USER GUIDE FOR CRYO-TEM ON THE TALOS L120C TRANSMISSION ELECTRON MICROSCOPE

Bio-Imaging Center (Updated 11/10/2023)

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Cryo-TEM User Manual for the Talos L120C

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Section 1: Microscope Prerequisites

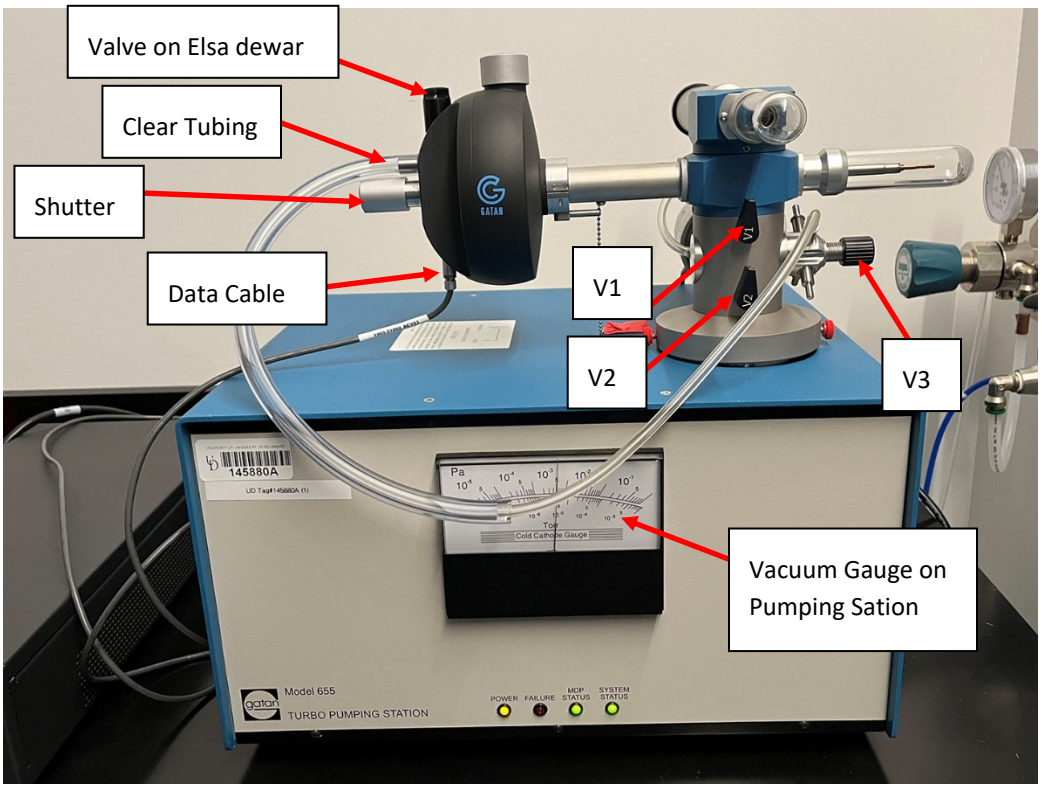
1. The anti-contaminator must be filled with liquid nitrogen. This will not only cool the anti-contaminator to improve the vacuum of the microscope, but it will also cool the cryo-box which is necessary for cryo-TEM imaging.
2. Direct alignments should be performed with a room temperature specimen before starting cryo.
    a. Perform direct alignments (or auto-tuning) for microprobe mode
    b. Perform direct alignments (or auto-tuning) for nanoprobe mode
    c. Microprobe and nanoprobe have a separate set of alignments, so they must both be performed as both modes will be used for low dose imaging.
3. Set the microscope to LM 210X. This is the minimum mag that can be used without the cryo-box being visible on the FluCam. Ensure the beam is centered and adequately spread out to fill the field of view.
4. Ensure the pre-specimen shutter is used for the camera. This will prevent the grid from being continuously irradiated between camera exposures.
5. Close the column valves, reset the holder, and commence freezing grids and cooling the cryo-holder.

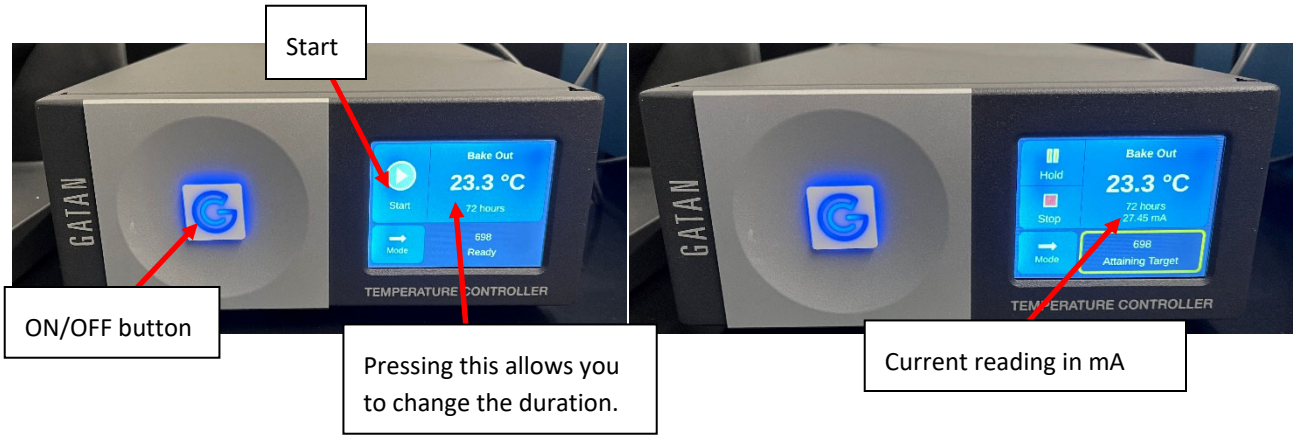
Section 2: Regenerating the Elsa Holder

The Elsa cryo-TEM holder must periodically be baked out to regenerate the vacuum of the holder. If the holder is not properly baked out, it will begin to sweat with condensation when it is cooled, and it will not maintain temperature.

