



Protocol for using the Talos L120C

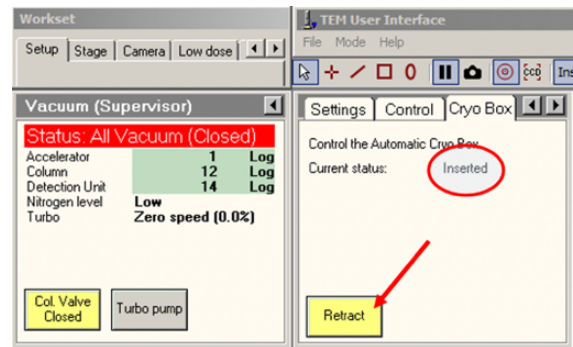
location: Room 236B, Biochemistry connector, Rollins research center




Important

- This protocol is intended as instruction guidelines for users. It assumes previous user training and approval by Robert P. Apkarian Integrated EM Core (IEMC) staff.
- This protocol requires knowledge of the controls at the microscope.
- Cryo-EM grid loading and microscope alignments will be carried out by **IEMC staff** unless the user has been properly trained and approved to do it.
- Please contact IEMC staff for assistance in case of any troubles.

Checklist before you start working on the Microscope:

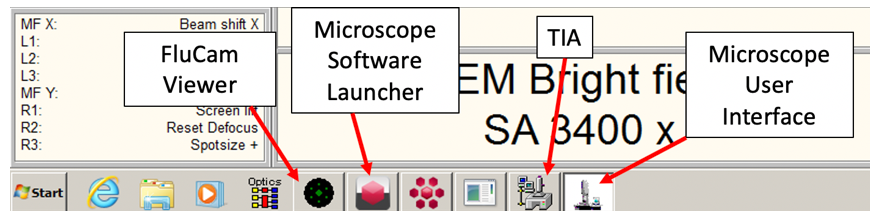
1. Check the vacuum status and temperature status. Everything should be green.
 - a) Write these values down on the logbook if required.
 - b) If some of the values are not green, then alert IEMC staff.
 - c) The Vacuum Overview OCX should indicate that the Cryocycle has been completed successfully.
2. Check the status of the microscope:



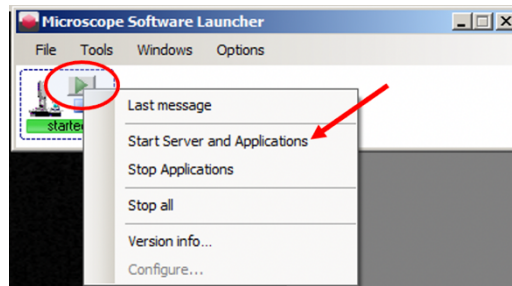
		
Microscope stage: no sample present. If a sample was left in the microscope, please inform IEMC staff.	Cold trap (a.k.a. Anti-Contamination Device or ACD) Dewar has been removed.	Water flow is working properly. Ensure that the indicator is floating and not all the way to the bottom.



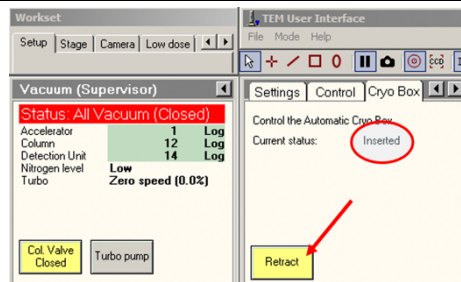
3. Fill the Dewar with LN2 and wait at least 10 min monitoring both the LN2 level and the state of the column vacuum (it should show a reduction in the log).
4. All of the appropriate software should already be running (Microscope User Interface, TEM imaging and analysis (TIA), and Flu Cam Viewer).



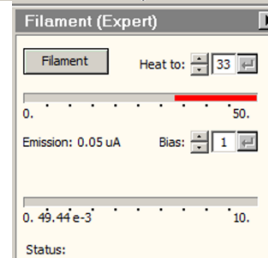
5. If the softwares are not running:
 - a) Open the Microscope Software Launcher.
 - b) Right click on the green triangle to open the selection menu.
 - c) Click on Start Server and Applications.
 - d) Start the FluCam Viewer if it did not start automatically.



6. Check the "Current status" of the Cryo Box ("Cryo Box" tab of the Vacuum OXC). If you are working with a cryo sample, insert the automatic cryo box.

