

20241204 (ver. 1.1)

Okayama University

Cryo-Electron Microscope Manual

Titan Krios G4 and Falcon 4i

For tomography

This manual is based on the KEK Cryo-Electron Microscope Initial Training Text for External Users (ver.3)

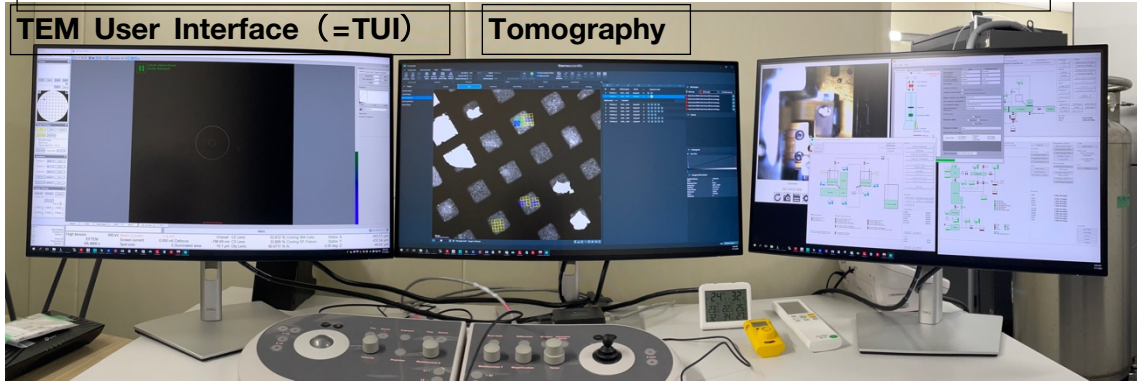
https://www2.kek.jp/imss/sbrc/230203_KEKTitan_TrainingText_v3.pdf

and has been modified to include sections on daily adjustments and routine data measurements to accommodate tomography operations.

**International Center for Structural Biology,
Research Institute for Interdisciplinary Science,
Okayama University
Nobutaka Numoto**

I. From sample exchange to Atlas acquisition

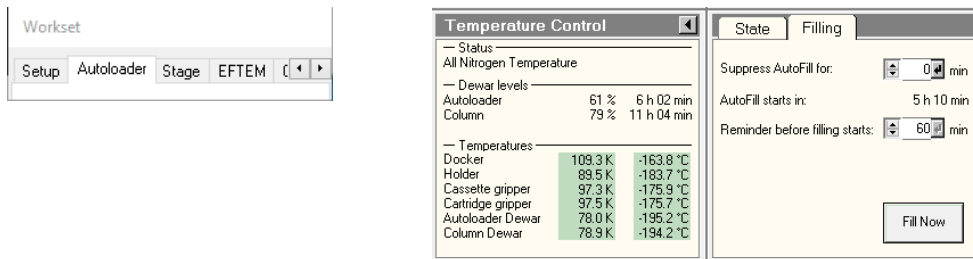
*TEM User Interface is version 3.16.1, Tomography is version 5.14.0.5704REL



1. Inventory

Use TEM User Interface (=TUI)

- TUI > Autoloader tab > Temperature Control



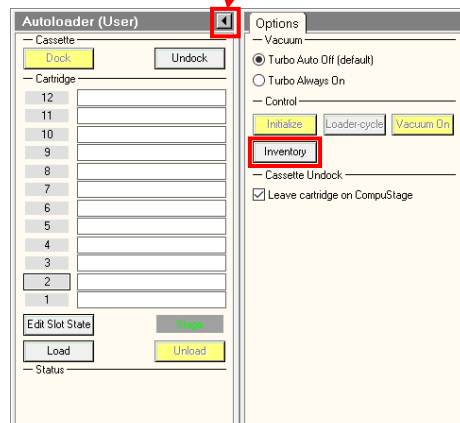
- When you're done with the NanoCab Dock (don't forget to take out the NanoCab!)
Wait until the temperature display is all green, and then wait until everything drops below -160°C (a few minutes)

If the Option window is not open, click here.

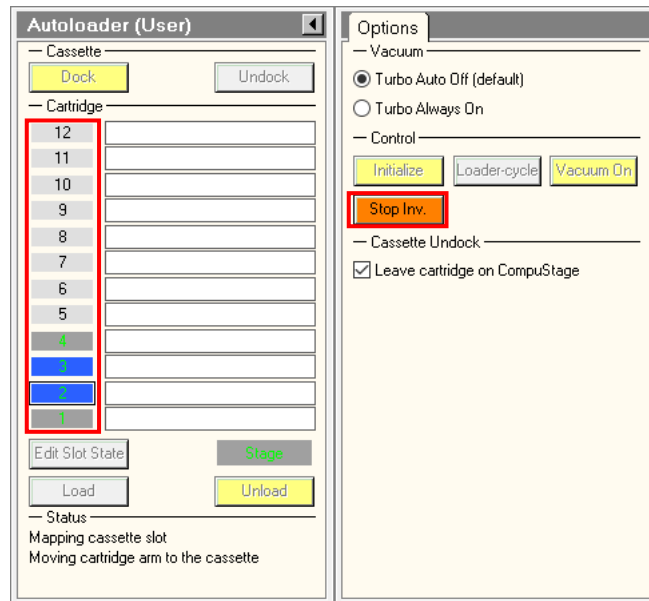
- TUI > Autoloader tab > Autoloader > Option



- Press the Inventory button.
(After the Dock, it takes a while to become active)



- If all the grids you put in are recognized correctly, OK



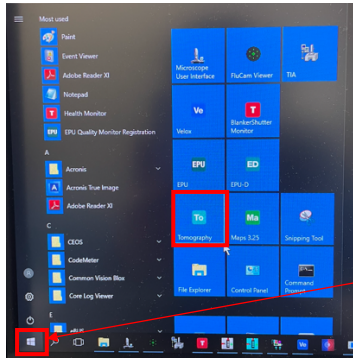
Dark gray: Judged to be missing a grid
 Blue: Grid is determined to be present
 Light gray: Recognition work has not yet been done

(If it is not recognized correctly, Inventory again, and if there is still a discrepancy, take out the Cassette once and check it.)
 Is the grid falling?
 If you're still wrong, contact FEI.)

- When the number of grids you put in +2 is checked, press the Stop Inventory button to stop.
- The name of the grid is entered after the Inventory is finished (or Unload if there are any grids left in the stage)
 - * It's not good to have a name in an empty slot
 - * This name will not be reflected in the folder name of the result. Leave a note.

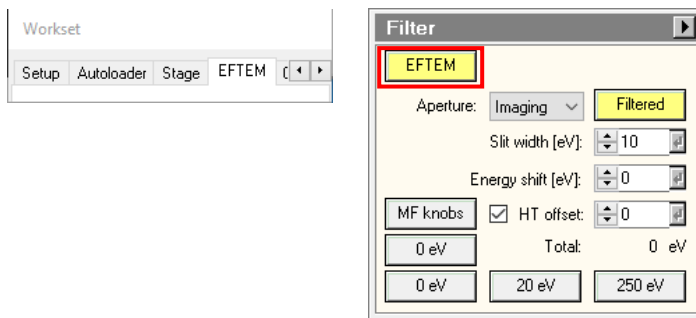
2. Approximate alignment (No need to do it every day?)

- Starting Tomography (hereinafter Tomo)

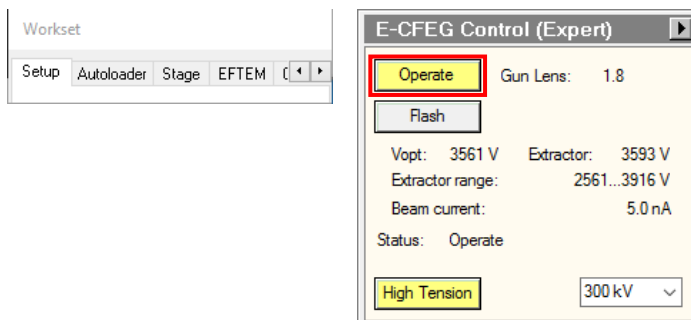


From Start menu of Windows OS

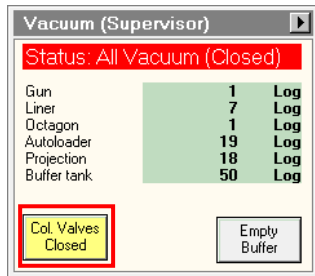
- View the TUI > EFTEM tab > Filter
- **Verify that the EFTEM button is yellow.**
(If it is not yellow, press the EFTEM button to turn it yellow.)



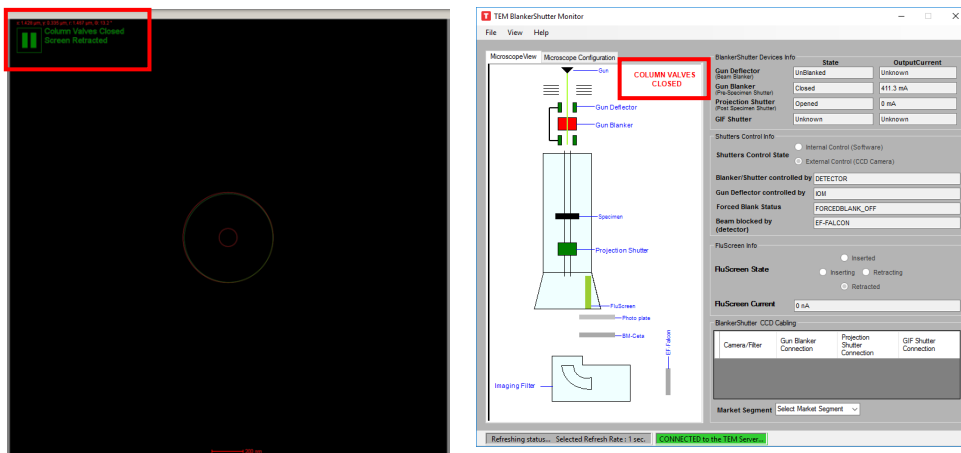
- TUI > Setup > E-CFEG
- **Verify that the Operate button is yellow.**
(If it is not yellow, press the Operate button to turn yellow.)



- TUI > Setup tab > Vacuum
- Press the Col. Valves Closed button to open the Column valve.



The open/closed status of the column valve can be checked on the screen of the TUI or with the "TEM BlankerShutter Monitor" on the right monitor

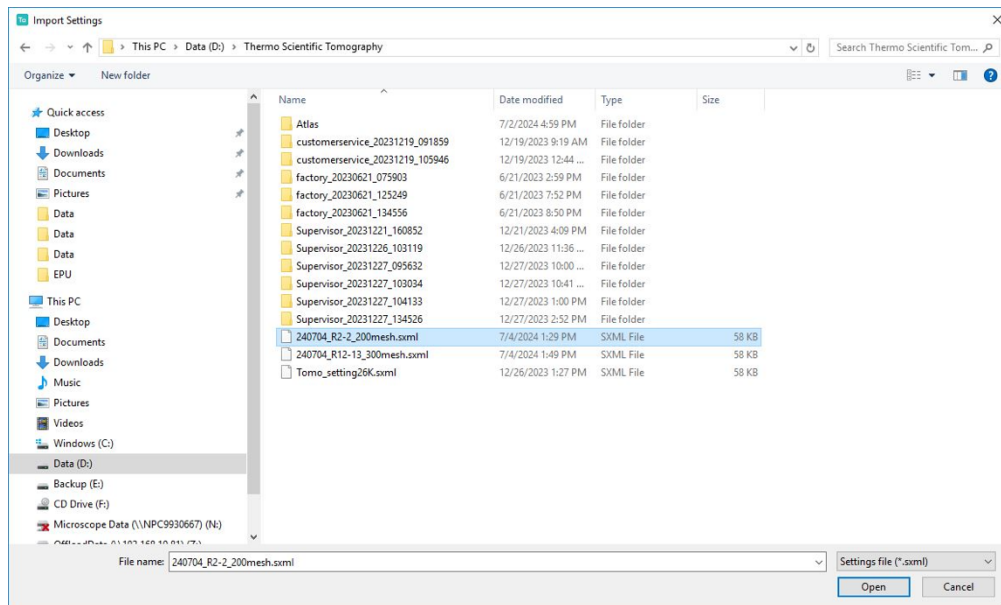
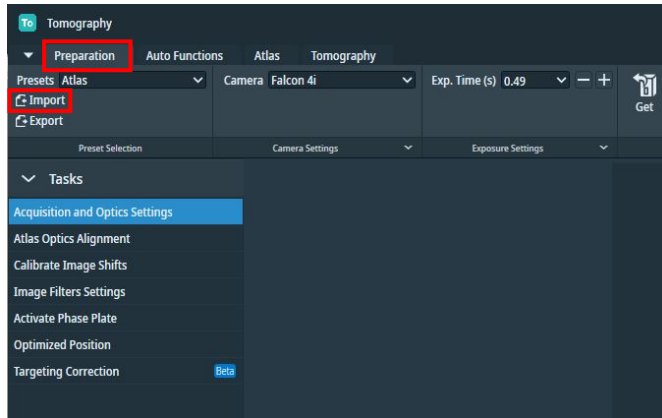


- Tomo > Preparation tab, press Import and open the following .sxml file

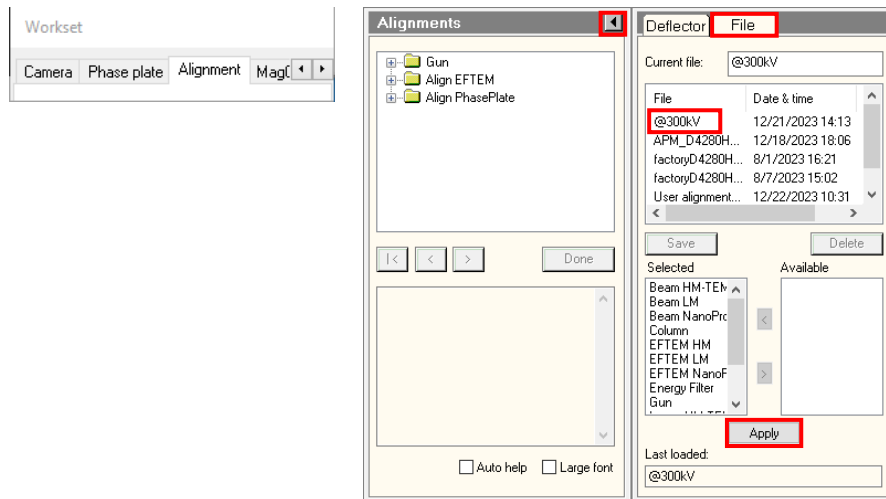
D:/Thermo Scientific Tomography/YMMDD_*.sxml**

*Hole *Files are created for each hole and mesh size (magnification settings, etc. are different). Select the most recent file for the grid you are using.

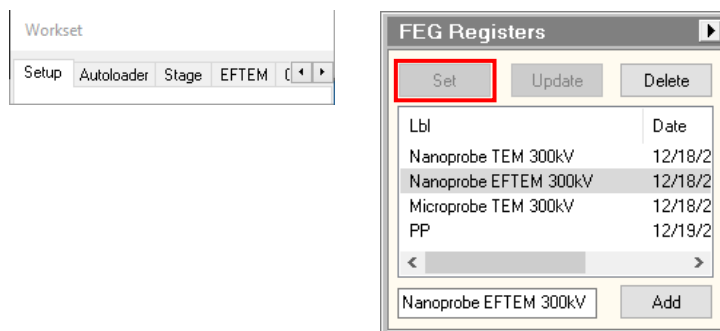
*If not present, create one separately.