Procedure to Bake-out the Elsa Holder:

1. Insert the Elsa holder into the port on the Gatan Model 655 Turbo Pumping Station.
2. Attach the clear tubing to the port on the back of the Elsa holder.
3. Turn on the Turbo Pumping Station (switch is on the back).
4. Ensure V1 and V2 are open (pointed up). Wait until the vacuum is about halfway on the gauge.
5. Open V3. Open V3 counterclockwise until the knob stops and then rotate one-half turn clockwise. Wait for the vacuum to recover to about halfway on the gauge.
6. Open the valve on the Elsa holder. Open it counterclockwise until the knob stops and then rotate one-half turn clockwise. Wait for the vacuum to recover to about halfway on the gauge.
7. Attach the data cable to the Elsa holder.
8. Start a bake-out cycle on the temperature controller.
    a. If the Elsa holder has been used regularly, an overnight 16-hour bake-out is sufficient.
    b. If the Elsa holder has not been used for several weeks, perform a 72-hour bake-out.
    c. It is good practice to keep a log of the stable readout current (current reading the next morning)
        i. If the readout current starts to vary, there could be an issue with the cryo-holder, and it may need maintenance.





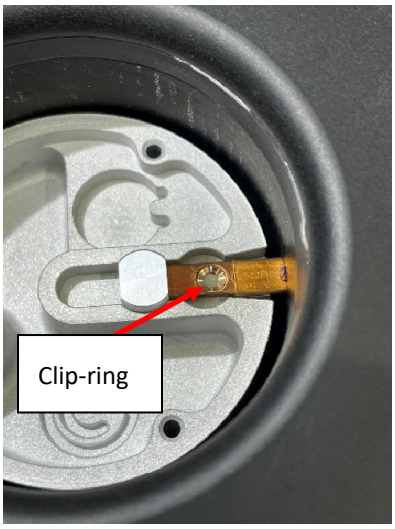
Procedure to Remove Elsa Holder After Bake-out:

It is important to follow these steps in this order or you may ruin the vacuum to the Elsa dewar.

1. With the turbo pumping station still running, close the valve on the Elsa holder.
2. Close V3 on the pumping station.
3. Turn off the Turbo Pumping Station.
4. Remove the clear plastic tubing from the back of the Elsa holder.
5. The Elsa holder can now be removed from the Turbo Pumping Station. It will put up some resistance as it is under a vacuum.

Section 3: Cooling the Cryo-TEM Holder and Loading a Vitrified Grid

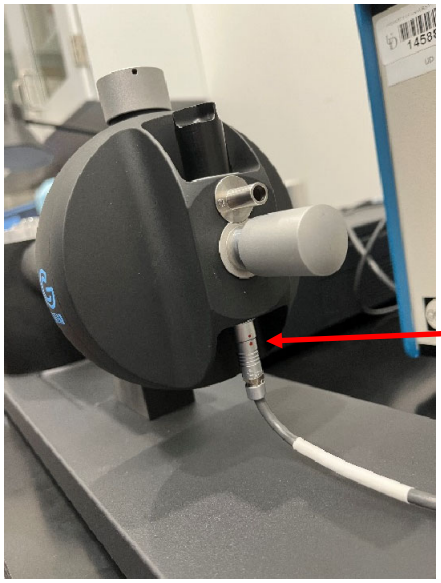
1. Insert the cryo-holder into the cryo-transfer station. Ensure the shutter is open and remove the clip-ring with the clip-ring tool. Remove any grid that may still be present.



Open shutter

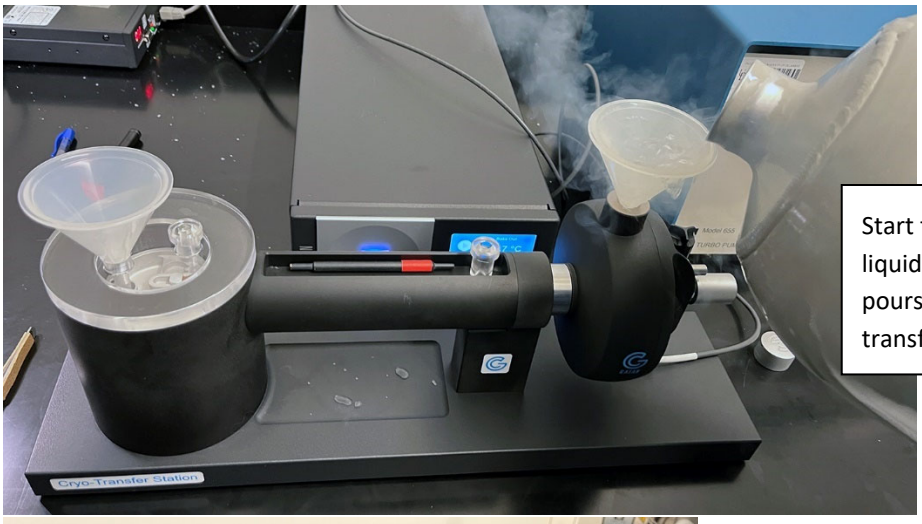
Remove clip-ring and any grid that may be in the holder.

2. Attach the data cable from the temperature controller to the Elsa holder.

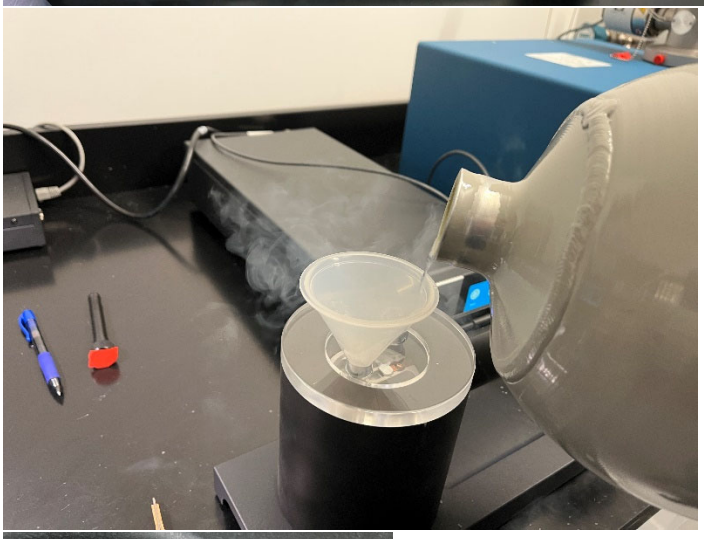


Red dot on data cable aligns with the red dot on Elsa holder.