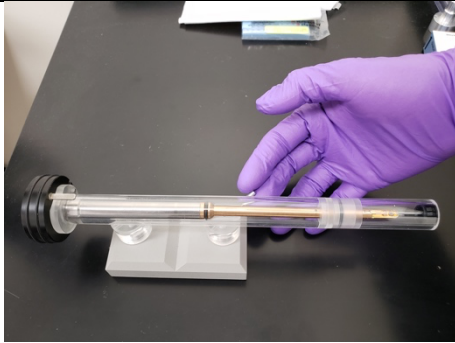


7. Start the filament from the "Filament (Expert)" OXC available from the Setup tab of the Microscope User Interface.

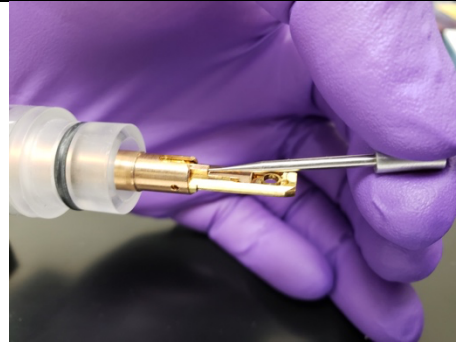




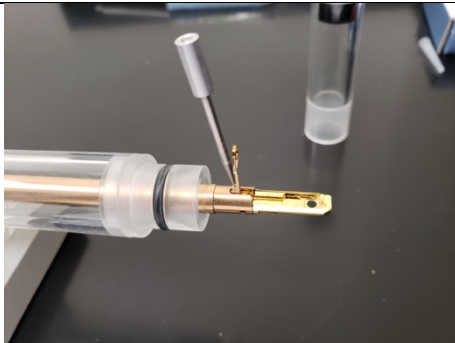
8. Loading a grid onto the single tilt holder



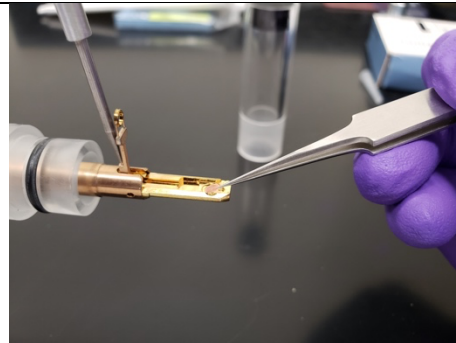
Find the holder tool



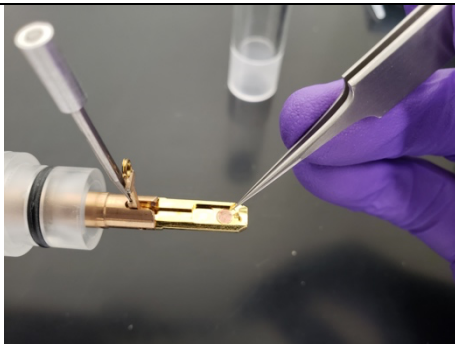
Insert the tool into the holder



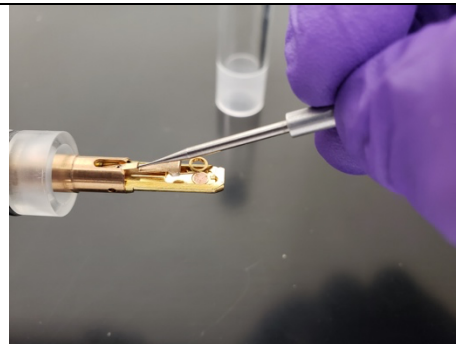
Use the tool to open the holder for grid insertion. Do not let go of the tool while opening the holder



Load your sample. Remember that the holder is upside down with respect to its orientation once inside the microscope