- TUI > Alignment tab > Alignments > Option > File tab Select 300kV (latest created on 11/27/2024) and select everything in Available and click < button to move to Selected, then press Apply



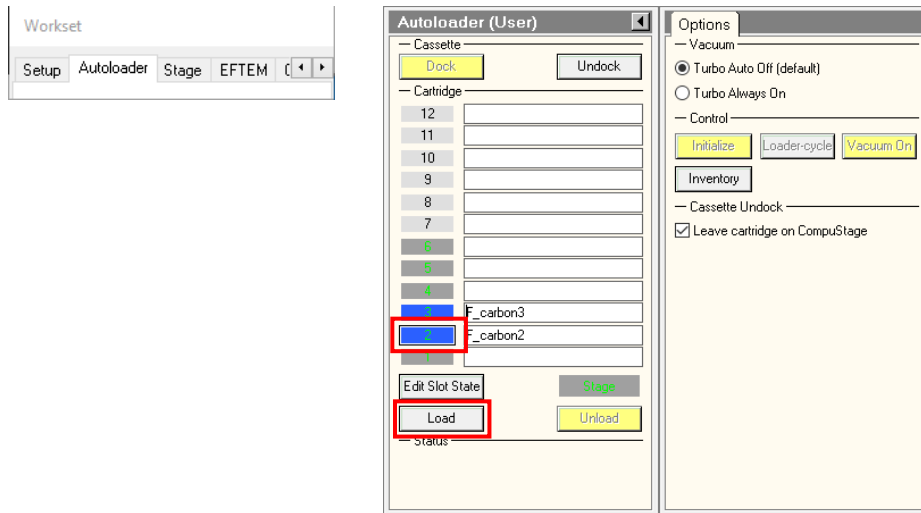
- TUI > Setup tab > Select Nanoprobe EFTEM 300kV (Latest updated on 11/27/2024) from FEG registers and Set (It is also effective when the beam is lost.) However, once you have done this, you will have to start the adjustment from scratch.)



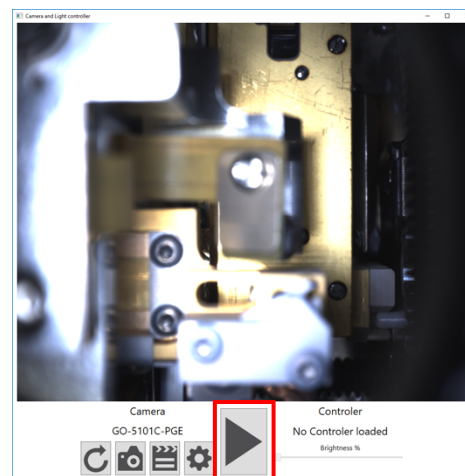
3. Grid load and preview, Atlas retrieval

• TUI > Autoloader tab > Autoloader

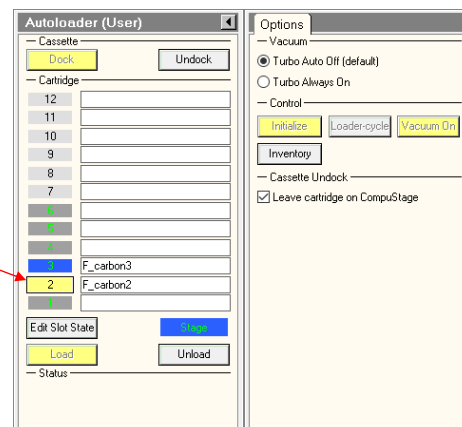
- Click on the number in the grid you want to see (highlighted with a black line)
Press the Load button.



If you press the play button of "Camera and Light controller" on the right monitor, you can see the live view of Autoloader.
This screen automatically stops after 15 minutes. If you want to watch it again, press the play button.



Correctly loaded grids
turn yellow

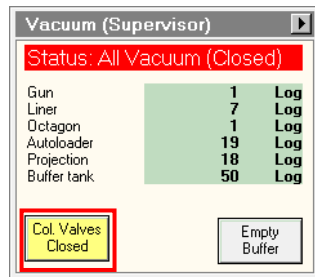


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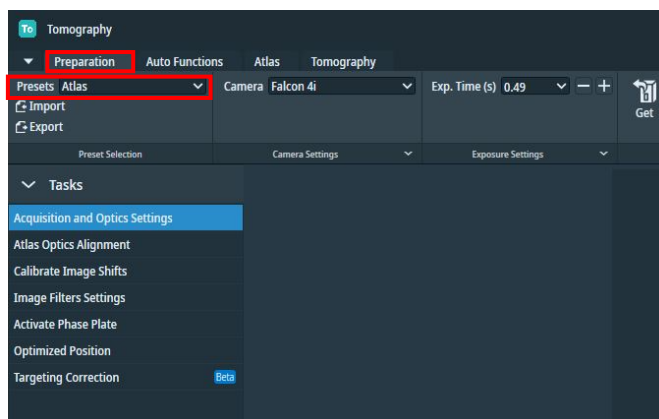
- TUI > Setup tab > Vacuum

- Press the Col. Valves Closed button to open the Column valve.



- Tomo > Preparation tab

- Select Atlas from the Presets pull-down menu in the upper left corner.



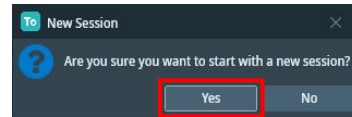
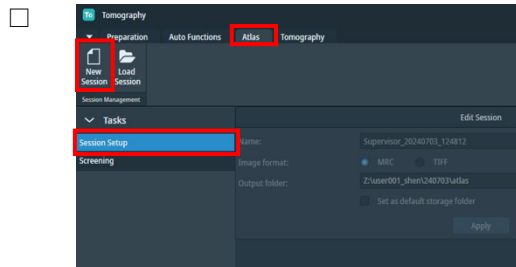
- Press Preview on the upper right edge to take a picture.

*If it is completely dark at this stage, check again that the column valve is open and that the E-CFEG is set to Operate.

*If the ice is too thick, it will be almost pitch black, but you should be able to see the grid square faintly.

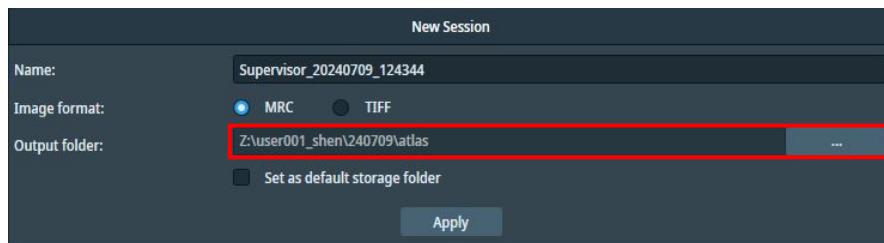
• Tomo > Atlas tab

Session Setup > New Session > Yes

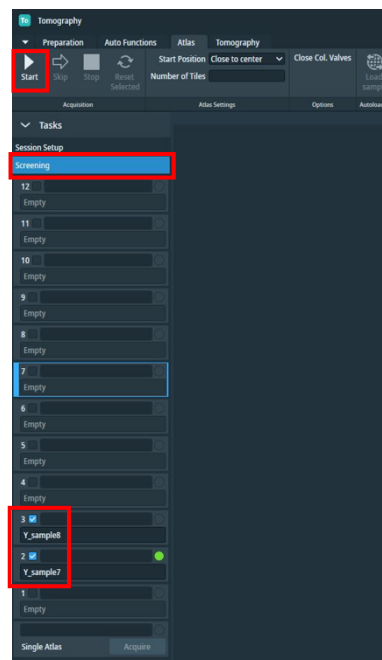


Specify the Output folder (save destination: Z:/userxxx/yymmdd/atlas/) and

Apply

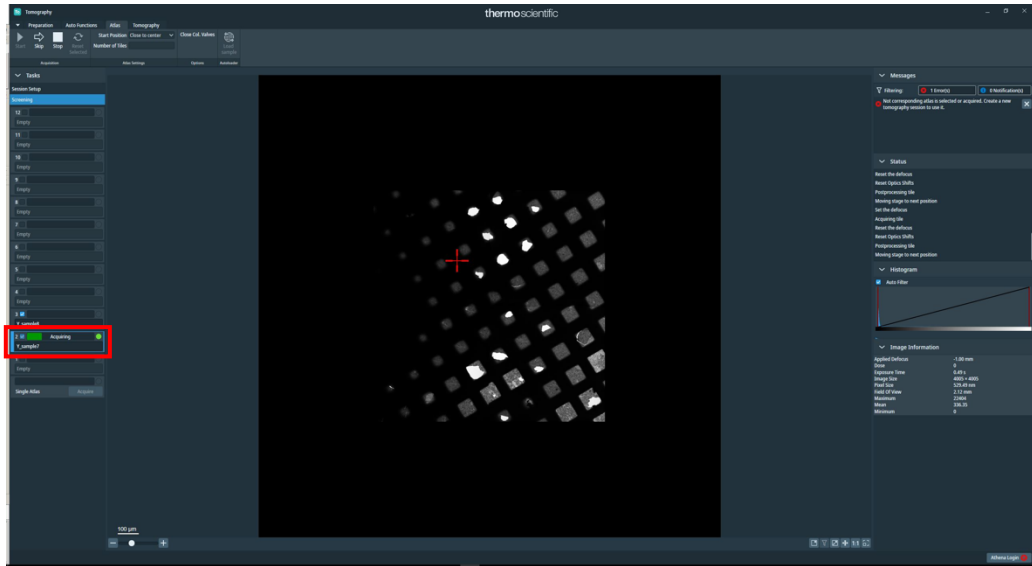


Press Screening, check the number of Grid (green light) you want to shoot, and press the Start button to start shooting as Atlas



- Click Acquiring to view the image.

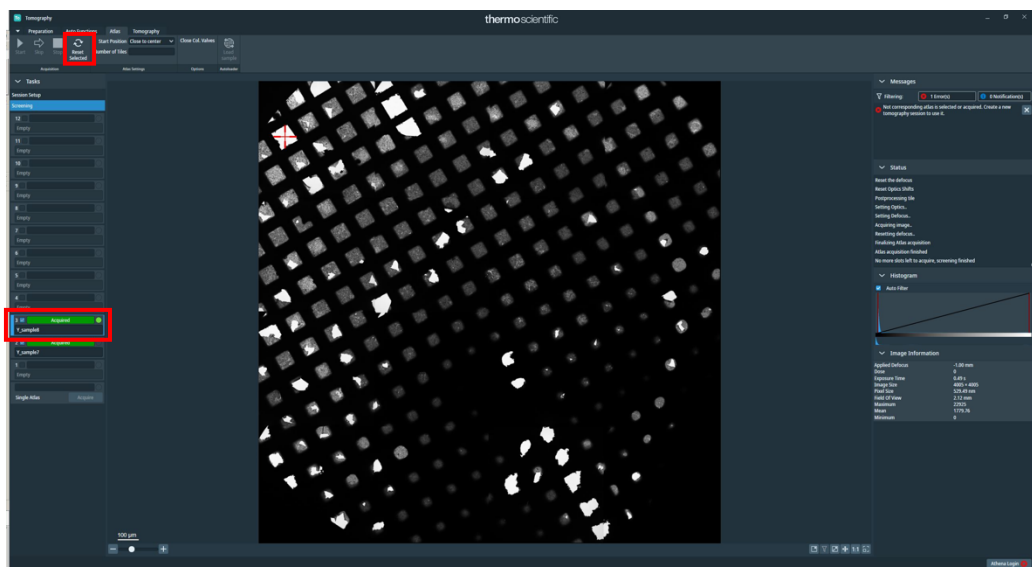
(* Wait a while to shoot the whole picture with 4 x 4.) Including the time it takes to replace the grid, it takes less than 10 minutes per sheet? From 2024/11/29, the recommended magnification for Atlas is 155x)



*If, for some reason, you want to retake Atlas for a specific grid

Select the grid in the Atlas tab and press the Reset Selected button in the upper left corner.

The data will disappear, so check that grid and Start

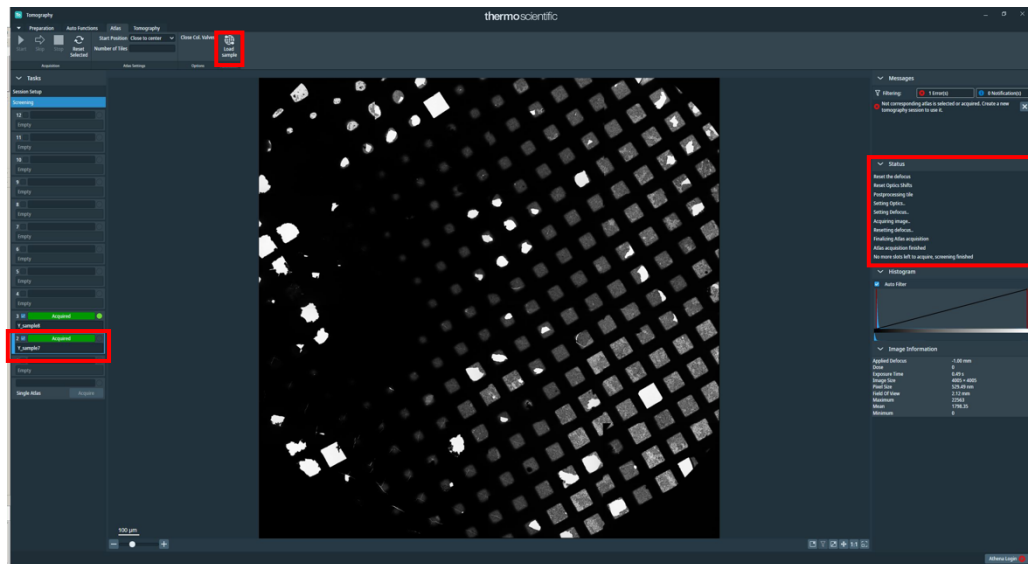


II. Beam adjustment (daily)

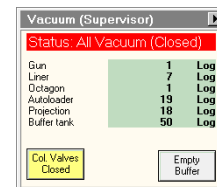
1. Move to the broken square in the grid

- ☐ Tomo > Select the desired grid on the Atlas tab and press Load Sample.