3. Add liquid nitrogen to the dewar on the cryo-holder using the funnel. After several fills, switch to filling the cryo-transfer station. Do not fill the cryo-transfer station above the seam in the black plastic.



Start filling the Elsa holder with liquid nitrogen. After about 3 pours, switch to filling the cryo-transfer station.



Start filling the cryo-transfer station with liquid nitrogen.

Do not fill above this seam in the black plastic of the cryo-transfer station.

4. Repeat filling both the cryo-holder and cryo-transfer station until they are filled with liquid nitrogen.
5. Wait until the temperature stabilizes to -170˚ C.
    a. Note, if you submerse the tip of the holder under liquid nitrogen or pour liquid nitrogen onto the tip of the cryo-holder, it will temporarily give an artificially low reading. Let the tip equilibrate to get an accurate reading.
    b. Cooling the holder takes about 40 min.
    c. The holder should be cooled to a minimum -160˚ C, but -170˚ C is preferred. The devitrification temperature when the vitrified ice turns crystalline is at -150˚ C.
    d. Once cooled, the cryo-holder maintains its temperature for approximately 8h.

Section 4: Loading a Vitrified Grid

For the following steps, a surgical mask should be worn to prevent the moisture from your breath from crystallizing onto the surface of your grid or contaminating your liquid nitrogen.

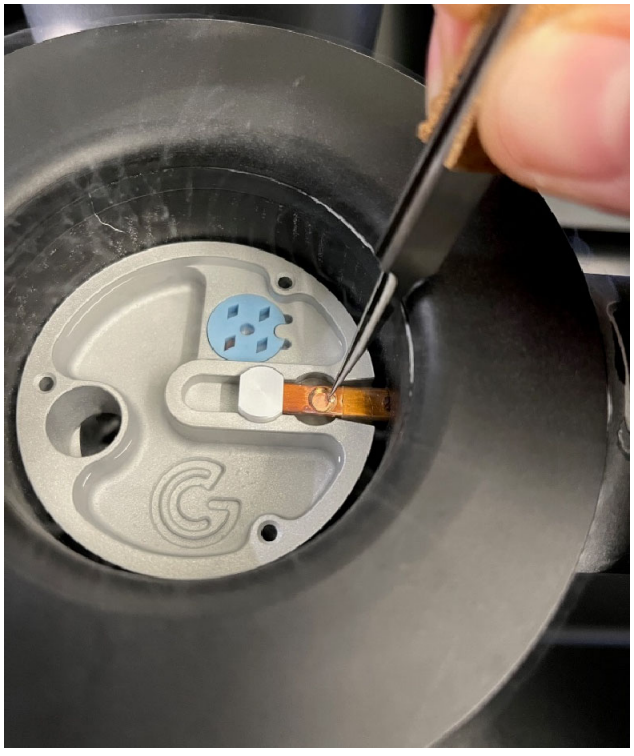
1. When the temperature has stabilized to -170˚ C or colder, it is safe to load a grid. First, lift the plexiglass lid to the cryo-transfer station and quickly transfer your cryo-grid box from the dewar to the cryo-transfer station.

Ensure the stable temp is -170˚C or colder.

Quickly transfer the cryo-grid box from the small LN_2 dewar to the cryo-transfer station

2. Any object that will contact your vitrified grid must be pre-cooled to liquid nitrogen temperatures to prevent re-crystallization of ice. Pre-cool your forceps by immersing them in the adjacent liquid nitrogen and wait until the violent bubbling subsides. I prefer to use forceps insulated with cork. Using the pre-cooled forceps, move the grid from the grid box to the tip of the holder. Ensure the grid is flat and centered.

Pre-cool the forceps in LN_2. Wait until the violent bubbling has stopped.



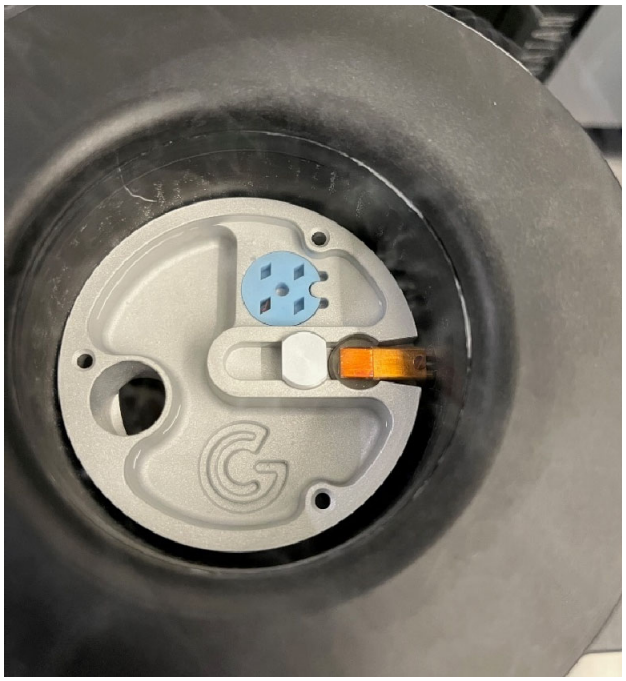
Move the vitrified grid from the cryo-grid box to the cryo-holder.

3. Pre-cool the clip-ring and clip-ring tool by immersing them in the adjacent liquid nitrogen and wait until the violent bubbling subsides. Position the clip-ring over the grid and press down until you hear a click. To separate the clip-ring from the clip-ring tool, rotate the clip-ring tool counterclockwise to unthread it from the clip-ring.

Pre-cool the clip-ring and clip-ring tool in LN_2. Wait until the violent bubbling has stopped.