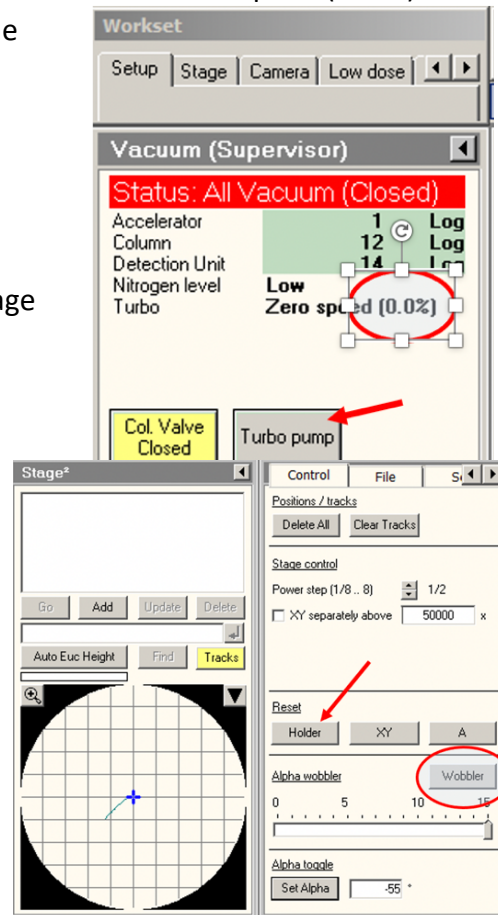
Use the closed tweezers to ensure the grid is properly positioned



Use the tool to close the holder. Do not let go of the tool while closing the holder

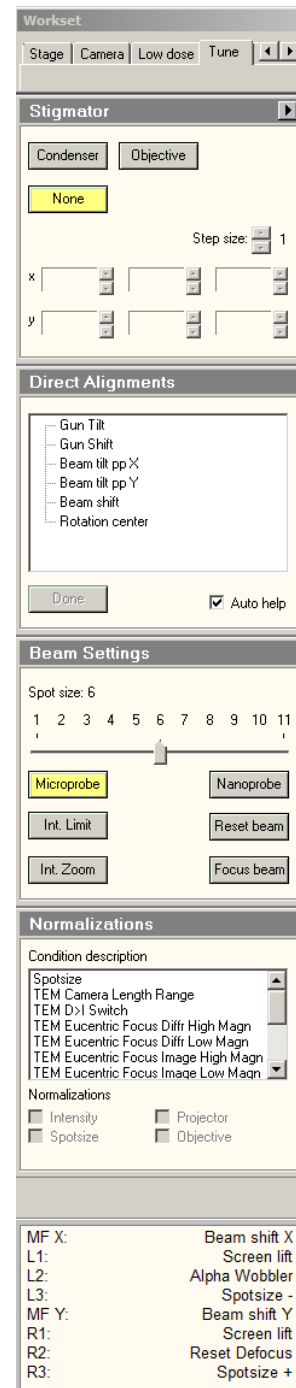


9. Insert the holder into the microscope.
 - a) Wear gloves when handling a sample holder to prevent contamination. Never touch the brass part of the holder. There are two types of sample holder available for different applications:
 - Single-tilt: negative stained samples, morphology observation.
 - Gatan 626 cryo-EM holder: for screening of cryo-EM samples
 - b) Start the Turbo pump (Vacuum OCX) and wait for it to reach full speed (100%).
 - c) Open the Vacuum overview OCX to monitor the state of the vacuum during holder insertion.
 - d) Reset the holder from the Stage OCX.
 - e) If the cryo-EM holder is being inserted, tilt the stage to -55 degrees by typing this value and pressing "Set Alpha."
 - f) Align and insert the holder straight into the stage load lock area (holder pin towards the closed position). Nitrogen will pour out of the holder – BE CAREFUL.
 - g) Wait for the load lock to pump down.
 - h) Rotate the holder towards the open position and fully insert it into the microscope column area.
 - i) For cryo-EM, press the "Set Alpha" button again to tilt the stage back to 0 degrees.
 - j) Reverse the above steps to remove the holder.
 - k) Select the appropriate holder from the drop-down menu when prompted and acknowledge it.
10. Open the column valves and find your beam. If it is not already visible try moving the stage, lowering the magnification, or checking that the fluorescent screen is down for the Fluorescent Cam.
11. Set the microscope to ~3400x magnification.





