20260423（ver. 2.3）

Okayama University

Cryo-electron Microscope Manual

Titan Krios G4 and Falcon 4i

For Admin

Acknowledgements:
This manual was created with reference to the

KEK Cryo-Electron Microscope Initial Training Text for External Users (ver.3)
https://www2.kek.jp/imss/sbrc/230203_KEKTitan_TrainingText_v3.pdf

We would like to express our sincere gratitude to all the authors of KEK text.

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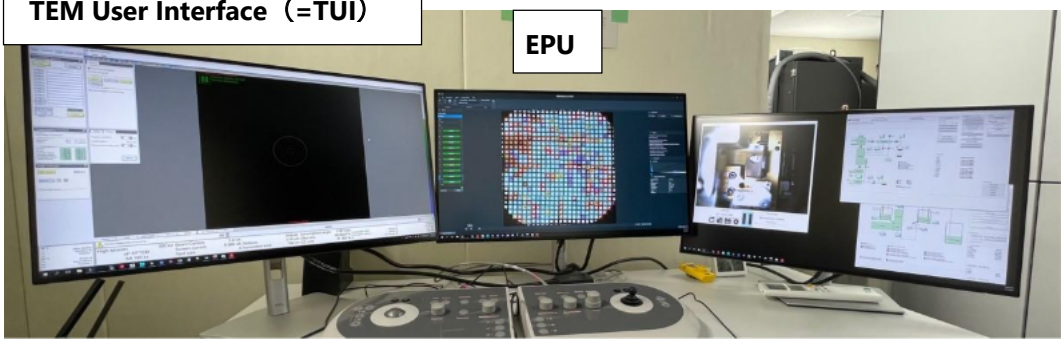
I. Sample Exchange

*TEM User Interface is version 3.22.1, EPU is version 3.14

TEM User Interface（=TUI）

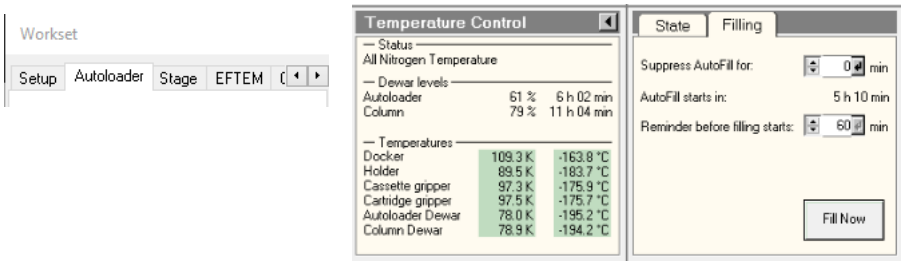
EPU



1. Inventory

Use TEM User Interface (=TUI)

TUI > Autoloader tab > Temperature Control



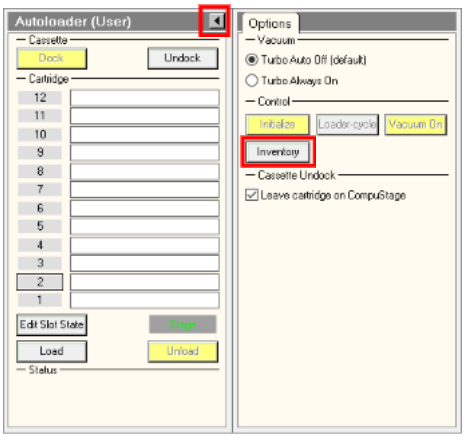
When the NanoCab Dock is finished (don't forget to take out the NanoCab!), wait until the temperature display is all green, and then wait until everything is below -160°C (a few minutes).

TUI > Autoloader tab > Autoloader > Option

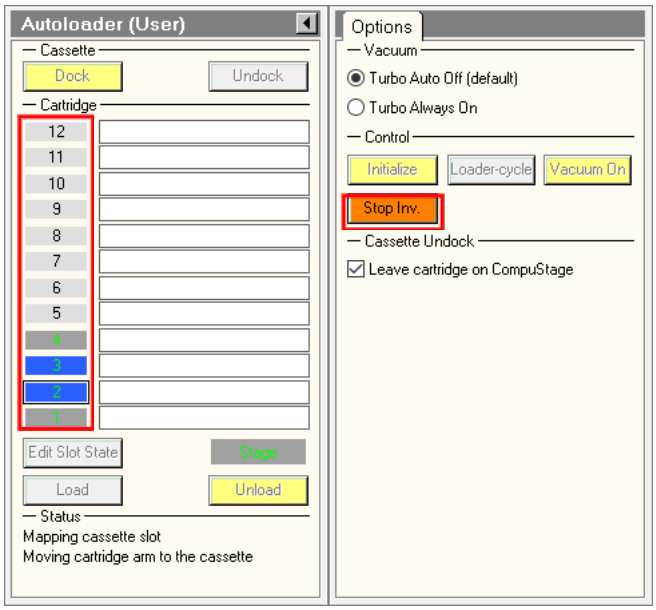
click the **Inventory** button

*After docking, it will take some time for it to become active

If the Option window is not open, click here



Make sure all grids you have entered are correctly recognized.



Dark gray: Judged as having no grid
Blue: Judged as having a grid
Light gray: No recognition work has been

*If not recognized correctly, **Inventory** again.

If there is still a discrepancy, take out the Cassette and check it.

Is the grid falling?

If you still feel strange, contact FEI

When the number of grids entered is checked to +2, click the **Stop Inventory** button to stop.

Enter the name of the grid after the inventory is finished (**Unload** if there is a grid left on the stage)

* It's not good if an empty slot has a name

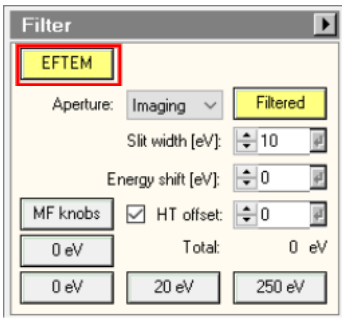
*This name will not be reflected in the resulting folder name, etc. Leave a note.

2. Restarting the EPU

View TUI > EFTEM tab > Filter

Make sure the EFTEM button is yellow

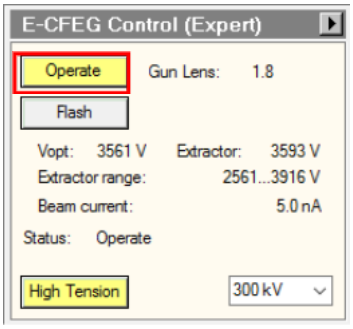
If it is not yellow, click the **EFTEM** button to turn it yellow.



TUI > Setup > E-CFEG

Check if the Operate button is yellow

If it is not yellow, click the **Operate** button to turn it yellow.



EPU Restart

Click the × in the upper right corner to drop it. Wait about 30 seconds. Launch from the pin icon

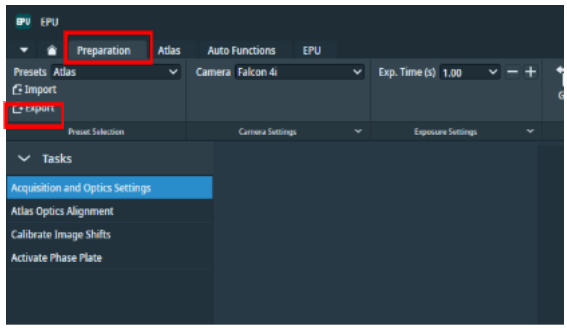
Only if the magnification changes from the previous time

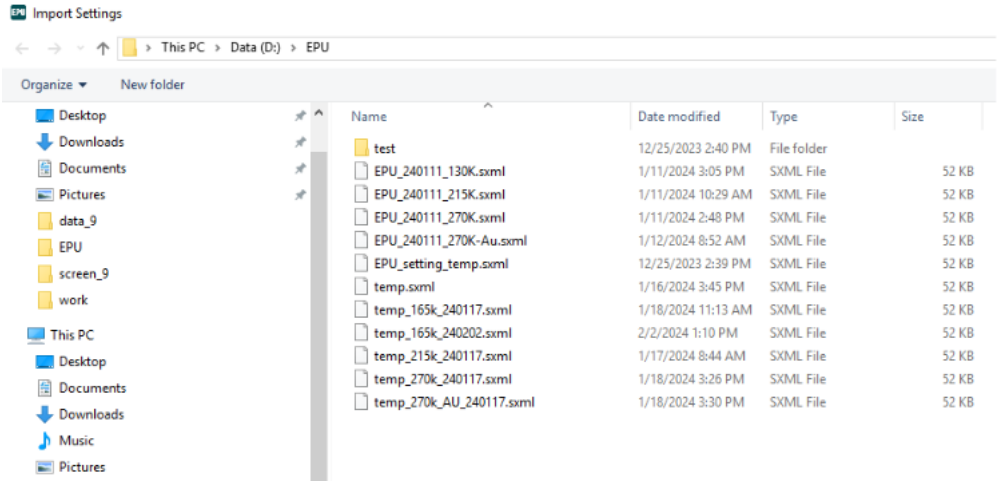
*Or when you want to restore some set value.

EPU > Preparation tab, click **Import** to open the .sxml file below.

D:/EPU/temp_xxxk_YYMMDD.sxml

(xxx=magnification. Select the latest date at the magnification you want to use)



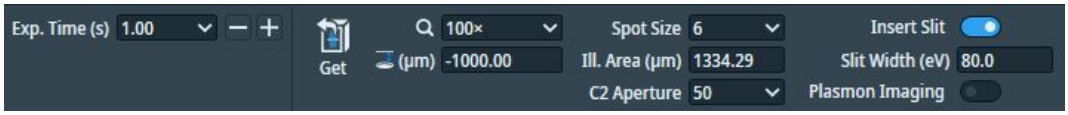


*If there is no change in magnification or settings from the last time, it is better not to do it.

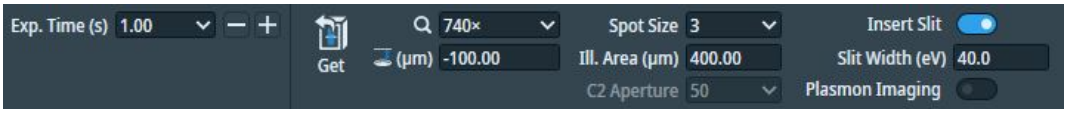
Check the settings at each magnification.

If it has changed, return to the our default values below.

EPU > Preparation tab > Presets > Atlas

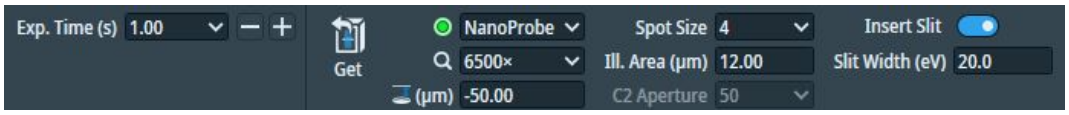


EPU > Preparation tab > Presets > GridSquare



*Magnification should be 740x for 300 mesh, 470x for 200 mesh

EPU > Preparation tab > Presets > Hole/EucentricHeight

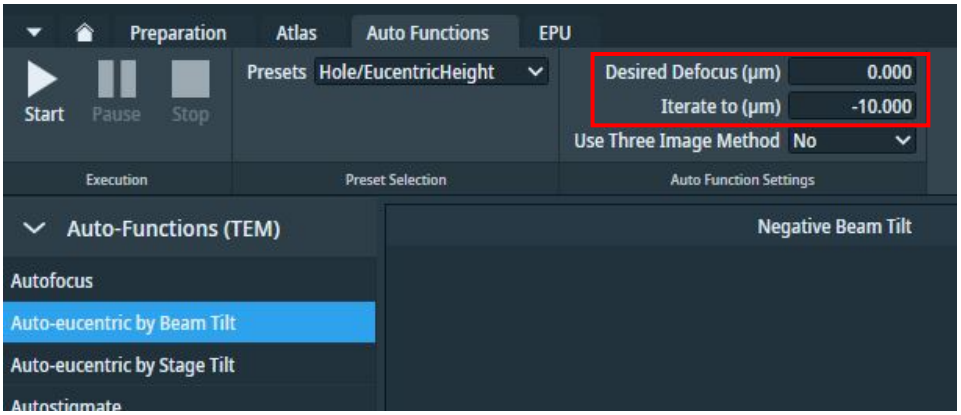


EPU > Preparation tab > Presets > Data Acquisition

*The above is for the case of 165k magnification.

It will be different when reading .sxml of other magnifications.

EPU > Auto Functions tab, **Auto-eucentric by beam tilt**

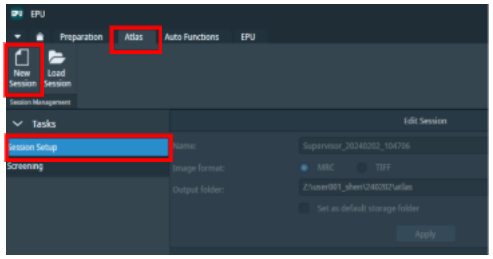


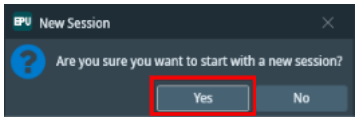
*Set Desired Defocus = 0, Iterate to = -10.

3. Get Atlas for adjustment

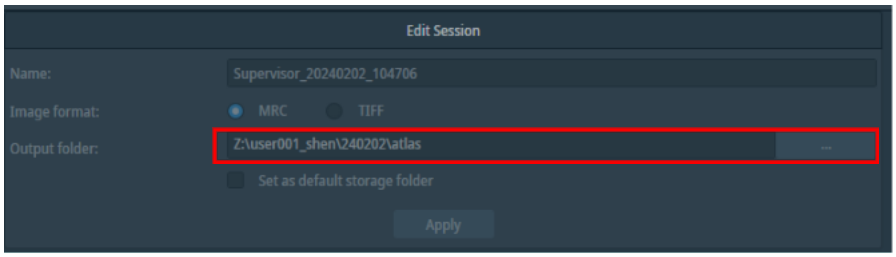
EPU > Atlas tab

Session Setup > **New Session** > **Yes**

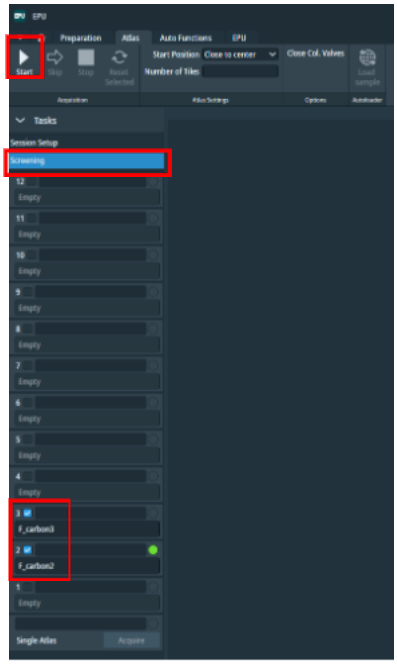




Specify the Output folder (save to: Z:/userxxx/yymmdd/atlas/) and click **Apply**

