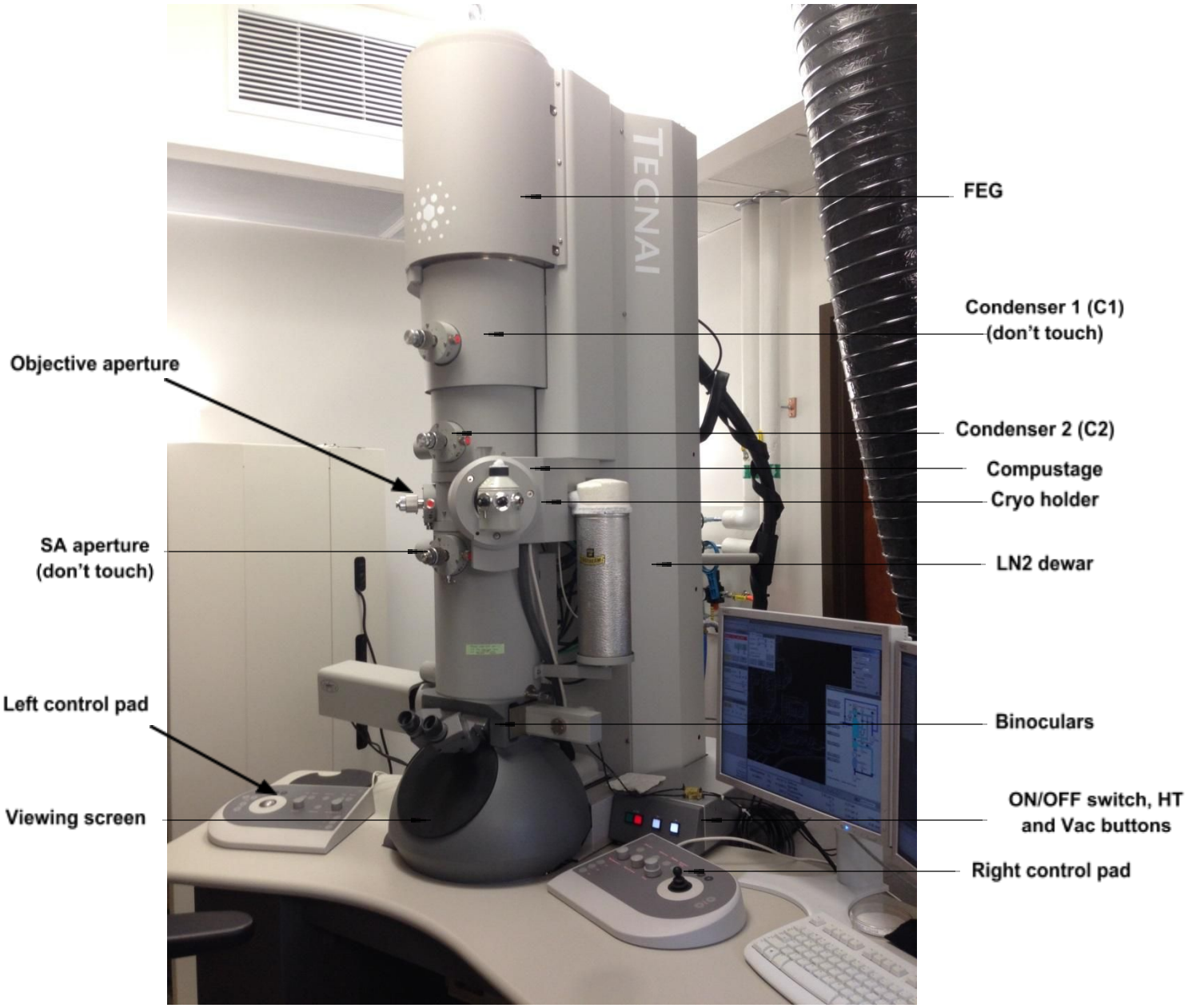
The Tecnai F20 cryo electron microscope is a high resolution electron microscope that can be operated at 120 or 200kV. This microscope is equipped with a Field Emission Gun (FEG) as its illumination source and is suitable for data collection of negative stained as well as cryo preserved samples. Three different types of holders are available for use: a room temperature (RT) for setup and 0-degree tilt data collection, a Fischione RT holder for collecting random conical tilt negative-stained images, and two cryo TEM holders for vitrified samples (Gatan 626). The TF20 is equipped with a 4k by 4k Gatan UltraScan CCD camera for digital image collection. The compustage of the microscope allows for precise movements and tilts to up to +/- 70 degrees.

The microscope's main parts are shown below:



The microscope is controlled by a computer seating underneath the desk. It is critical that no foreign objects are inserted in this computer (software and especially thumb drives or any external drive) as they could contain viruses/malware that would put the computer and microscope at risk.

A user account will be created for you when you have completed training and proven that you can safely operate the microscope. Data can ONLY be retrieved the next day as it is copied nightly to the support computer that is behind the curtain. This computer is supported by the CSB and external drives can be installed on it to save data. Each micrograph is ~65MB in size, thus, data retrieval and removal of it is important to keep data collection uninterrupted. Each month (or as needed) the Lab Manager will send a reminder for people to remove their data. Both the support computer and the microscope computer have limited storage space and they are not for long-term data storage.

Two pieces of software control the microscope and digital camera, the Tecnai User Interface which connect the user with the microscope, and Gatan Digital Micrograph (DM). Each day a cryo cycle (or bake out cycle) is run at the end of the day and the camera must be retracted.

Before leaving the room: Close the Column Valves and put the screen down. This ensures that contamination and irradiation to the samples are kept at a minimum and also protects the microscope.

