Session Plan

Basic Vitrobot training

Summary

1. Basics of cryoEM grid preparation
2. Overview of equipment, grid manipulation, cryogenic handling
3. Glow discharge
4. Vitrobot startup and interface
5. Ethane container handling
6. Grid freezing process
7. Grid storage and recording

Review

General notes and prior knowledge

Cryoelectron microscopy can be a powerful research tool but it is far, far more complex than running a gel. You need to be mentally and emotionally prepared to invest several weeks of your time into learning the method. Sample preparation is essential, including both the quality of protein itself and that of vitrified ice.

Principle

While it is referred to most often as CryoEM, the method is in fact cryoTEM - cryogenic *transmission* electron microscopy, emphasizing that the images of protein molecules are generated by focusing electrons that diffract upon going through the specimen. For a number of reasons, the thickest ice that a 200keV electron can penetrate is about a quarter of a micron. Hence ideally we would like to suspend protein molecules in a very thin layer of ice. To avoid formation of crystalline ice, the sample is flash frozen by plunging it into liquid ethane, and to create the thin water layer solution is placed on a grid (copper or gold are common materials) that carries a perforated carbon film. Traditional method places 1-3 ul of protein sample on a grid that is then blotted using filter paper and rapidly plunged in liquid ethane. The thickness of the remaining water layer just prior to plunging will determine ice thickness. Several obvious factors influence the outcome here, including blotting time and tightness of contact with filter paper,

ambient temperature and humidity, buffer composition, etc. Unfortunately, one's ability to control these factors is limited. Fortunately, a single "good square" on one grid may produce enough data to solve a structure, and this could often be accomplished by simply freezing enough grids.

Equipment

All of the sample preparation equipment is located in room 1122. As you enter the room, you will find a sink on your right hand side. LED illuminated magnifying glass is mounted on both sides of the room around the bench workspace. You will find a toolbox, ethane container and other items on the shelves above the bench on your right. Vitrobot is at the far end of the bench, next to the hood. Under the counter, there is a styrofoam box that can be used to dump unwanted liquid nitrogen.

On your left side by the door you find the glow discharge unit. Dry box is across the room on the left side, and a small black under-counter refrigerator can be used to keep your samples cold. Three 4L transfer dewars are either under the counter or on it while drying up.

On the right side by the hood, there is the storage dewar that holds 6 empty canisters and the cryopuck holder. Across from it by the dry box there is 50L transfer dewar from which you can get liquid nitrogen.

Grids

CryoEM grids are round metal wafers that also carry a perforated carbon film. These are handled using thin tweezers. It is important to make sure to grip these by the rim in order to avoid damaging the carbon film. Only one side has the carbon film, and it is to that side that you will apply protein solution. It is therefore important to know which side it is. Luckily, the most commonly used grids, the Quantifoils, have the carbon side (dull) that is easy to distinguish from the metal side (shiny).

Cryogens

Protein samples must be frozen for reasons that are rather obvious - to immobilize the protein molecules and prevent sample vaporization while exposed to vacuum inside the microscope. In cryoEM, samples are stored in liquid nitrogen. Another cryogen used is liquid ethane (to accelerate the freezing process to obtain amorphous ice. While effects of liquid nitrogen on the human body are not quite as dramatically instantaneous as depicted in movies, you are nonetheless dealing with materials at temperatures that are 250C below that of your body, so extreme caution needs to be exercised in order to avoid serious injury.

Wear gloves. Cotton gloves covered by latex gloves work great when handling objects cooled in liquid nitrogen directly (storage pucks, ethane container brass cup, etc). These provide excellent but temporary protection, so handle these as quickly as possible. Latex gloves alone will protect well against liquid nitrogen spills, since liquid nitrogen boils off hot surfaces such as your glove protected hand. It is only recommended to use latex gloves only in situations where maximum dexterity of movement is required.

Wear goggles. In various circumstances such as pouring liquid nitrogen into a warm container, liquid may be ejected in small droplets.

Extreme caution is needed when handling liquid ethane. Unlike liquid nitrogen, it is maintained during cryoEM sample preparation at temperatures below its boiling point, so if spilled, it will not boil off your skin and will result in instant burn.

Always remember that your protein sample when trapped in a thin layer of vitrified ice needs to stay cold, usually under liquid nitrogen. Whenever you are handling grids, tools that come in direct or near contact with them should be cooled prior to use. For example, when moving a grid, lower tweezers into liquid nitrogen away from the sample, and hold them down until no active boiling is visible. Keep in mind that if you withdraw a tool from liquid nitrogen, it will rapidly ice up at room temperature. To avoid depositing extra ice on the grid, warm the tool up using a hair dryer if repeated use is expected. Use common sense (e.g. it is not necessary to fully dry the pencil to unscrew the box lid since you are not making contact with the grid) and err on the side of caution.