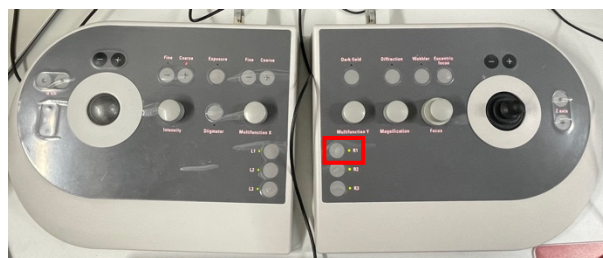
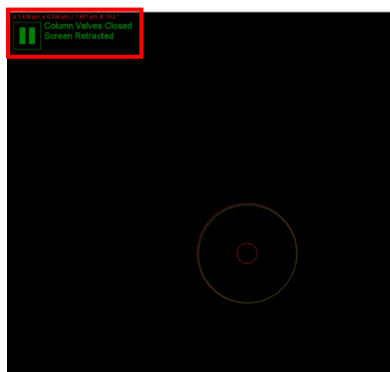
Verify that the Tomo > Status displays "Cartridge at slot x is now on the stage"



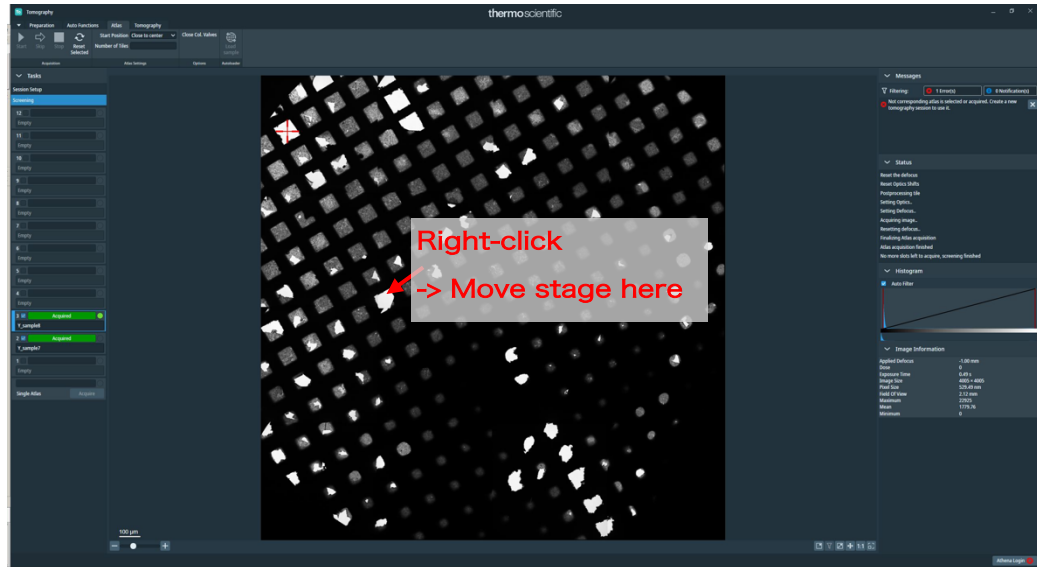
- ☐ If the Column valve is closed, Press the Col. Valves Closed button to open the Column valve.



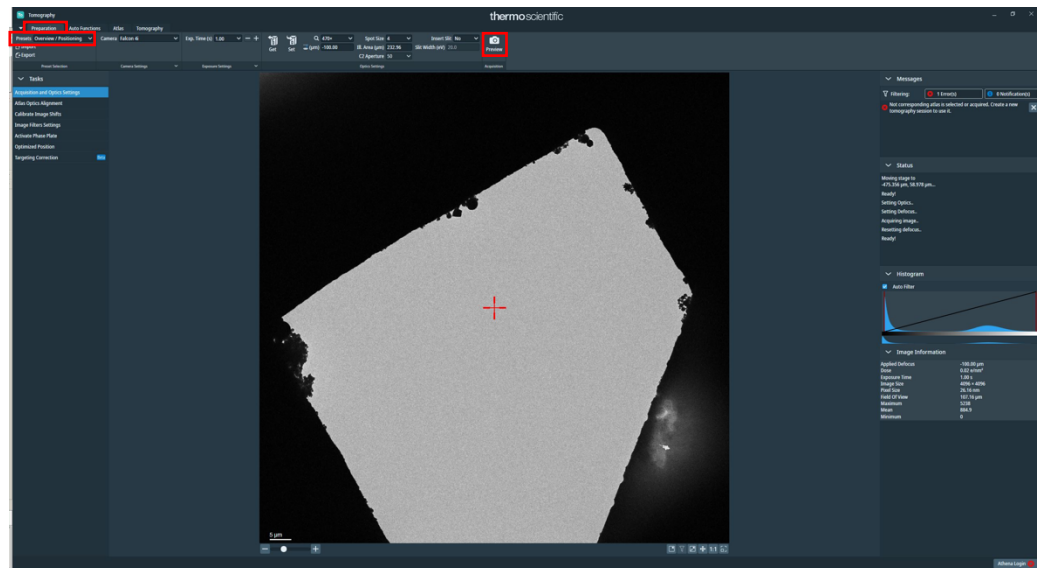
- ☐ If the fluorescent plate is not raised, press R1 on the operation plate to raise the plate (= Screen Retracted).



- Tomo > Right-click on a broken square in the Grid in the Atlas tab > Move stage here

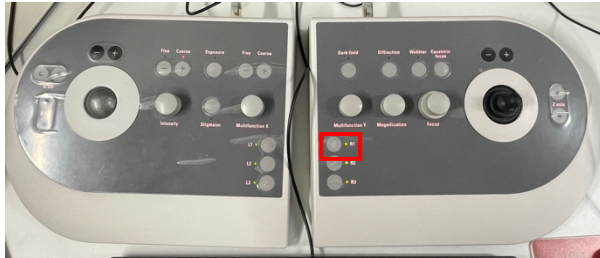


- Tomo > Preparation tab > Presets, select Overview / Positioning and Preview



- Make sure you're in a broken square
 - * If the position is off, right-click again on the square image > Move stage here
 - * Overview / Positioning = Grid Square in EPU

- Press R1 to lower the fluorescent plate.

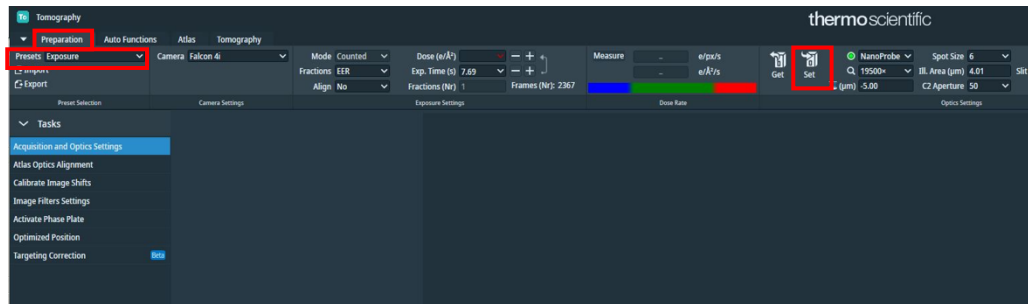


- Flucam Viewer is now in EF (=EF mode is displayed as a green circle),
Confirmed that it is Natural, High Resolution



2. Adjusting the Pivot Point

- Be in the broken square in the grid and make sure the fluorescent plate is down
- Select Exposure from Tomo > Preparation tab > Presets and press "Set"

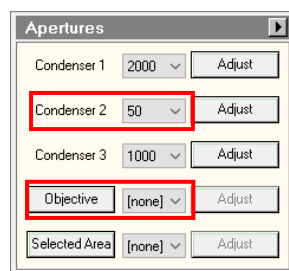


*Exposure = Data Acquisition in EPU

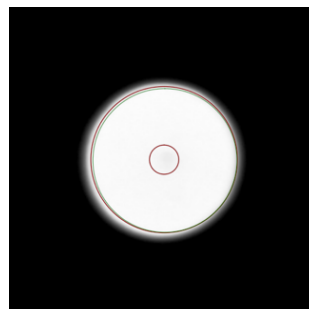
- Press the Eucentric Focus on the control board.



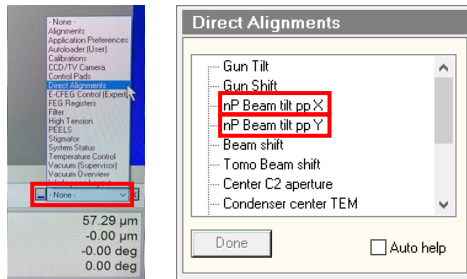
- TUI > Stage tab > Apertures and see that C2=50, Obj=none
(*C1=2000, C3=1000 do not need to be touched at all in the future)



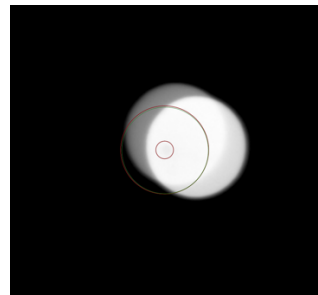
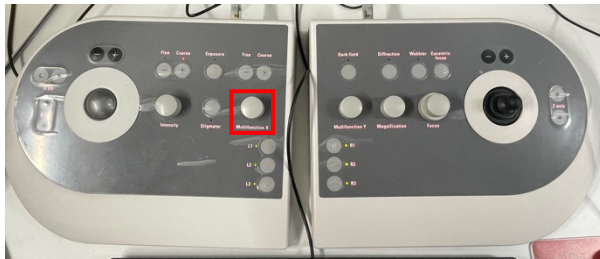
- Turn the Intensity dial to make it as large as the larger circle in the center of the Flucam Viewer screen.



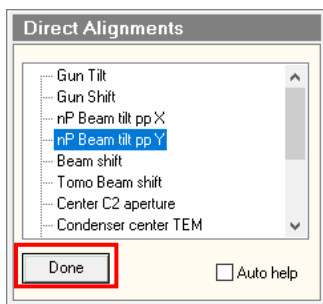
- The lower right of TUI > Direct Alignment > Select nP Beamtilt pp X.



- Use only Multifunction-X on the control panel to adjust the two flashing lights so that they overlap.

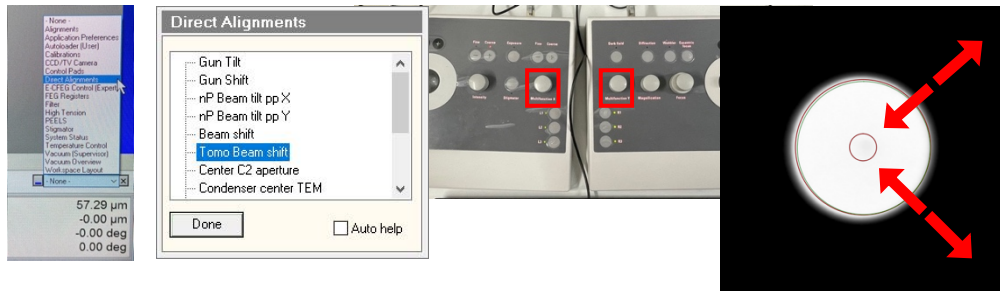


- The lower right of TUI > Direct Alignment > Select nP Beamtilt pp Y.
- Use only Multifunction-X on the control panel to adjust the two flashing lights so that they overlap.
- Press Done

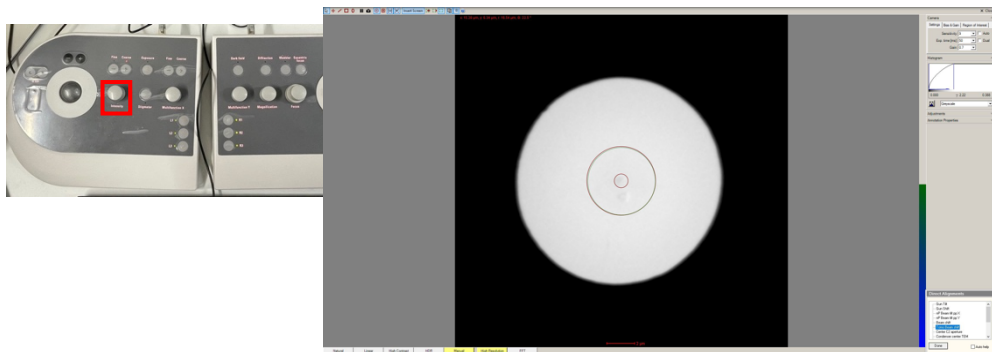


3. C2 Aperture Centering

- Be in the broken square in the grid and make sure the fluorescent plate is down
- TUI Bottom right> Select Direct Alignment > Tomo Beam shift
- Use the Multifunction dial to center the light.



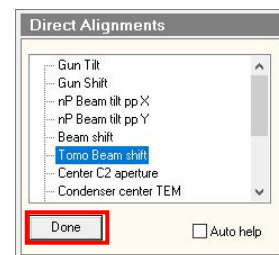
- Turn the Intensity dial to the right to spread the light. Check that the light spreads in concentric circles.



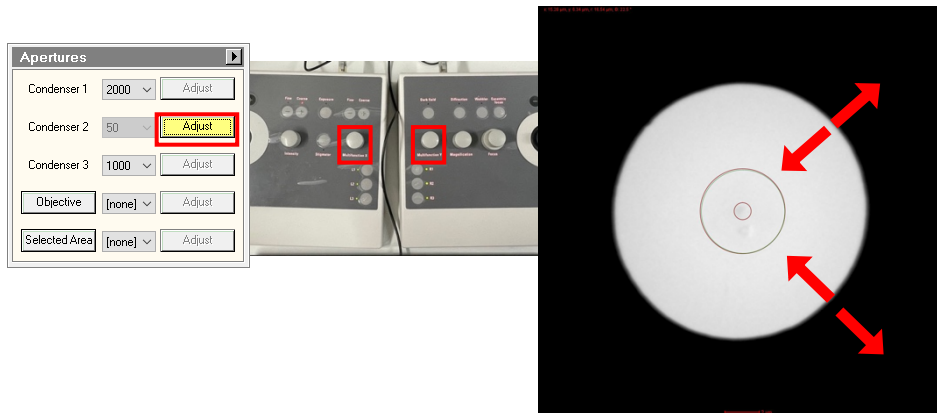
The contrast of the light can be adjusted with the mouse wheel.
 Due to magnification issues, it cannot be made large enough to touch the black screen.

As long as it spreads out roughly in concentric circles, there's no problem, so click "Done" of Tomo Beam shift in Direct Alignment.

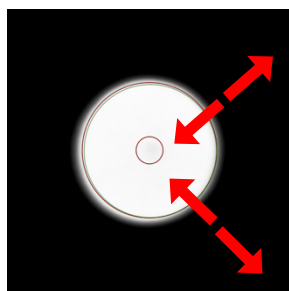
If not, make the following adjustments.



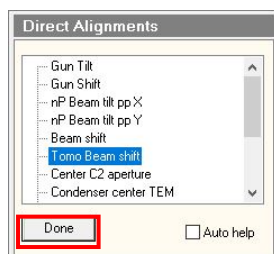
- Press Adjust on the right side of C2 and use the Multifunction dial to move the aperture so that the circle of light is circumscribed on a black background.



- Press Adjust again to the right of C2
- Squeeze the light again to about the larger circle in the center, and move the light to the center with the Multifunction dial.

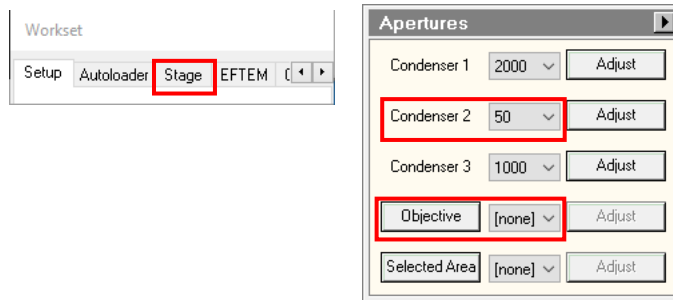


- Turn the Intensity dial to the right to see if the sound spreads in concentric circles.
- If the light circles are almost concentric, it's OK. If not, repeat the above steps until the light circles are concentric.
- Click "Done" of Tomo Beam shift in Direct Alignment.