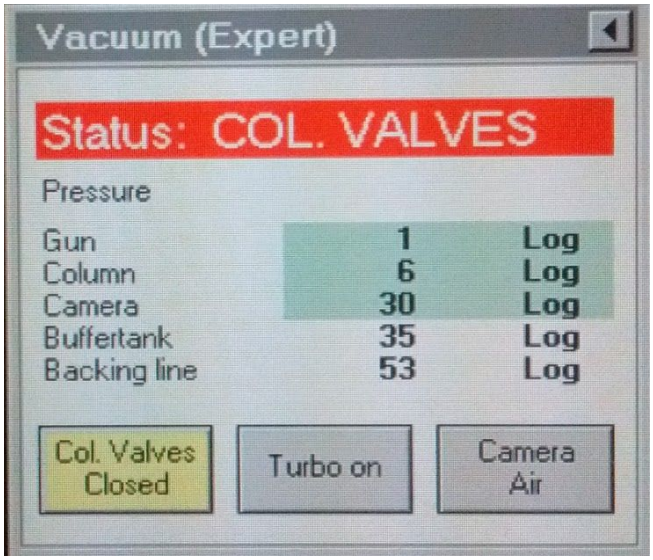
The Lab Manager is available to assist with data collection when needed and is always available for troubleshooting. Any problems or concerns must be notified immediately, this ensures that repairs happen in a timely manner and keeps downtime to a minimum.

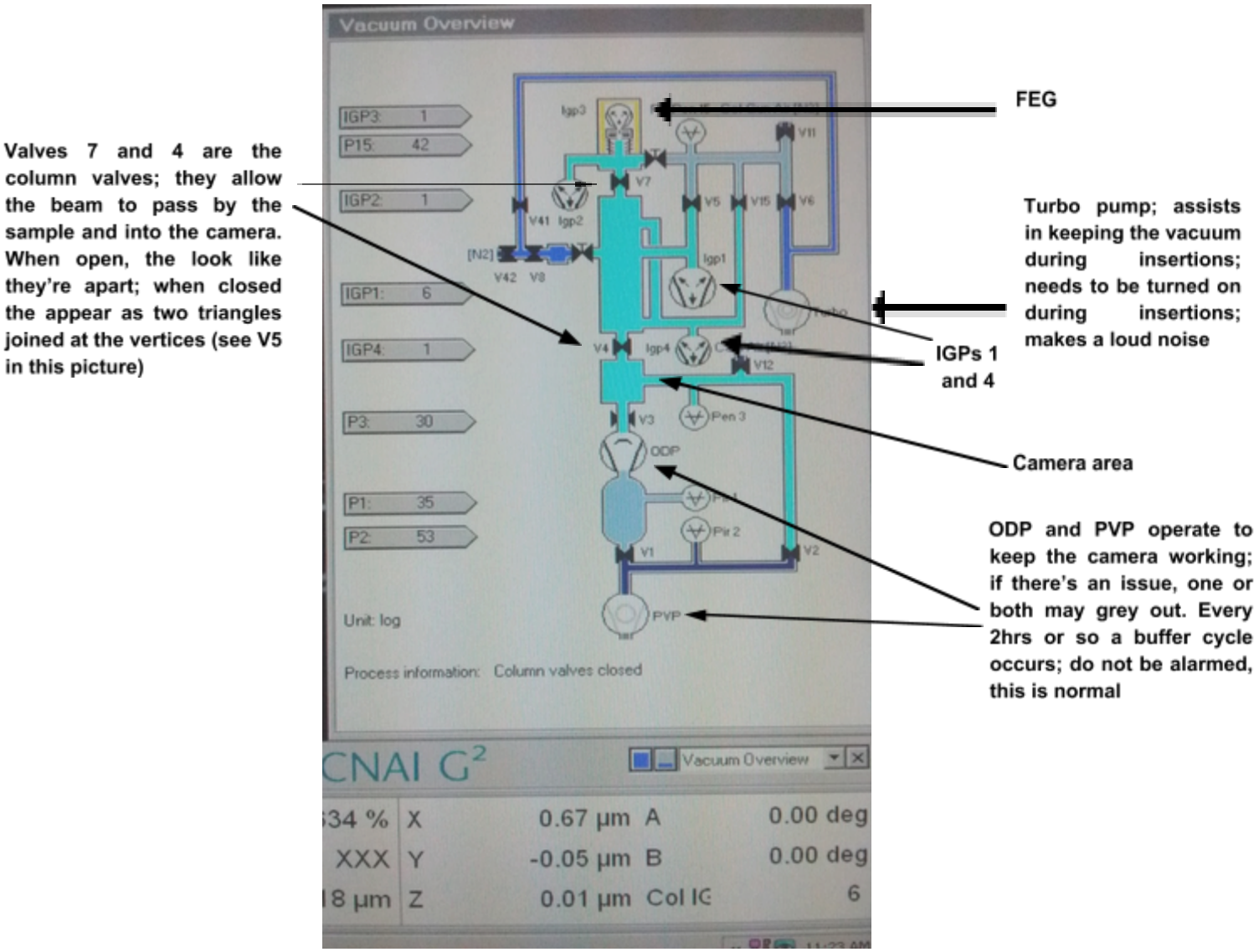
F20 Protocol for inserting RT holder and Gatan 626 cryo holder

I. **Start Up**
II. **Sample loading (at RT)**
III. **Alignments**
IV. **Sample loading (cryo)**
V. **End of Session**

I. **Start Up** (Do not proceed if these conditions are not met. Please remember to notify EM manager of any concerns or problems)