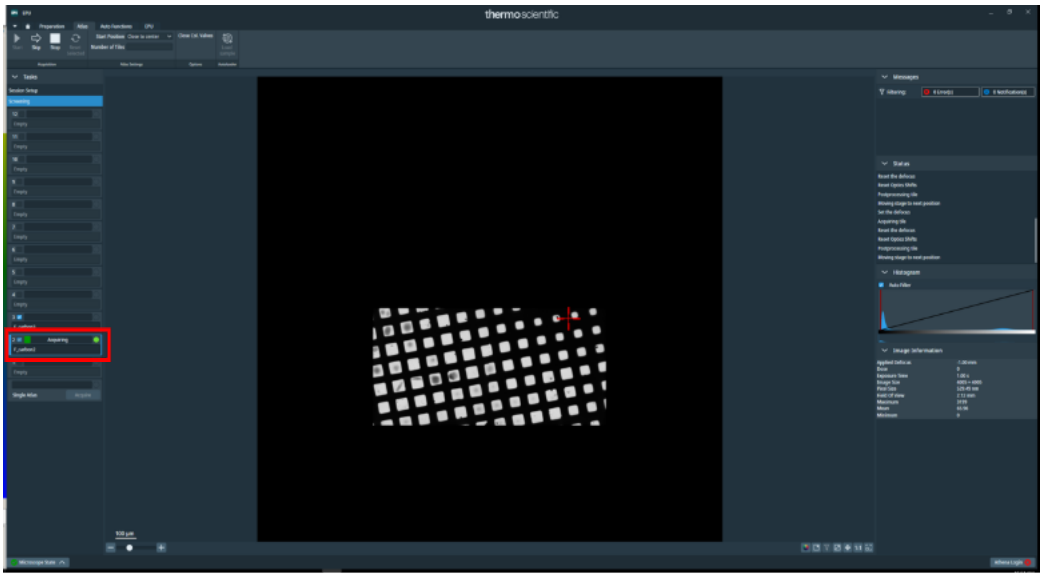


Click **Screening,** check the number of the grid you want to shoot (just one is enough), and click **Start** to start shooting Atlas



Click **Acquiring** to view the image

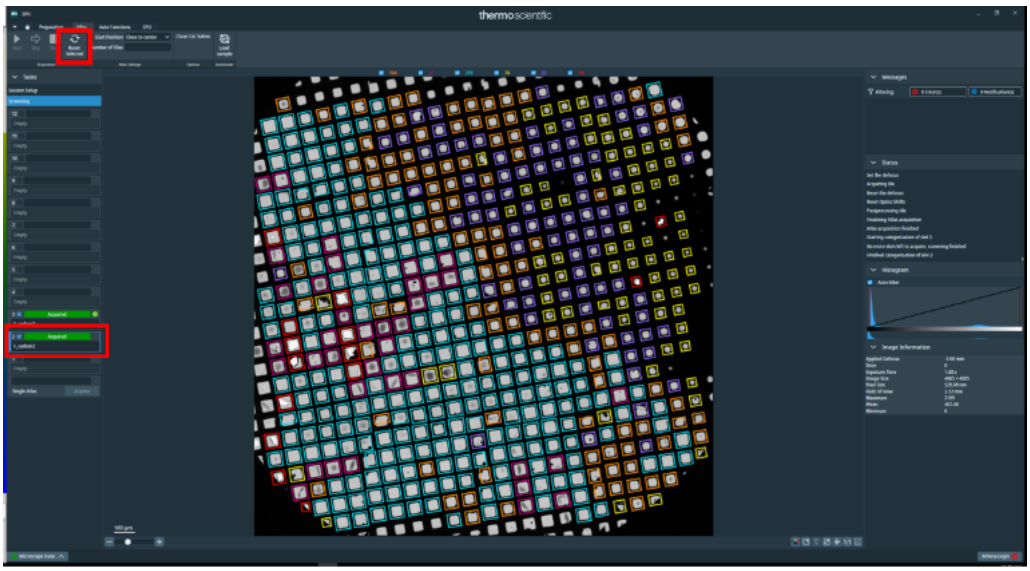
* Wait a while to shoot the whole picture with 4 x 4. Including the time to replace the grid, 7-8 minutes per grid? You can stop as soon as you have enough tile for beam alignment.



* When for some reason you want to retake Atlas for only certain grids

Select the grid in the Atlas tab and click the **Reset Selected** button in the upper left corner

The data will disappear, so check the same grid and click Start

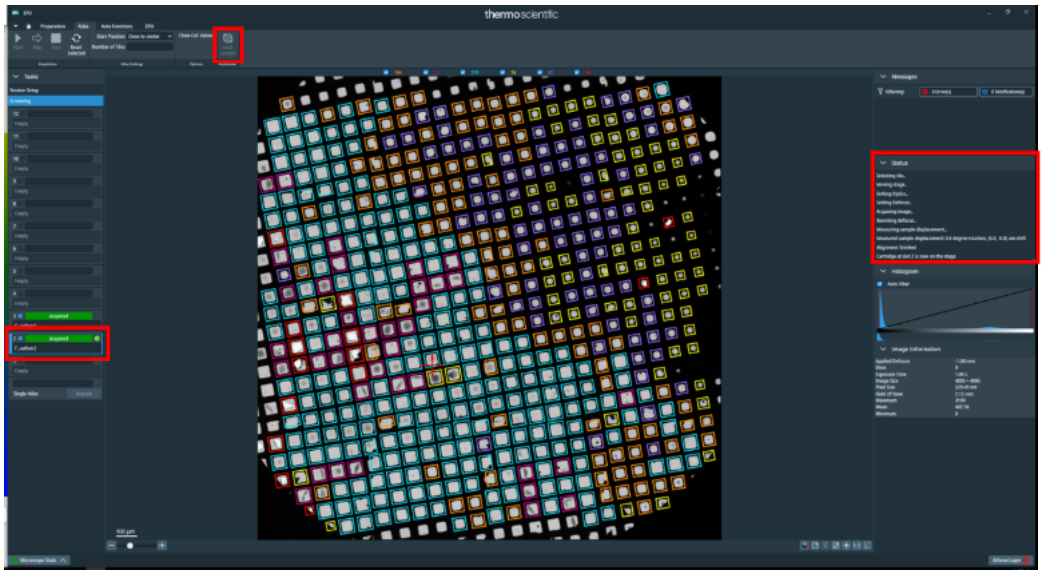


II. Beam adjustment (daily)

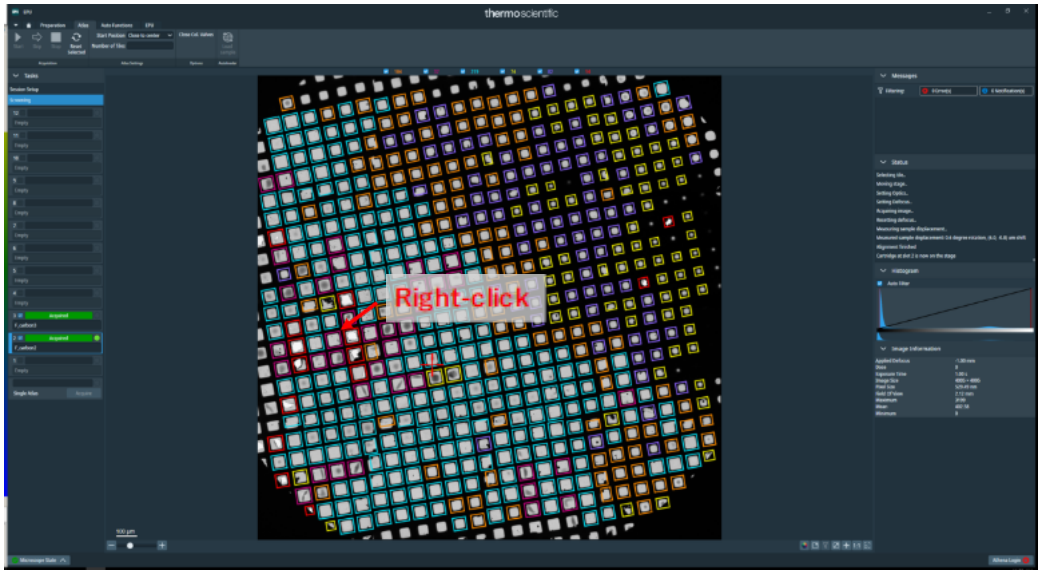
1. Move to the broken square in the grid

EPU > Atlas tab, select the desired grid and click **Load Sample**

*Confirm that "Cartridge at slot x is now on the stage" is displayed in EPU > Status



EPU > Atlas tab, right click on a broken square > **Move stage here**

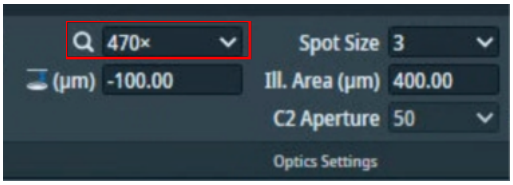


EPU > Preparation tab > Presets -> Grid Square

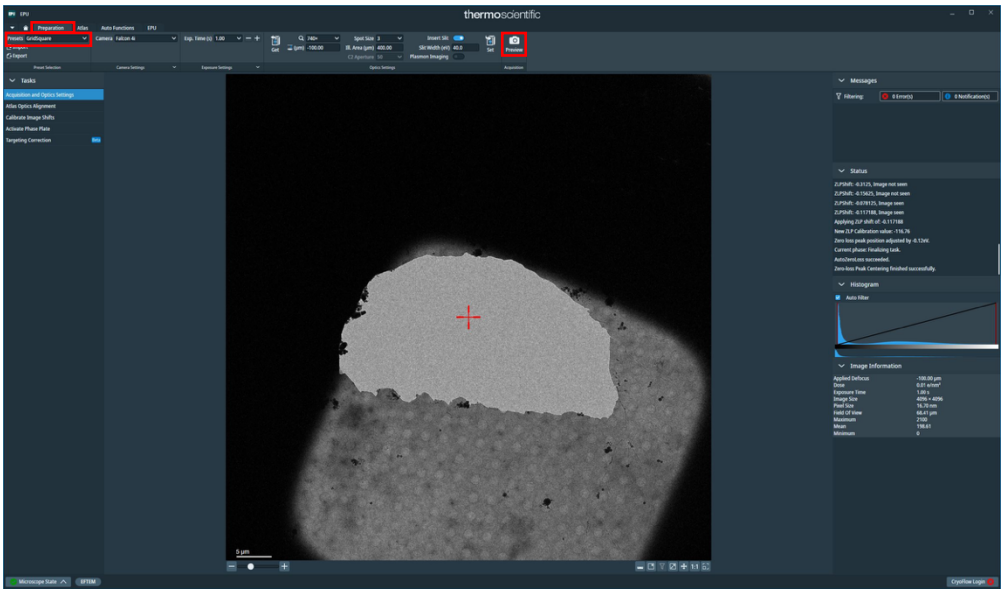
For a grid of 300 mesh, select 740x

For a grid of 200 mesh, select 470x

*Spot size, Ill. area can be left as it is.



Click **Preview**

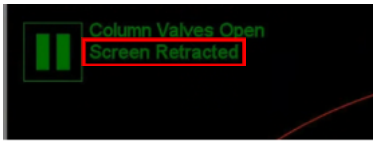


Make sure you are in near center of the broken square

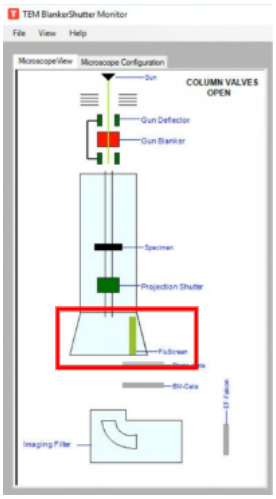
*If it is out of position, right-click again on the square image > **Move stage here**

Press **R1** to lower (insert) the fluorescent plate.

*The status of the fluorescent plate can be checked on the TUI Flucam Viewer screen or the TEM BlankerShutter Monitor screen.



*These are the states in which the fluorescent plate is raised (not inserted)

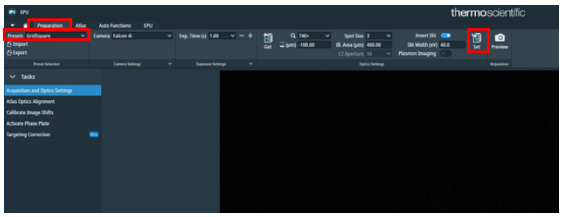


In the Flucam Viewer, confirm **EF** (= The center of the Energy filter is shown in a green circle)、**Natural,** and **High Resolution** buttons are active

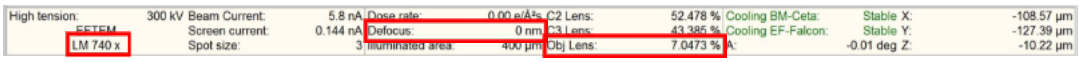
2. Pivot Point Adjustment

Make sure the stage is in the broken square and the fluorescent plate is down

EPU > Preparation tab > Presets, select Grid Square and click **Set**

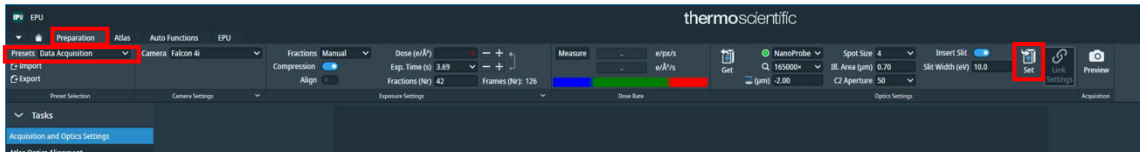


Press the **Eucentric Focus** button on the control board (*At x740, Obj lens = around 7%)



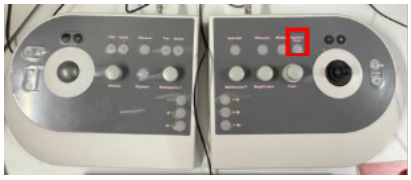
Bottom of TUI, make sure that Obj lens = around 7% at x740. If Defocus is not 0, set it to 0 on the control board R2 (Reset Defocus).

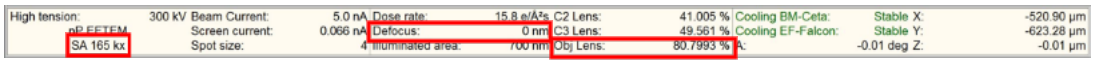
EPU > Preparation tab > Presets, select Data Acquisition and click **Set**



Press the **Eucentric Focus** button on the control panel (*Obj lens = around 80% at the set magnification)

*If Defocus is not 0, set it to 0 on the control board R2.

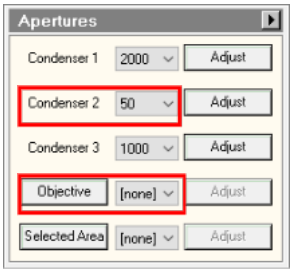




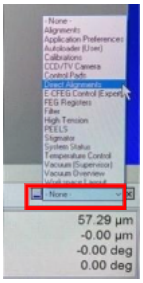
*The above operations are performed to reset the defocus of LM and SA.

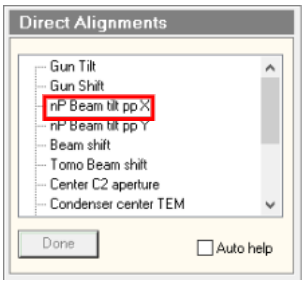
TUI > Stage tab > Apertures, make sure that C2=50, Obj=none

*Check also C1=2000 and C3=1000, and don't change these at all in the future.

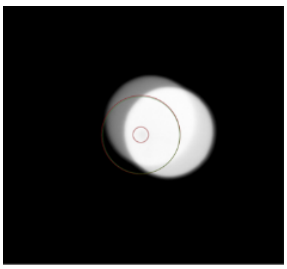


TUI (at the bottom right) > **Direct Alignment**, select **nP Beamtilt pp X**

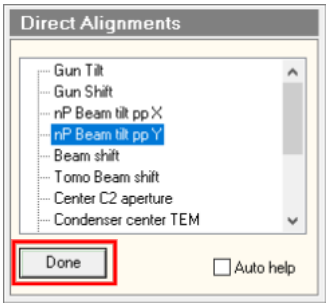




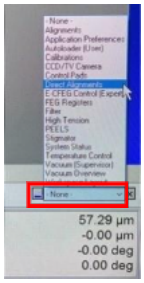
Use <u>only **Multifunction-X**</u> on the control board to adjust the two flashing lights so that they overlap.