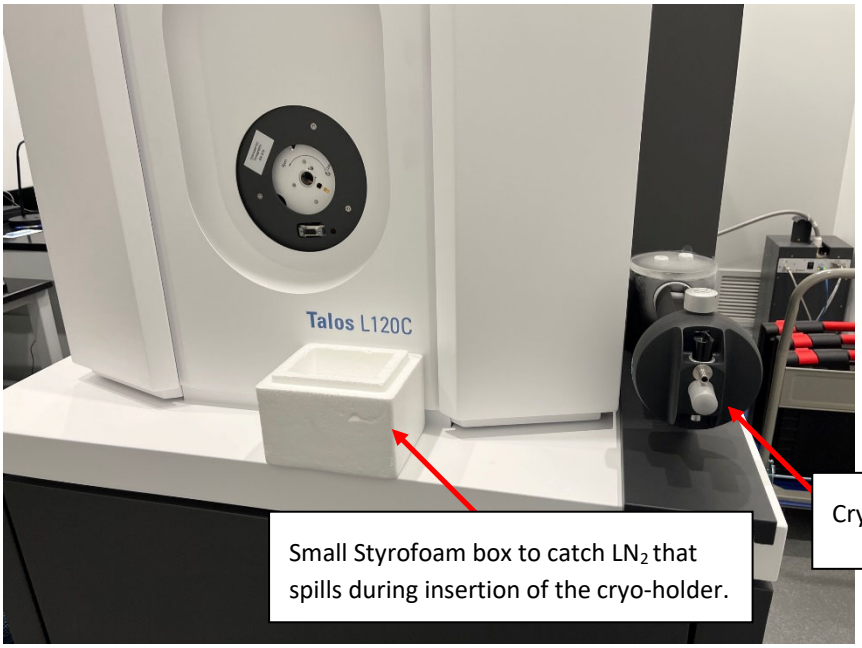
Use the clip-ring tool to press the clip-ring onto the grid. When it is engaged, you will hear a click. Rotate the clip-ring tool counterclockwise to disengage it from the clip-ring.

4. Close the shutter on the Elsa holder. This will help to protect the grid from getting atmospheric ice contamination during the sample insertion into the microscope.



Shutter is closed. Note: To close the shutter, you may have to slightly back the rod out of the cryo-transfer station due to the tight fit.

5. Pour liquid nitrogen onto the tip of the holder to cool it an additional amount just before transferring the cryo-holder into the microscope.
6. Place the plexiglass lid back on the cryo-transfer station.
7. Move the cryo-transfer station to be next to the Talos L120C TEM. You want to minimize the time it takes to get the cryo-holder into the vacuum of the microscope, so it is best to move the cryo-transfer station to be next the microscope. When moving the cryo-transfer station, please support the cryo-holder to prevent it from falling out of the cryo-transfer station during transport.



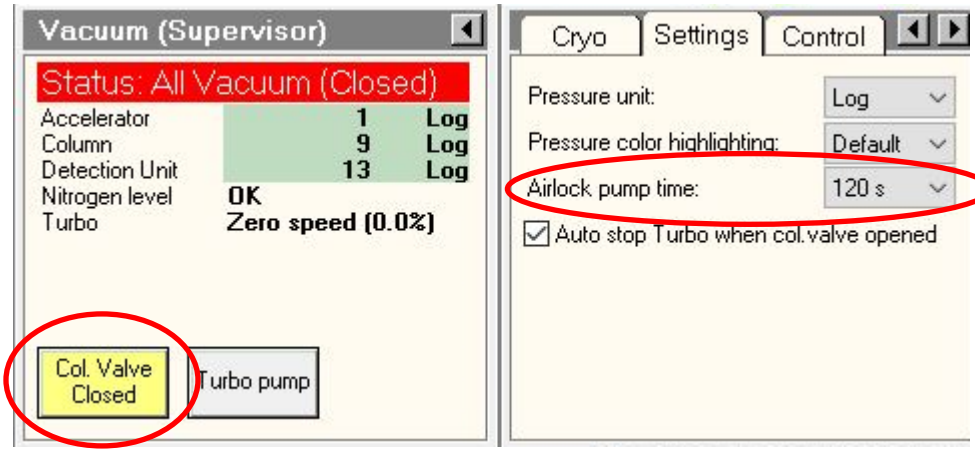
The cryo-holder should be next to the microscope when loading a frozen grid.

Cryo-holder in the cryo-transfer station.

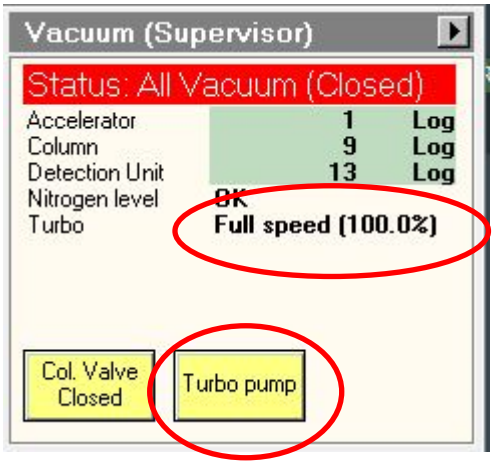
Small Styrofoam box to catch LN_2 that spills during insertion of the cryo-holder.

Section 5: Inserting the Cryo-Holder into the Microscope

1. Ensure the column valves are closed.
2. Ensure the air lock pump time is 120s.
    a. This is the **minimum** pump time for cryo-TEM.
    b. A 120s air lock pump time has worked well on our system with a cooled Elsa holder. Some systems need longer air lock times. If the vacuum regularly crashes during sample insertion of the cryo-holder at a 2 min air lock time, then this means the air lock time may need to be increased to 3-4 min.