1. Check that the **Vac** and **HT** buttons are lit on microscope panel. If the **Vac** and/or **HT** buttons are not lit, please contact lab manager immediately, do not proceed any further.
2. Ensure that the camera unit is on (check side cabinet, on the left of the microscope). All 3 switches on the box should be on (in the up position) and temperature should read -25°C. (Double check with EM Manager if unsure)
3. Log in to the **microscope computer** using your username and password. Do not insert foreign objects on this computer, **it is essential for controlling the microscope** (do not charge your phone or other electronic devices; do not insert USB or external hard drives; only retrieve data from support computer sitting behind the curtain (data is copied nightly)).
4. Open the Tecnai User Interface and Digital Micrograph applications by double clicking on the icons.
5. Check that the **High Tension** (HT), **Operate** and **Power** buttons are lit (yellow) on the Tecnai User Interface. **If they are not, please contact the lab manager immediately, do not proceed any further.**
6. Verify that the microscope is at the appropriate operating voltages (HT at 200kV and extraction voltage at 3850V).
7. Check that the microscope status says **Column Valves** (button on user interface is yellow). This means that the column valves are closed (For the column valves, yellow = closed; grey = open).
8. Check the **Vacuum Overview** page by clicking on the Tecnai User Interface lower right menu. IGP1 should read 6 and IGP4 should be at or near 1 (unless it's the summer, in which case it will be hovering around 6-10). The IGPs maintain the vacuum on different parts of the microscope. IGP1 and IGP 4 maintain the vacuum on the column itself, therefore, to minimize contamination of the sample and (more importantly) of the column, the microscope should ONLY be operated when these values are in the optimal range.



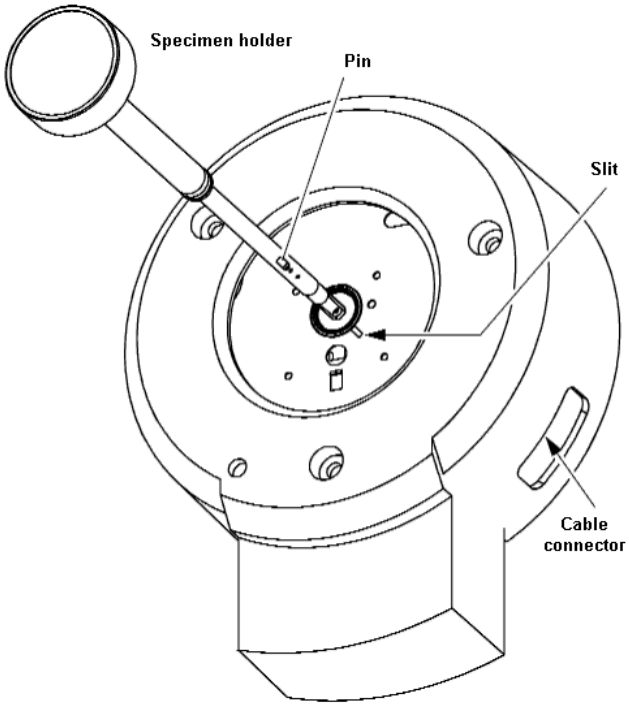


9. Fill the liquid nitrogen dewar. You'll need to fill it 2x initially. The first time a lot of LN2 will quickly boil off. Wait 10-15 mins after initial fill up and then top off. This should last ~4hrs. In total, for an 8hr work day, you'll need to fill the anti-contaminator 3x. **Check every 2-4 hours** and add nitrogen as needed.
10. If all these conditions are met, you are OK to proceed.

II. Room temperature (RT) holder insertion:

The compustage:

The compustage is composed of the airlock and the stage itself. The stage is computer controlled (can move in X, Y and Z as needed, and also tilt). The airlock separates the holder from ambient. A timer is set when inserting samples so as to evacuate (with assistance from the Turbo pump) the airlock and ensure that the vacuum of the microscope is maintained. The part of the holder beyond the O-ring will be in contact with the high vacuum of the scope. Do not, under any circumstances touch this area. The oil from your fingers will contaminate the vacuum as well as your sample. This will cause the vacuum to crash or at the very least degrade (the readings in IGPs 1 and 4 will not be optimal). **Do not touch anything beyond the O-ring.**



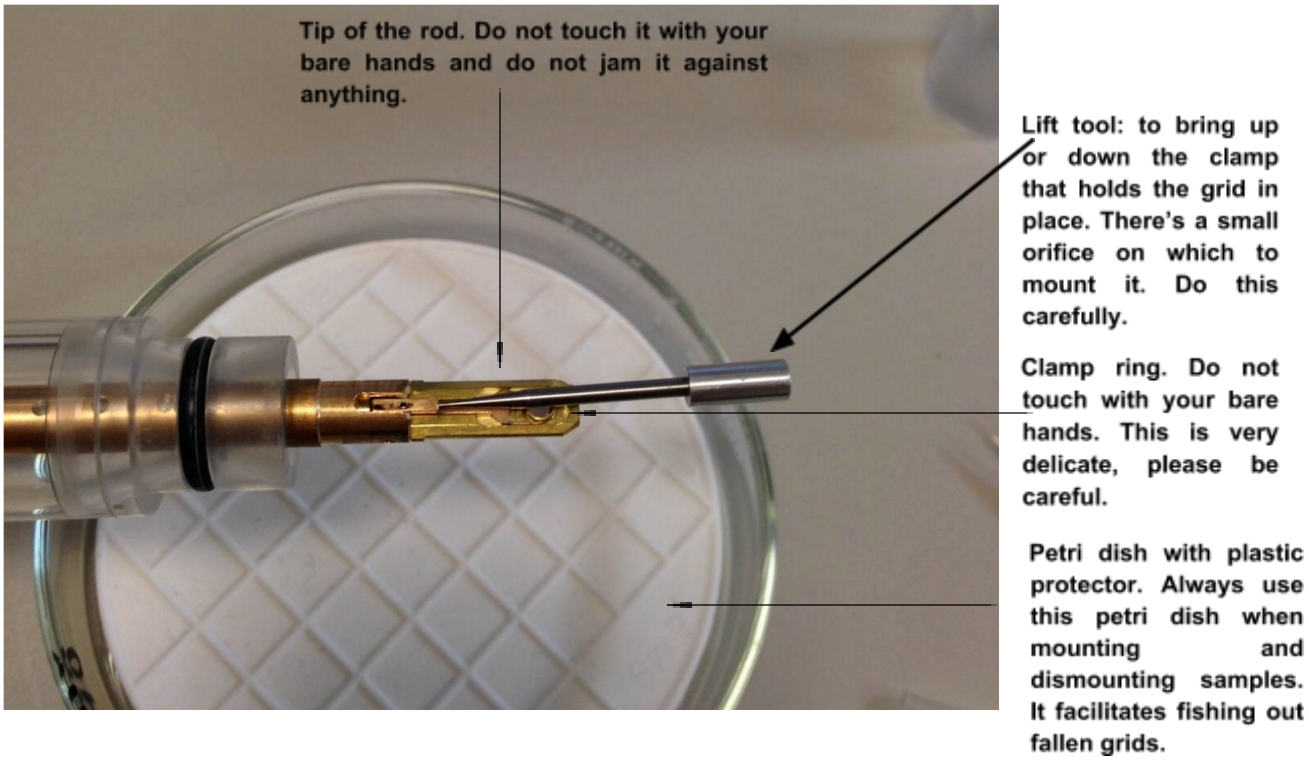
Schematic of the compustage (from: http://zhanglab.net/tem_help/tem/spechand.htm)