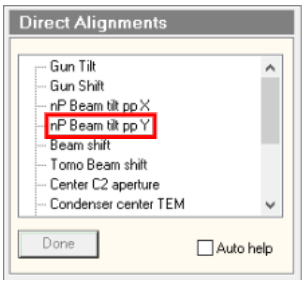


Click Done



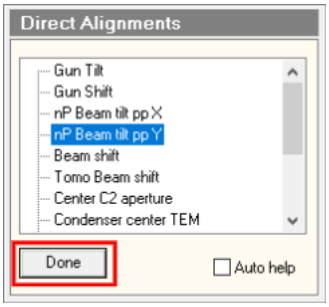
TUI (at the bottom right) > **Direct Alignment**, select **nP Beamtilt pp Y**





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

Click **Done**

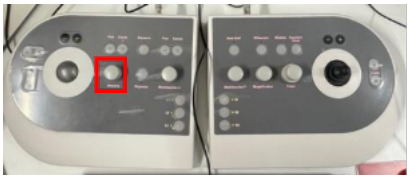


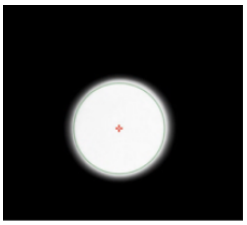
3. C2 Aperture Centering

Make sure the stage is in the broken square and the fluorescent plate is down

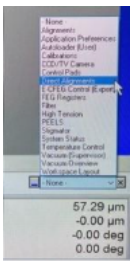
1st time

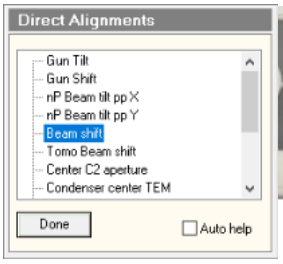
Turn the Intensity dial to the left to narrow the light to the size of the green circle in the center of the Flucam Viewer

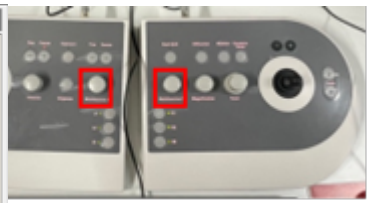


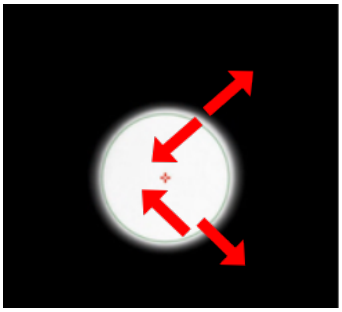


TUI (at the bottom right) > **Direct Alignment**, select **Beam shift** and center the light with the **Multifunction** dials

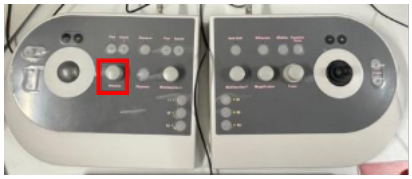






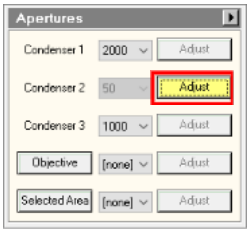


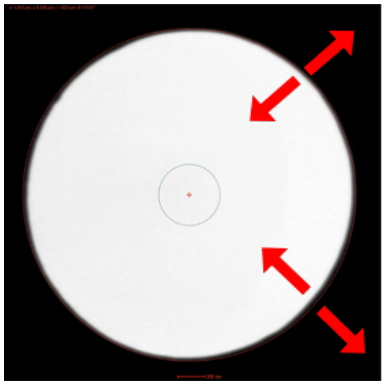
Turn the **Intensity** dial to the right and expand the light until it is the size of the outer red circle



Light contrast can be adjusted with the mouse wheel

Click **Adjust** on the right side of C2, and use the **Multifunction** dial to move the aperture so that the circle of light is inscribed in the red circle





Click **Adjust** again on the right side of C2

2nd time

Narrow the light again to about the green circle and move the light to the center with the **Multifunction** dial

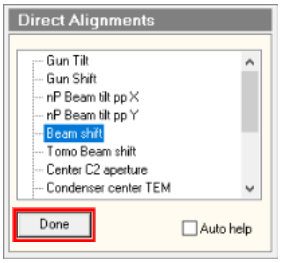
Turn the **Intensity** dial to the right and expand the light until it is the size of the outer red circle

Click **Adjust** on the right side of C2, and use the **Multifunction** dial to move the aperture so that the circle of light is inscribed in the red circle

Click **Adjust** again on the right side of C2

Again, narrow the light to about the green circle. At this time, it is OK if the ring of light spreads out almost concentricly. Repeat the above operations until the light spreads in concentric circles.

If you're okay, click **Done** on the Beam shift in Direct Alignment



*C2=150 is used for Atlas, so if you feel a problem with Atlas, it may be a good idea to make the same adjustment with C2=150.

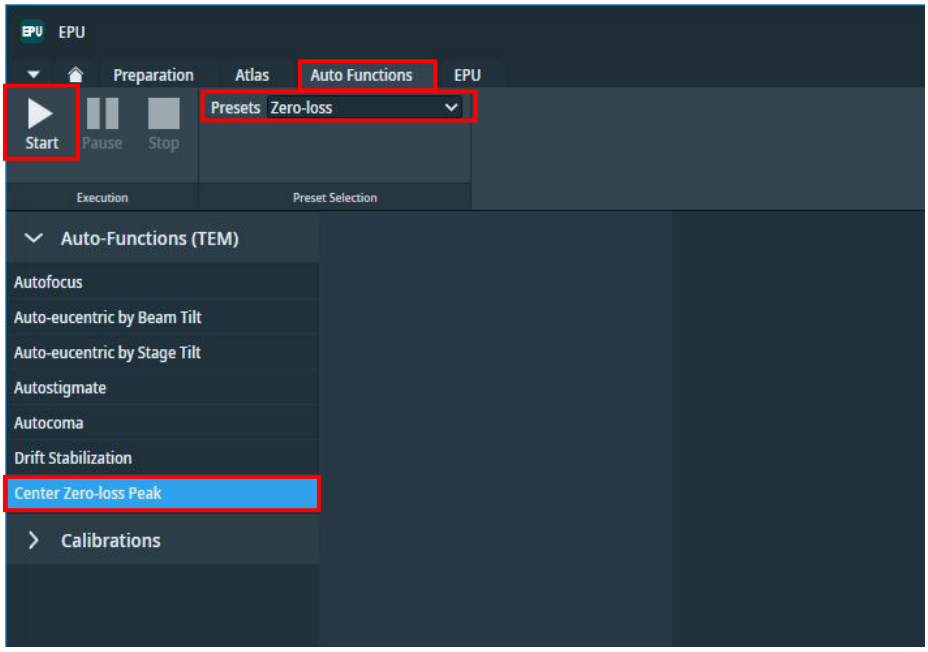
4. Center Zero-loss Peak (Auto Zero-loss)

After conditioning, adjust the energy filter according to Section III-2 (p. 41 onwards).

*It is assumed that it is a continuation of the previous part (i.e. the stage is in the broken square and the beam is aligned).

EPU > Auto Functions tab > Auto Zero-Loss

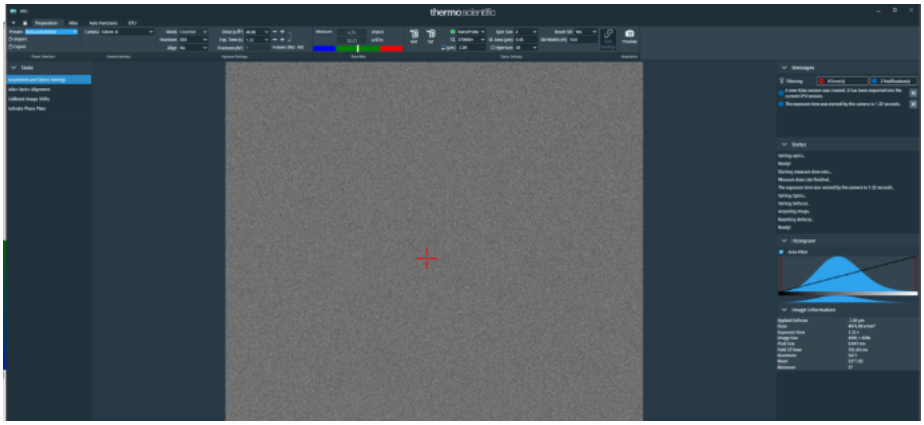
Make sure the Presets are Zero Loss and click **Start**



* Centering the energy filter

EPU > Preparation tab > Presets, select Data Acquisition and click **Peview**

Make sure the image is flat



*If it is not flat, start over from the beginning of this page

5. Alignment of the objective diaphragm (Omit if not using UltrAufoil)

When using UltrAufoil, make this adjustment with UltrAufoil

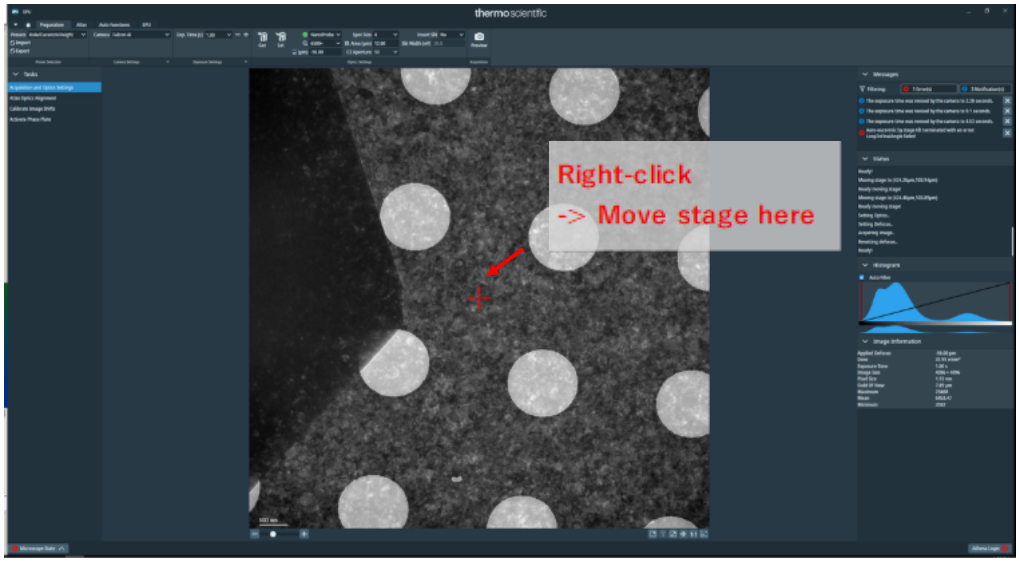
EPU > Atlas tab, select a clean square and **Move stage here**

EPU > Auto Functions tab, select **Auto-eucentric by beam tilt**

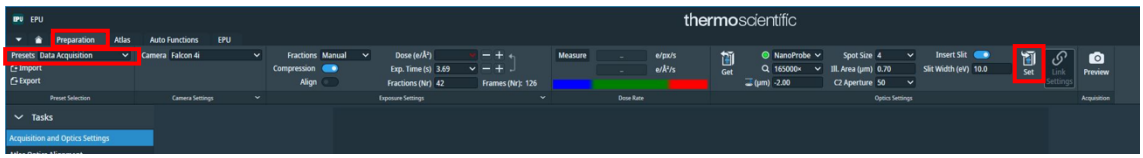
Presets > Hole/Eucentric, click **Start**

EPU > Preparation tab > Presets, select Hole/Eucentric and click **Preview**

Between holes, right-click on the trash-free area and click **Move stage here**



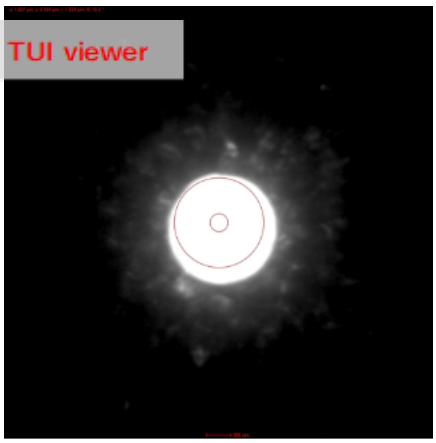
EPU > Preparation tab > Presets, select Data Acquisition and click **Set**

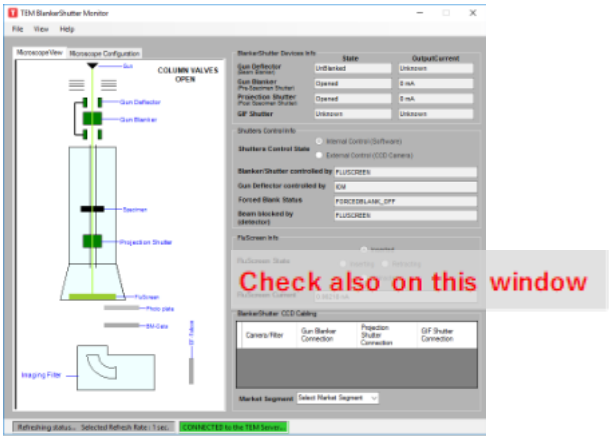


TUI > Stage tab > Apertures, make sure that **C2=50, Obj=none**

Be sure to press R1 here to put down the fluorescent plate!

***Pressing the Diffraction button without inserting the fluorescent plate may break the camera**



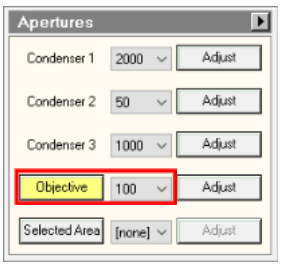


Press **Diffraction** button on the control board

Look bottom of the TUI and confirm that it displays "D = xx m" etc.

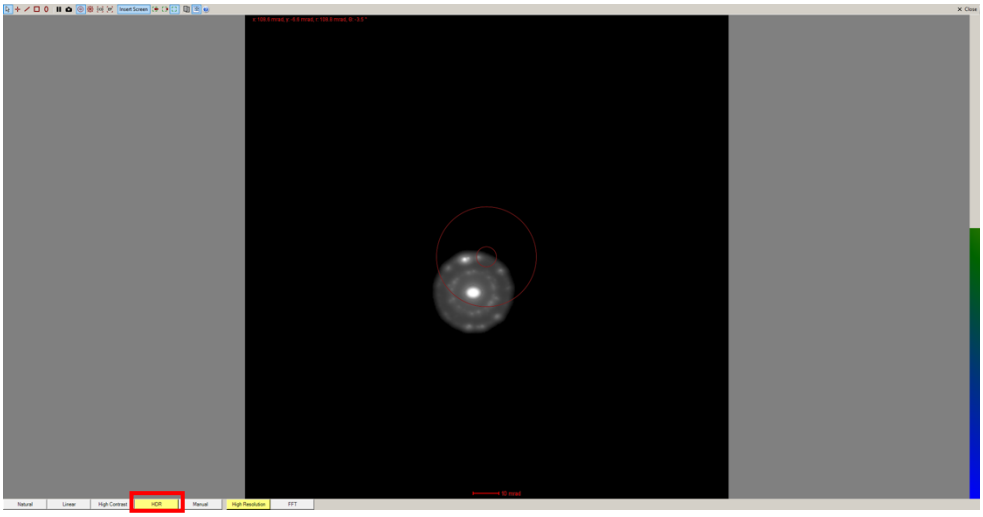
High tension:	300 kV	Beam Current:	5.3 nA
nP EFTEM		Screen current:	0.065 nA
D 2.5 m		Spot size:	4

TUI > Stage tab > Apertures, set **Obj=100**

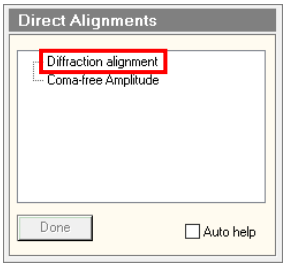


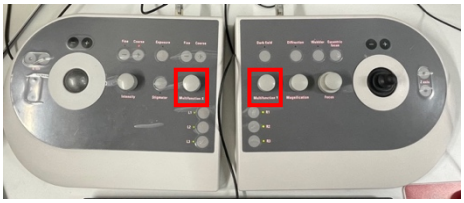
TUI in HDR mode

*If it is difficult to see the shadow of the objective aperture, adjust it with the mouse wheel.