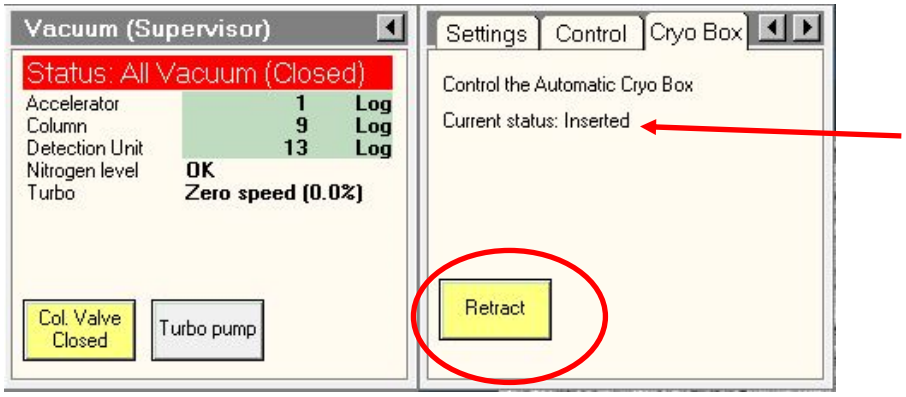


3. Turn on the Turbo Pump
    a. The turbo pump should be at 100% speed before you insert the cryo-holder to make sample insertion faster. You want the holder at high vacuum as soon as it is inserted into the microscope to prevent devitrification of the grid.

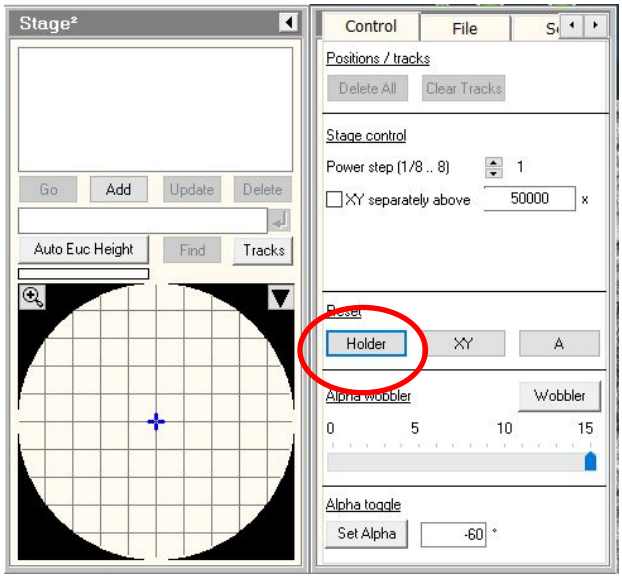


Very important! Ensure Turbo Pump is at full speed before inserting the cryo-holder.

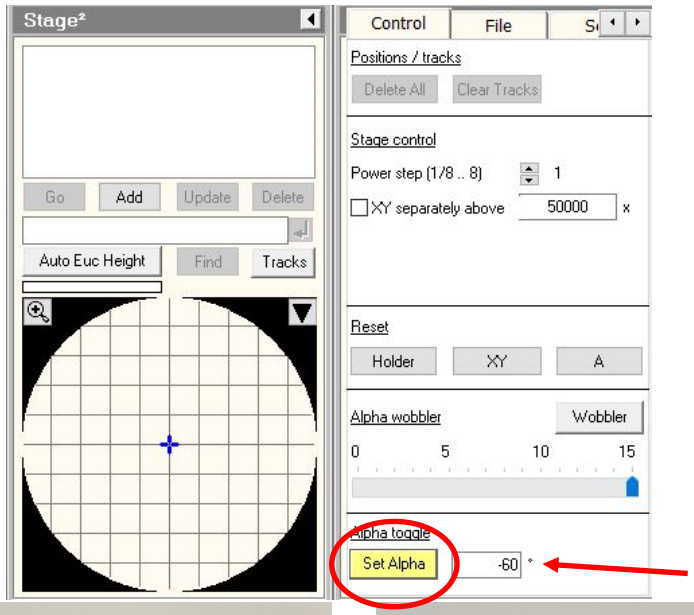
4. Insert the cryo-box
    a. The cryo-box is an aperture that sits above the sample.
    b. The cryo-box is cooled via the liquid nitrogen from the anti-contaminator, and it will provide a cold surface that will stick contaminants and prevent ice from re-crystallizing on top of the grid.



5. Remove the objective aperture.
    a. There should be adequate room for the cryo-holder with the objective aperture inserted, but it is a good practice to remove the objective aperture as an added safety measure. Some TEM systems have a very small polepiece gap so there is very little room between the sample and the objective aperture.
6. Reset the holder.
    a. This will ensure the X, Y, Z, and alpha- tilt of the stage are reset to zero coordinates.



7. Set the stage to -60˚ tilt.
    a. This pre-tilts the stage so less liquid nitrogen is spilled from the cryo-holder during sample insertion.
    b. Set alpha button
        i. The set alpha button allows you to toggle between a 0˚ tilt and a user-defined tilt value (ie. -60˚ tilt for cryo-holder insertion).

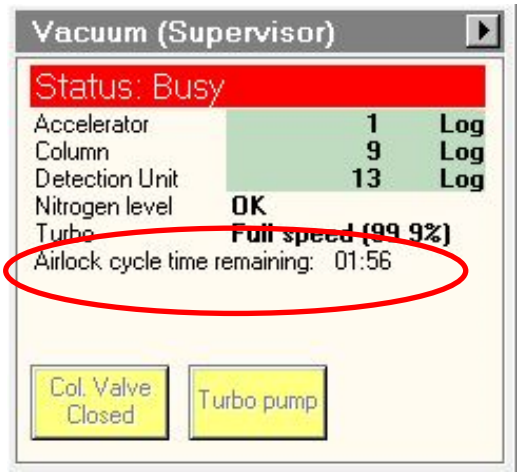


Alpha tilt is 0˚.

Alpha tilt is -60˚.