- Alignments should be done with either a platinum iridium specimen (PtIR) or a waffle grid (with or without latex spheres). This grid should be carefully mounted on the room temperature (RT) holder and returned to its proper receptacle once you're done aligning the microscope. If you can't find either grid or you lose it, notify the EM manager immediately. **Do not touch anything in the rod with your bare hands, especially beyond the O-rings, or you'll contaminate the rod, your sample and the microscope.**

Procedure:

During specimen insertion and retraction you should **not** need to use considerable force to load specimens. **Do not force or jam anything.** It could damage the compustage and/or holder. Contact the EM manager if you notice anything unusual (noises, smells, sounds, anything out of the ordinary could signal trouble. When in doubt, please ask).

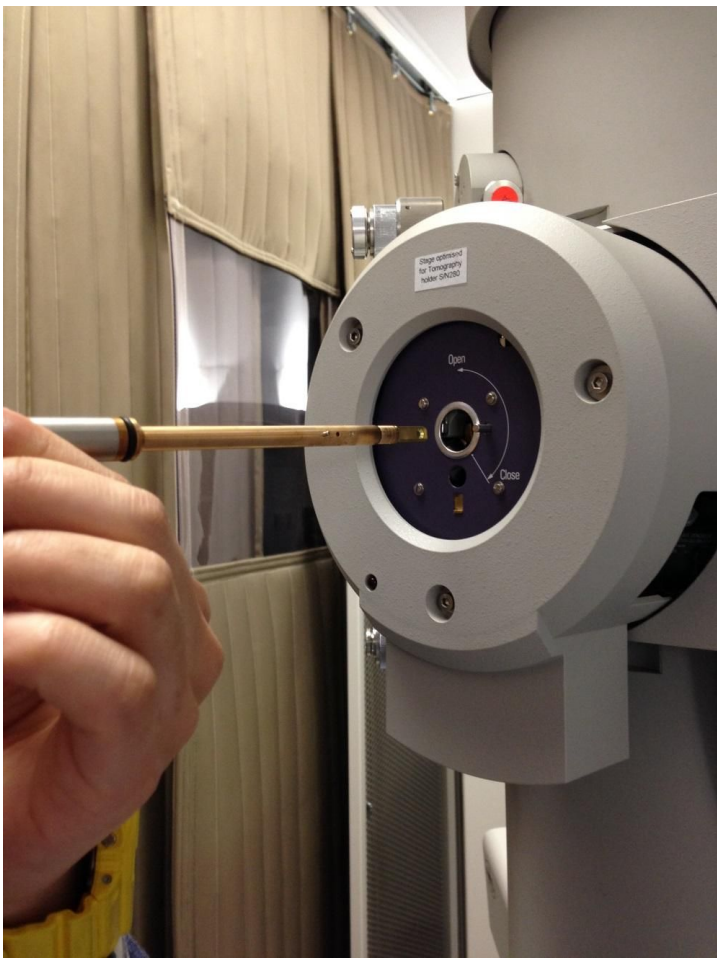
1. Make sure that the Column Valves are closed (Setup Tab, Column Valves closed is yellow).
2. Verify that the red light on the compustage is off.
3. Make sure the stage is at 0,0,0 in X, Y and Z by going to the Search Tab, Stage 2 menu, flaput window, and click on Holder. This will 'Home' the stage.
4. Turn on the Turbo pump on the Setup Tab (click on Turbo On, the button will go from grey to orange and when the turbo is ready, it will be yellow).
5. Get the RT holder, remove the plastic covering at the end.



6. With the tool that's in the back part of the mounting station, place it in the small orifice in the tip area (see picture) and carefully lift the clamp (from 3 o'clock to 12 o'clock).