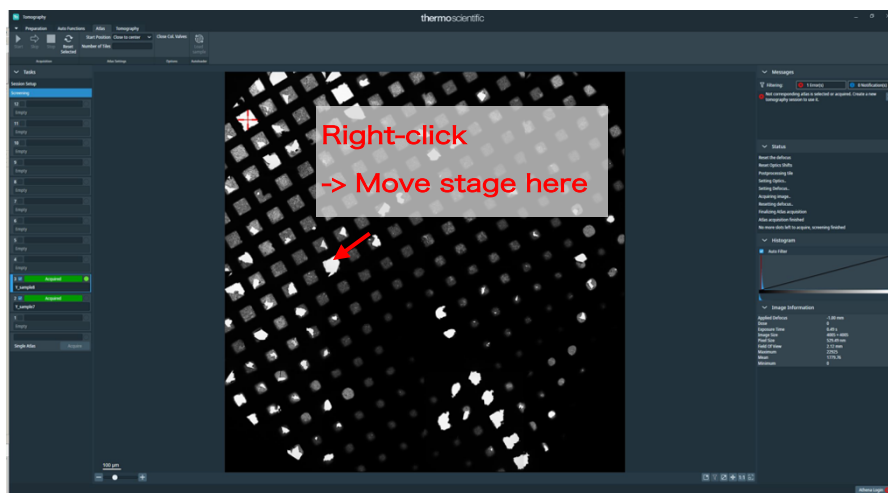


4. Adjusting energy filter

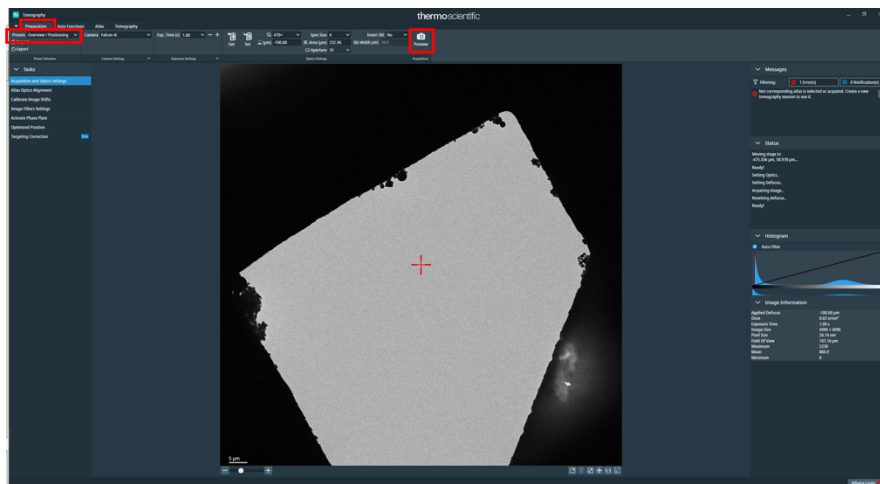
- Press R1 to raise the fluorescent plate.
- TUI > Stage tab > Apertures and select, **C2=50, Obj=none**



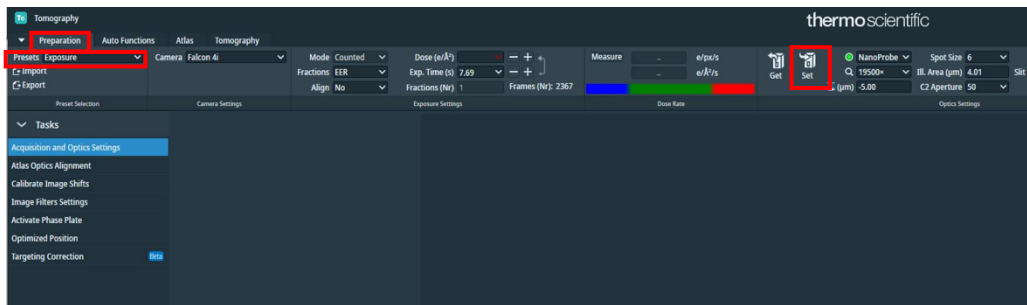
- Tomo > Right-click on the hole in the Grid in the Atlas tab -> Move stage here



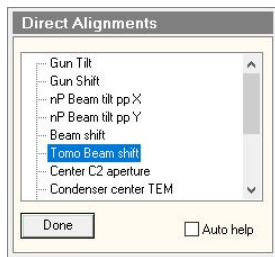
- Tomo > Preparation tab > Presets > Overview / Positioning, and click Preview (Confirm that you are in the hole)



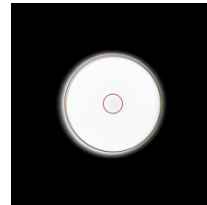
- Tomo > Preparation tab > Presets > Exposure, and click "Set"



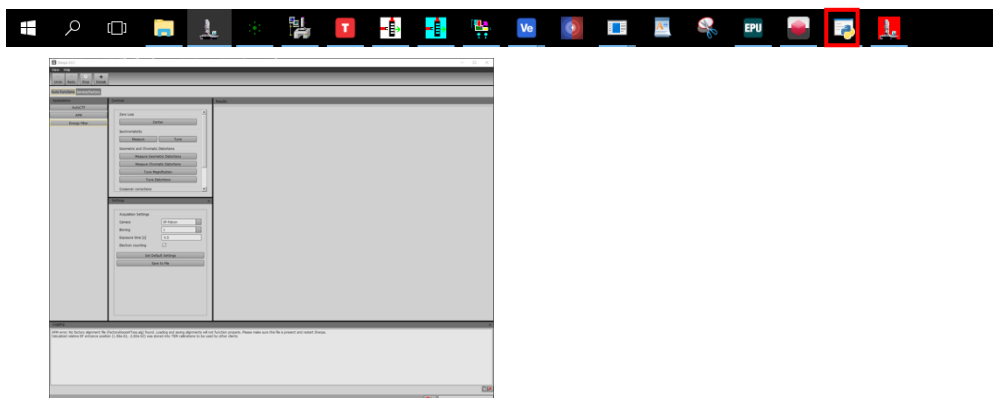
- Press R1 to lower the fluorescent plate
- If the beam is not centered, center it with Direct Alignment > Tomo Beam Shift
- Then, Done



- Make sure the beam is hitting the entire green circle.
- Press R1 to raise the fluorescent plate.

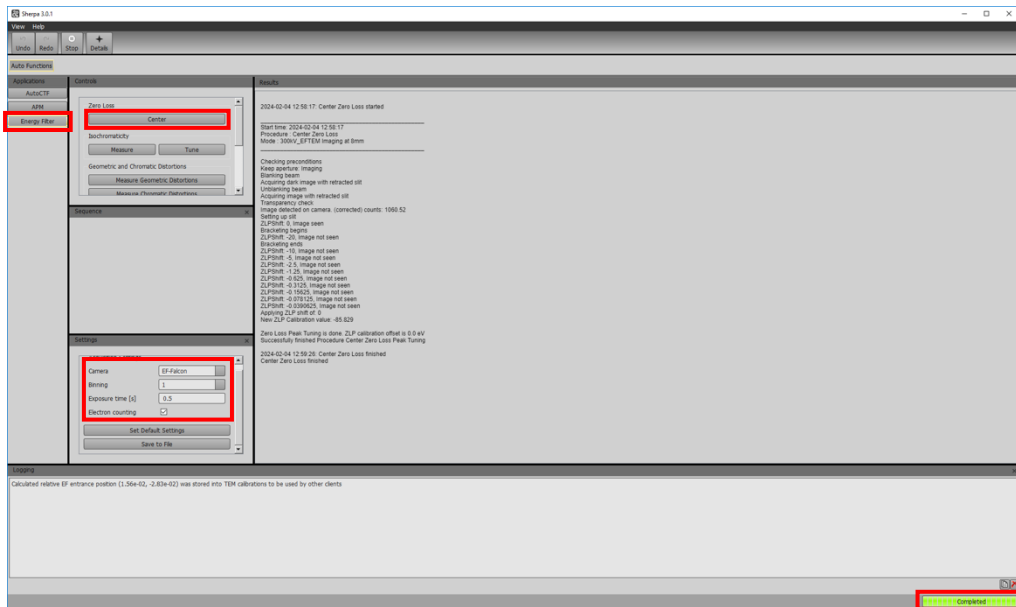


- Normally, Sherpa should already be launched, so put the window on the surface

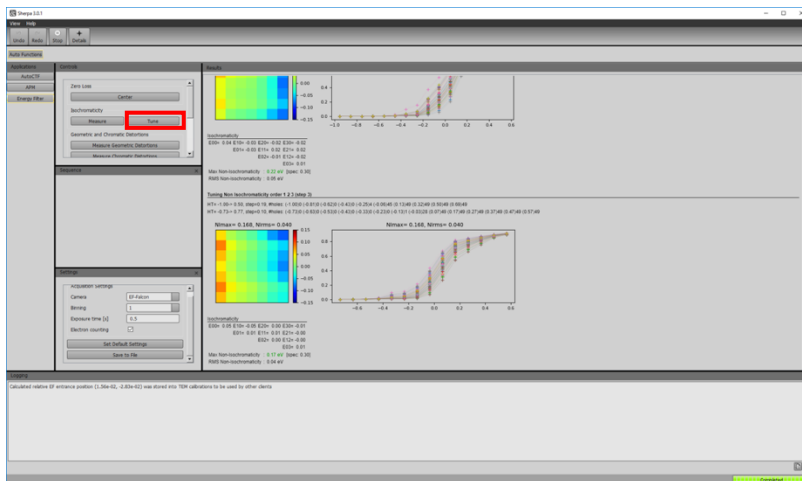


(If it is not launched, start Sherpa from Tools of the Microscope Software Launcher.)

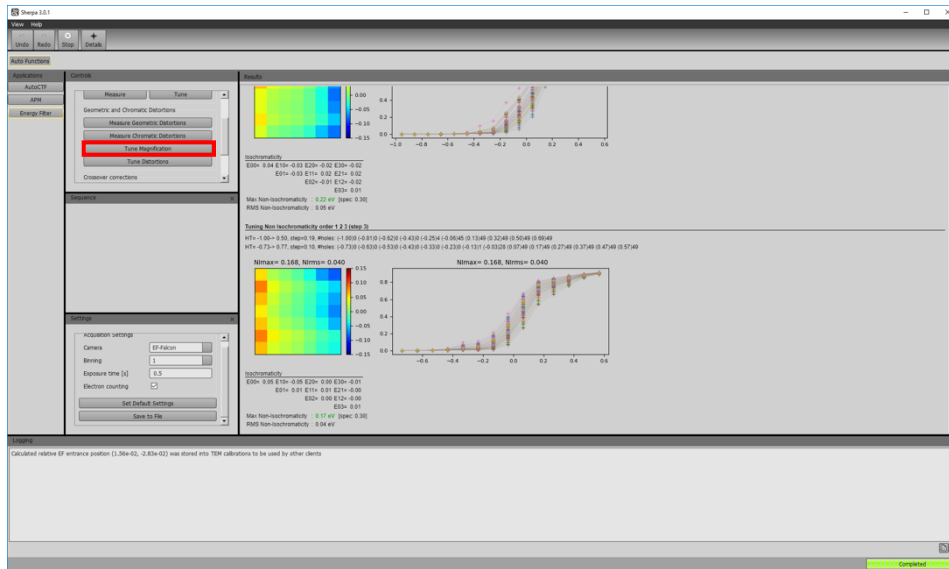
- Press the Energy Filter button on the left side of the Sherpa Window.
- Look at the Settings square at the bottom left of the Sherpa Window
- Check EF-Falcon, Bin=1, Exp time=0.5sec, Electron counting
- Look at the Controls square in the upper left corner of the Sherpa Window
- Zero loss: Press the Center button to pass the beam through the center of slit.
- Confirm that completed is displayed at the bottom right of the screen (the same applies below).



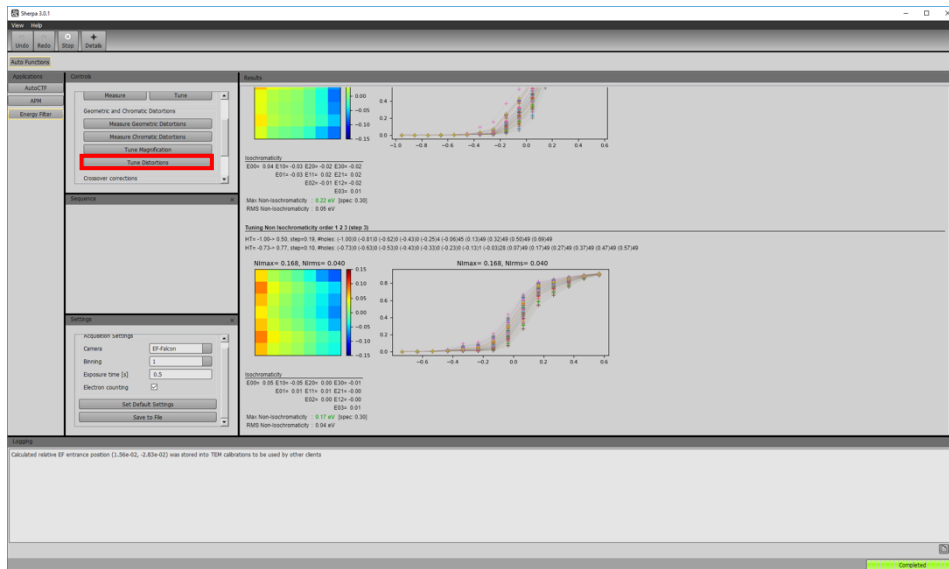
- Isochromaticity: Press Tune to equalize the energy hitting the camera. After a while, a red-green image appears. Wait a few minutes for the color of this screen to be uniformly green



- Geometric and Chromatic Distortions: Press the Tune Magnification button



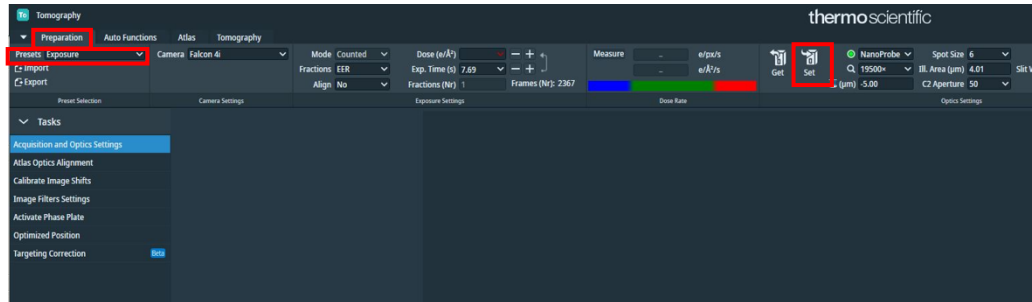
- Geometric and Chromatic Distortions: Press the Tune Distortion button.



- Zero loss: Press the Center button again to adjust the turn.

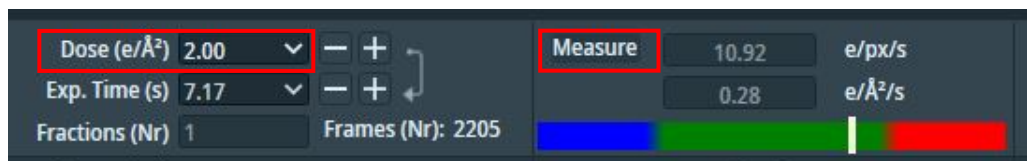
5. Dose estimation

- (Make sure you are in a broken square)
- Tomo > Preparation tab > Presets > Exposure, and click "Set"



- Enter the value of Dose ($e/\text{\AA}^2$) and press "Measure"

*For tomography, a total dose of about $100 e/\text{\AA}^2$ is recommended. If you use $\pm 60^\circ$ and 3° increments, that's 41 movies. Therefore, each movie should be about $2 e/\text{\AA}^2$.