Glow discharge

The purpose of this operation is to make your grids hydrophilic. Place the grids you plan to use today on a proper support - the easyGlow model we use is designed to fit a standard cover glass in the groove of the metal stand inside the glow discharge chamber. Wrap the cover glass in paradigm - this will make it easier to pick up the grids by pushing down on the parafilm with the tweezers.

To run the glow discharge unit, place the grids on the stand, and cover it with cylindrical thick glass cover (be careful not to damage the glass. Recommended parameters are 20 second glow at about 20 mA. Use the stylus to activate the cycle. The screen shows the current step in the protocol, do wait until they are all complete and the chamber is vented before opening the cover and removing grids.

Opinions vary on how much time you have before grids need to be glow discharged again. If you see that a protein drop applied to the grid forms a bead instead of being pulled into it, repeating the glow discharge step is certainly recommended. If you prefer not to waste a good grid, put a bare grid (these have no carbon film on them and cost about $0.20 each) together with your regular ones and use it to test how hydrophobic it has become over prolonged wait. Alternatively, just glow discharge your grids again if in doubt - it does no damage and is fast. So despite this being the first step described in this tutorial, it makes sense to wait until Vitrobot is fully set, either before or after filling up a liquid ethane container.

Running Vitrobot

1. ***Start the instrument.*** The power button is in the back right side. If nothing happens after you flip the switch, the previous user might have shut down the Vitrobot using the touchscreen but didn't power the unit down. Just flip the switch again. You should see the touchscreen come to life and start what appears to be Ubuntu Linux.
2. ***Set the temperature.*** Interface features 2 screens switched by the bottom tab - console and options. Set the desired temperature first. In most cases 4C is used and it might take 20 minutes or so for the chamber to cool off, so do it as soon as possible. In the console screen, temperature is controlled by the vertical bar. The allowed range is 4-50C, so touch the bar near the bottom. It's usually difficult to make it exactly 4C, so you may need to use the scroll arrows to get it exactly right.
3. **Set the humidity.** To the right of the temperature control there is humidity control. Set humidity to 100%. Select the manual radiobutton to check whether the humidifier is working - you should see some misty wind appear inside the chamber. If that does not occur or the word "Empty" appears above the settings radiobutton, do fill the humidifier tank as described below. *Set the humidifier to "off" for now.*
4. ~~***Attach the humidifier tank.*** This will be the metal cylinder. It fits into the round port underneath the chamber. There is a label on the cylinder indicating which side should be facing forward. Lift it up and rotate clockwise until it clicks in place. Attach the control cable that is found below the tank and behind the moving platform. Red dots on the connector and the plug should line up which is essentially impossible to do when the tank is already in place, so just turn it around until it slides up with ease (do not apply any excessive force).~~
5. ***Fill the humidifier tank.*** Only do this if/when the humidifier fails to maintain 100% humidity and/or reads Empty in the console tab. There is a short piece of silicon tubing on the bottom of the tank, use it to connect a large syringe filled with about 30mL of deionized

water. Inject water into the cylinder and then withdraw about the same amount of air to complete the process.

