Protocol for use of Gatan 626-DH Cryotransfer System
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Step 1. Insert the holder into the appropriate adapter on the Model 655 Dry Pumping Station. Evacuate and heat the cryoholder dewar to at least 110°C for more than two hours according to the procedure in Section 5 of the cryoholder manual. If the cryoholder is to be routinely used, the standard protocol is to maintain the cryoholder in the dry pumping station when not in use in the microscope.

Step 2. Add liquid nitrogen to the cold finger and Gatan model 651 anticontaminator on the microscope and monitor the vacuum in the microscope.

Step 3. At the start of any experiment the transparent cover of the workstation should be in place together with the two plastic caps covering the access ports.

Step 4. The workstation may now be filled with liquid nitrogen. Place an object under the non-dewar end of the workstation to raise it approximately 1” in height. Insert the plug stem in the side entry port of the workstation. Remove one of the plastic caps and use the funnel to add the liquid nitrogen until it reaches the opening of the side entry port of the workstation. Replace the plastic cap immediately, remove the plug stem and insert the specimen rod in the side entry port to minimize the ingress of water vapor which would contaminate the liquid nitrogen with ice crystals. No frosting should occur when the specimen tip cools because all air in the workstation has been displaced by dry nitrogen.

Step 5. The specimen holder dewar may now be filled with liquid nitrogen. First add a small quantity of liquid nitrogen to cool the dewar and then half fill the dewar when the initial boiling has subsided. Connect the control unit to the AC power and to the dewar. Depress the power switch. The temperature reading at the tip of the specimen rod should now be displayed. As the temperature falls, the outside surface of the dewar will become cold to the touch until the zeolite cryopump starts to cool and pull the vacuum in the dewar down to the low 10⁻⁵ Torr range. The outer surface of the dewar will then warm up towards room temperature.

Step 6. When the specimen rod reaches its minimum temperature it is ready to accept specimens stored in the specimen grid holder in liquid nitrogen, grids from an ultracryotome or from another cryostation. The grid holder and the transfer tool should be in contact with liquid nitrogen when specimen grids are loaded and the transfer tool is screwed into place. Any forceps or ancillary tools introduced into the workstation should also be in contact with liquid nitrogen before coming in contact with specimens. Before transferring the grid holder one of the plastic caps on the workstation cover should be removed and the cover rotated until the access port is positioned over the grid holder recess. Add more liquid nitrogen if necessary to fill up the reservoir in the support platform. The grid holder can now be transferred quickly to its recess in the workstation. The transfer tool should be unscrewed and the cap of the workstation replaced to prevent ice crystals from falling into the liquid nitrogen.

Step 7. If the specimen rod has been inserted into the workstation with a Clipring™ in place, the specimen Clipring™ must now be removed from the specimen tip. The cryoshield should now be retracted by pulling on the knob at the rear of the dewar. Insert the Clipring™ tool through the filler port and hold it perpendicular to the Clipring™ while screwing it onto the threads of the insertion tool. The Clipring™ can now be pulled out of the specimen tip and stored in the liquid nitrogen reservoir while still on the insertion tool.

Step 8. The second plastic cap on the workstation cover should now be removed to provide entry for fine-tipped tweezers used for specimen loading. Adjust the level of liquid nitrogen above or below the level of the specimen rod depending on the need to load the sample in liquid or gaseous nitrogen. Dip the points of the tweezers in the liquid nitrogen reservoir for about one minute to cool or until the liquid nitrogen stops bubbling, then pick out a specimen grid and load it into the recess in the specimen tip.
Check that the grid is properly seated before pressing the Clipring™ down onto it. The Clipring™ insertion tool can now be unscrewed and removed. Check that the Clipring™ is properly seated. It should not move when probed with the cold tweezers. If the Clipring™ is loose it can be pressed into place using the spherical tip of the back end of the insertion tool. Do not forget to cool the tip of the insertion tool before pressing it against the specimen and Clipring™. If the user finds it difficult to load specimens through the holes in the plastic cover, specimen loading can be done with the cover removed. In this case the liquid nitrogen will become contaminated with ice crystals; the styrofoam-insulated vessel of the workstation should be emptied of liquid, the metal tray removed, warmed to room temperature and dried before the next specimen is loaded.

Step 9. After loading the specimen the cryoshield is pushed gently forward over the grid using the external shutter control knob. Liquid nitrogen is added to the reservoir to cover the tip of the rod. When the temperature reading on the control unit reaches approximately -193°C, disconnect the control unit cable from the holder and move the control unit and workstation into convenient positions on the microscope console.

Step 10. The specimen rod may now be inserted into the microscope. To avoid an increase in specimen temperature during the transfer two methods can be used. A) Empty the dewar into a styrofoam cup prior to loading the rod into the TEM airlock. The airlock should then be pumped out as quickly as possible and the dewar should be refilled with liquid nitrogen as soon as the specimen is in the TEM. There is enough thermal capacity in the dewar even when empty to maintain a low specimen temperature for this short period of time. B) Alternatively one can transfer the holder with some liquid nitrogen kept in the dewar. Make sure that the level of liquid nitrogen inside the dewar is below the cross tube. Fashion a styrofoam plug for the opening of the dewar that will allow the outflow of gaseous nitrogen but impede the flow of liquid nitrogen. Pre-tilt the goniometer around to 60° so that the holder can be inserted with the dewar opening close to horizontal. As soon as the airlock is pumped out, simultaneously bring the goniometer tilt back to zero degrees and bring the holder dewar to its upright position so that the holder can be fully inserted into the microscope. On the CompuStage of Philips microscopes it is not possible to manually tilt the goniometer. The following procedure is recommended: use the controls to pre-tilt the CompuStage to 60° tilt (counterclockwise). Insert the holder into the airlock. When the airlock indicator light switches off, partially open the airlock by rotating the holder dewar to its upright position. Then press the “Reset A” switch on the CompuStage page while holding the holder dewar upright. When the goniometer is at zero, the holder can be gently inserted into the microscope.