EM grid

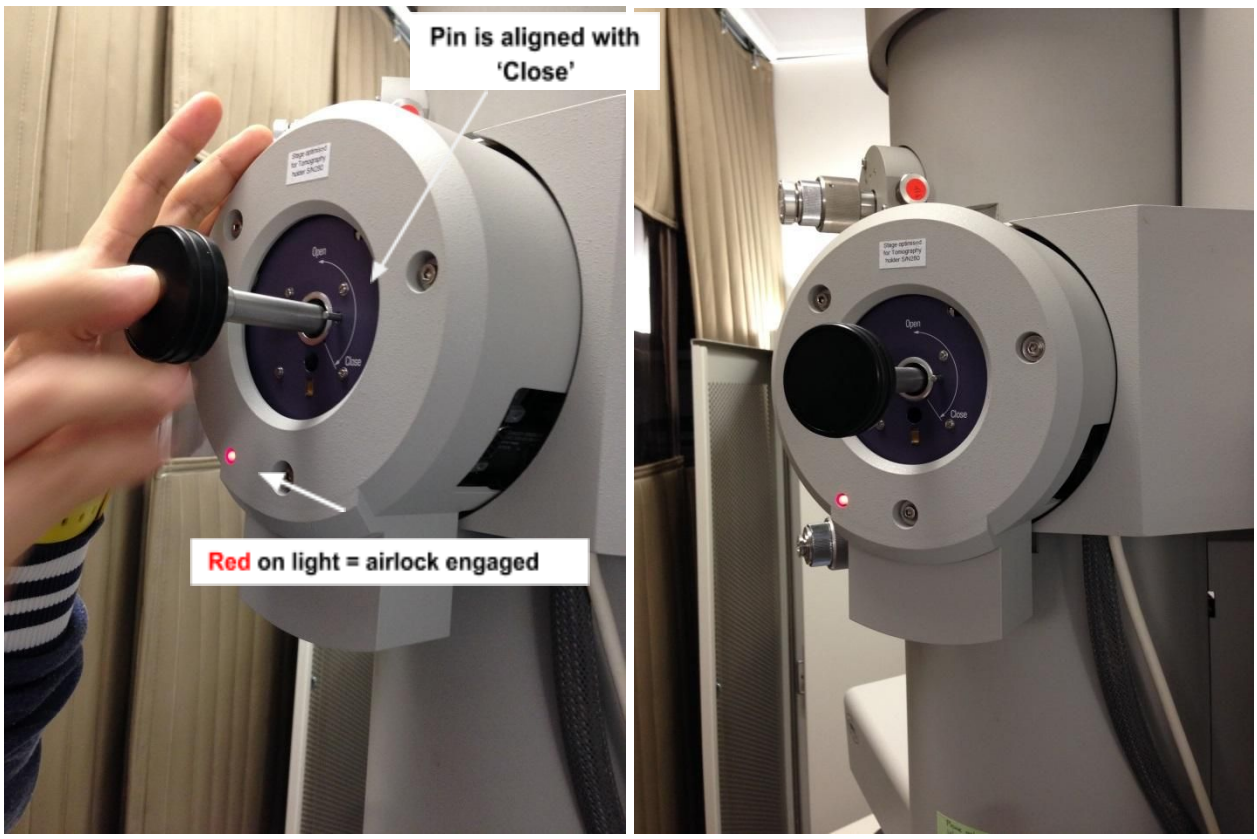
7. Carefully place your grid in the opening on the tip. Make sure it resides in the circular recess of the tip.
8. With the tool, carefully bring down the clamp until it securely fastens the grid and clamp. Do this with care so as to avoid jamming the ring onto the grid.
9. Return the tool to its resting place. Replace the plastic covering on the tip. Turn the holder upside down and gently tap the back (black part) a few times to verify that the clamp was positioned properly and that the grid is mounted correctly and stays in place.
10. Take the holder out of the plastic cover and align its pin to the 3 o'clock position.



Pin is at 3 o'clock position; the pin is facing the right

Airlock light. It will turn red while doing the insertion and when the airlock is engaged

11. Carefully insert the holder in this position, then turn to the 5 o'clock position (this aligns the pin to the 'Close' word on the outside of the compustage.



12. If the airlock is engaged, the red light will light up on the compustage. Once the airlock is engaged, do not push, jam or otherwise force the holder into the compustage.
13. Select Single Tilt Holder on the Tecnai User Interface.
14. There will be a wait time of 60-90s while the airlock is evacuated.
15. Watch out for when the red light turns off and the counter on the User Interface reaches 0s.
16. Securely turn the RT holder from the 5 o'clock position to the 12 o'clock position and gently guide the rest of the holder into the column.

17. Make sure the black part of the holder is flush with the compustage.
18. Turn off the Turbo pump.
19. Wait ~10 minutes to open the column valves, so as to give time to the stage to stabilize

III. Alignments

- The purpose of the alignment of the microscope is to ensure that the beam is parallel to the column and that the apertures are properly centered.
- Alignments should be done before every imaging session.
- Only load the most recent alignments file, made by the FEI engineer. Do not save your own alignment file. It will be deleted. The recent alignment made by FEI will only need to be touched up and it's a good place to start.