12. Spread the beam so that the size of it is slightly larger than the fluorescence screen (i.e. the edges of the beam are not visible). If you are looking at a cryo-EM sample, the dose rate should be kept at $0.3 \text{ e}/\text{A}^2$ or lower. The beam must become larger when turning the intensity knob clockwise. If this is not the case, then condense the beam and pass the cross over point to make it happen.
 - a) To get optimal performance and reliable diffraction patterns for focusing, bring your specimen to Eucentric height:
 - b) Find a reference object.
 - c) Activate the stage “Wobbler” and observe the movement of the reference object.
 - d) Minimize the movement using the Z up or down buttons on the right-hand panel.
 - e) If necessary, repeat the previous steps at higher magnification to tune the Z position.
14. If you are screening a cryo-EM sample, move the stage to an area of the grid that you do not necessarily care about and make the beam roughly the size of the flu screen (i.e. just until the beam edges are not visible). This will prevent you from damaging a good area during the following TEM alignment steps.
15. In the “Beam Settings” OCX, click on “Int Zoom”, this will keep the beam diameter the same (not the intensity) when you increase magnification.
16. Set the microscope to the high magnification to be used or 120kx if the magnification to be used is not yet known.
17. Press the “Eucentric focus” button on the right-hand panel.
18. Condense the beam to a spot, the beam must become brighter (smaller) when turning the intensity knob counterclockwise. It must become dimmer (larger) when turning the intensity knob clockwise. If this is not the case, keep condensing the beam (i.e. making it smaller) and pass the cross over point (smaller beam size) until the beam expands while turning the intensity knob clockwise.





19. If the beam is not round, find the Stigmator OCX (Tune tab), click on Condenser (the Multifunction knobs are now set for Condenser stigmator. click on “None” on the “Stigmator” OCX, to get out of stigmator adjustment.
20. On the “Direct Alignments” OCX (Tune tab), click “Beam Shift” and center the beam with the multifunction knobs. This is an alignment step so be mindful of how much you turn the multifunction knobs. Press “Done” when the beam is centered.
21. Pivot Points (PP): On the “Direct Alignments” OCX, select “Beam Tilt PP X”. Adjust the beam using the multifunction knobs until the two points overlap. Do the same with “Beam Tilt PP Y”, then click “Done”. For both “Beam Tilt PP X” and “Beam Tilt PP Y” always start with the multifunction X to adjust the PP.
22. Click “Beam Shift” again and center the beam using the multifunction knobs, then click done.
23. Expand the beam again just until the edges are not visible.
24. If you are screening the cryo-EM sample, directly do to step **number 30.**
25. Lift the fluorescent screen.
26. Insert the Ceta camera in CCD/TV OCX and hit the “Search” button, you should now see an image of your specimen on the right-hand screen (where the TIA software is open). Click on the “Live, FFT” button. You should now see the Fourier transform (FFT) of your live image.
27. Adjust the focus so that you see the first few rings of the FFT (underfocus not overfocus).
28. Go to the “Stigmator” OCX and make sure that Objective stigmator is selected. Use the multifunction buttons to adjust the FFT so that the rings are perfectly circular. Then hit the Stigmator button again or click on “None” on the “Stigmator” OCX, to get out of stigmator adjustment.
29. Start screening the grid by taking images with the Acquire button in the CCD/TV OCX.
30. For cryo-EM samples, click on the “Low dose” tab and click the low dose button.
31. Click on the flap out arrow (upper right of the OCX) and select the “Settings” tab. Set the different microscope states (Search, Focus, Exposure) to whichever imaging method you prefer. I recommend, Screen for Search and Focus, and your detector for Exposure.
32. Go to the Expose state by clicking on the “Exposure” button (Do not click on “Expose” because this will take an image), this puts the microscope in the state that you want for image collection (magnification, beam diameter, spot size). If the flu screen is retracted, insert the screen so that you can see the beam.



33. Set the microscope to the magnification at which you wish to collect data.

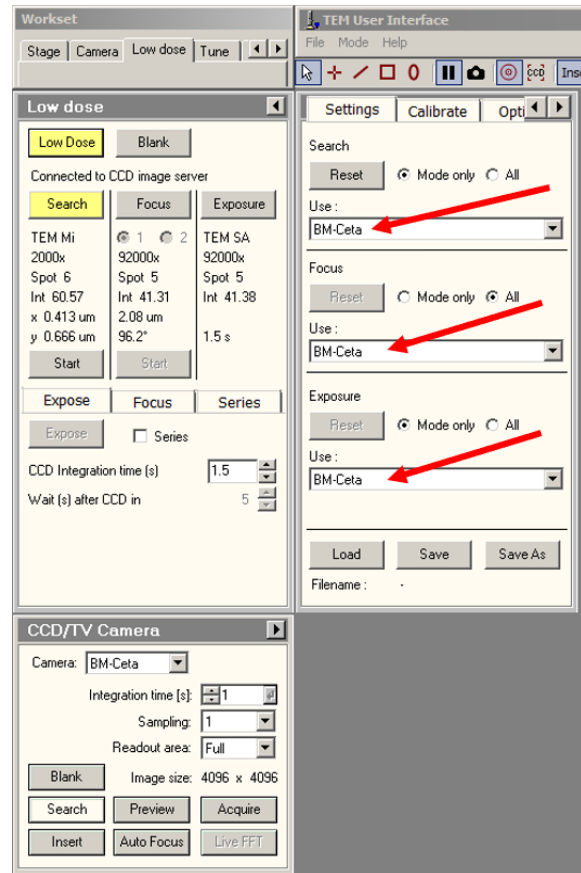
Note: Here is where you should consider what “resolution” and dose you wish to subject your sample to, as this will determine how you will set up the Exposure state.