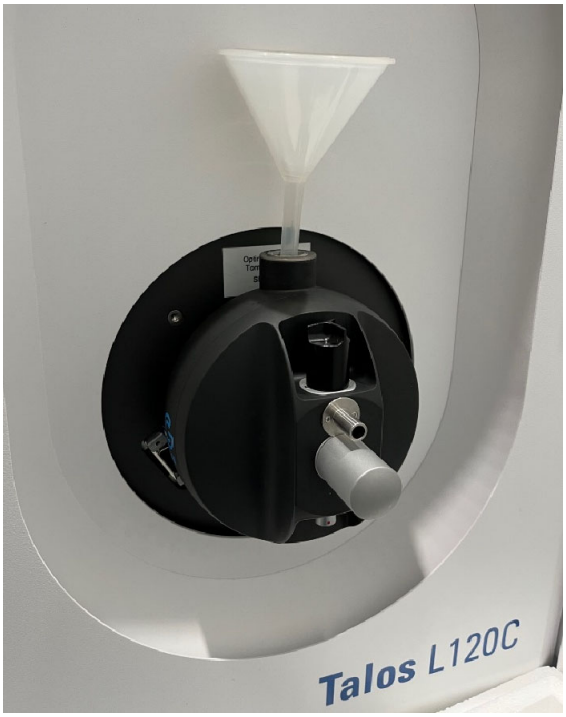
8. Insertion of the cryo-holder. For insertion of the cryo-holder, you will want to work quickly but in a controlled manner so as not to damage any of the equipment.
    a. Remove the cap to the cryo-holder as a lot of liquid nitrogen will spill during the insertion. There is a small Styrofoam container that can be used to catch the liquid nitrogen as it pours out.
    b. Since the stage is tilted to -60˚, you will have to rotate the holder 90˚ clockwise (the opening to the dewar will be pointed to the right) to be properly aligned for sample insertion.
    c. Once the holder is inserted, select cryo-holder from the dropdown menu in the TEM user interface.
    d. Wait for the 2-min air lock time. The red-light will turn off when you can complete the sample insertion.
    e. As soon as the red light turns off, immediately rotate the holder counterclockwise and slowly insert it into the column.

Some LN_2 will pour out of the cryo-holder dewar during sample insertion.



Wait for the airlock cycle time to countdown to zero and the red light on the stage to turn off before inserting the cryo-holder completely.

9. Once the holder is fully inserted, return the tilt to 0˚ by pressing “Set alpha”.
10. Refill the liquid nitrogen on the cryo-TEM holder using the funnel.

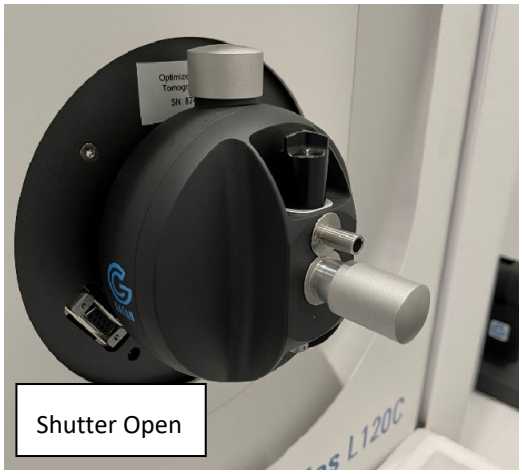


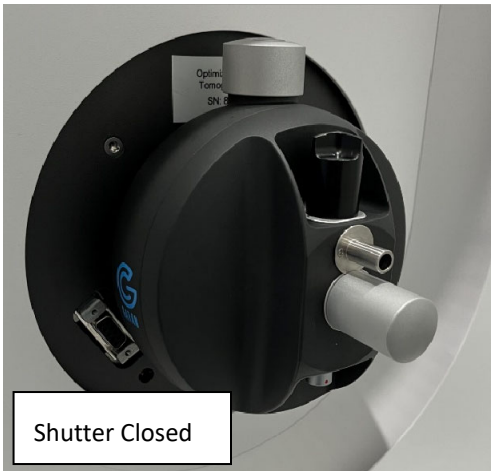
Refill the cryo-holder dewar with LN_2 using the funnel.

11. Open the shutter on the Elsa holder.
    a. Always open the shutter fully. Don’t leave it in a half-way state.
    b. Always support the cryo-holder with one hand while you open/close the shutter so as not to break the vacuum.
12. Let the holder stabilize for about 15-20 min before imaging. The temp of the holder must equilibrate in the vacuum of the microscope. If you try to image too early, there will be significant drift.

Section 6: Removing the Cryo-Holder from the Microscope

1. Close the shutter on the cryo-holder.
    a. Always close the shutter fully. Don't leave it in a half-way state.
    b. Always support the cryo-holder with one hand while you open/close the shutter so as not to break the vacuum.


Shutter Open


Shutter Closed

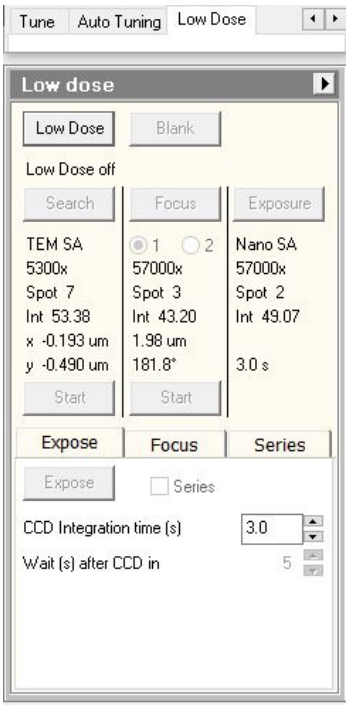
2. Ensure the column valves are closed.
3. Remove the objective aperture.
4. Reset the holder.
5. Remove the cap from the cryo-holder dewar. Liquid nitrogen will spill out of the holder during removal. This is normal.
6. Slowly pull back on the cryo-holder until you have room to press two fingers against the plate surrounding the sample rod. Slowly pull back on the cryo-holder while applying pressure to the surrounding plate until you feel it stop. By pressing against the plate with your fingers, this minimizes movement and reduces the odds of breaking the vacuum.
7. Rotate the cryo-holder slowly clockwise until you feel it stop.
8. As you continue to apply pressure to the plate with your fingers, slowly and gently pull back on the cryo-holder in a controlled manner until you feel it come out freely. If you plan on imaging another sample, quickly reinsert it into the cryo-transfer station and top off both the dewar and the cryo-transfer station with liquid nitrogen. If you will no longer be using the holder, remove the remaining liquid nitrogen from the cryo-holder by pouring it into the wide-mouth dewar. Place the holder in the pumping station and bake it out as described in Section 2.

