

Plunge Freezing of Particulate Specimens

Needed Materials (Some of the numbers correspond to labels in Fig. 1 below):

1. Liquid nitrogen in a Taylor-Whorton LD4 liquid nitrogen dewar or equivalent
2. Several pairs of self-closing tweezers or tweezers with locking O-rings
3. Pair of fine-point, normal-closing tweezers. The user may want to tape a thin layer of insulating material to the sides of these tweezers so that they may be more easily handled in liquid nitrogen.
4. P20 Gilson Pipetman and pipet tips
5. Copper EM Grids, 400mesh ([Electron Microscopy Sciences](#) or other EM supply company) coated with a perforated carbon film. [Quantifoil](#) grids may be used instead. These grids should be freshly [glow-discharged](#).
6. Whatman #1 filter paper cut to rectangular strips of approximately 2 cm X 9 cm size
7. 350 ml vacuum flask
8. 50ml conical test tube for storing grid boxes
9. Gatan covered grid box with cover-rod. Non-Gatan cryo, grid boxes may also be used.
10. Eye protection
11. Ethane gas (CP grade) with regulator, tygon tubing and a pipette tip
12. Aluminum foil-lined styrofoam cooler
13. Laboratory "freezer" tape
14. Freeze-plunge device. Our device is in-house made but commercially-made, automated devices such as the [FEI Vitrobot](#) or the [Gatan Cryoplunge](#) are available.
15. Two transfer cups and wire support ("spiders"), one for liquid nitrogen and the other for liquid ethane. Ours were made from test tube caps (VWR Scientific products SP16) with a holder that suspends them over the 350ml vacuum flask.
16. Particulate sample for freezing



Fig. 1. Setup needed for plunge-freezing samples.

Caution: Eye protection is a necessity. Liquefied ethane can do serious damage to the eyes. If liquid ethane comes in contact with liquid nitrogen it can violently splash. Ethane is also flammable so it must be used in an area with adequate ventilation. No open-toed shoes are allowed when handling cryogenics and protective lab garments should also be worn. Standard practices for handling infectious materials should be followed.

Preparation:

1. To save a great deal of effort it is advisable to check the quality of the specimen by preparing a negatively-stained preparation prior to examination with cryoelectron microscopy. In our lab, with a virus specimen of about 50 nm diameter, we have found that the sample concentration should be about 3-5mg/ml of protein. On a good quality negative-stain preparation the particles would then cover the field of view and be touching or even slightly overlapping. Of course a specimen of much greater or smaller size would require different concentrations which would have to be determined by trial-and-error. The amount of solute in the buffer is also important and a solute content of <300mM is advisable. We have not found much of a difference when using different buffers but some materials such as glycerol or detergents may affect the quality of the frozen prep.
2. Perforated carbon support films are preferable over standard films if the images of the sample are to be used for calculating a three-dimensional reconstruction. This is because the amorphous carbon of the films adds additional noise to the images. However, if the sample concentration is somewhat low then standard support films may be preferable because the sample has a tendency to adhere to the carbon when allowed to sit for several minutes before blotting and plunging. In recent years [Quantifoil](#) films have been preferred over in-house made films. *Quantifoils* have holes of a standard size and periodicity, a characteristic not easily achieved in in-house-produced perforated films. Quantifoils, however, are quite a bit more expensive than normal grids and so they may not be the choice for inexperienced users with a less-than refined technique. Films should be freshly prepared and not be more than a few weeks old because the hydrophilic nature of the films changes over time which affects the ability of the sample to evenly spread over the film. Various techniques have been used to get an even spreading. Some researchers will glow discharge their films prior to use but we have found that too much of the sample then adheres to the carbon instead of being suspended over the holes. This problem has sometimes been alleviated by double blotting; that is, putting a drop of the sample on the grid, blotting it nearly dry and then adding another drop before the final blotting and plunging. Experience has shown that *Quantifoils* generally need glow discharging before use.
3. The temperature and humidity in the room make a big difference in evaporation of the sample and on how much moisture contamination occurs in the nitrogen. It is best to work in a walk-in cold cooler at 4°C to prevent this ice contamination from forming and getting on the grids.

Procedures (Click on each thumbnail on the right for a larger image. Click on the large image to close.):

1. Prepare your work surface area by placing everything that you will need within arm's reach and removing anything that you will not need. It is preferable to work in a chemical hood or another area with adequate air flow because of the explosive nature of ethane.
2. Check to make sure that the height of the freeze-slam device is correct. The proper positioning of the rod is when, at the press of the foot switch, the grid is under the level of the liquefied ethane but not so far down that the grid might slam into any frozen ethane that may be in the cup. Most of the time the height on the freeze-slam device need not be modified because most of our vacuum flasks are the same size. Put on eye protection and fill both the vacuum flask and the styrofoam cooler with liquid nitrogen. Place a conical test tube, a Gatan grid box and cover tool, and the liquid-nitrogen transfer cup into the cooler.
3. Slowly lower the ethane transfer cup into the nitrogen in the vacuum flask. If liquid nitrogen bounces into the cup, empty it because it will cause difficulty in liquefying the ethane and could cause the freezing ethane to pop and splash (Fig. 2).
4. Make sure that the small black valve nearest the tygon tubing on the ethane regulator is