6. ***Set proper Vitrobot options.*** These are found in the options screen. The following settings are recommended. (1) Use foot pedal (on): individual steps of vitrification protocol can be activated by pressing on the foot pedal, leaving your hands free. (2) Turn humidifier off during process (on): humidifier creates airflow in the chamber when running, turning it off during blotting makes the process more predictable. (3) Skip grid transfer (on): After the ethane container is lowered and tweezers detached, the platform will move about an inch towards you, presumably making it easier to transfer the grid into the blue box as you have more space. In practice, most people move the entire container off the platform to the bench, rendering this step useless. Since a small chance remains of accidentally activating this step while tweezers are still attached, it is better to skip it. (4) Autoraise ethanelift (off): This will result in the platform being elevated immediately after the grid is lifted into the chamber. If you plan to move the ethane container off to the bench for manipulation (as most people do) this option needs to be off unless you are sure you will not forget placing the ethane container on the platform immediately after inserting the tweezers.
7. ***Run the gap check.*** During the blotting cycle, pads with attached filter papers will close on your grid, blotting away the extra liquid. The desired result of this process is an ultra thin layer of water remaining on the grid that will turn into a super thin layer of ice upon plunging it into liquid ethane. Many different factors play the role here and need to be optimized for your protein sample. One important one is the blotting force. It is not the actual force applied to the pads but rather the distance between them when fully closed. Larger numbers correspond to smaller gaps between the pads and thus stronger force with which pads are squeezing the grid. Unfortunately, blotting force number produces different distances between the pads on different Vitrobots and at different times. Furthermore, since pads are not perfectly flat, different blotting force values will be optimal at 16 positions of the pads that Vitrobot cycles through. This requires performing a calibration procedure that is fortunately simple and fast. To perform it load the protocol named “gap”. It is set up to cycle through all 16 pad positions and multiple blotting force values. Do not attach the tweezers and do not insert the filter papers. As it does so, carefully watch the light strip below the pads. When the pads are spread too far apart (blot force too low), the line of light will be unbroken. When the pads close too tight, a solid dark patch appears in the middle of the light strip. Everything in between is an acceptable value of the blot force, resulting in pads just barely coming into contact. Tabulate your observations on the provided sheets - these will guide your selection of blot force. (This is often a source of confusion - you need to develop your own sense of

what constitutes a “good gap” and what other parameter selections work best for you and your sample.

8. ***Prepare liquid ethane.*** Gray round styrofoam assembly is used to prepare liquid ethane and plunge grids into it. *Alternative to ethane is eutectic propane:ethane mix which has the advantage of having its melting point below liquid nitrogen boiling point.*
    - ***a.*** To assemble it, start with the main piece and place the brass cup into the round opening in the center.
    - ***b.*** Lower the aluminum table that has 4 depressions for blue grid boxes.
    - ***c.*** Place enough boxes for all the grids that you plan to make.
    - ***d.*** Notice that eventually liquid nitrogen will be making no direct contact with the brass cup - this is to delay the freezing of ethane as its melting temperature is above that of liquid nitrogen. However, such contact is needed during the liquefaction process. That is the role played by the “spider” - 4-legged aluminum support that is placed on top of the brass cup.
    - ***e.*** Pour liquid nitrogen into the assembly to cool off all of its components. You will have to top it off a few times as liquid nitrogen boils off. Continue until you can no longer see nitrogen gas bubbling around the metal. This includes the inside of the brass cup, so pour some liquid nitrogen into it as well. To minimize the amount of condensation, cover the assembly using a perfectly sized round CD spindle cover. Before proceeding to the next step, use the back of the plastic hex key to splash out and boil off liquid nitrogen from the cup, leaving it cold and dry.

      THE FOLLOWING four sections assume that you are not using the Nanosoft ethane condenser tool - if you are, please follow directions here https://www.nanosoftmaterials.com/ethane-condenser

    - f. Roll the ethane cylinder close enough and position it in such a way that you can reach the valves on the pressure regulator with your non-dominant hand. Open the main valve first. Open the output valve and place the pipet tip inside the piece of tubing that is inserted in a 50mL tube filled with mineral oil. Using the pressure regulator knob, open the flow just enough so that gas begins to bubble through the oil. Retrieve the pipet tip and turn the regulator knob about 1/16 of a turn - this will establish the proper ethane flow.
    - g. Quickly place the nozzle into the brass cup. First stick it into the corner near the bottom of the cup. Hold it still until the pool of liquid ethane begins forming. You do want to lift the tip off the bottom of the cup eventually to reduce obstruction to accelerate the process, but be careful not to pull the tip out of liquid entirely as this will result in ethane mist.

h. Fill the brass cup to about one millimeter from the top. Now close the output valve on the ethane cylinder and then withdraw the tip. Cover the ethane container with a CD box and roll away the ethane cylinder.

i. (*This step does not apply when using ethane:propane mix as it won't freeze. It is recommended to wait at least 5 minutes to give enough for the temperature in the cup to lower sufficiently*). Wait until ethane begins to freeze. Watch for whitening around the perimeter. Once you start seeing some white,remove the spider using a pair of tweezers. It is possible that the spider will freeze to the top of the brass cup (this is more severe in the summer when humidity goes up). If this happens place the nickel coin on top of the spider above the brass cup. This provides enough heat to melt the ice. Pull the spider up and out. Cover with a CD box.