1. DO NOT DO ALIGNMENTS IN LOW DOSE
2. Make sure that “Low Dose” is turned off (the box will be gray)
3. Remove the Objective Aperture: Move the small pin on the Objective aperture to the right or to where the point is pointing to the specimen holder. This allows you to do an alignment without the objective interfering
4. Add a spacer to the sample holder in the compustage. ***this must be done with the column valves closed!*** The spacer is typically a BIC pen cap. This spacer allows for the sample to be removed from the beam path without breaking the seal of the sample chamber. The spacer makes the alignment process easier.
5. Wait for the column vacuum to recover after inserting a sample. This takes ~10 minutes. You want IPG1=6 and IGP4=1. These values can be found in “Vacuum Overview.” This is displayed on the right side of the left computer monitor (If it is not there then it can be selected in the bottom right corner of the left computer monitor).
6. Open column valves.
   Check for the beam; If there is no beam try one of the following:
   1) Move the stage (only if a sample is in the scope/beam path)
   2) Lower the magnification
   3) If you can’t find the beam call/find me. There may be a bigger problem
7. Eucentric Focus: Click on Eucentric Focus button located on the right panel, this should take you within a few microns of true focus.
8. Load Alignment file: Go to the Alignments tab, click on the flapout window and under File, find the most recent alignments file for the appropriate voltage (120 or 200kV). Click on the file, on the Available box under the alignments file, double-click on the all the alignments, then click apply. *The beam may go away but will come back when you load the FEG registry.
9. Load FEG registry: Go to the Search tab. Under Registries, find the most recent file. Click on the file, then click Set. This will change your spot size to 3. Change the spot size back to 5.
10. Verify Settings: Verify that you your settings are correct (magnification, spot size, beam position). *Typically I use Mag= 135,000x, Spot size = 5, and center the beam using the trackball. ** The intensity of the beam will need to be adjusted when changing the magnification
11. Make the beam round. Check this at high magnification (~135kx). Condense the beam using the intensity knob to the size of a dime or slightly smaller. At high magnification (~135kx) the beam may look triangular. Go to Alignment tab and click on Condenser. Right click on the current setting and copy the setting to another location (ie. copy 3 to 1). Use the Multifunction X and Y knobs on the control panel to make the beam round. Only adjust one multifunction knob at a time. Click “none” when finished.

12. Select C2 aperture: Determine which C2 aperture to use (as of 3/28/16).
    Position 1 = 50
    Position 2 = 70
    Position 3 = 100 *most commonly used for negative stain
    Position 4 = 150
13. Center the Condenser aperture (C2): Using the intensity knob, condense the beam and go through crossover and monitor that the beam spreads evenly about the center. If adjustments are needed, center the condenser aperture (C2-manually) by very slightly turning the center and left-side knobs of the C2 Aperture until the beam is centered on both sides of crossover.
14. Check that the beam is still round. If not follow the procedure outlined in step #11
15. Gun Tilt: Lower the magnification to ~10,000x. To adjust the gun tilt (minimize exposure time) go to the Tune tab and select "Gun Tilt" under Direct Alignments. Bring the large screen down, move the small screen into the beam path (physical tab to the left of the microscope). Using the multifunction knobs (set to gun tilt) adjust the brightness. Keep an eye on the exposure time ("Meas. Exp." on the monitor) to make it as low as possible. Click done when finished with alignment. Move the small screen back to its original position (out of the beam path).
16. Gun Shift: Perform the gun shift in Alignments. Click on Gun Shift and follow the directions. The magnification and spot size will be set automatically. The idea behind this alignment is to condense the beam and using the multifunction X and Y knobs to center the beam. Follow the instruction in the left hand panel in the FEI user interface. In order to get a more accurate alignment be sure to click "Normalize All" (typically set to L2 on the left control pad) before and after each step. Repeat the entire procedure 2-3 times until the beam remains in the center. Click "done" when finished with alignment.
    *The gun shift needs to be done with a sample in the microscope (or a sample in the scope with a spacer/pen cap in place). IF not then the the microscope will beep and you will get an error message which says "x-ray safety: Spotsize clipped to 5."
17. Beam Tilt X and Y: Adjust beam tilt pivot points. Change the magnification to 10,000x. Condense the beam to a dot. Click on Beam Tilt ppX under Direct Alignments. Adjust the beam so that the brightest spot is around the center, not the edges (with multifunction knobs). Click "done" when finished. Do the same for ppY and click "done" when finished. Change the magnification to 135,000x and re-do both pivot points X and Y.
18. Beam Shift: Adjust beam center by clicking on Beam Shift under Direct Alignments, then center with multifunction knobs. Click "done" when finished.
19. Remove spacer: Close the column valves and then remove the spacer/pen cap from the compustage
20. Wobbler: Go to the Search tab, flapout menu and click on Wobbler. This is to set up the proper Z-height (eucentric height). The goal is to minimize the movement (wobbling) and only have the sample move evenly about the Z axis. Adjust Z by clicking on the +/- buttons on the right control pad until the movement is minimized (it is suggested that you find a feature on the grid or a grid bar to keep track of the movement). Once done, click on Wobbler (the button will go from yellow to grey, it takes a moment to come to a full stop).
21. Rotation center: With the screen down and the small screen inserted, click on Rotation Center under Direct Alignments and minimize the beam movement, making it wobble evenly about the center. Set the multifunction knobs to the lowest movement possible and carefully minimize the wobbling.

22. Select Objective aperture: Determine which objective aperture to use (as of 3/28/16).
    Position 1 = 10
    Position 2 = 20
    Position 3 = 40 *most commonly used for Negative stain
    Position 4 = 100
23. Diffraction: If the objective aperture is not inserted, insert it to the appropriate setting (if the small pin is pointing to the right/toward the specimen holder then the objective is out, if the small pin points to the left the objective is in). Click on the Diffraction button on the right control panel and condense the beam to a small point. Verify that the aperture is centered appropriately by observing the halo around the small point (it should be even and continuous). Adjust as needed (usually very minimal change) and exit diffraction mode by clicking the diffraction button.
24. Check C2 stigmation: Condense the beam, go through crossover and verify that the beam is round and even. Correct if needed by going to Tune and select Condenser. (To ensure that you can get back to where you started right click on the highlighted settings and copy them to another panel ie. “copy 3 to 1”) Use the multifunction knobs to adjust the beam to make it round. Click done when finished
25. Check objective stigmation (done with the CCD): Find focus (can be done using the Live View on Digital Micrograph or by inserting the small screen and adjusting the focus until all contrast has disappeared). Once focus has been found select reset defocus (typically R2 on the right control board), start live view and go between 0.25-0.5nm underfocus (negative value). Observe the shape of the FFT of the image. If it isn't round, go to Stigmator, select Objective and adjust with the multifunction knobs until the FFT of the image is an even circle. Select none when finished
26. Turn on Low Dose (If working in Low Dose mode) by clicking on the LD button, goes from grey to yellow). This is where you will want to change/determine which magnification to use while imaging. Most negative stain projects will use a focus magnification of 100 kx with an exposure magnification of 62 kx-80 kx (*These values are good starting points). With the large screen down, verify that the beam is centered in all imaging modes (Search, Focus, Exposure). *Center the beam in exposure first!!! Verify that the beam is centered and spread to the desired level in all modes and check that the spot size is correct. Once the beam settings are correct, click through the 3 modes a few times while centering the beam to minimize hysteresis (beam movement), when changing from one mode to the next. *If the beam isn’t staying centered ask for help!
27. Check the liquid nitrogen in the dewar (fill every 2-3 hours).
28. When leaving the microscope for any reason do the following:
    a Close column valves
    b Stop the camera
    c Retract the camera
    d Put the screen down