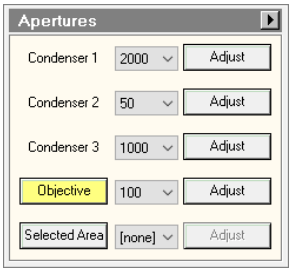
If the beam is not in the center, it will be centered with a **Direct Alignment > Diffraction Alignment on the Multifunction dial**



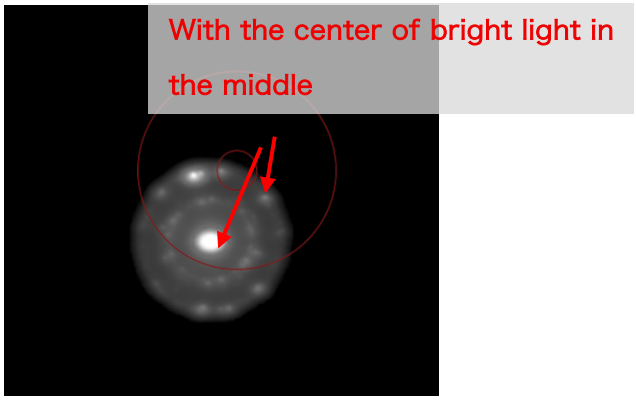


(Is it safe not to do the following three points unless they are very off?)

Click the **Adjust** button next to Objective



Use the **Multifunction** dial to align the bright center of light with the surrounding fuzzy center of light



Click the **Adjust** button next to the Objective again

Press the **Diffraction** button on the control board again to exit diffraction mode.

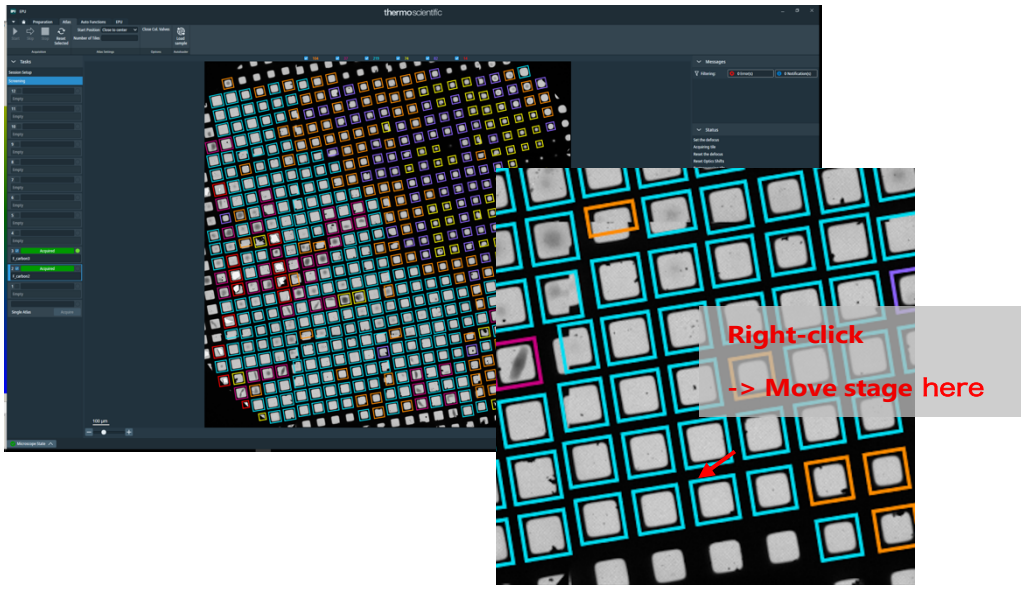
*The FluCam Viewer display should automatically return to Natural

Observations with UltrAufoil can be made with **Obj = 100**

6. Center alignment between EPU magnifications

*When using UltrAufoil, it is better to make this adjustment with UltrAufoil

Move stage here where there is a noticeable garbage by looking at the EPU > Atlas tab



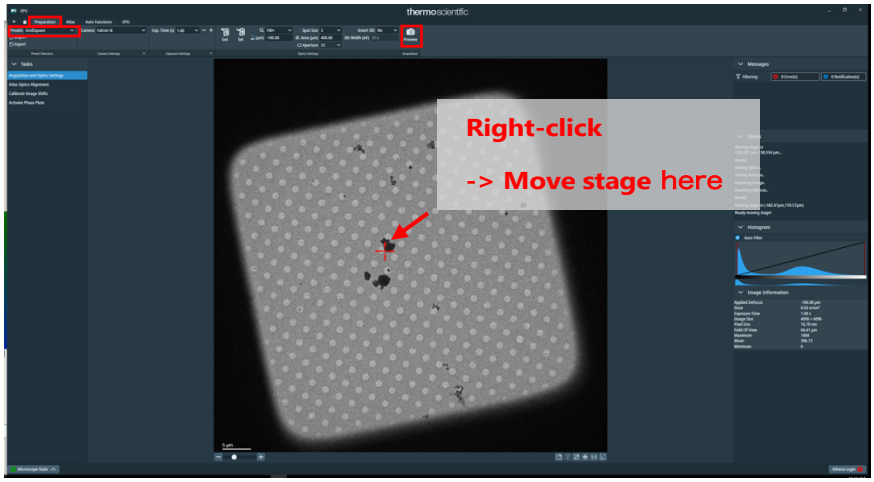
* Choose garbage that is not too large. Zoom in/out of images with the mouse wheel

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

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

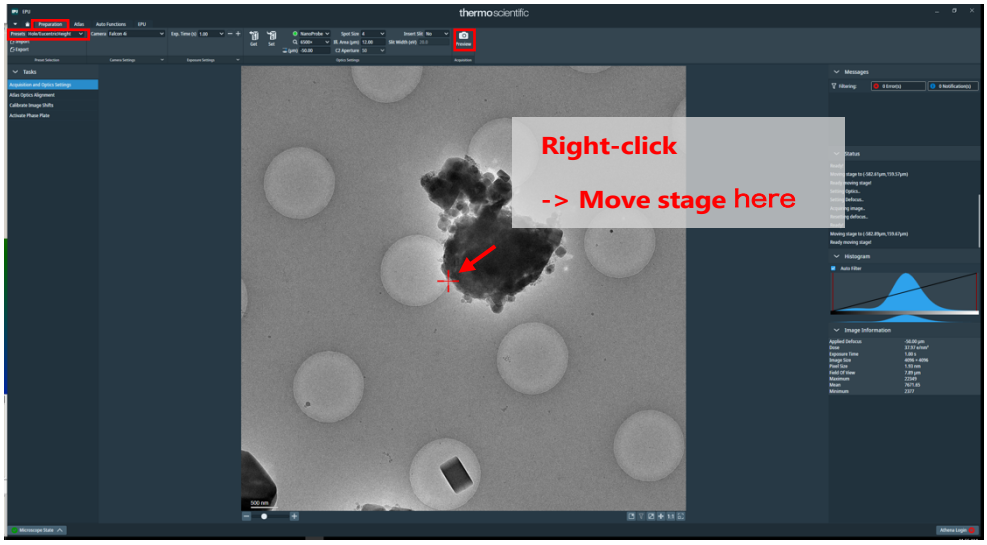
EPU > Preparation tab > Presets, select Grid Square and click **Preview**

Right-click on a conspicuous piece of garbage > **Move stage here**



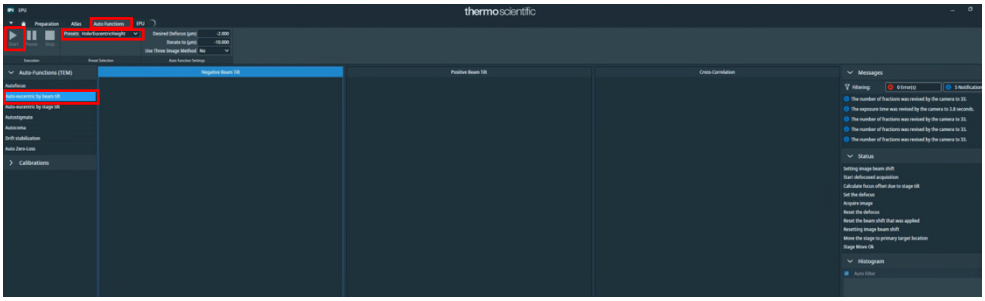
EPU > Preparation tab > Presets, select Hole/Eucentric and Preview

Right-click on a conspicuous piece of garbage > **Move stage here**

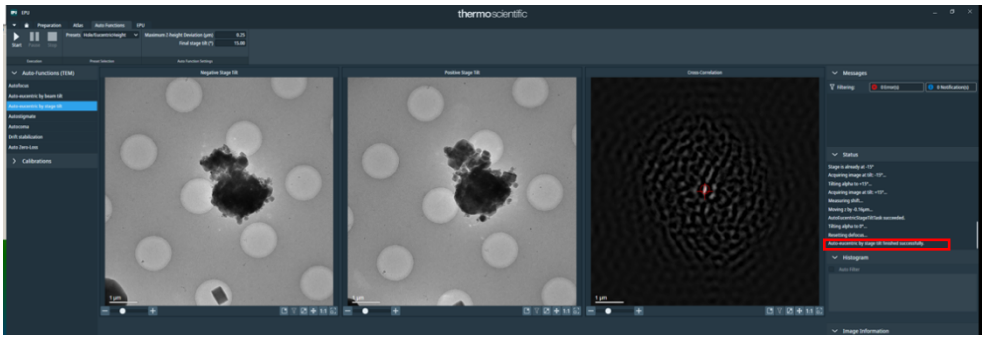


EPU > Auto Functions tab, select **Auto-eucentric by beam tilt**

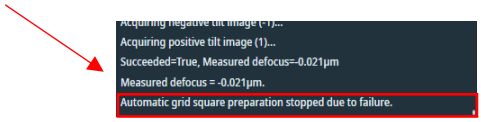
Set Presets to Hole/Eucentric and click **Start**



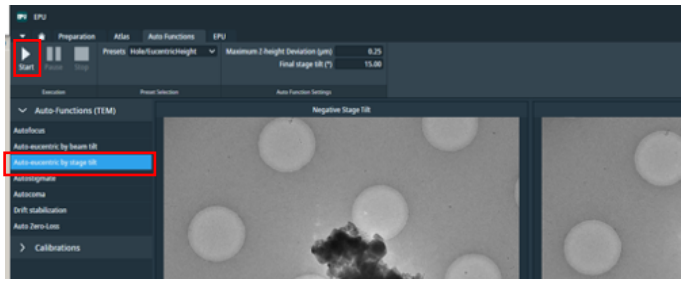
Check the display of "Auto-eucentric by stage tilt finished successfully" in Status



*If Auto-eucentric fails,



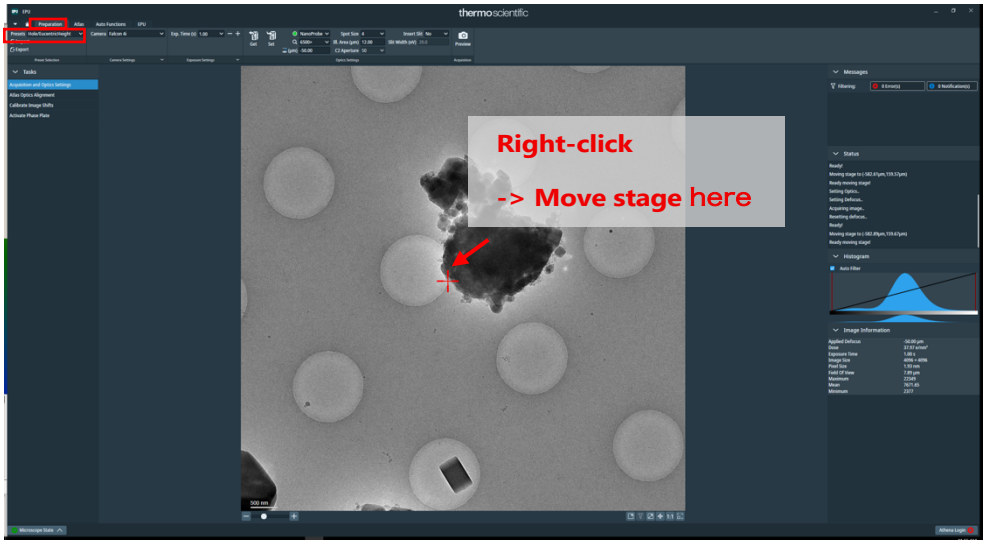
Select **Auto-eucentric by stage tilt** and **Start** again



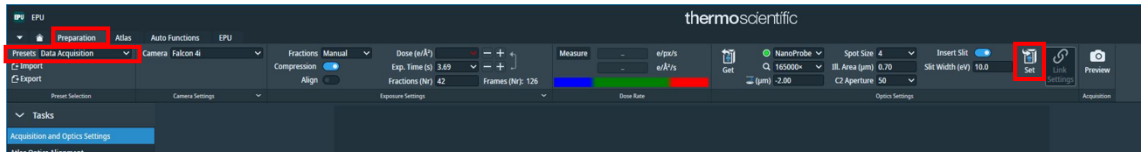
* You only need to succeed in one or the other

EPU > Preparation tab > Presets, select Hole/Eucentric and click **Preview**

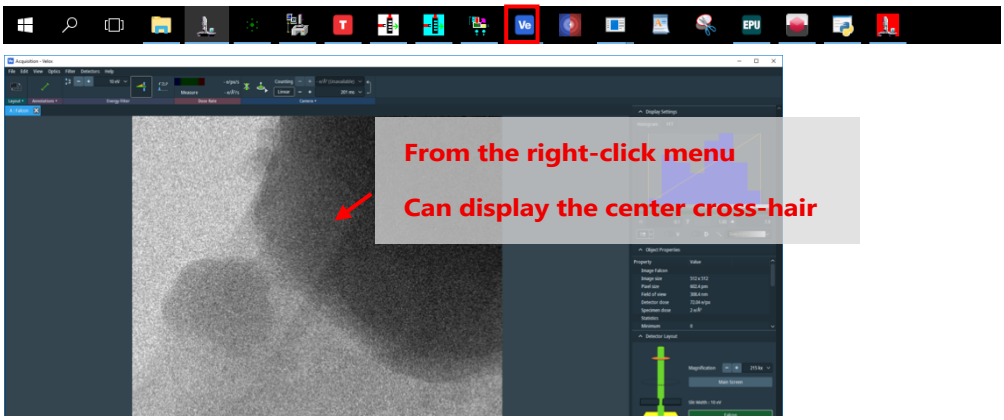
Move stage here to the corner of the garbage or the intersection of the garbage and the hole.



EPU > Preparation tab > Presets, select Data Acquisition and click **Set**



Start Velox (two windows, but only use Acquisition)



Switch the EPU screen to the Atlas tab. Otherwise, it will cause an error as they will compete for Falcon.