Note: Each time you change the magnification, center the beam and adjust the size to fit the flu screen.

34. To remove any ‘User beam shifts’ that may have been introduced, go to “Direct Alignments” and press “Beam Shift”. The theory here is that we start with our Exposure state as the ground state, and any beam shifts that will occur in Focus state will be added on top of this. Use the System Status OCX if necessary, to determine whether there is any ‘User beam shift’ applied in exposure mode.

The ‘user beam shift’ value should be (0.0000).

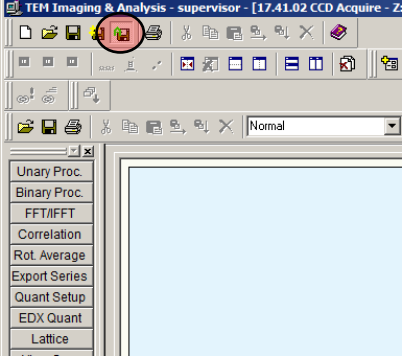
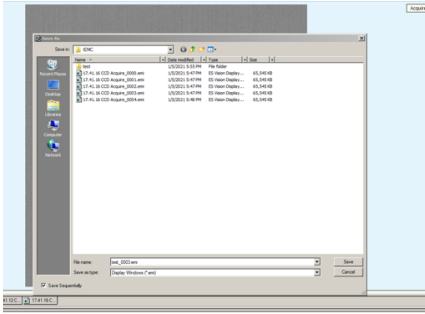
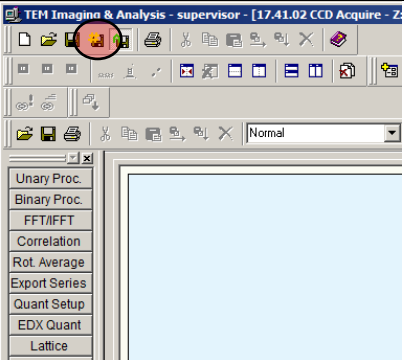
35. In the Direct Alignments OCX click on Beam Shift and center the beam. Unclick when you are done. The beam should now be set up for this state.
36. Set the beam intensity that you require for your particular sample. For cryo samples, this value should be between 40-60 e-/A².
37. Next click on “Focus”, this will put the microscope into the state that you want it for focusing your sample. Under the Focus button you can set the beam in position 1 or position 2, these two positions should be 180° rotated from each other (0 and 180, for example) and they should be more than 2 microns away from the Expose position. Use the slider to adjust both distance and angle under the Focus tab. Start with position 1 and set the microscope at a magnification equal or higher than your Exposure magnification. Set the beam to a diameter that just covers the screen, and center it with the **track ball**, **not the multifunction buttons**.