Step 11. Remove the styrofoam plug from the opening of the dewar and check the level of liquid. A small flashlight is useful to detect bubbling in the dewar, or the back end of the Clipring insertion tool can be used to detect the level of nitrogen. More liquid nitrogen may be added to the dewar at this time, or the debubbler can be used to remove excess liquid nitrogen and suspended ice crystals. Reconnect the control unit cable to the holder and monitor holder temperature. Place the plastic cap over the opening of the dewar to retard frosting on the cross tube. Allow the vacuum in the TEM specimen chamber to fully recover before withdrawing the cryoshield. A full dewar should last about five hours.

Step 12. At the end of the experiment the specimen can be transferred cold back into the workstation, removed from the specimen holder and discarded or replaced in a storage grid holder. If conditions permit, the next frozen specimen may be loaded into the holder without having to warm and dry the specimen rod and workstation.

Techniques for obtaining the highest resolution and stability
1. Clamp the control cable to the microscope column to prevent transmission of room noise and vibration to the specimen rod. If the heater is not being used the control box cable should be disconnected from the dewar.
2. Vibration can be minimized by keeping the liquid nitrogen level below the cross tube in the dewar.
3. If bubbling is seen in the dewar, take a cotton swab and gently rub the spot generating the bubbles to dislodge any ice crystals at that point. Use the debubbler to eject the crystals from the dewar.
4. Tapping lightly on the dewar towards the microscope column once the temperature has stabilized will help seat contacting surfaces and minimize drift.
5. The height of liquid nitrogen in the dewar can be checked by inserting the rounded end of the Clipring™ tool in the liquid nitrogen in the dewar for a few seconds. After removing the tool from the dewar, breathe on the tool to generate a layer of frost on the cold surface. A ruler then gives an accurate measurement on the tool of the level of liquid in the dewar.
6. Drift can occur if the specimen rod o-ring is not properly lubricated.
7. Severe vibration of the specimen is usually caused by inadequate specimen clamping. To test the Gatan Clipring™ use tweezers to verify that the specimen grid is properly clamped. Ice crystallization in the specimen is also a sign of poor specimen clamping.
8. Thermal instability caused by air drafts around the cryoholder can be minimized by placing a sleeve of bubble-wrap packing material around the collar of the goniometer and cryoholder.
9. Verify that the drift performance of the single tilt holder provided with the microscope is within manufacturer’s specifications.

**Gatan protocol for testing the performance of cryoholders in Philips CompuStage microscopes**

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Before drift measurements are made with any cryoholder the reproducible performance of the Philips single tilt holder to its drift specifications will have been verified according to the procedure in the Philips manual. The generalized procedure is as follows:

Step 1. Determine that the Philips single tilt holder attains a 10Å/min or less drift rate at room temperature in one hour or less after insertion. If the drift rate is greater than 10Å/min, check the room temperature as read from the control unit of the cryoholder while on the bench. Room temperature should be around 20°C for best performance. Insert the cryoholder in the microscope to read the internal column temperature; accurate readings can take hours. The difference in temperature between the room and column should be 1°C or less. If a temperature difference exists, adjust the water chiller temperature and make further readings the following day. Once temperature stability is achieved, test for 10Å drift: if the rate is higher, check for drafts and shield the goniometer with aluminum foil, if the rate is acceptable, proceed to step 2.

Step 2. Insert the cryoholder cold as instructed in the protocol in the cryoholder manual. After the holder has reached a stable temperature for at least 30 minutes, drift readings can be taken.

Equipment needed for test:
Specimen: graphitized carbon or gold-sputtered latex calibration standard grid from an EM supply company
CCD camera: bottom mount Gatan SlowScan or MultiScan camera
Software: DigitalMicrograph™ with drift measurement script

1. Evacuate and heat the cryoholder to at least 110°C overnight in preparation for the test. Allow the holder to cool to room temperature.
2. Place the grid in the room temperature cryoholder and insert the holder in the microscope. Center the grid in the CompuStage field.
3. Take an image of graphitized carbon or gold-sputtered latex to calibrate the images for the drift measurement script. The magnification on the computer monitor should be ~325,000X for graphitized
carbon. The magnification should be chosen such that the drift over the chosen time interval is approximately one half of the viewing field or less. If higher drift rates are encountered the script will terminate and print less than ten readouts in the results window. Set the drift measurement script to take 1024 X 1024 images binned 2X for a 0.5 sec exposure at 20sec intervals for a total of ten measurements over 200 seconds. Alternatively use M. Pan’s script that automatically rescales the readings if drift is greater than half of the viewing field.

4. Remove the cryoholder from the microscope and place it in the workstation. Cool the holder with liquid nitrogen in the workstation such that the tip sensor reads -190-196°C. Transfer the holder into the microscope. Adjust the liquid nitrogen level in the dewar below the rod and remove any ice crystals. Confirm that liquid nitrogen is quiescent in the dewar. Wait until the holder temperature stabilizes.

5. Check the grid square for tears in the support film and run the test in a region away from large holes. Measure drift by means of the drift measurement script in DigitalMicrograph™. Disconnect the control box cable before making the drift measurements. Note the speed and direction of drift. The direction of drift into the column can be determined by directing a jet of air from a dry air can on the back of the dewar. Set the beam intensity for reasonable counts on the camera. Take measurements at 15 minute intervals, or more frequently if necessary. The script can be run after repositioning or tilting the cryoholder and allowing the cryoholder and goniometer to stabilize a specified length of time to determine drift rates at different tilt angles and locations on the grid.