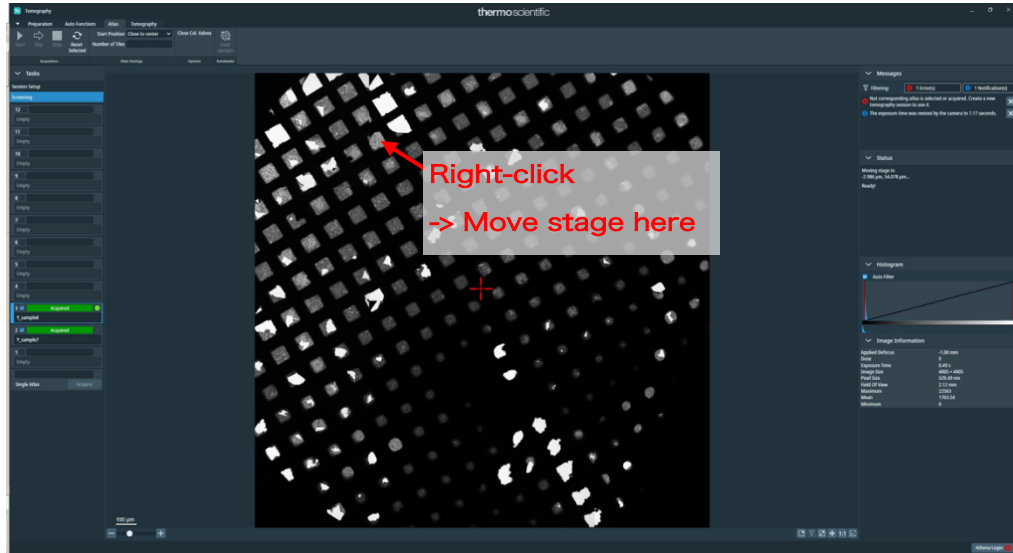
*Adjust Spot Size and Ill.Area to keep the exposure time as short as possible without going into the red area of the bar.

However, the Ill. Area should be wide enough compared to the field of view (FOV).



6. Center alignment between EPU magnifications

- Tomo > Atlas tab, where there is conspicuous trash -> Move stage here

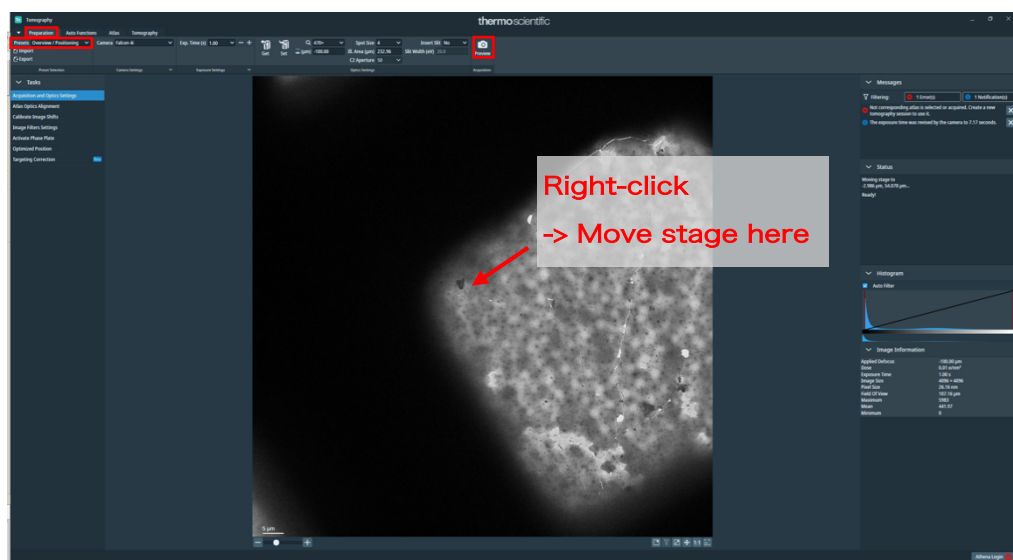


* Choose a litter that is not too large. You can control the zoom in/out of the image with the mouse wheel.

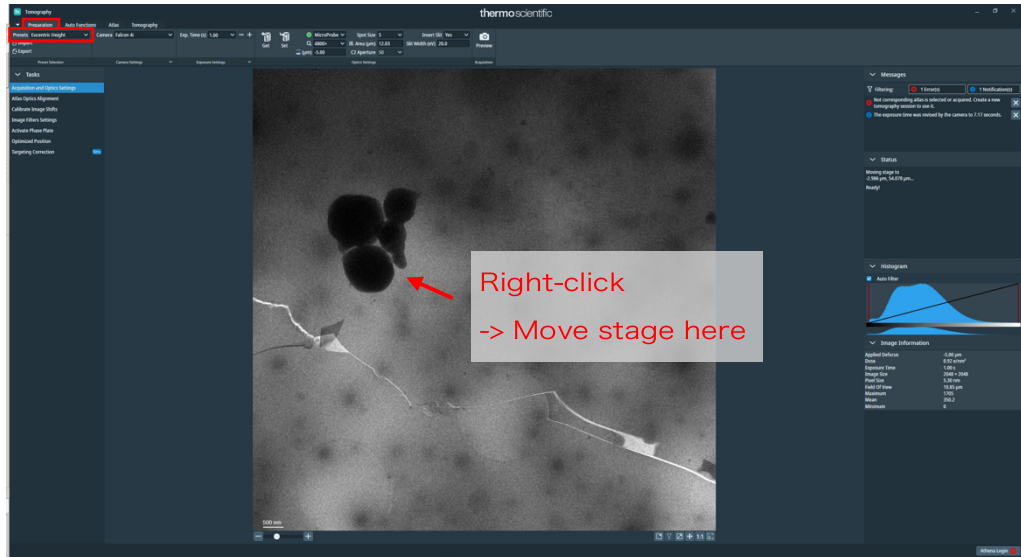
A relatively clean square is easier to make the following adjustments.

It is better to bring the center on the boundary between the garbage and the hole

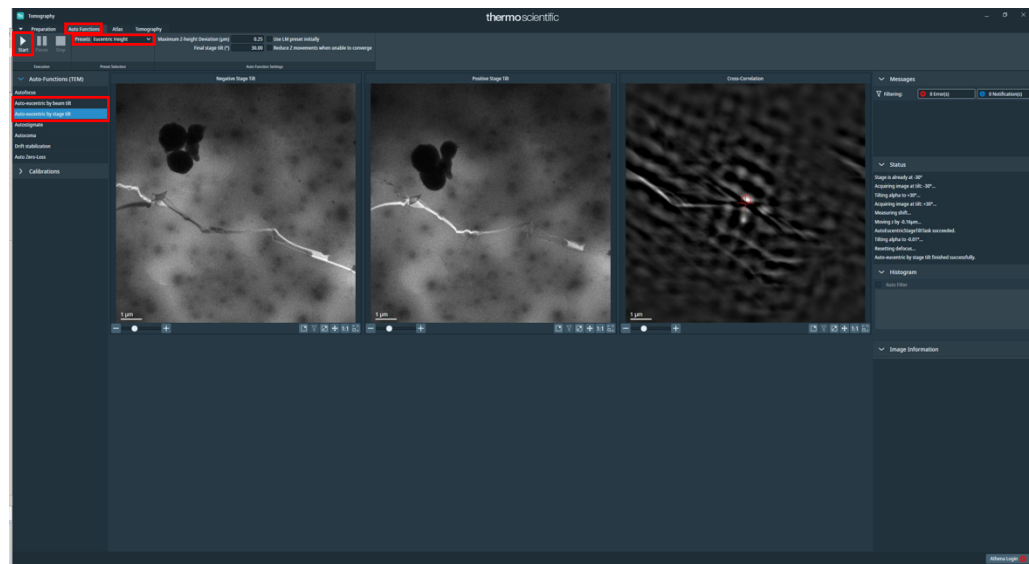
- Tomo > Preparation tab > Presets > Overview / Positioning -> "Preview"
- Right click where there is noticeable garbage -> Move stage here



- Tomo > Preparation tab > Presets > Hole/Eucentric -> "Preview"
- "Move stage here" to the corner of the garbage or the intersection of the garbage and the hole

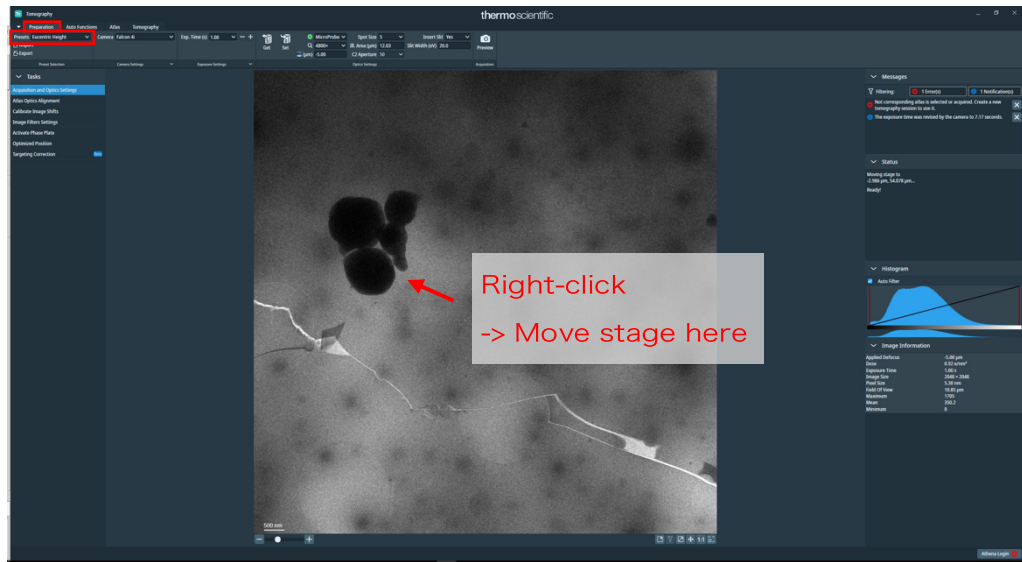


- Tomo > Auto Functions tab, select "Auto-eucentric by stage tilt" or "Auto-eucentric by beam tilt"
- Presets > Eucentric Height, and click "Start"

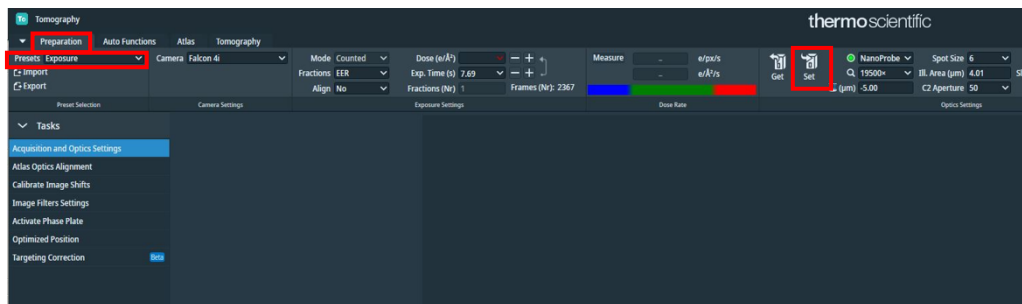


* It's fine as long as either beam tilt or stage tilt is successful.

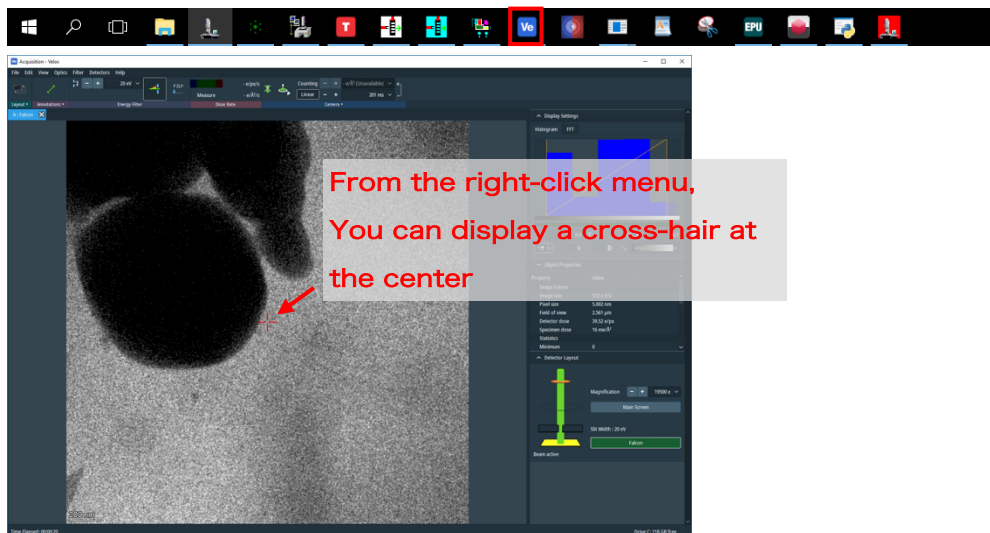
- Tomo > Preparation tab > Presets > Eucentric Height, and click "Preview"
- Move stage here at the corner of the garbage or at the intersection of the garbage and the hole




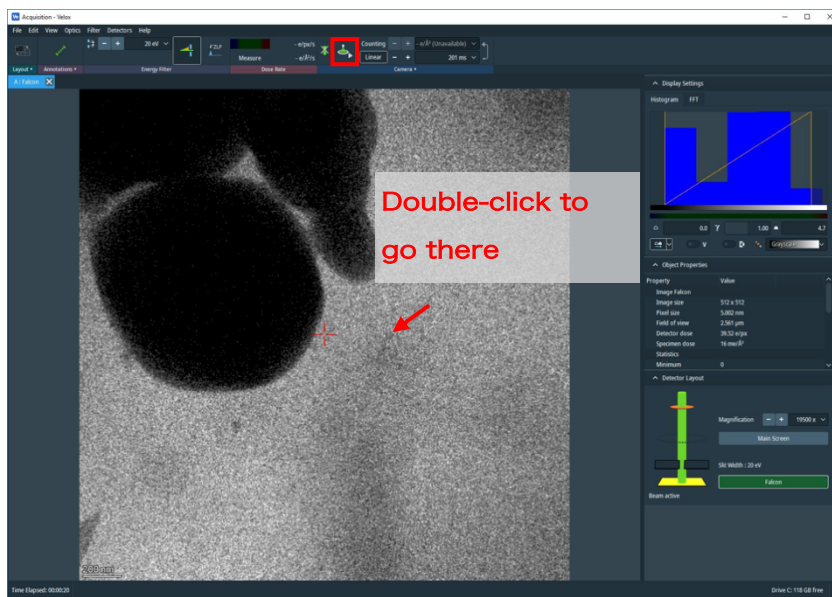
- Tomo > Preparation tab > Presets > Exposure, and click "Set"




