dry, room temperature object into the ice, or by bubbling in fresh ethane gas.

10) Transfer the cryogen assembly to the Vitrobot. Immediately before starting to use the Vitrobot, carefully remove the spider legs from the assembly, place the float into position if it is not already there and move the reservoir to the Vitrobot. Once the assembly leaves the Styrofoam box, ambient humidity will start to freeze on any available cold surface and so it is important to do this transfer only immediately prior to use of the Vitrobot. You should also cool the “button” for grid storage at this time by placing it into the metal holder in the liquid nitrogen reservoir.

NOTE: You may want to do one or more “dry runs” of the Vitrobot before transferring the cryogen assembly to the device. This ensures that all the parts are working properly and can prevent bad things from happening with a real (and sometimes valuable) specimen.

Plunge-Freezing Grids

11) Pre-treat sufficient grids for freezing before cooling the cryogen. Most users want their grids to be hydrophilic and so the carbon support films are glow-discharged within an hour or so of freezing. Grids can also be lightly plasma cleaned immediately prior to freezing. Other treatments can also be done to the carbon support films. In any event, these steps should be taken before cooling the cryogen in order to minimize contamination/condensation of ambient humidity onto any exposed liquid nitrogen cooled surfaces. Always prepare a few more grids than you intend to freeze, in case something happens to a grid during the freezing process.

12) Attach a grid to the special "Vitrobot tweezers". Grasp as little of the grid as possible with the tweezers (but enough that it will not fall out during freezing). The further the tweezers reach into the interior of the grid, the more likely the possibility that the tweezers will wick away the specimen when it is applied to the grid. Such wicking can be prevented by treating the tweezers with beeswax prior to freezing. As a matter of consistency, always place the same side of the tweezers facing the carbon coated side of the grid (and in the next step, always clamp the tweezers into the Vitrobot in the same orientation). Lock the tweezers shut by moving the black slider to the 1st or 2nd position (where it will not interfere with the blotting pads).

13) Put the Vitrobot into the "Place next grid" state and attach the tweezers to the Vitrobot's metal rod. If you have followed these instructions to this point, the Vitrobot will be in a state where the metal rod extends 5 or 6 cm out the bottom of the blotting chamber. The "Place next grid" state occurs when the rod is retracted so that only a few cm project out the bottom of the chamber. This allows the user to attach the tweezers to the rod without banging the grid into the assembly that holds the cryogen.

14) Raise the tweezers into the blotting chamber. Simply pushing the "Continue" button on the Vitrobot controls will retract the tweezers into

the blotting chamber and seal it from the outside atmosphere. If you are controlling the humidity inside the chamber, wait at this point for the humidity to reach 100% (or whatever your target is).

15) Raise the cryogen. Simply pushing the "Continue" button on the Vitrobot controls will raise the liquid nitrogen reservoir and cryogen cup so that it is contact with the bottom of the blotting chamber. This will start to cool the bottom of the chamber and may cause the humidity in the chamber to condense on the bottom (and lower the overall humidity in the chamber). For this reason, avoid leaving the Vitrobot in this state for any longer than is necessary.

16) Apply specimen to the grid. Again, pushing the "Continue" button will cause the next step in this sequence to happen: the tweezers will move lower inside the blotting chamber, allowing the user to reach in from either side and apply the specimen to the grid using a Pipetteman or Hamilton syringe. Most people use a 3 to 5 µl droplet of specimen. You should see the droplet cover the grid and spread out into a large droplet. If an aqueous specimen does not behave this way, the grid is still too hydrophobic to use.

17) Blot the specimen. The "Continue" button will initiate the blotting procedure. Any "Wait time" will happen between pushing the Continue button and the start of the blotting and the blotters will close around the grid for the length of time specified in step 7). If you have specified a "Drain time," the blotters will open and nothing will happen for the drain time duration. If there is no drain time, the blotters will open and the grid will be plunged immediately into the cryogen. The tweezers, liquid nitrogen reservoir and cryogen cup will then drop away from the blotting chamber. The timing of these steps are set using the "Options" menu (but cannot be changed once the grid has been raised into the humidified chamber) and their progress can be monitored by watching the "Options" page.

18) Detach the tweezers from the metal rod and store the frozen grid in the liquid nitrogen cooled storage button. Perform these actions as quickly as possible and keep the grid within the layer of cold dry

nitrogen gas that the float creates. You may want to pause for a brief instant before putting the grid into the storage button. This will allow excess liquid ethane to evaporate from the grid. Alternately, you can blot the grid using a small piece of filter paper that has been placed near the storage button. If you do not do this, you may see that your grid turns white as soon as it touches either the storage button or the liquid nitrogen surface. This white material is solid ethane. Some people believe that solid ethane is bad for frozen grids while others think it protects the surface from contamination by humidity in the atmosphere as the grid is manipulated during loading into the microscope.

19) Continue or shutdown. At this point, you can freeze more grids by repeating steps 13) through 18). Before replacing the tweezers for step 13, warm them up and dry them off briefly using the hair dryer. If you have frozen your last grid, shut down the system using the following steps.

Shutting Down the System

20) Turn off the Vitrobot. Remove the tweezers from the device. Press the "Exit" button in the Vitrobot controls and follow the instructions. After the Vitrobot program shuts down, turn the computer off and then power off the device.

21) Tidy up. Close the main valve on the compressed air cylinder and make certain that the ethane cylinder's main valve has also been closed. Move the liquid nitrogen reservoir and cryogen cup to the back left corner of the fume hood in SI 034 and allow it to warm naturally to room temperature. Detach the humidity reservoir by un-doing the steps used to attach it, disconnect the cable and empty any remaining water from the cylinder: turn the cylinder upside down, attach the 60 ml syringe to the plastic tube and pull on the syringe plunger to remove any remaining water in the sides of the cylinder. Leave the cylinder on its side to dry.

22) Sign the log books and store your frozen sample. There are log books for both Vitrobot use and the storage dewar(s). Please use them.