IV. End of Session

1. Close column valves
2. Retract the campera
3. Put the screen down
4. Home the stage (go to Search-Stage and select the tab - Navigate to Control and click on Holder, this ensures that the stage is at 0um in X, Y and Z.
5. Remove the holder carefully.
6. Click on cryo cycle. Make sure the cryo cycle will run for 365 minutes or more.
   Wait ~5 minutes or for Turbo to turn yellow before removing the liquid nitrogen dewar
7. Dismount the specimen from the holder and dispose of it (or save it) properly.
8. Once the Turbo turns yellow, remove the dewar from the microscope and dispose of the remaining LN2 in the foam container under the table.
9. Place the protective fabric underneath the anti-contaminator.
10. If using the RT or RT tomography holders, return them to their proper receptacle.
11. If using the Gatan cryo holder(s), return to the dry pumping station and follow the instructions there on how to warm it and pump it properly.
12. Please sign up on the user's sheet. Record the actual time spent imaging.
13. Return the next day to retrieve Data. Data will be moved overnight to the computer located behind the curtain in the F20 room

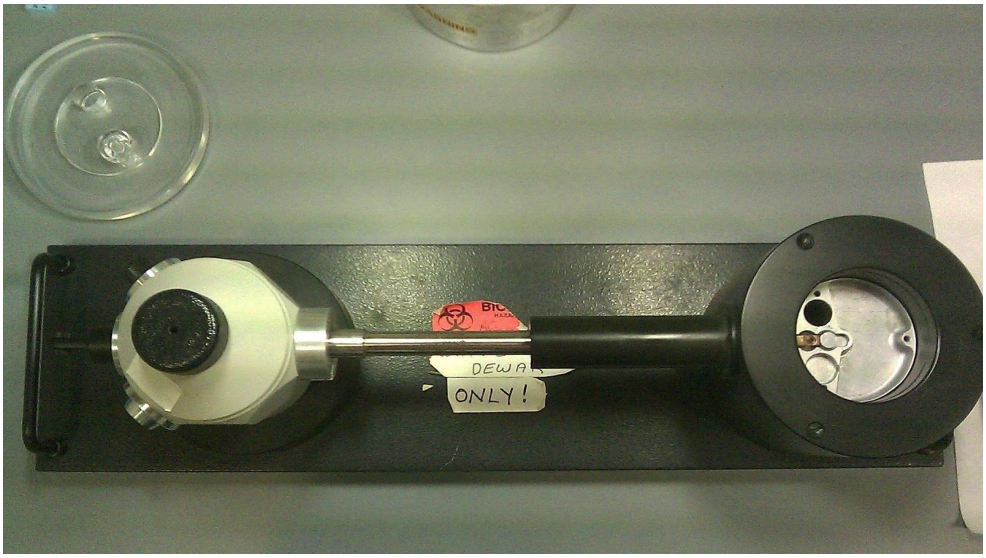
V. Imaging

1. Open Digital Micrograph
2. Set exposure time to 1 second

3. Want the mean value to equal ~1000

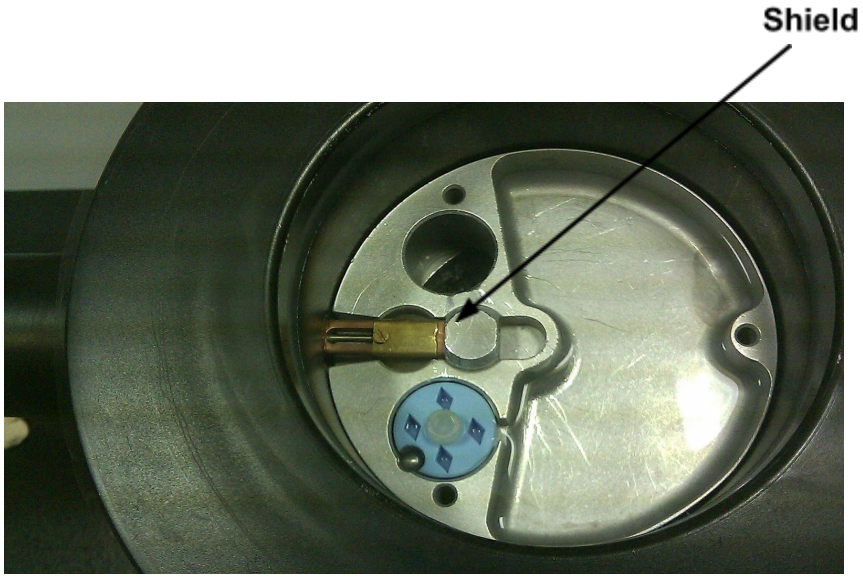
VI. **Sample Loading (cryo)**

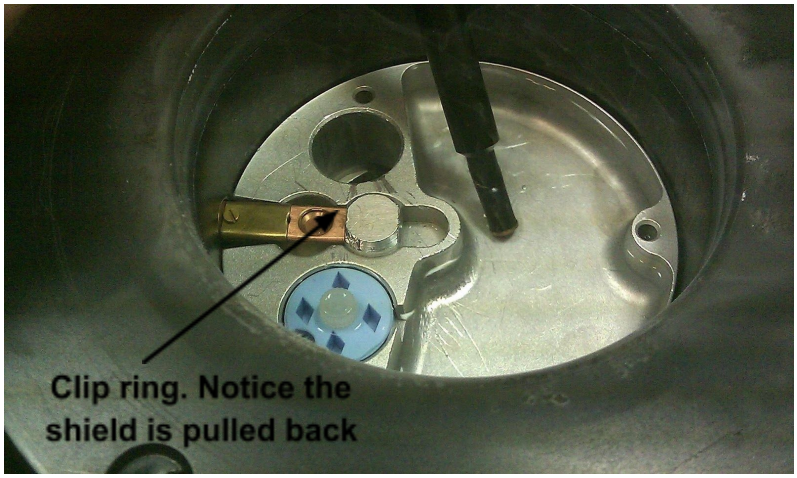
1. Make sure the anti-contaminator dewar is filled with liquid nitrogen (LN2).
2. The cryo holder should be pumped at least 2hrs in the Dry Pumping Station (see separate protocol) before use. Preferably, you'll have pumped the holder the day before for at least 6hrs.
3. Place the cryo transfer station on the table top. Insert the cryo holder into the station and remove the clip ring and previous grid if present.



4. Make sure you have gathered your cryo sample in transfer dewar, forceps, large forceps for grid box transfer, and anything else needed and place it near the transfer station.
5. Add LN2 to both the cryo transfer station and the cryo-holder. These will boil at different times. Do not overfill the holder nor the station, this will only cause the holder to become icy and the transfer station to frost.
6. Plug in the control unit cord and turn on the temperature control unit.
7. Tilt the compustage to -60° by going to the Search tab, flapout window, Control tab, Alpha toggle, type in -60° and click Set Alpha. Turn the Turbo on.
8. Check that the airlock pump time is set to 60-90s (Setup tab, flapout window, Settings tab, Default airlock time: 60s).

9. Cool the large forceps and transfer the grid box into the position in the transfer station. Cool the screwdriver if needed and loosen the top on the cryo grid box and place the cover on the transfer station.
10. Continue adding LN2 as needed. You want to keep the temperature at least -175°C or colder (to ~ -197°C).
11. Cool the following in the recessed bowl area: clipring tool (silver with black knobs) and attached clipring and forceps for the grid transfer .