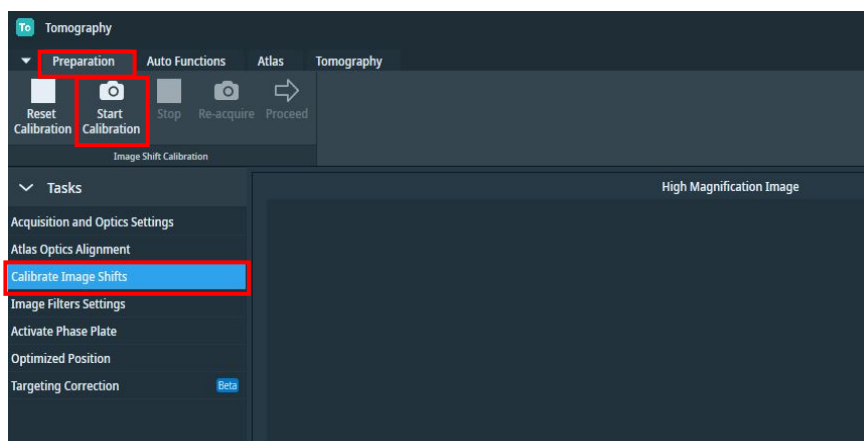
- Launch Velox (there are two windows, but only Acquisition is used)



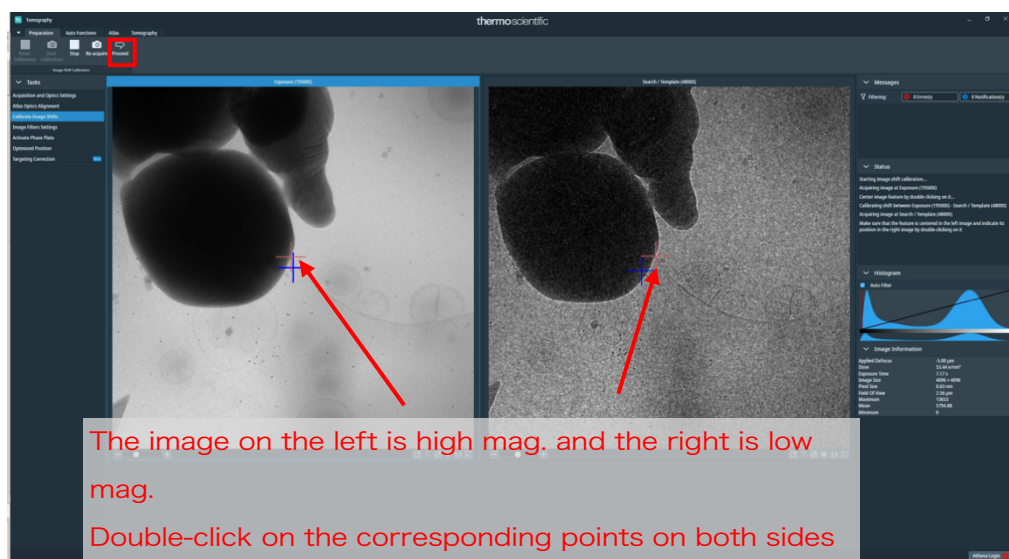
- Click the  icon. The beam irradiation begins, and an image is captured.
 - * Continue irradiation until manually stopped
- Double-click to move where you want to go in the image.
- Bring it to the corner of the garbage or the intersection of the garbage and the hole
 - * Don't go to the center you thought you would do without repeating a few double-clicks?



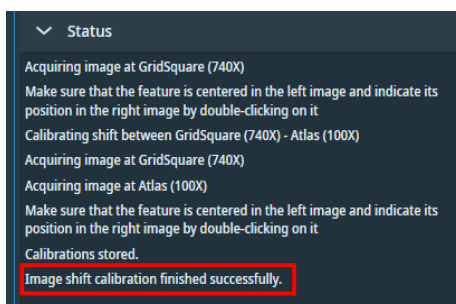
- Click the  icon again to end beam irradiation.
- Tomo > Preparation tab > Calibrate Image Shift, and click Start Calibration



- View the highest magnification (Exposure) image on the left side of the screen.
 - * If the center is off, double-click to match it and then click Re-acquire
 - Should I not do it unless it is very off?
- "Proceed"
- An image at one level lower magnification (Search = Eucentric) will be obtained on the right.
 - Double-click on the corresponding locations on both images to specify them and press proceed.



- The image on the right side will change to the left side, and a new image with a lower magnification (Overview) will be acquired on the right side.
- Again, double-click on the corresponding points on both sides and "Proceed"
- Finished when the Status says finished successfully



* If you change the magnification, try again.

That's all for daily beam adjustments.

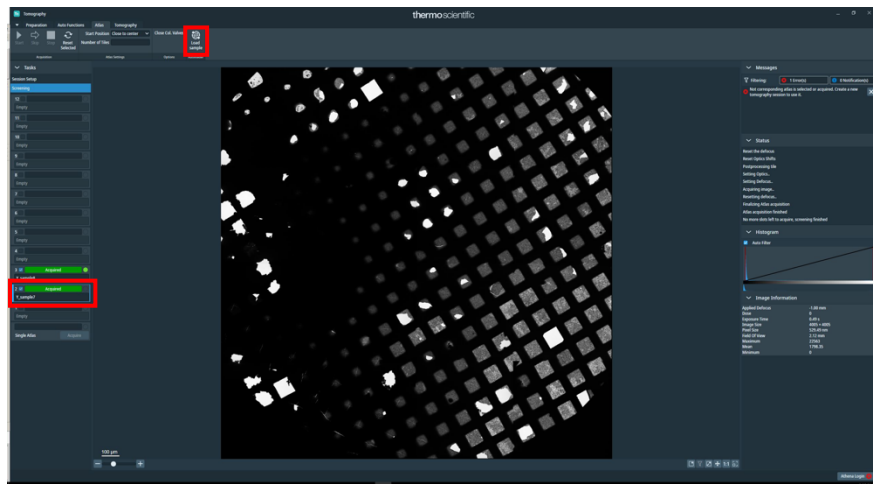
*Auto CTF does not produce a Thon ring at the tomography exposure magnification (around x20k), so if you want to make adjustments, do so at a higher magnification.

Or adjust the rotation center?

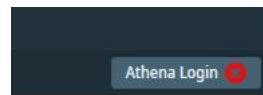
III. Data Measurement

1. Setting up a session (using Tomo Live)

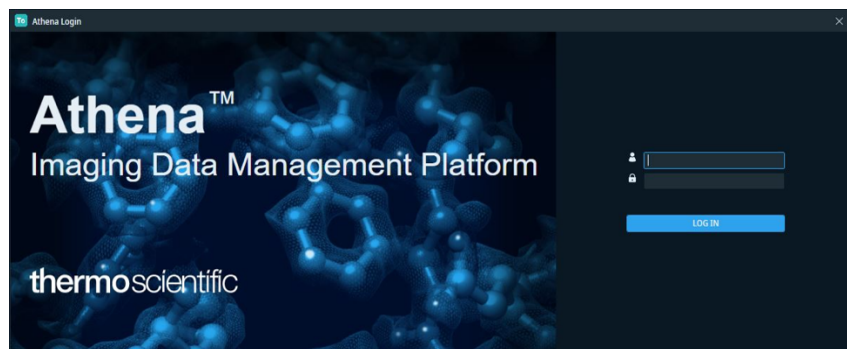
- In the Tomo > Atlas tab, select the desired grid and click "Load Sample"



- Tomo > bottom right, click the login icon to log in to Athena



Click Here



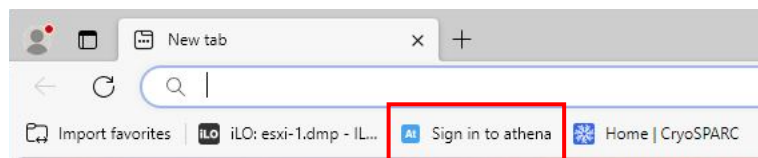
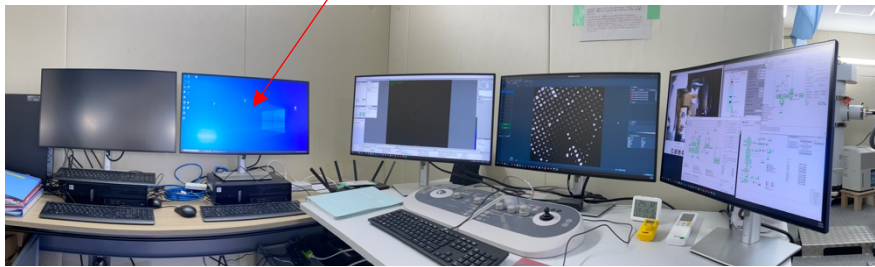
Log in window will be displayed. ID: epu, Password: epu

- If you can log in, the display at the bottom right will change.



Login ID

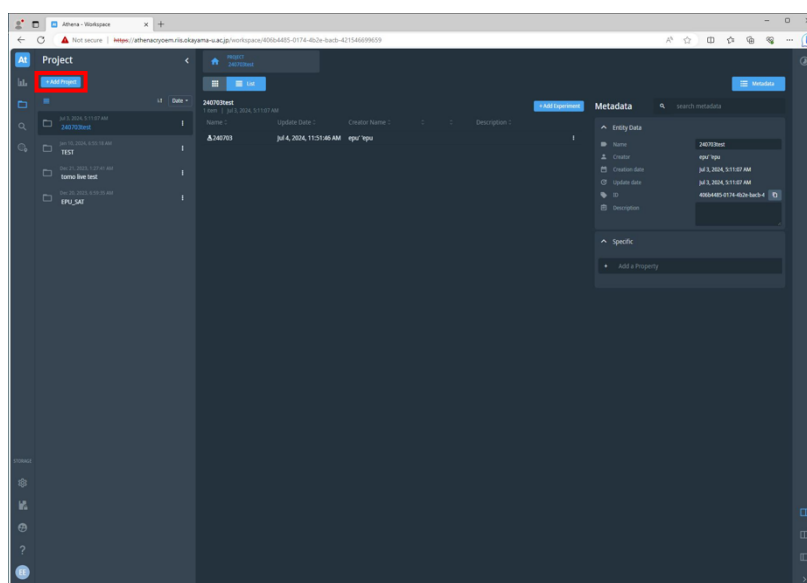
- On your Tomo Live monitoring PC, run "Edge" > Bookmark > Sign in to athena



- When the login screen is displayed, ID: epu, Password: epu

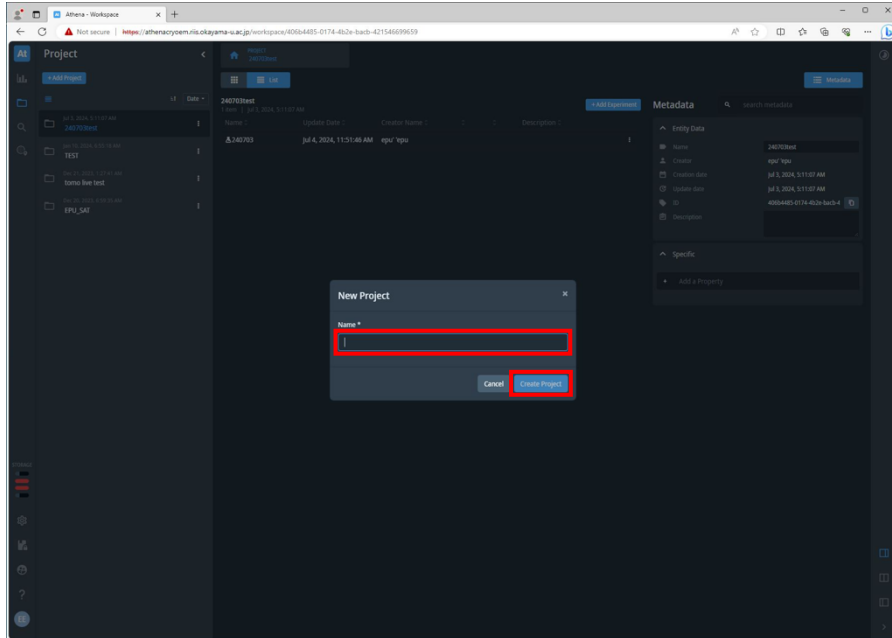


- After logging in, Athena > "+Add Project"



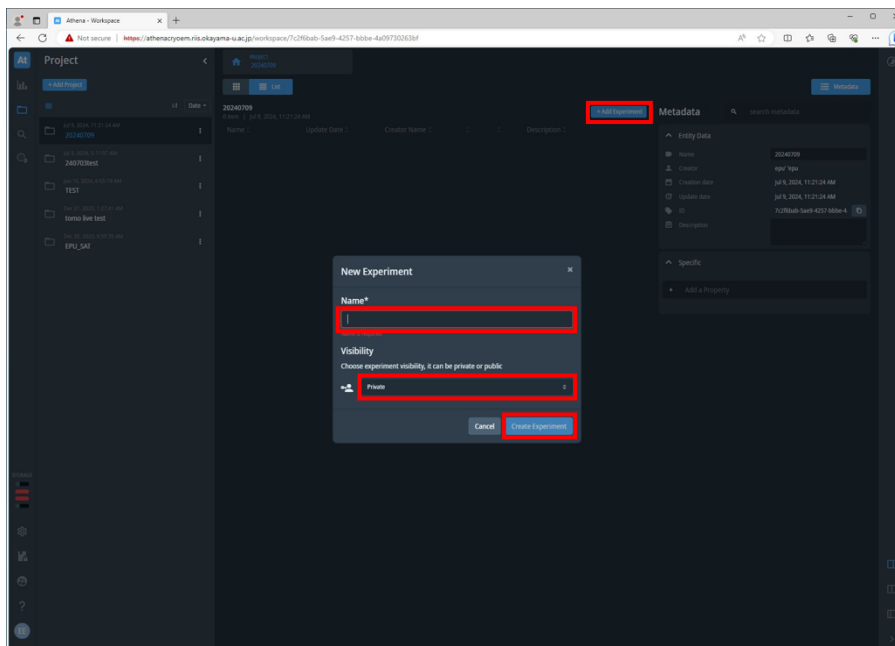
- Enter the project name and click "Create Project"

* You can create it for each sample name.

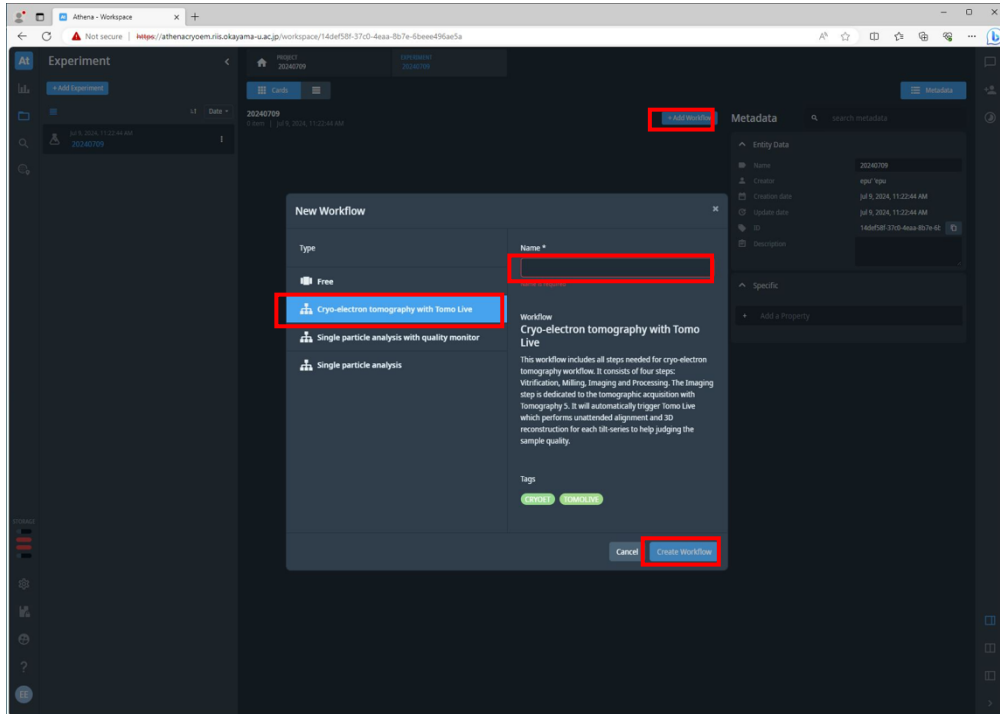


- Athena -> "+Add Experiment" > Enter the name of the experiment (e.g. date).