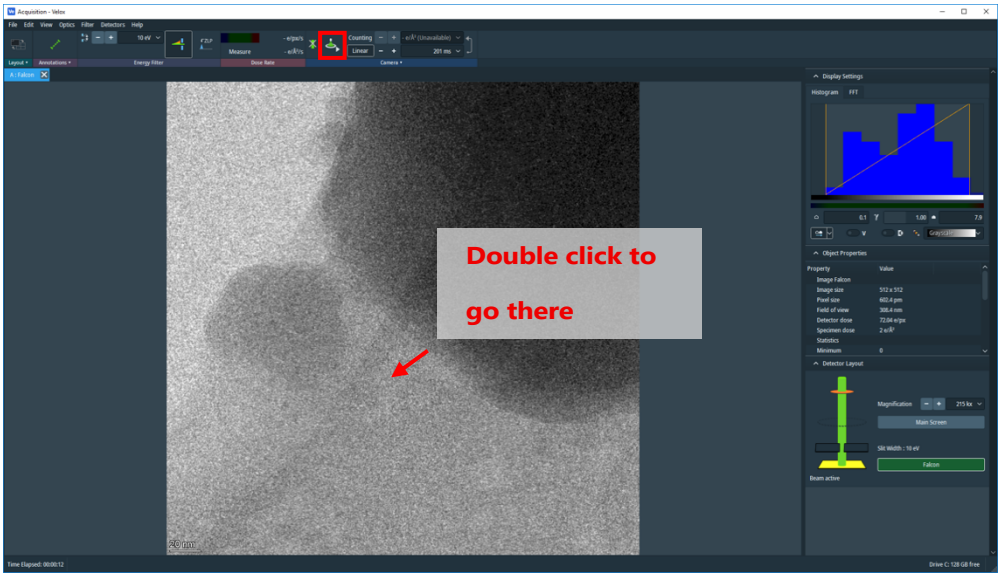
 Click on the icon. The beam irradiation begins and the image is captured.

*Be careful as it will continue to irradiate until you manually stop.

Double-click to move where you want to go to the image

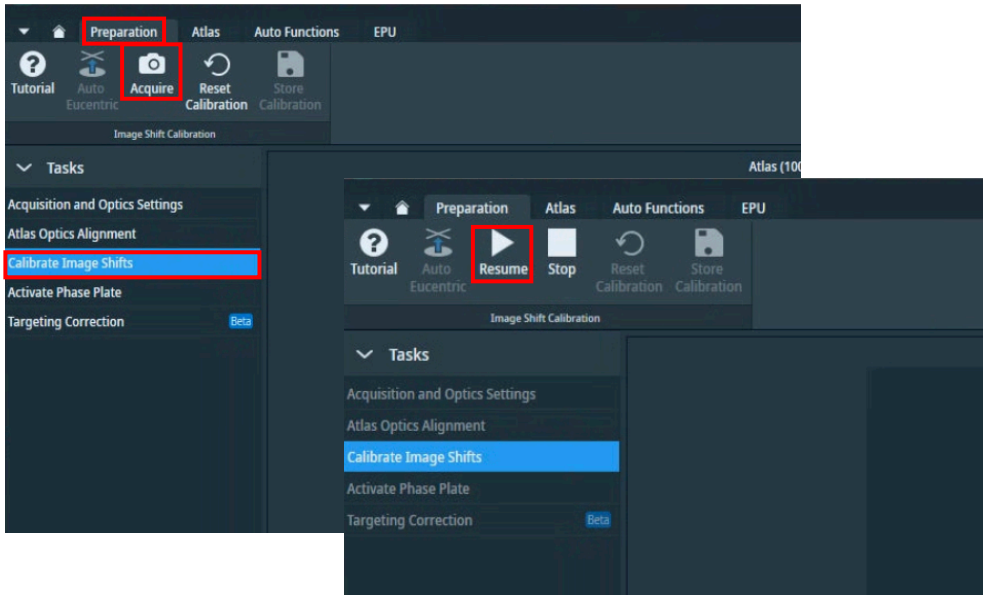
It comes mainly to the corner of the garbage or the intersection of the garbage and the hole.

*I thought I wouldn't double-click several times?

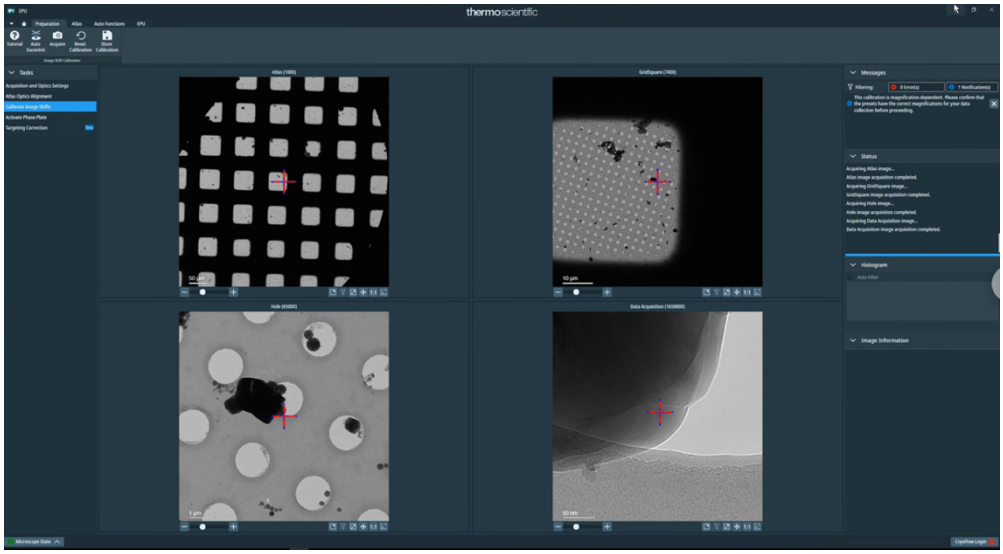


 Click the icon again to end the beam irradiation

EPU > Preparation tab, select **Calibrate Image Shift** and **Acquire** > **Resume**

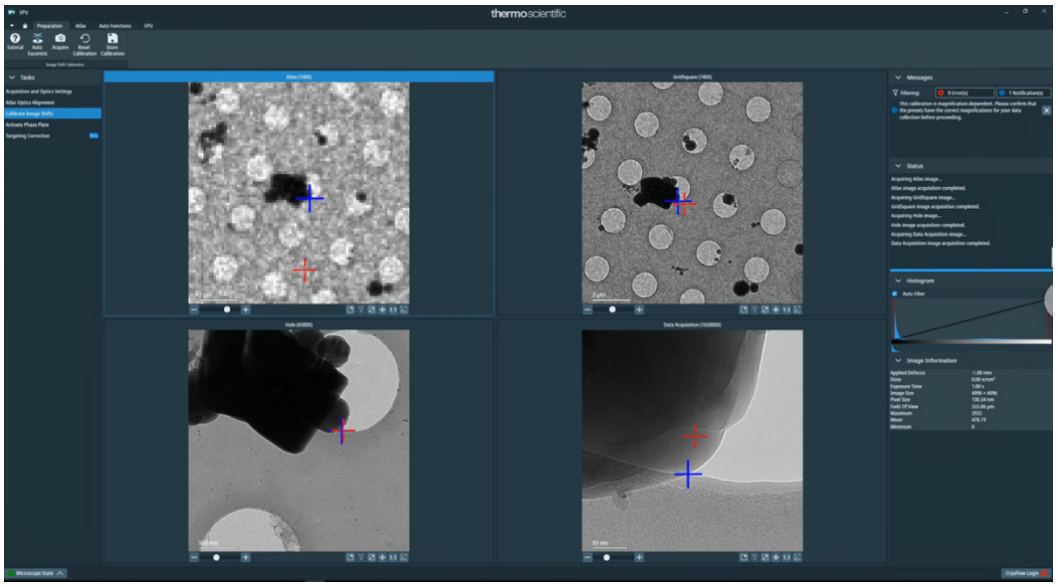


Atlas, Grid square, Hole/eucentric, and Data acquisition are taken sequentially at the magnification set.

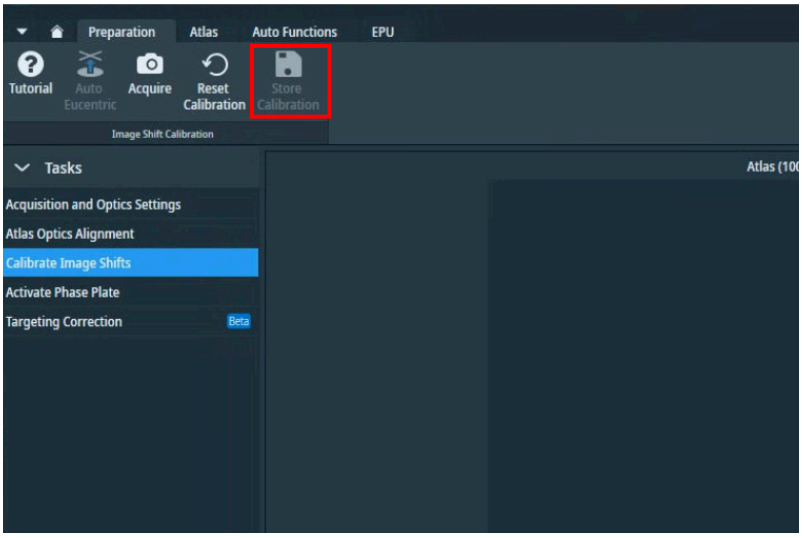


In the image at each magnification, specify the corresponding point by double-clicking.

*Performed in order from the high-magnification image. It is a good idea to match the scale of both images as much as possible and double-click on the corresponding point.



Click **Store Calibration**



*If you change the magnification, you will start over each time.

7. AutoCTF (Correction of Astigmatism and Coma Aberrations)

*This correction can only be done with a carbon film grid.

*For the adjustment method using Sherpa, refer to Section III. S4.

EPU > Preparation tab > Presets > Hole/Eucentric, there should be a garbage image left. Right-click on **the nearest carbon surface** and **Move stage here**

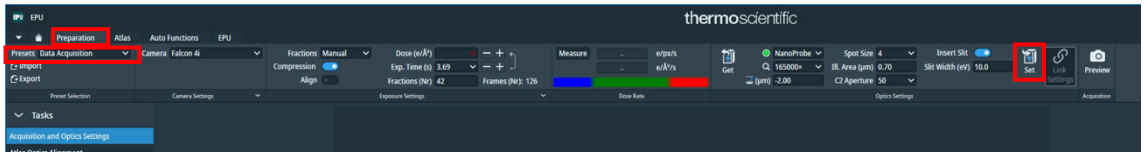
Be careful if the observation sample is a fibrous polymer!

In many cases, the sample is also densely covered with carbon surfaces, and there is a risk that the thon ring will be significantly distorted due to the regularity of the sample.

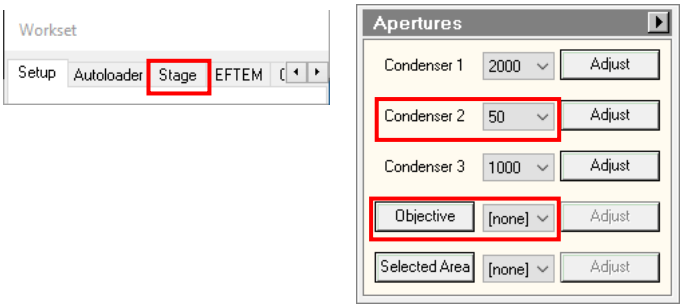
If you proceed with the adjustment as it is, it will be very strange

Adjustment using Sherpa (III. 4.) Somewhat safer because you take a Snap first

EPU > Preparation tab > Presets, select Data Acquisition and click **Set**

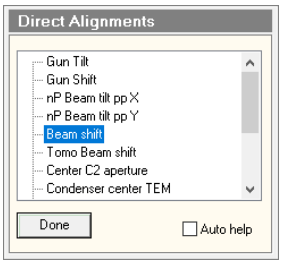


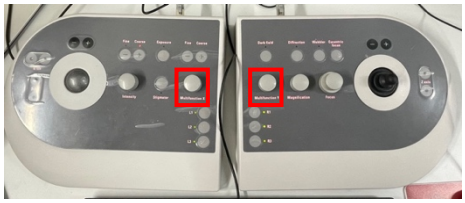
TUI > Autoloader tab > Apertures, make sure it's **C2=50, Obj=none**



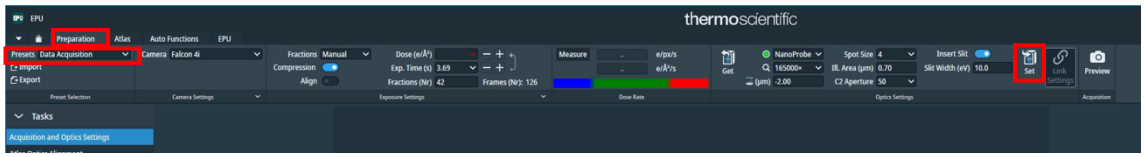
Press **R1** to lower the fluorescent plate

If the beam is not centered, center it with **Direct Alignment** > **Beam Shift** and click **Done**

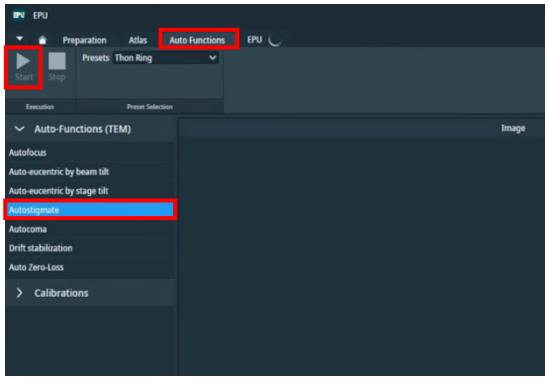


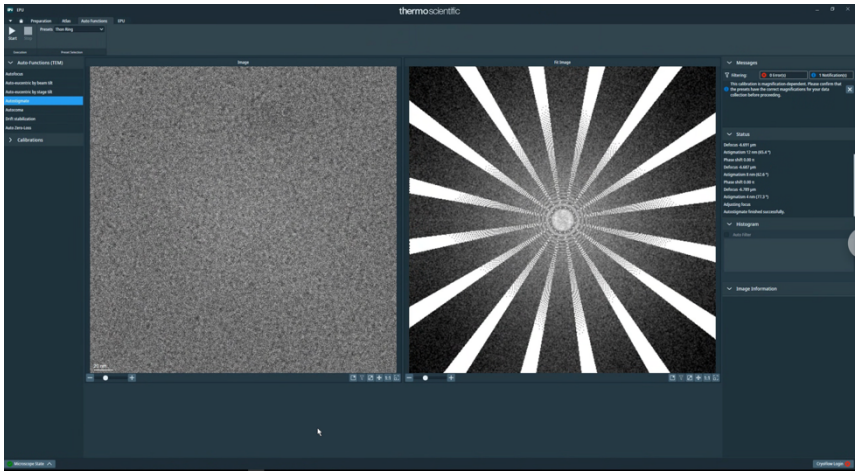


Set again in Data Acquisition and press **R1** to raise the fluorophore



EPU > Auto Functions tab, select **Autostigmate** and click **Start**



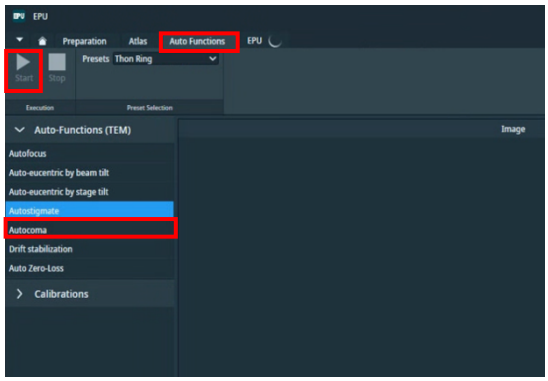


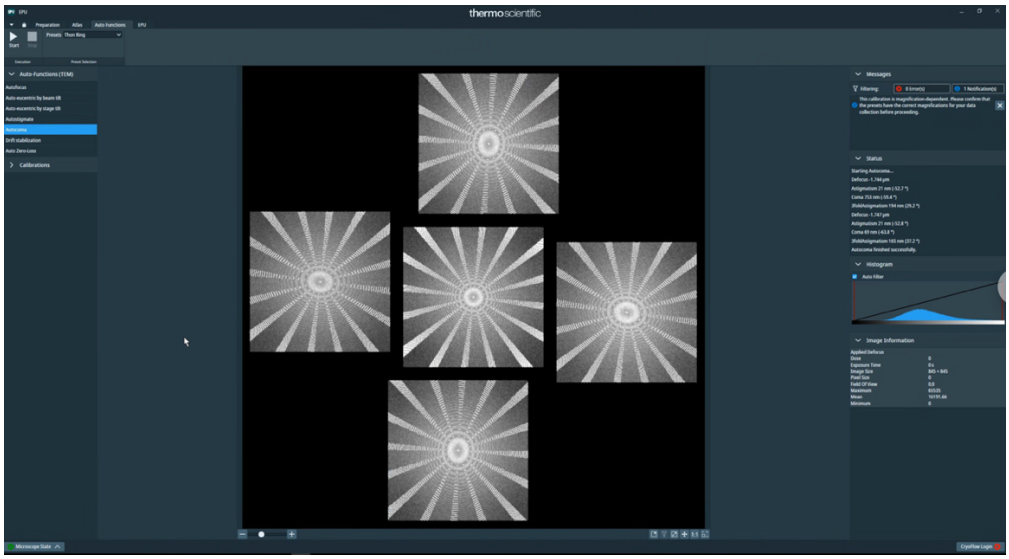
*Adjustment of astigmatism is made

*Thon ring should be a perfect circle, so it should be adjusted to be that way

*Check the display of "Autostigmate finished successfully"