Apply firm pressure to the plate while removing the cryo-rod to minimize the chances of breaking the vacuum.

Some LN_2 will pour out of the dewar during sample removal. It is okay! You can refill it as soon as the rod is returned to the cryo-transfer station.

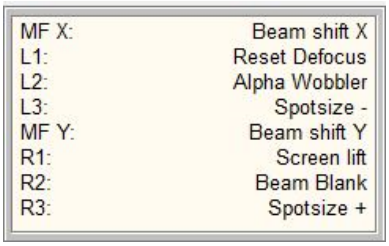
Section 7: Low Dose Imaging Module

1. Prior to imaging in Low Dose, direct alignments should be performed for both microprobe and nanoprobe modes as each mode has their own independent set of direct alignments.
2. Turn off Intensity Zoom if it is activated.
3. Locate grid squares with the appropriate ice thickness in LM.
    a. LM and the Cryo Box:
        i. At LM 115X, you will see the edges of the cryobox on the FluCam.
        ii. At LM 155X, most of the cryobox will not be visible (maybe just a corner).
        iii. At LM 210X, none of the cryobox will be visible on the FluCam.
    b. Use spot size 6-7 to limit the electron dose hitting the sample.
    c. Grid squares of interest can be stored under Stage^2.
    d. When in the LM magnification range, use the largest C2 aperture.
4. Increase the magnification to SA mode and blank the beam.
    a. Change the aperture to the smallest C2 aperture size, 50µm.
        i. The C2 aperture size controls the electron dose (flux) hitting the sample. The smaller the C2 aperture, the lower the electron dose.
        ii. The dose scales x^2 as a function of the C2 aperture diameter
5. Insert the objective aperture.
    a. Contrast in cryo-TEM is due to phase contrast rather than amplitude contrast.
    b. The objective aperture enhances amplitude contrast. There is still some limited amplitude contrast in cryo-TEM so the objective aperture will improve contrast in the image.
    c. The objective aperture can limit resolution in cryo-TEM images since it cuts off high scattering angles, which contain the highest resolution information in the images.
        i. However, the Talos L120C TEM is mostly limited in resolution by the LaB6 filament, and you can safely use the 100µm objective aperture without limiting resolution in the images.
6. Activate the Low Dose Server

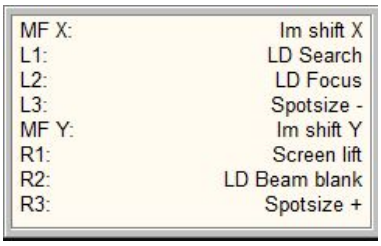


Low Dose Server. There are 3 modes: Search, Focus, and Exposure.

a. Attention: When the Low Dose Server is activated, the hot keys are reassigned to control functions within the low dose server. My personal preference is to use the mouse clickable buttons in the low dose server software and re-assign the shortcut keys L2 for alpha wobble and L1 for reset defocus.



Hotkey assignment under normal usage.



Hotkey assignment when the low dose server is activated.

b. Activate Search Mode
   i. Find the eucentric position with stage wobble.
   ii. Pick a spot size that provides enough signal so you can just see the holes in the quantifoil. You don't want to use too much dose to avoid damaging the sample, but you need enough dose to see features on the grid. Spot size 7-8 is a good starting point.
   iii. Search Mode must be in microprobe mode because search is done at lower magnifications. Nanoprobe is only useful at >10,000X magnification.
   iv. Note: The parameters for search, focus, and exposure modes in the low dose server update when you click off and then re-click back. If you decide to change magnification, intensity, spot size, etc when you are using search, focus, or exposure modes, when you click off, the new settings will be stored as the new parameters.
c. Activate Focus Mode
   i. Focus mode moves the beam to an area adjacent to your region of interest.
   ii. With Focus Mode, you want a lower spot size so you have enough signal to focus the image. You don't care about beam damage since the focus area is away from your region of interest. Bubbling due to beam damage may actually help with focusing.
   iii. Focus Mode is done in Nanoprobe Mode.
      1. Nanoprobe Mode allows you to achieve a smaller beam diameter at a much lower dose.
      2. If you condense the beam to the same diameter in microprobe mode that you have in nanoprobe mode, the dose will be much higher in microprobe than in nanoprobe.
   iv. Keep the beam diameter just larger than the FluScreen to minimize the area of the sample getting irradiated by the beam.
   v. A typical defocus range for cryo-TEM imaging is -0.5µm to -2.5µm. For small, low contrast samples, you may need to use a defocus value as high as -5µm.
   vi. You want the magnification settings for Focus Mode to be the same as Exposure Mode so that the focus is more accurate and to circumvent lens normalizations (normalizations cost more time)
d. Activate Exposure Mode
   i. The beam is automatically blanked until you press Exposure.
      1. The beam should be blanked above the specimen to protect it.
      2. Beam blanking can be checked by going to Microscope Software Launcher→Tools→Blanking

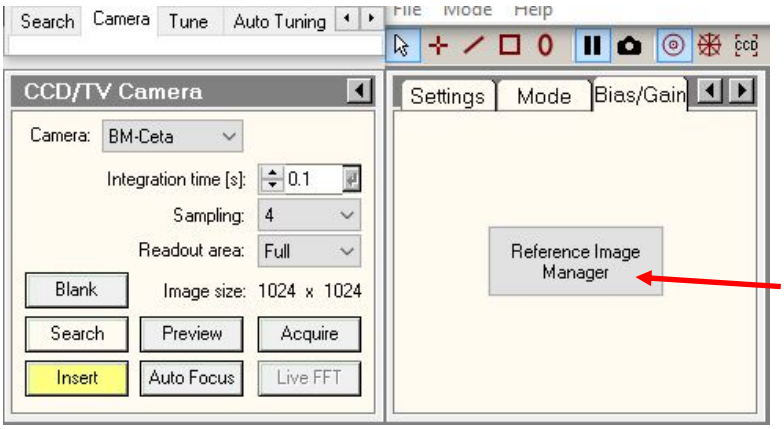
ii. Exposure Mode settings should be roughly the same as Focus Mode
    1. Magnification should be the same for focus and exposure modes for focus in the final acquired image to be accurate.
    2. Exposure mode should also be done in Nanoprobe Mode.
    3. Intensity of the beam should be similar in focus and exposure modes as long as the camera does not get saturated.
iii. Press exposure
    1. There will be a 5s wait time, and then it will take an image of the area of interest on the Ceta camera
    2. You must find a balance between exposure time and spot size.
        a. You can expose up to 3s, but too long of an exposure will suffer from drift in the image.
        b. Rather than having an exposure >3s, it is better to use a smaller spot size (to increase the dose) and a shorter exposure time.
iv. After you have pressed exposure, the Low Dose Server automatically returns to Focus Mode until you manually blank the beam.