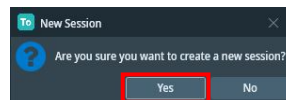
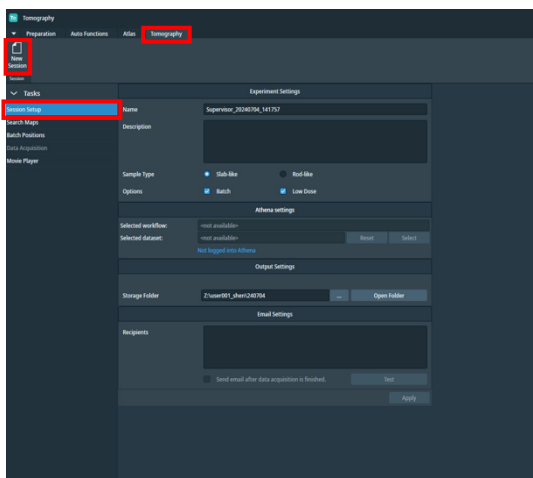
"Visibility" can be "Private" > click "Create Experiment"



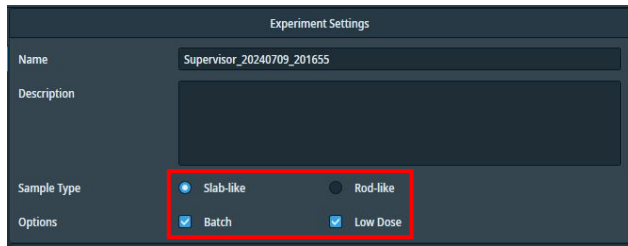
- Athena -> "+Add Workflow" > select "Cryo-electron tomography with Tomo Live" and enter a Workflow name (e.g. date) > "Create Workflow"



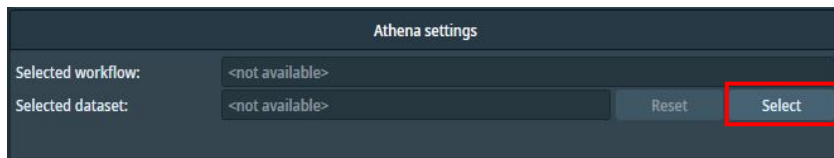
- Go back to Tomo's screen and go to Tomography tab -> "Session Setup" > "New Session" > "Yes"



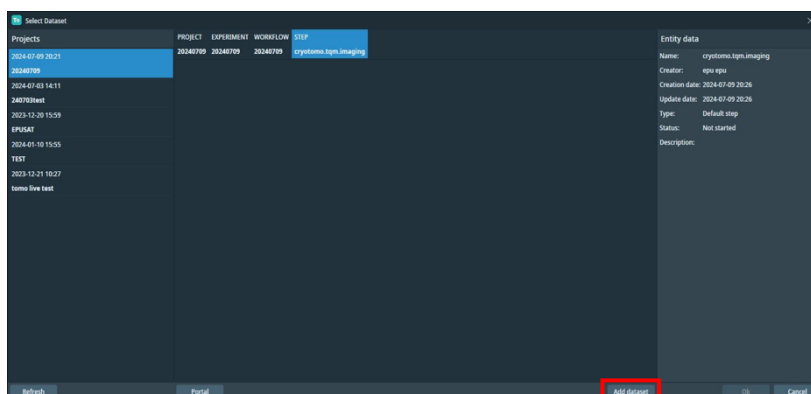
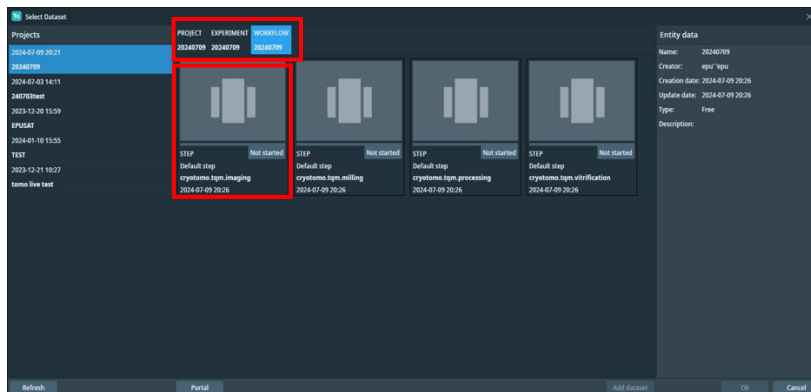
- In the Experiment settings, check Slab-like, Batch, and Low Dose.



- In Athena settings, click "Select"



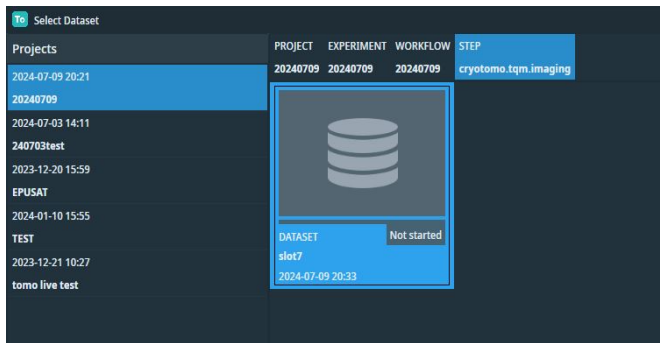
Select the Project, Experiment, and Workflow that you just created in Athena, in that order, and Select "cryotomo.tqm.imaging" in Step, and click "Add dataset"



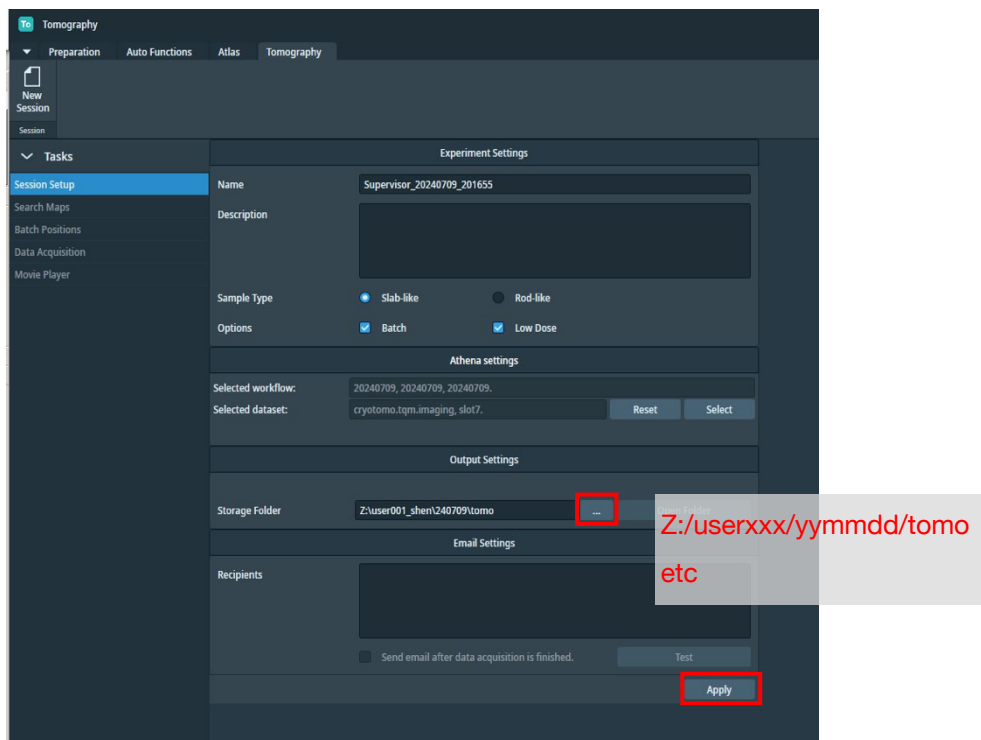
- Enter the Dataset name and click "OK"



- Verify that the Dataset has been entered



- In the Output settings section, select the destination directory.

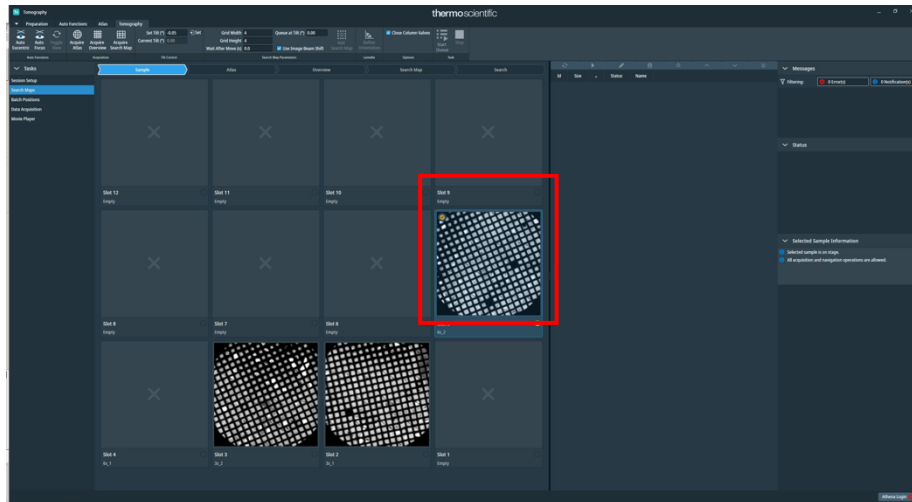


- Press "Apply"

2. Retrieving a Search Map

- Tomo > Tomography tab > Search Maps > Sample

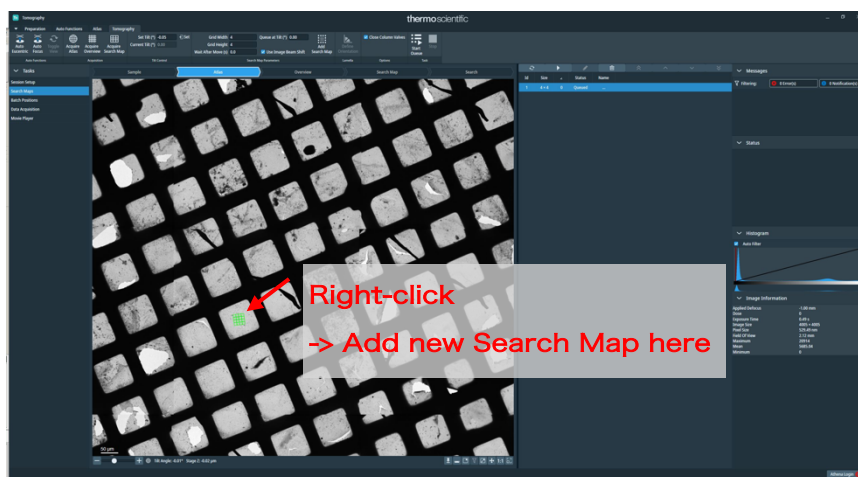
Ensure that the desired atlas is highlighted



* If the grid you want to observe is not loaded, click "Load Sample" from the Atlas tab.

*Tomo > In the Auto Function tab, set the eucentric height in an appropriate place ?

- Tomo > Tomography tab > Search Maps > Atlas, right-click on the square where you want to draw a search map and select "Add new Search Map here".



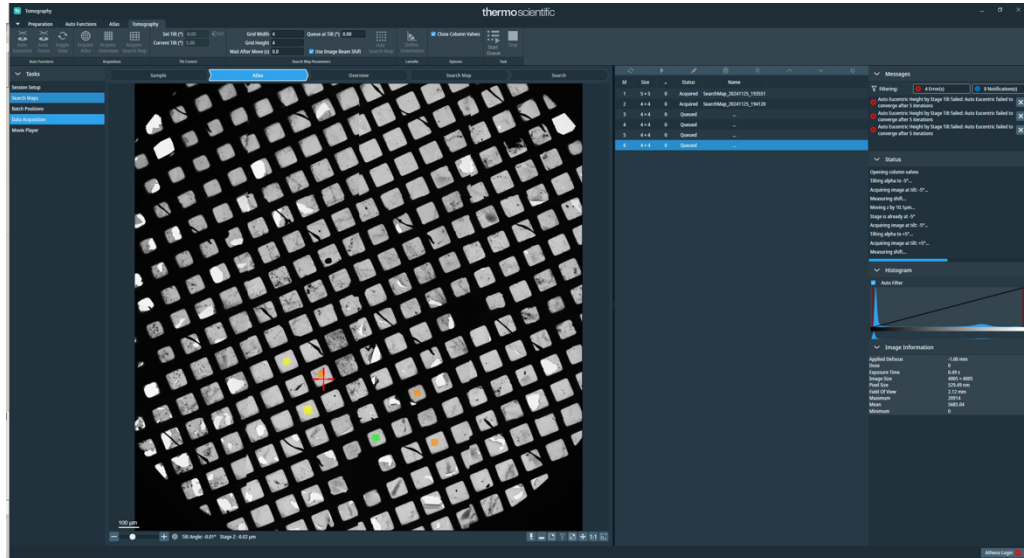
The search map is registered in the queue.

You may put it more and more on the square that you care about.

*Grid Width and Height can specify the number of tiles in the Search Map.