EPU > Auto Functions tab, select **Autocoma** and click **Start**





*Adjustment of coma aberration is carried out

*The ellipse of the top and bottom (left and right) Thon rings should be similar, so they should be adjusted so that they are

*Check the display of "Autocoma finished successfully"

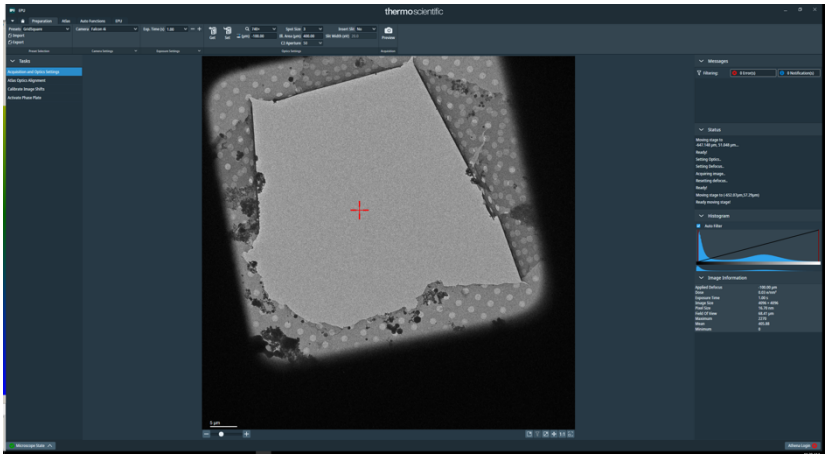
Select **Autostigmate** again and click **Start**

8. Setting up Dose

EPU > Atlas tab, right click on the hole in the Grid > **Move stage here**

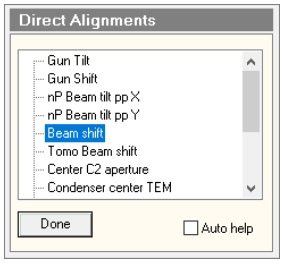
EPU > Preparation tab >Presets, select Grid Square and click **Preview**

(Make sure you're in the hole)

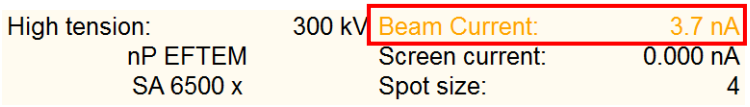


EPU > Preparation tab > Presets, select Data Acquisition, **Set** and Press **R1** to lower the fluorescent plate

If the beam is not centered, center it with **Direct Alignment** > **Beam Shift** and click **Done**



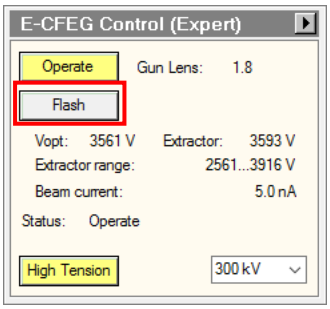
Check Beam Current with TUI



Flash if it looks below 5

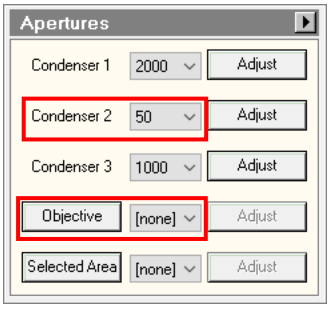
(TUI > Set up tab > E-CFEG Control)

*Close the column valve when flushing

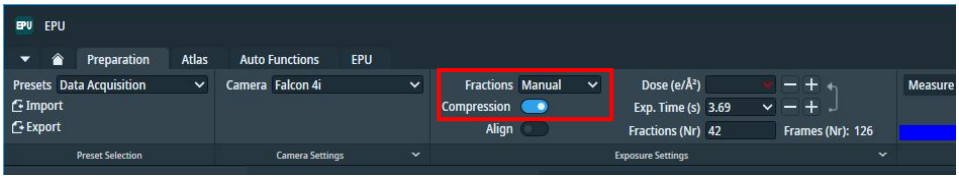


EPU > Preparation tab > Presets, select Data Acquisition and click **Set**

TUI > Autoloader tab > Apertures and make sure it's **C2=50, Obj=none**



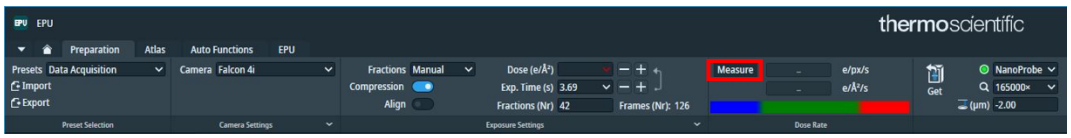
Make sure that fractions = **Manual**, compression is **checked**.



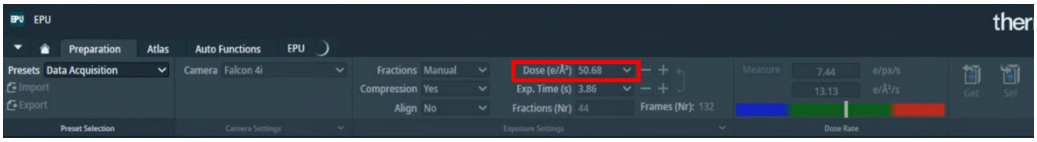
*Configuring in TIFF format

*If you want to shoot in EER format, please describe it separately.

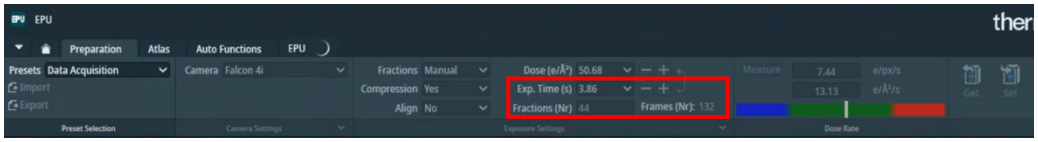
Click **Measure**



Select 50 from the Dose pulldown (this value is often better)



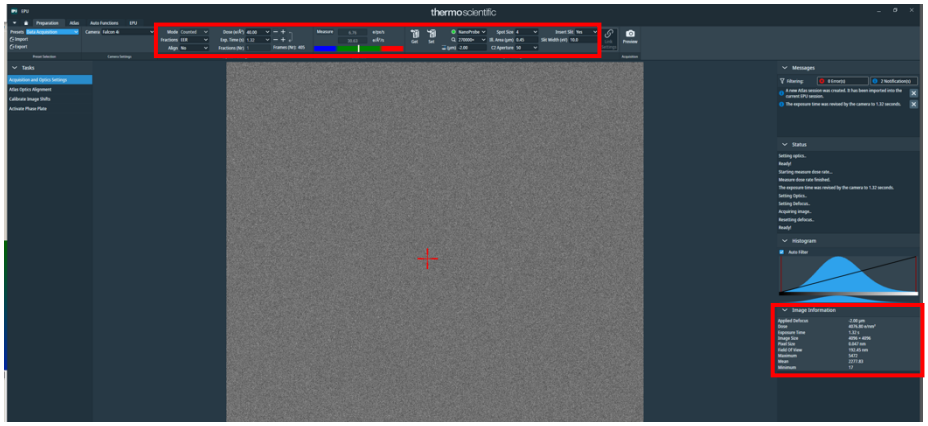
Fine-tune the Exp. Time so that **the value of Fractions (preferably 40-50) × (integer) = Frames.** The value of Dose also changes accordingly.



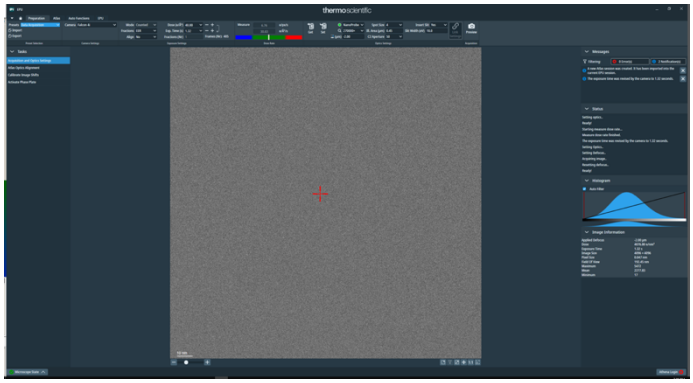
*If the value of Frames is not divisible by the value of Fractions, a problem occurs when Motion correction occurs.

Finally preview and then take a screenshot

*When you preview, information such as pixel size appears in the lower right corner of the screen.



Take the entire screen with a screenshot app



*Save to the user's directory on the day

That's all for the daily adjustment!

9. Acquisition of Gain Reference (Execute only after Conditioning)

*After conditioning, obtain the gain reference according to III-3. (p.46-).

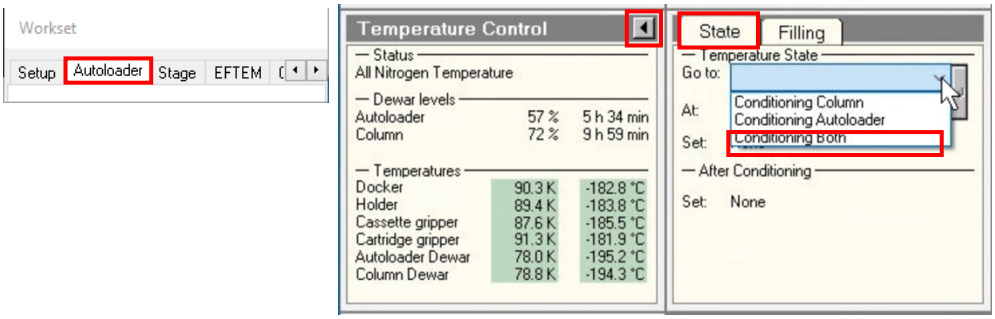
This will take about 20 minutes.

III. Adjustments and maintenance as needed

1. Conditioning

Basically, do it every two weeks

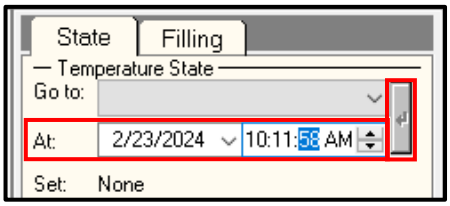
TUI > Autoloader tab > Temperature Control



Click the triangle to view the State tab

Go to: From the pull-down menu, select **Conditioning Both**

At: Enter the current date and time + about 2 minutes and click the enter button



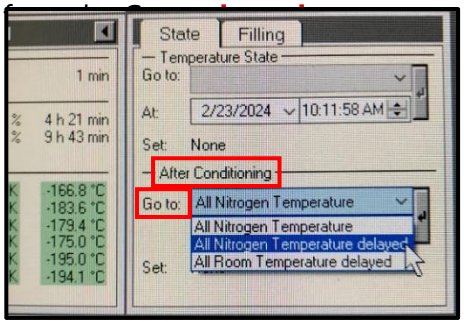
*Beware of mistakes in AM and PM

*Depending on the amount of liquid nitrogen remaining in the Dewar at the start, it may take up to 12 hours to return to room temperature

*You want to ensure that the room temperature continues for at least a few hours after it is fully returned to room temperature

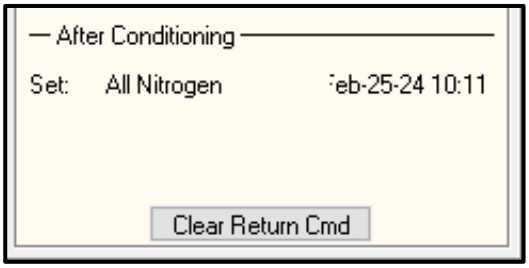
After the above settings are completed, the After Conditioning item will be configurable, so

Go to: From the pull-down menu, select **All Nitrogen Temperature delayed**



Set: Enter the date and time you want to start cooling and click the enter button

The set time is displayed



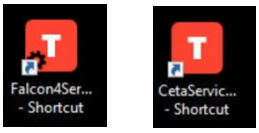
*You can redo the settings with **Clear Return Cmd**.

*Sufficient cooling is achieved in about 2 hours from the start of re-cooling

If the Autoloader and column are at room temperature for a long time (more than 12 hours), the detector will also stop cooling

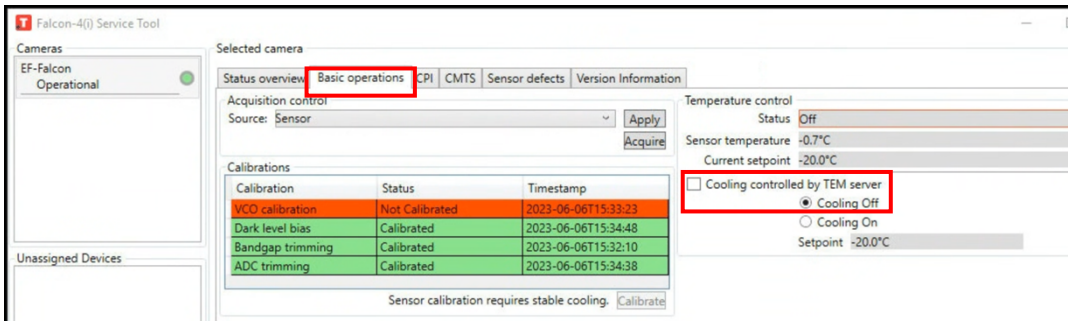
*If the column is at room temperature, the vacuum will be a little worse. If the detector is cooled in that state, it is easy for debris to stick to it

Double-click **Falcon 4i Service Tool** and **Ceta Service Tool** from the desktop shortcut icon to open it



Falcon 4i Service Tool > Basic Operations tab, clear **Cooling controlled by TEM server** checkbox

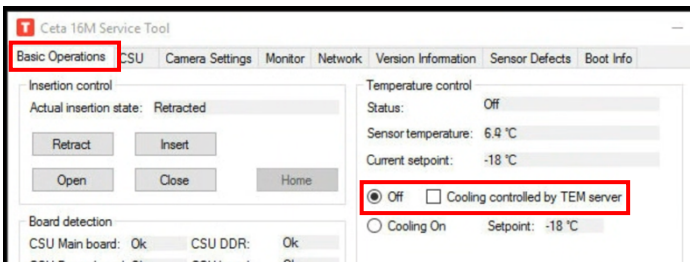
Change Cooling from **ON** to **OFF**



Ceta Service Tool > Basic Operations tab, clear **Cooling controlled by TEM server** checkbox

Change Cooling from **ON** to **OFF**

*This is automatically turned off.