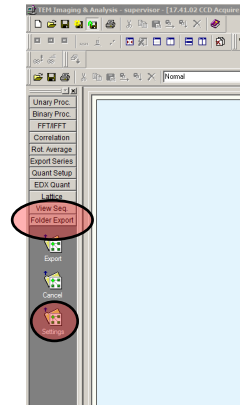
38. Next go to Focus position 2 and repeat the same thing.
39. Go back to “Exposure” and make sure that the beam is still centered nicely. If not, go to Direct Alignments, click on ‘Beam Shift’ and use the multifunction X and Y buttons to center it. Again, this is to avoid adding ‘User beam shifts’ in Exposure mode.
40. Next go to “Search” and set the beam to the appropriate search conditions. ~1000x to 4000x, spread the beam so that the Dose reads less than $0.1 \text{ e}/\text{A}^2$.
41. Lower the flu screen and center a recognizable feature by moving the stage (joystick).
42. Go to the Exposure mode and center the same recognizable feature (it will appear much larger) by moving the stage.
43. Go back to the search mode, open the options tab and make sure the “search shift” box is checked. Now use the multifunction X and Y buttons to center the same recognizable feature.
44. Make sure the Ceta camera status is **“Stable”** in the bottom status panel and there are no orange/red protector/status messages.
45. You are now ready to acquire low dose images.
46. Find a location (hole) in the search mode by pressing the start tab and start using the Ceta camera.
47. Focus in the focus mode by pressing the start tab. Start the Live FFT and set the required defocus.
48. Set the microscope to the “Exposure” state. Click the “expose” tab followed by the expose button to acquire an image
49. Saving single images on TIA: Right-click on the image and select Export. Select the format of the image to be saved and proceed.
50. Saving images sequentially: This method will save images in *.emi format. A folder export procedure needs to be done at the end of your session to generate images in any other format supported by TIA.



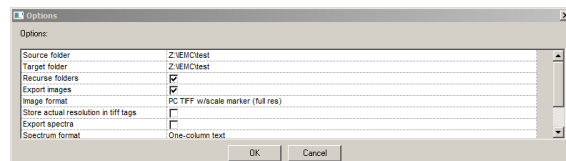
<p>a) On the top-left area of the TIA software click on the AutoSave Icon (with the small green letter A).</p>	
<p>b) Use the “Save As” window to select or create a folder where your images will be saved. c) Give a name for your images with the ‘.emi’ extension. An underscore and a sequential number (4 digits) will be added to each subsequent image and it is not necessary at this point. d) Press Save. This will save this first image as “..._0001.emi” and will activate the Save Sequential Icon next to AutoSave.</p>	
<p>e) From now on, simply select another image, or acquire a new image and click the Save Sequential Icon to automatically save the image with the setup name and the sequential number.</p>	



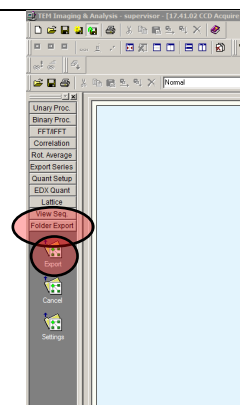
- f) Once your session is complete, it is necessary to use the “Folder Export” option to convert the emi files into one of the formats supported by TIA.
- g) Expand the Folder Export section. DO NOT PRESS EXPORT YET. Select the Settings option



- h) Type the information for the Source folder and the Target folder (no browse button available). From the windows explorer, one can copy the folder location and paste it using “Ctrl + V)
- i) Select the Image format to which the*.emi file will be converted.
- j) Press OK to exit the Folder Export - Settings window



- k) Press the Export button. TIA will generate the new, converted image files into the designated folder.





51. When you are finished imaging on the microscope:
- If you were using the Low Dose kit, unclick the low dose button.
 - Insert the fluorescence screen.
 - Go to 36000x magnification, spread the beam out so you don't see the edges of it.
 - If you used the objective aperture, remove it at this point. Find the Apertures OCX and click on Objective to remove the aperture. The button will go from yellow to gray.
 - Retract the CETA camera by pressing the Insert button in the CCD/TV Camera OCX. The button will go from yellow to gray.
 - Reset the holder coordinates back to X=0, Y=0 and Z=0. From the Stage OCX, click on Holder in the Reset Section. This will also reset the angle (Alpha) back to 0.
 - Close the column valves: Setup tab, Vacuum (supervisor) OCX. Press the "Col. Valve Closed" button to turn it yellow.
 - Start the Turbo Pump: Setup tab, Vacuum (supervisor) OCX. Press the "Turbo pump" button to turn it yellow. Wait for the pump to reach full speed (100%)
 - If you are finished with your sample, remove the holder to load the next sample. In case the cryo holder is being used, tilt the stage to -55 degrees by typing this value and pressing "Set Alpha".
52. If you are done for the day, and nobody else has signed up, you can remove the holder and leave it outside ensuring the cover is on to protect from dust falling on it.
- Turn off the filament.
 - Make sure there are no users signed up after you.
 - Remove the Dewar in the anticontamination device.
 - Start the Cryo-cycle from the main Vacuum OCX.