j. During the freezing session, you have to watch for liquid nitrogen level. It is important to make sure that it does not splash into ethane since ethane will freeze instantly and it is generally recommended to remake liquid ethane in such cases to avoid contamination. The floating styrofoam ring helps with that, so only add liquid nitrogen into the small ledges outside the ring, never over the top. In general, you do want to keep the blue boxes submerged to protect grids from icing up. (*This does apply to ethane:propane mix as well - while it won't freeze, contamination with liquid nitrogen even if it separates into a phase may negatively impact the freezing rate*)

k. Despite minimal heat transfer, liquid ethane will eventually freeze. To keep it liquid, stick the back of a tweezer into it to melt the ethane ice. It is recommended to check that you can see brass on the bottom of the cup prior to every grid. If ethane freezes too far up, the grid will hit it and fold in half. Folded grids cannot be clipped and must be discarded. At the same time, avoid fully melting the ethane in order to make sure that ethane is as cold as it can be (at its freezing point). (*This does not apply to ethane:propane mix since it won't freeze at all*).

9. ***Glow discharge grids.*** See above.
10. ***Insert filter paper.*** There are two plastic rings kept inside the Vitrobot chamber. Mount filter papers on them by pushing through the central hole in the same direction in which filter papers curl up. Click "Reset blotpaper" button on the interface, which will pull up the shaft. Push plastic rings into pads while holding them in place with your other hand to avoid displacing pad holders. Click the button again to confirm.
11. **Activate the humidifier.** Now is the time to set the humidifier to "on" mode. Make sure humidity reaches 100%.
12. ***Freeze grids.*** Repeat the following steps as needed.

a. Pick a single grid with Vitrobot tweezers. Make sure to pinch the grid by the rim to minimize damage to carbon coating. Lock down tweezers by pushing the black locking clamp down until finger tight. Do not overtighten as tips might separate and drop the grid. Check that the grid is properly gripped by gently knocking the tweezers against your finger.
b. Push the pedal once (equivalent to clicking “Place new grid button” on the interface). The shaft comes either down or slightly up depending on prior situation. Attach the tweezers to the shaft by inserting the triangular shaft tip into the opening in the brass connector of the Vitrobot tweezers. Make sure to feel it click so that the tweezers are aligned. Misaligned tweezers may get caught in the square opening in the ceiling of the humidity chamber (very bad idea). Make sure that the carbon (dull) side of the grid is facing your dominant hand side.
c. Push the pedal again. This will lift the tweezers into the chamber and interface will request that you place the ethane container.
d. Setup your freezing protocol. Define blot, wait and drain times, blot force and number of blots. If you use double blotting with different force values (based on gap calibration) and thus add multiple blotting events, make sure to check the “skip application” box for all steps but the first one (otherwise the robot will pause between blotting events to wait for you to apply extra protein).
e. Check nitrogen level and ethane condition. Top off and melt as necessary. Place an ethane container in the round holder below the humidity chamber.
f. Push the pedal again. This will lift the ethane container. Some liquid nitrogen may spill over as the protective styrofoam ring is pushed down, this is normal. Now is the good time to pipette the protein.
g. Push the pedal (equivalent to clicking on the process button). This will lower the grid down so that it’s level with the ports on the sides of the humidity chamber (keep the red plugs closed to help maintain temperature and humidity).
h. Apply the protein through one of the ports depending on your handedness. Standard volume is 3ul, but do experiment. Obviously, smaller volume will tend to produce thinner ice, but the relationship is not linear. Try not to touch the grid with the tip but with a liquid bubble instead. You might notice if you hesitate that the protein solution gets pulled into the tip, this is because air in the pipettor shrinks as it cools off.
i. Push the pedal one last time. This will lift the grid back up to pad level, blot it and plunge into liquid ethane. Vitrobot will now lower down the ethane container while keeping the grid submerged.
j. Transfer the frozen grid into the blue box. By most accounts, this is the most challenging manual part of the process. What follows is some advice, but do work

to refine your own technique. Make sure that at least your non-dominant hand is gloved.

i. Detach tweezers from the shaft. Keep the grid submerged. Gently push the brass connector towards you while holding the shaft in place with your other hand.

ii. Move the ethane container to the bench. This is not strictly necessary, but most users find it difficult to operate while an ethane container is still on the platform. If you prefer to keep it in place, make sure not to check the "skip grid transfer" option so that you can move the platform forward (push the pedal) at this point. (At this step, it helps to rest the tweezers against the side of the brass cup).

iii. Unlock the tweezers while keeping them closed. To accomplish this, hold down the tweezers with your left hand below the lock, then use your right hand to slide the lock up. Now hold the tweezers closed with your right hand and release the left hand. (If you are left handed, swap hands).

iv. Move the grid out of the liquid ethane and into the blue box. Be quick but calm. Some find it useful to transfer the grid into liquid nitrogen first, some go straight to the box. Just position the grid above the slot and let it go - it will fall into the slot under its own weight. Forcing it in might damage the grid. If you see that the grid turns white, do not worry - it's frozen ethane, not ice. It will vaporize once exposed to vacuum, and will protect your grid in the meantime.