12. Open the shield on the cryo holder and transfer a grid from the grid box to the recessed grid location on the cryo holder. Place the clip ring on (you should hear a click) and then hold the shaft and turn the knob until both feet of the tool are together. Gently pull up with the clipring tool.





13. Verify that the clip ring is securely held in place with the cold black rod (clipring should not move). If clipring is loose, cool down the knob end of the clipring tool and gently press on the clip ring.
14. Close the shield on the cryo holder and remove the tools from the transfer station.
15. Continue adding LN2 as needed to the cryo transfer station but do not overflow.
16. Tighten the grid box lid and remove from transfer station.
17. Carefully remove the cryo holder from the transfer station and insert it into the microscope.

18. The guide pin on the cryo holder will be at the 12 o'clock position and must be quickly rotated clockwise to the 3 o'clock position so that the airlock is triggered and the pre-
    pump begins. When the position of the holder is correct, the holder will slide into the airlock about 1 inch further.
19. Select the ST Cryo- holder option (bottom of the Tecnai User Interface).
20. Wait for the airlock pumping cycle to finish (60-90s) as indicated by the timer on the UI.
21. When the holder is upright, reset the stage tilt to 0° by going to the Search tab, flapout window, Control tab, Alpha toggle and click Set Alpha (this will return the stage to 0°).
22. Gently allow the vacuum to pull the holder into position.
23. Refill the dewar on the cryo holder. Use the de-bubbler (rubber stopper with tubing) to remove the any excess LN2 from the neck of the cryo-holder.
24. Remove any remaining LN2 from the transfer station and allow the tools used to warm up and dry off.
25. Wait ~10-15 minutes before opening the column valves. Because there is a lot of movement happening during insertion, the grid will drift a lot until it settles down. Ideally the column vacuum (IGP1) will return to 6 before viewing the sample.

VII. End of Session

14. Close column valves
15. Retract the campera
16. Put the screen down
17. Home the stage (go to the Stage tab - Control and click on Holder, this ensures that the stage is at 0um in X, Y and Z.
18. Remove the holder carefully.
19. Click on cryo cycle. Make sure the cryo cycle will run for 365 minutes or more.
    Wait ~5 minutes or for Turbo to turn yellow before removing the liquid nitrogen dewar
20. Remove the dewar from the microscope and dispose of the remaining LN2 in the foam container under the table.
21. Place the protective fabric underneath the anti-contaminator.
22. Dismount the specimen from the holder and dispose of it (or save it) properly.
23. If using the RT or RT tomography holders, return them to their proper receptacle.
24. If using the Gatan cryo holder(s), return to the dry pumping station and follow the instructions there on how to warm it and pump it properly.
25. Close Digital Micrograph and the Tecnai User Interface and log out.
26. Please sign up on the user's sheet. Record the actual time spent imaging.
27. Return the next day to retrieve Data. Data will be moved overnight to the computer located behind the curtain in the F20 room