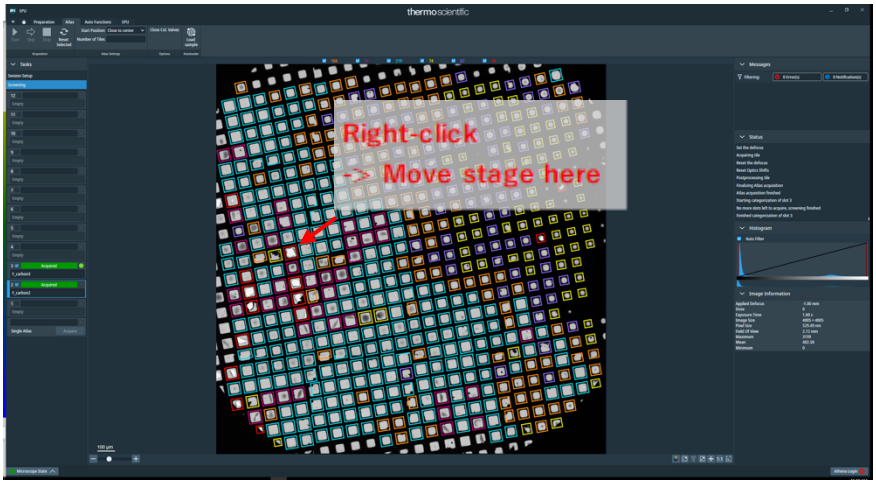
Falcon 4i Service Tool and Ceta Service Tool windows do not close

* When closed, the cooling control is transferred to the TUI, and cooling starts again.

2. Energy filter related (adjusted using Sherpa)

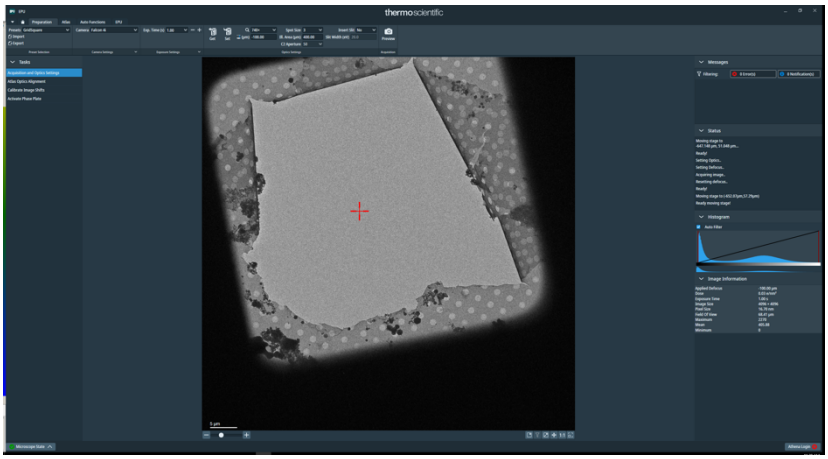
After Conditioning (every 2 weeks), make this adjustment during the first measurement

EPU > Atlas tab, right click on the hole in the Grid > **Move stage here**

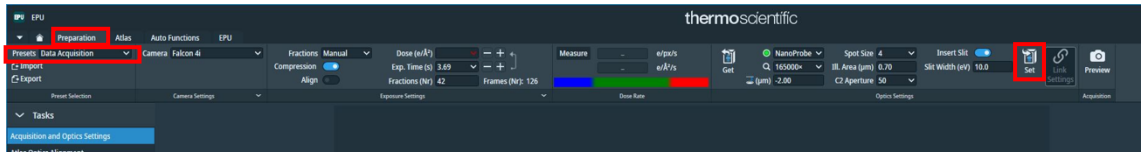


EPU > Preparation tab > Select Presets or Grid Square and click **Preview**

(Make sure you're in the hole)

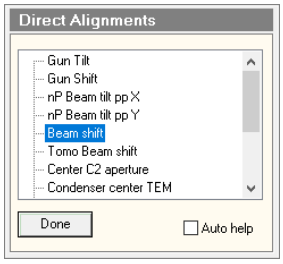


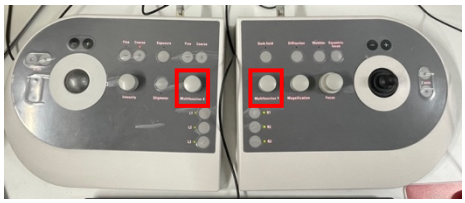
EPU > Preparation tab > Presets, select Data Acquisition and click **Set**



Press R1 to lower the fluorescent plate

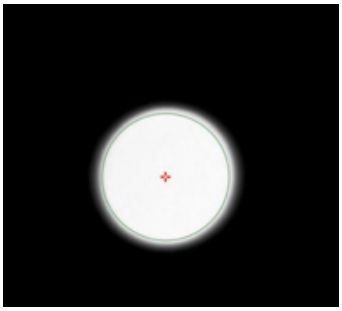
If the beam is not centered, center it with **Direct Alignment** > **Beam Shift** and click **Done**



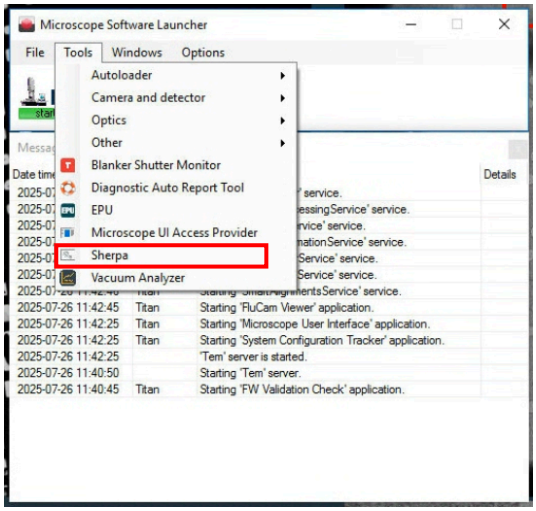


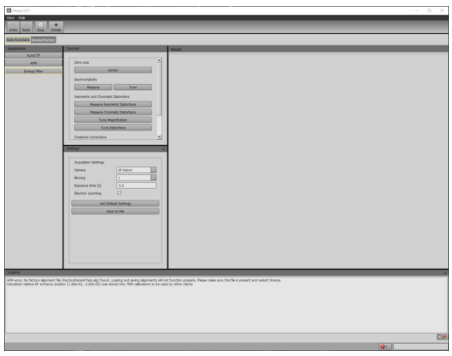
Make sure the beam hits the entire green circle

Press **R1** to raise the fluorescent plate



Microscope Software Launcher > Tools > Sherpa





Click the **Energy Filter** button on the left side of the Sherpa Window

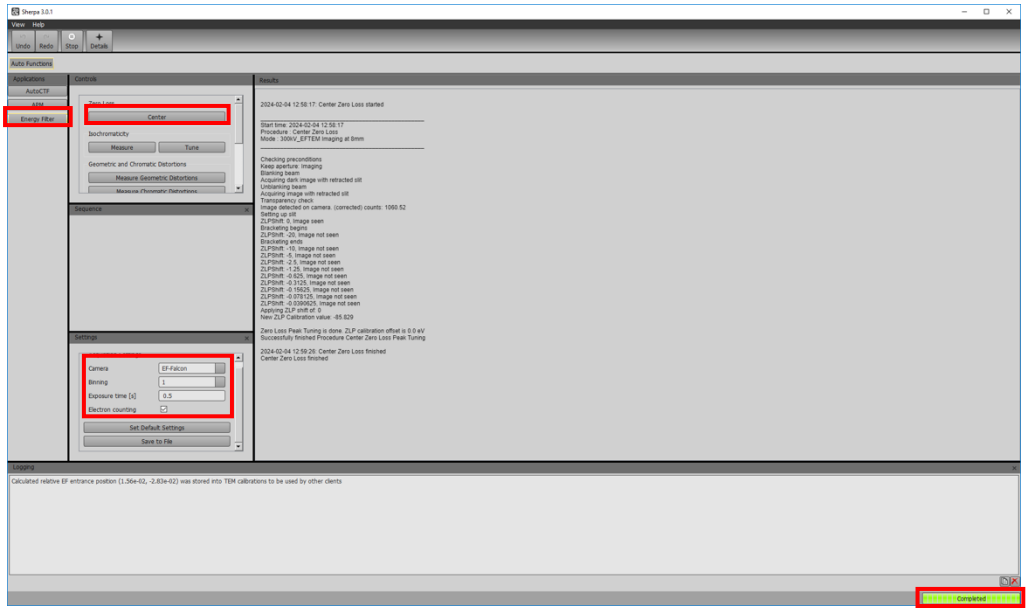
Look at the Settings square at the bottom left of the Sherpa Window

Check EF-Falcon, Bin=1, Exp time=0.5sec, Electron counting

Look at the Controls square in the top left corner of the Sherpa Window

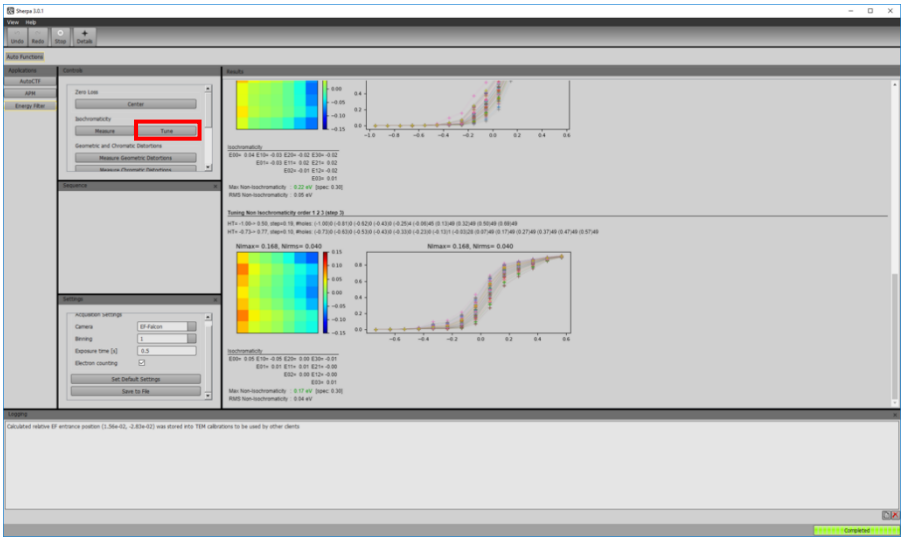
Zero loss: Click the **Center** button to pass the beam through the center of the slit

Confirm that it says completed in the bottom right corner of the screen (same below)

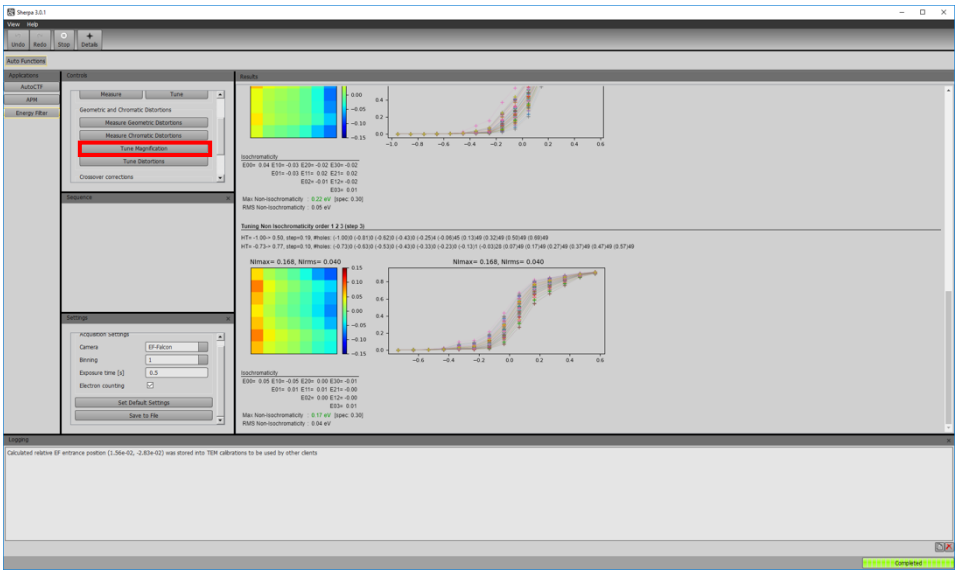


Isochromaticity: Click **Tune** to even out the energy hitting the camera

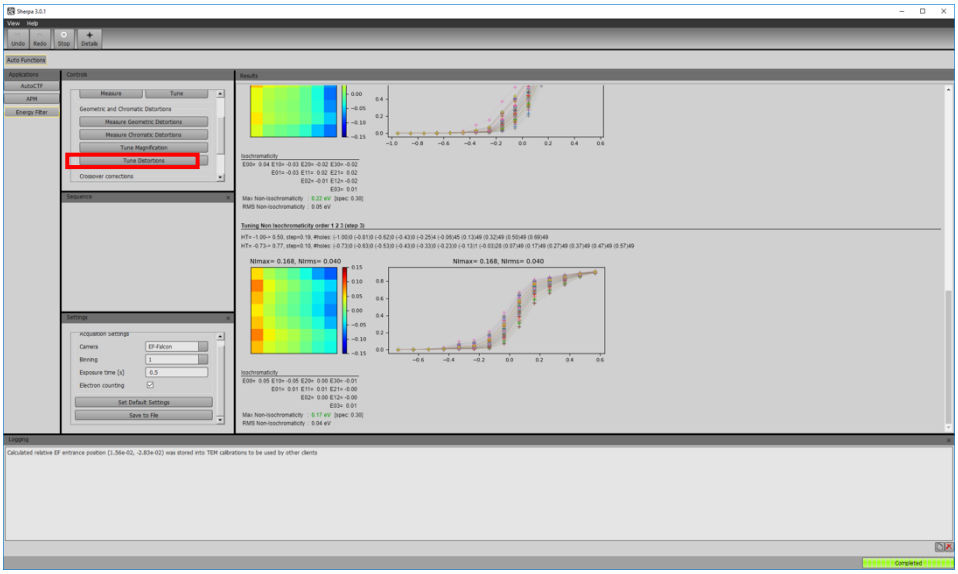
*After a while, an image of a red, green, blue image will appear. Wait a few minutes for the color of this screen to turn green uniformly