13. ***Turn off the instrument.*** Place everything that needs drying in the dry box and run it for 2 hours at 32C. Remove filter papers and place plastic rings in the humidity chamber. Remove the humidifier and empty it. Use the exit button to shut down the Vitrobot then flip the power switch off.

Storing and recording grids.

Grid boxes are stored in pucks. Puck container is in the large storage dewar. Retrieve the puck pulling up the holding shaft and pulling the puck out with large forceps. Place it in a small foam dewar, lock down the container and return it to storage dewar. Now transfer your grid box(es) into the puck and notice its position. Return puck to the container.

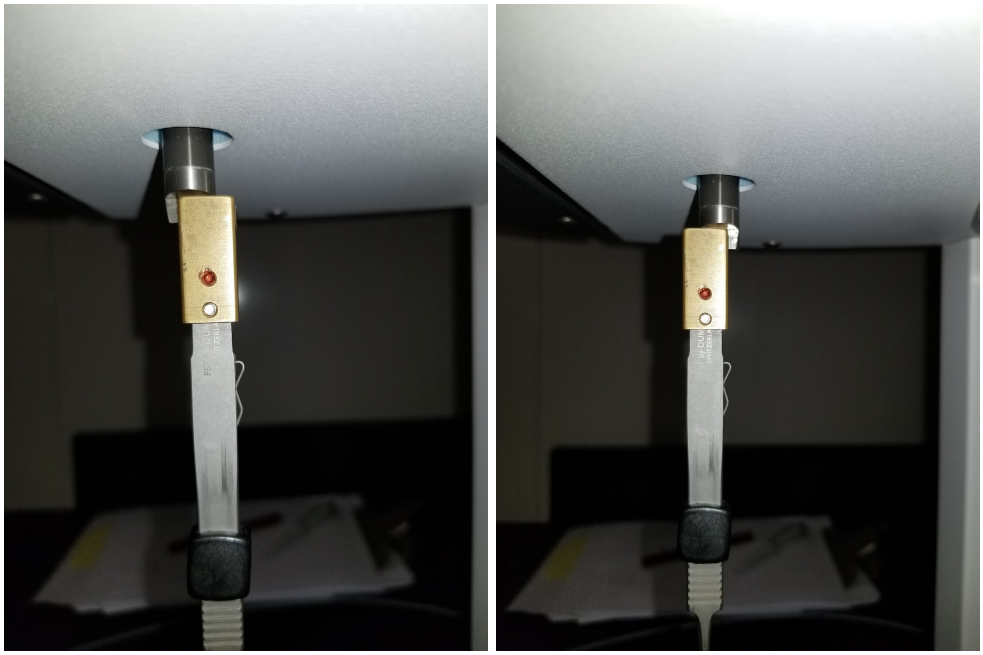
To record your grid position, add them to a request in cryodb.ibbr.umd.edu.

Appendices

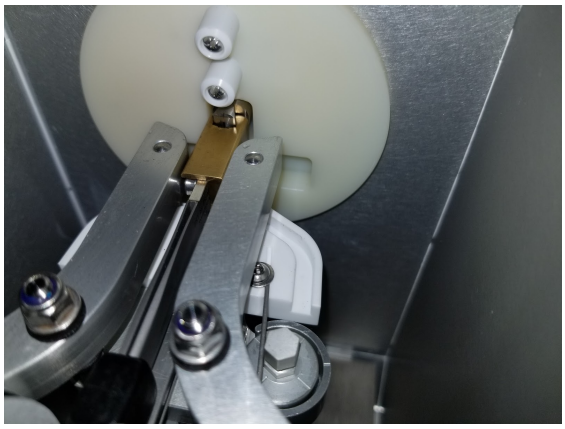
Appendix 1. Misaligned Vitrobot tweezers

When you attach Vitrobot tweezers to the shaft, they have to be co-axial. This is good

This, on the other hand, is very bad



When tweezers move up, they will get caught either in the round opening in the bottom of the chamber or in the square opening on the top of it



Vitrobot will lock down. If this ever happens, **do not** power cycle, since you can damage the motors by running them into a hard limit. Slowly step away from the instrument, put away your samples and tools and call for help.