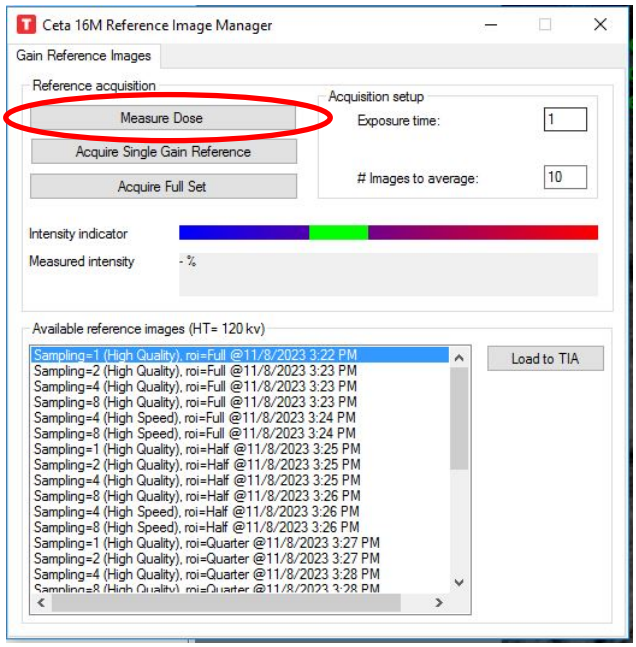
e. Notes on Search, Focus, and Exposure Modes
    i. The Low Dose Server was originally designed so that Search and Focus are done on the FluScreen and the final exposure is done on the Ceta camera using TIA software.
    ii. You can trick the Low Dose Server so that you can do all 3 modes on the Ceta camera in Velox. You just need to activate one of the modes, lift the FluScreen, and then click Play in Velox.

7. Troubleshooting the Low Dose Server
    a. If you go to Focus Mode and there is no beam:
        i. Drop magnification until you see the beam.
        ii. Use the **trackball (user-defined beam shift)** to center the beam.
        iii. Go back up in magnification.
    b. If you go to Exposure Mode and there is no beam in the acquired image:
        i. Go to Exposure Mode and unblank the beam.
        ii. You may have to drop the mag to find the beam.
        iii. Use the **Direct Alignments beam shift** to recenter the beam.
        iv. Go back up in magnification.
    c. Sometimes you must reiterate steps i and ii above to get Low Dose to work properly.
8. Dose
    a. For most cryo-TEM imaging, the total dose on the sample should not exceed ~40 electrons/$Å^2$/s.
        i. Too high of a dose causes beam damage.
        ii. Initially beam damage makes the proteins appear less defined and blurry.
        iii. At a high level of beam damage, you will see bubbling.
    b. Measuring Dose
        i. The dose should be measured on the Ceta camera for the most accurate reading.
        ii. Procedure
            1. Lift FluScreen
            2. Go to Reference Manager under the Camera Box in the TEM User Interface
                a. Click Measure Dose
                    i. It will provide a dose reading as electrons (pe) per pixel per second (pe/p/s).
                    ii. You must convert the pixels to angstroms based on your pixel size.

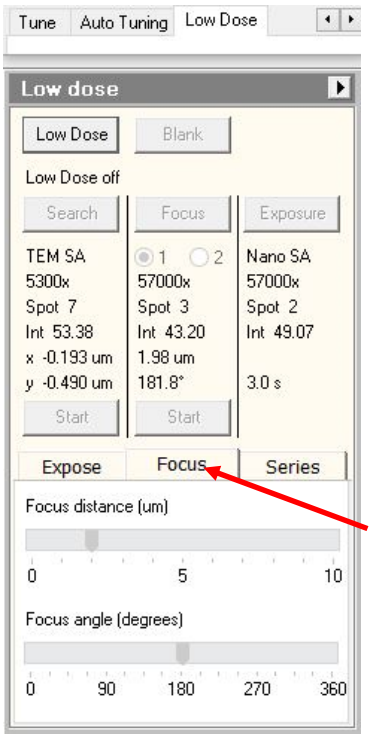
iii. Example: If the dose reading is 13 electrons/pixel/second and you did a 3 second exposure at a pixel size of 2.5 Å, the total dose is $(13/(2.5\ Å)^2)$ x 3s = 6.24 electrons/ $Å^2$

3. For the most accurate dose reading on the sample, the dose reading should be done on a hole (no sample) with the objective aperture removed. This will give the most accurate value of the dose hitting the sample.





9. Setting up the Focus Mode
    a. In Focus Mode, you are focusing away from where you want to take an image.
    b. You can set up the focus distance (distance away from where you want to take an image) and focus angle.
        i. In general, you don't want to be more than 3µm away from where you want to take an image for focus mode.



A focus distance <3 µm should be used for most circumstances. You do not want your focus area to overlap with your exposure area. A 180˚ angle works well.

10. Cryo-TEM Data Export
    a. Cryo-TEM data in particular should be exported as a 16-bit Tif. 16-bit images have a much larger dynamic range than 8-bit images.

Section 8: Drying Liquid Nitrogen Dewars

- To minimize ice contamination on your vitrified grids, the liquid nitrogen should be clean and free of ice contamination. Liquid Nitrogen dewars can be dried and kept clean of moisture by streaming nitrogen gas into them overnight the night before a cryo-TEM session.