Fig. 2. Vacuum flask in position on the freeze-slam device. The flask is filled with nitrogen and has the two transfer cups, the one on the left is filled with liquid nitrogen and the one on the right with liquified ethane. Some solidified ethane may be observed in the bottom of the cup.

closed and the main valve on the ethane tank is open. You will be using the small black valve to carefully regulate the flow of ethane into the cup. Place the tip of the ethane gas Pasteur pipette into the bottom of the ethane transfer cup and slowly open the small black valve. The ethane will first come out as a gas but will soon liquefy in the bottom of the cup. Listen for the "gurgling" sound of the ethane as it starts to liquify. Keep moving the pipette tip around the bottom of the cup to prevent ethane from freezing in the tip. Use extra caution whenever you add ethane because if the pipette tip freezes or ethane is added too quickly, liquid ethane and nitrogen could be forcefully blown out of the cup and could cause serious eye injury. As the ethane liquefies it may be possible to increase the flow of the gas. Allow the transfer cup to fill up until a meniscus of liquid forms at the top. Do not overfill the cup and allow ethane to flow over the side and freeze. Anything frozen outside of the cups could freeze the nitrogen cup in place thereby making it difficult to transfer the frozen grid.

5. Place the liquid nitrogen transfer cup into the vacuum flask next to the ethane cup. Do this slowly to prevent nitrogen from splashing into the ethane. Take a pair of self-closing tweezers, pick up a grid, and secure the tweezers into place on the plunging rod with "freezer" tape. Raise the rod so that the tip of the tweezers is approximately 8 cm above and centered over the surface of the ethane liquid.
6. With the Pipetman put about 3.5 μ l of the sample onto the grid. Keeping the volume of the drop the same makes the technique more reproducible. If the drop does not spread evenly over the grid you may need to pull it around the grid with the pipette tip. Alternatively, you may put the sample on the grid before attaching the tweezers to the rod.
7. Take a filter paper strip and hold it in position so that it is parallel to the plane of the grid in front of the sample drop. Do not touch the paper against the grid yet. Position yourself so that you are looking through the grid and paper strip from the back side.
8. Touch the filter paper flat against the sample and the grid for several seconds (Fig. 3). Observe from the back side how the sample liquid is wicked onto the paper. When the wicking appears to cease, quickly hit the foot switch to plunge the grid into the ethane. The time between the initial wicking and the plunging is at best 2-4 sec. If it is difficult to see the wicking a strong light may need to be placed in front of the grid. If the wicking is done for too long of a period the grid will be dry. Conversely, if the wicking is too short the ice will be too thick. The paper must be held parallel and firmly against the grid without damaging the grid. If any part of the grid pulls away from the paper before plunging, the sample will begin to evaporate and the sample particles will be bunched together near the periphery of the holes in the carbon film.
9. Carefully remove the tape that holds the tweezers in place on the plunging rod and very quickly transfer the grid to the nitrogen transfer cup. If done quickly enough a thin layer of liquefied ethane remains on both sides of the grid and provides a momentary insulation during the transfer.
10. Pick up the nitrogen transfer cup and the tweezers and move both to the styrofoam cooler and place into the liquid nitrogen (Figure 4). Release the grid into the Gatan grid box. Try not to breath onto the surface of the nitrogen because the resultant fog will make it impossible to see the grid box. This procedure may be done somewhat more easily by picking up the grid box with a standard pair of tweezers and holding the box nearer to the top of the liquid nitrogen. Care must be taken to avoid exposing the grid to the ambient air. The Gatan grid boxes and cover rods are in limited supply. If you plan on storing your grids in liquid nitrogen for any length of time please use the square grid boxes instead.
11. If additional grids are needed, place the tools back into their proper positions and top off all liquids. If the ethane has frozen re-melt it by adding more ethane gas. Use a dry pair of self-closing tweezers for the next grid. Make sure that all tools are dry before being cooled down. Wet tools will freeze and make the process much more difficult. For example, it will be impossible to remove a frozen grid without damaging it if wet tweezers are used. A hair dryer may be used to warm up and dry frozen items.



Fig. 3. Blotting the grid before plunging.



Fig. 4. Placing the grid in the Gatan grid-storage box.

12. When sufficient grids have been frozen, screw the grid box cover rod onto the top of the grid box and then place both into the conical tube filled with nitrogen. The conical tube and samples can then be placed into one of the vacuum flasks and carried to the microscope or placed into storage in a liquid nitrogen cryo freezer.
13. Close the valve on the ethane bottle and clean up the area for the next person. Pour the liquid gases out of the dewars, transfer cups, and cooler into an appropriate place in a well-ventilated area.