- "Start Queue"

Eucentric height will be determined and take a map (if eucentric fails, it will be skipped)



- *Yellow -> acquired map
- * Orange -> could not be obtained (eucentric could not be obtained) map (still Queued).
- * Green -> the map you are currently selecting

- If Eucentric fails, delete the queue and take the eucentric manually via Auto Functions tab, then

Tomo > Tomography tab > Search Maps > Atlas,

Right-click and select "Move stage here and Acquire Search Map"

Search map is acquired and added as Acquired

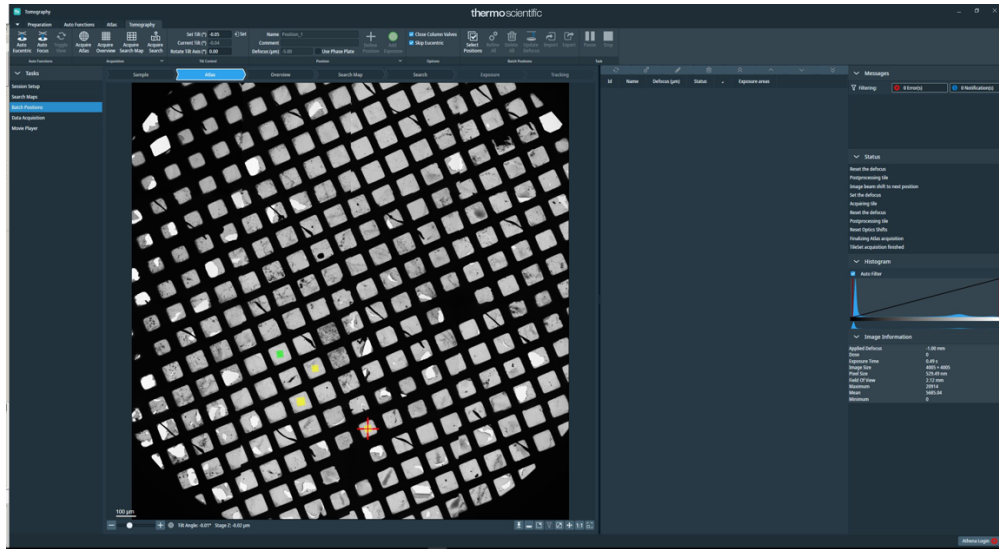
- Tomo > Tomography tab > Search Maps > Search map,

check if obtained correctly

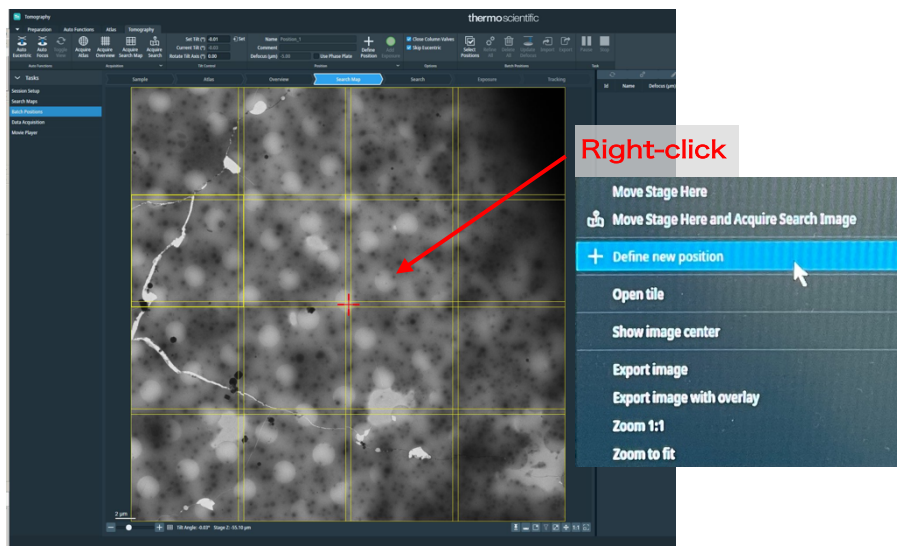
*At the bottom of the screen, the Stage Z value, the one where eucentric is automatically determined is green, the others are white.

3. Data collection

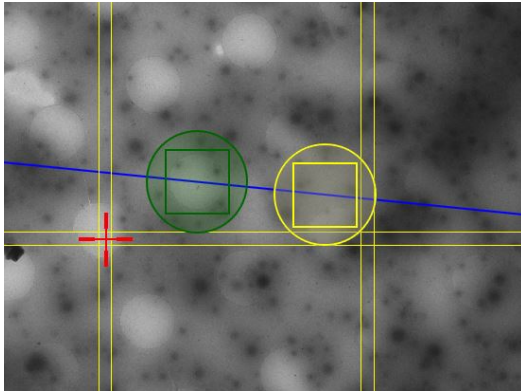
- Tomo > Tomography tab > Batch Positions > Atlas, select one of the maps



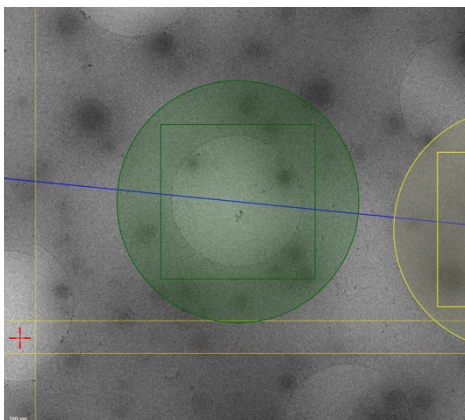
- Tomo > Tomography tab > Batch Positions > Search Map
Right click on the position you want to collect data, and select "+Define new position"



- The irradiation position (green) and focus position (yellow) for data collection come out, so adjust them.

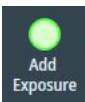


The blue line is the central axis of the stage tilt

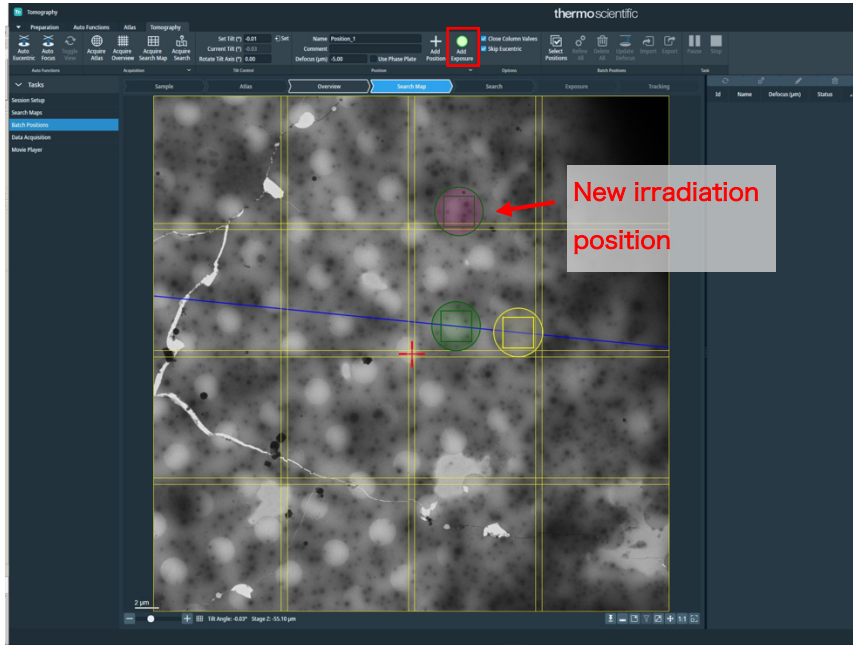


*The irradiation position (green) should be so that the hole fits in the center, and the peripheral part (colloidal gold as Fiducial markers) should also be firmly in the field of view.

*Focus position (yellow) can only be positioned on the tilt axis.

-  "Add Exposure" can increase the irradiation position (magenta)

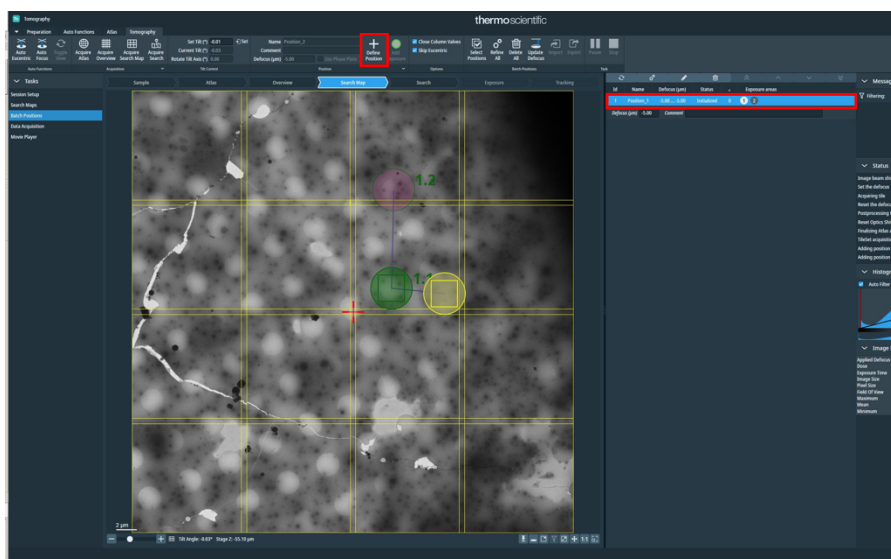
* Up to 5 irradiation locations per search map



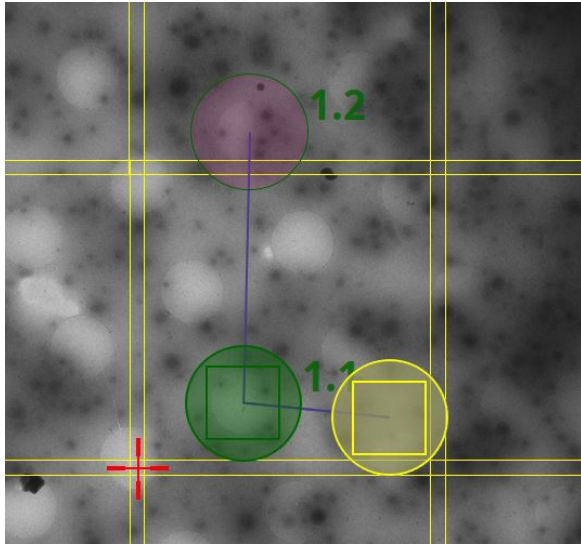
*For additional irradiation positions, data collection will be performed by AFIS

*Be sure not to choose a position too far from the initial irradiation position (green = on the tilt axis)

- Once the irradiation position and focus position are arranged, click "Add Position" to queue



* The order of the irradiation positions is displayed and reflected in the queue.



Id	Name	Defocus (μm)	Status	Exposure areas
1	Position_1	-5.00 ... -5.00	Initialized	0 1 2

Defocus (μm) -5.00 Comment

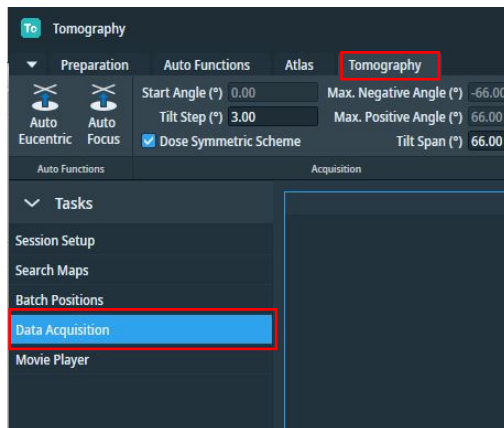
A red arrow points to the trash can icon in the top toolbar of the interface.

*If you want to delete a queue, select it and click the trash can icon

- Acquire a search map, determine the irradiation position, and add it to the queue repeatedly, and cover the area you want to collect data

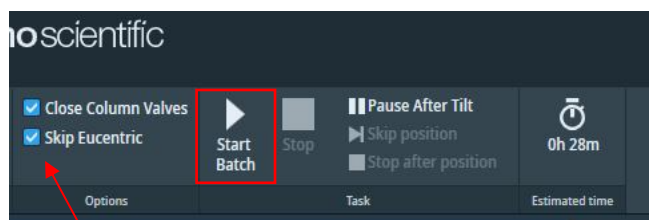
* If you use a maximum of 5 locations on one search map, it will take about 1 hour to take pictures of those 5 locations.

- Tomo > Tomography tab > Data Acquisition, set data collection parameters



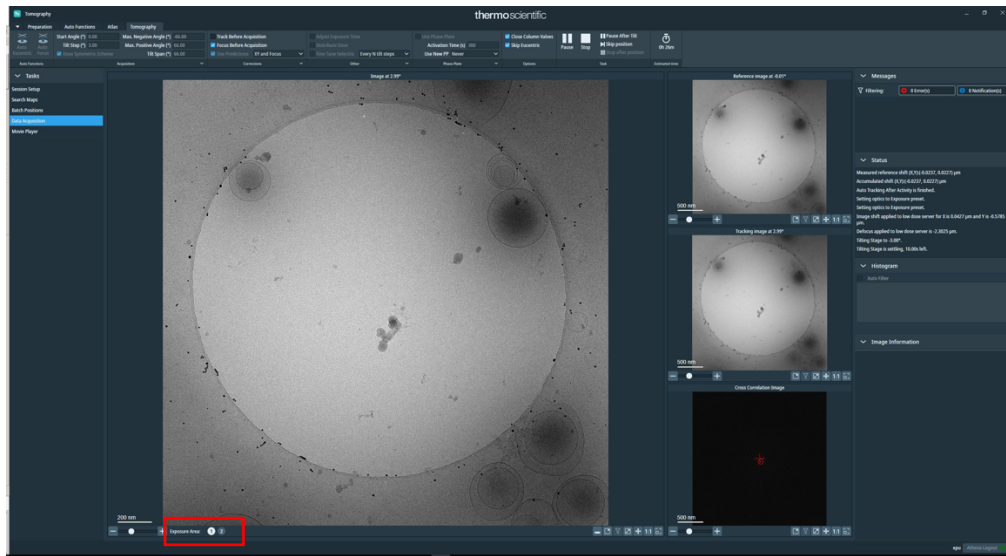
- Tilt step (°): Angle step of the data acquisition
- Tilt Span (°): Total angle range (positive and negative) of the data acquisition
- Dose Symmetric Scheme: Check the box
- Track Before Acquisition: Check the box
- Focus Before Acquisition: Check the box
- Use Predictions: Check if Holder Calibration is performed under the current shooting conditions.

- Start data collection with "Start Batch"

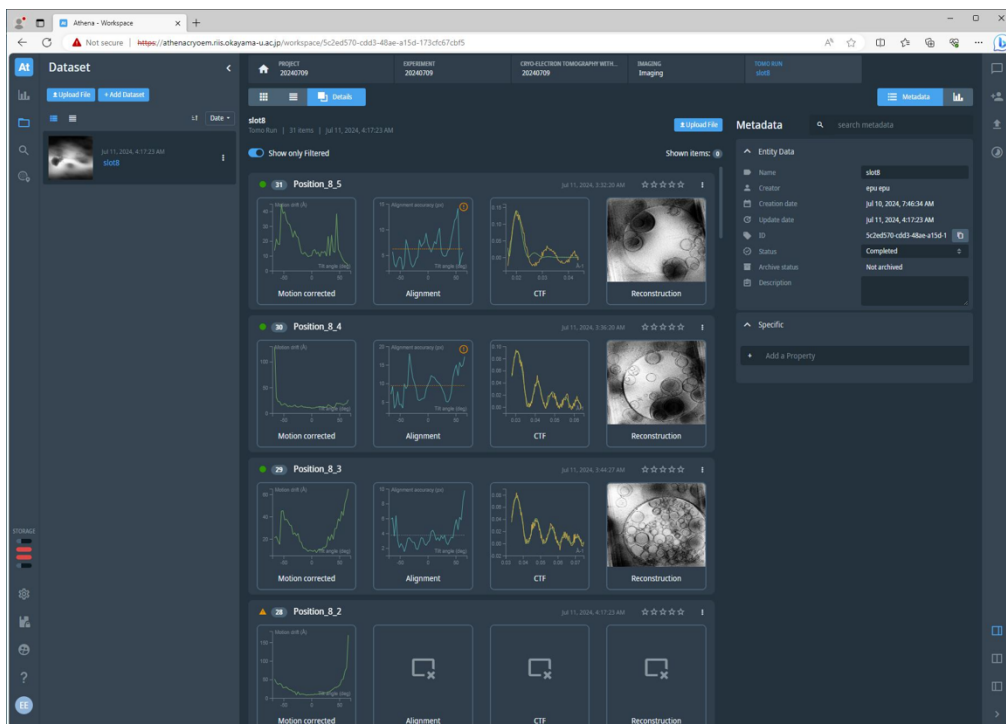


*Check both boxes.

- While data collection is in progress, each exposure area can be toggled by the number at the bottom left of the screen.



- You can check the results of the on-the-fly process with Athena's Tomo Live.



*3D reconstruction is completed 10-20 minutes after the data acquisition of the tilt series is completed.