Geometric and Chromatic Distortions > **Tune Magnification**, click the button



Geometric and Chromatic Distortions > **Tune Distortions,** click the button

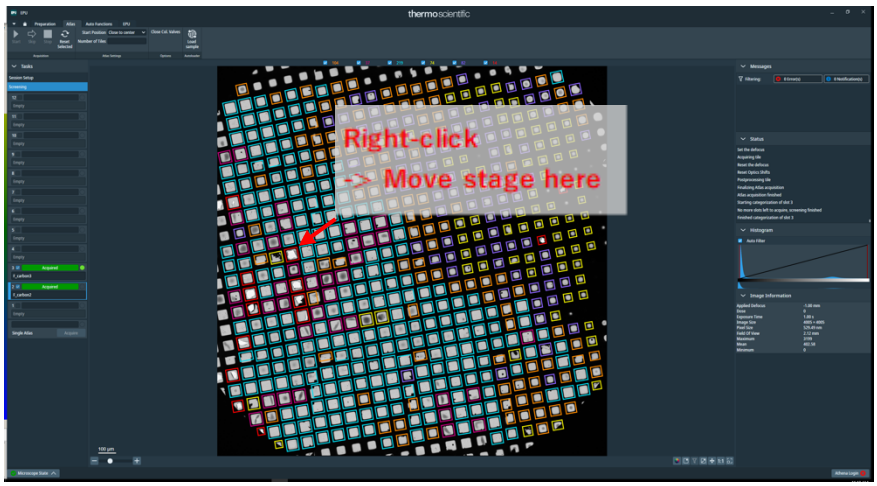


Zero loss: Click the **Center** button again to adjust the turn

3. Acquisition of Gain Reference

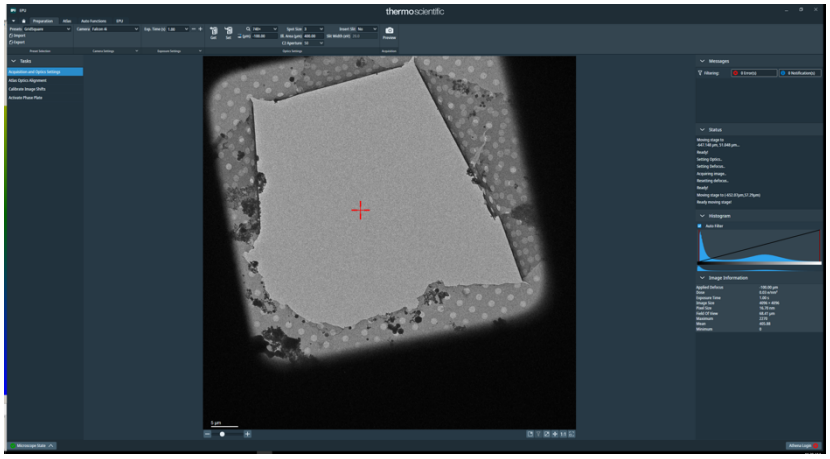
After each conditioning (every two weeks), acquire the gain reference.

EPU > Atlas tab, right-click on a grid area with a hole > **Move stage here**



EPU > Preparation tab > Presets, select Grid Square and click **Preview**.

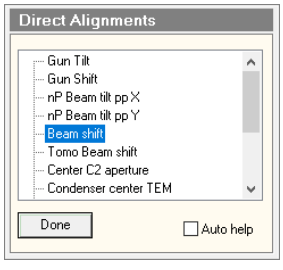
* Confirm that you are positioned over a hole.



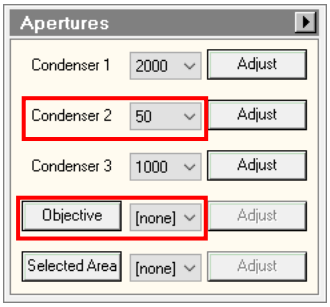
EPU > Preparation tab > Presets, select Data Acquisition and click **Set**.

Press **R1** to lower the fluorescent screen.

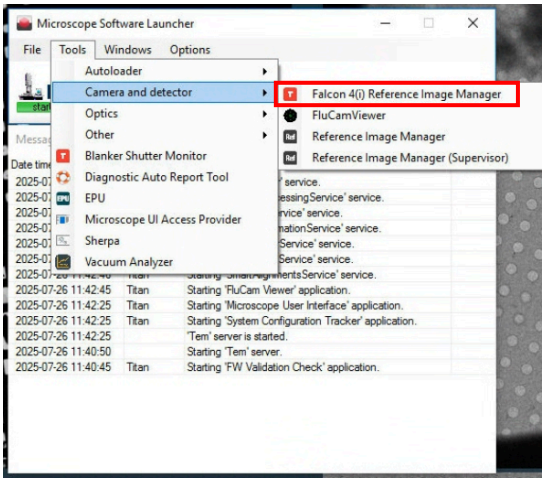
If the beam is not centered, use **Direct Alignment** > **Beam Shift** to center it, then click **Done**.

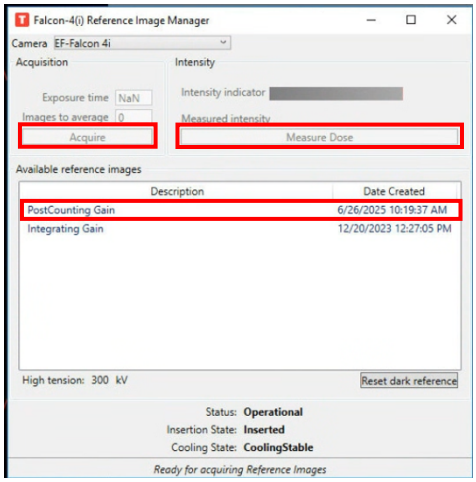


Check that TUI > Autoloader tab > Apertures is set to **C2 = 50** and **Obj = none**.



Microscope Software Launcher > Tools > Camera and detector > Falcon 4i Reference Image Manager





Since it is in counting mode, select **Post-Counting Gain**.

Press **R1** to raise the fluorescent screen and click **Measure Dose**.

Confirm that the bar is within the green range.

*For Falcon 4i, 1–12.5 e/px/s is appropriate. If it is outside this range, check the settings in Data Acquisition.

Click **Acquire** (this takes about 20 minutes).

Confirm that the date of the Post-Counting Gain has been updated.

EPU > Preparation tab > Presets, select Data Acquisition and click **Preview**.

Confirm that the obtained image is flat.

*.gain file is copied into OffloadData/ImagesForProcessing/EF-Falcon/300kV/ when measurement is started in EPU.

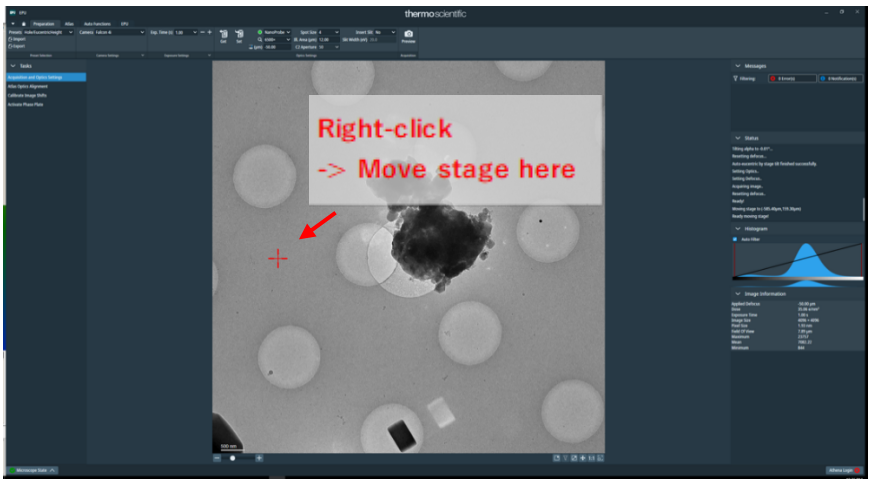
4. Sherpa-based AutoCTF

* This correction can only be performed with a carbon film grid.

* It is assumed that the operations up to this point have already established the eucentric height.

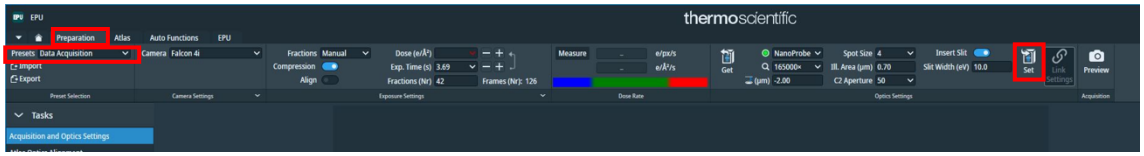
EPU > Preparation tab > Presets > Hole/Eucentric, there should be a garbage image remaining.

Right-click on the nearest clean carbon area and select “Move stage here.”

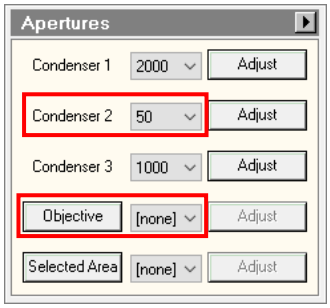


Be careful if the observation sample is a fibrous polymer! In many cases, the carbon surface is also densely covered with the sample, and the regularity originating from the sample may severely distort the Thon ring. If you proceed with the adjustment as it is, the results will become extremely incorrect.

EPU > Preparation tab > Presets, select Data Acquisition and click **Set**.

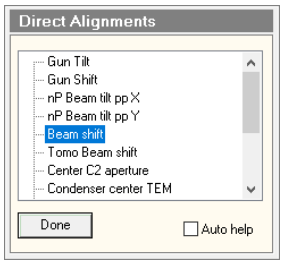


Check that TUI > Autoloader tab > Apertures is set to **C2 = 50** and **Obj = none**.



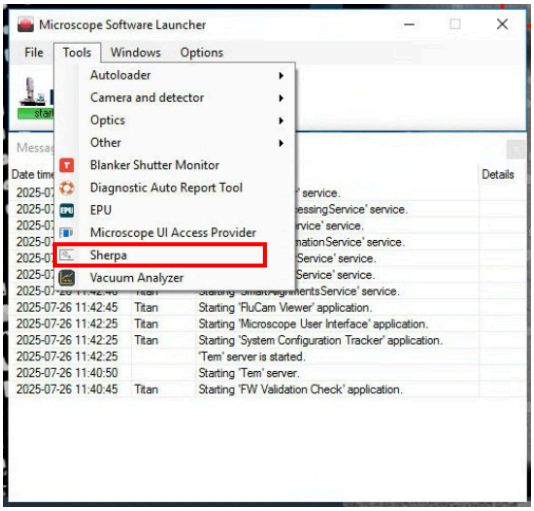
Press **R1** to lower the fluorescent screen.

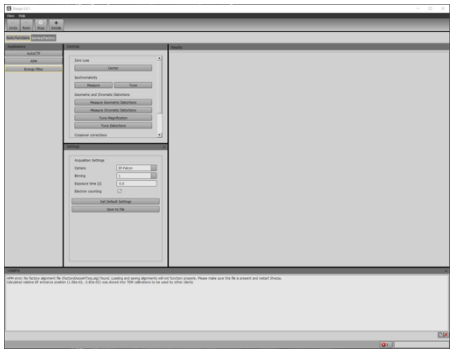
If the beam is not centered, use **Direct Alignment** > **Beam Shift** to center it, then click **Done**.



Set it again in Data Acquisition, then press **R1** to raise the fluorescent screen

Microscope Software Launcher > Tools > Sherpa



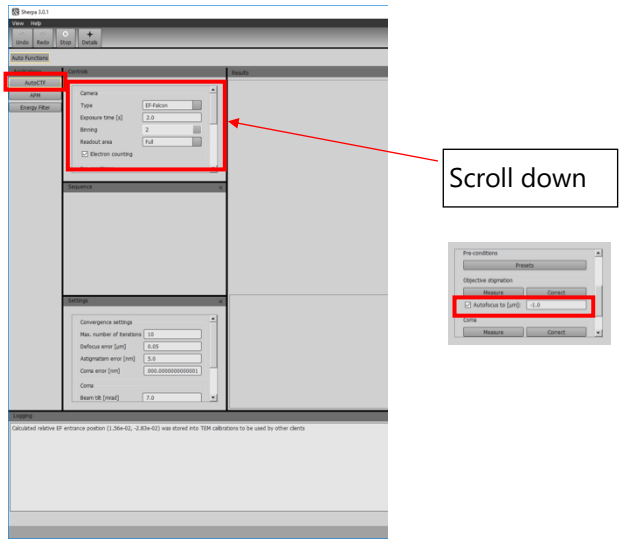


Click the **AutoCTF** button on the left side of the Sherpa Window.

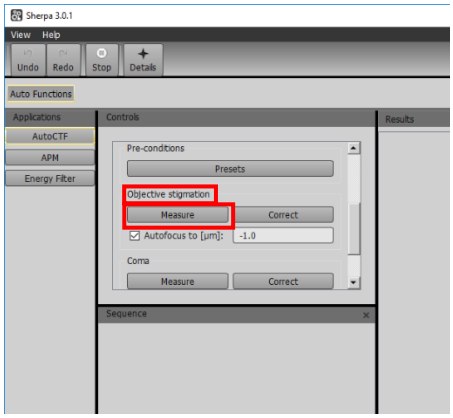
Set **Camera > Type** to **EF-Falcon**.

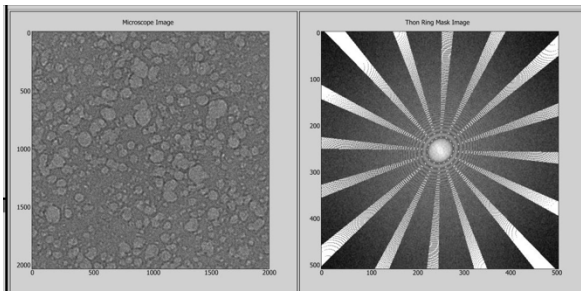
Since it is in EC mode, check the box for **Electron counting**.

Set **Exp. Time = 2**, **Binning = 2**, **Readout = Full**, and check **Auto-focus-to**, then set it to **–1.0**.



Click "**Measure**" for Objective Stigmation.





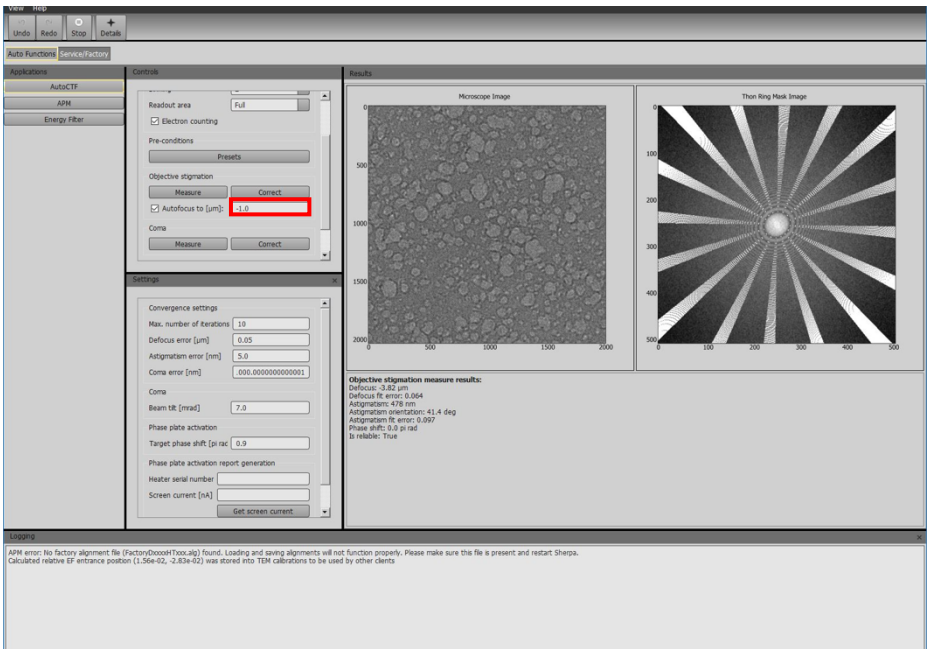
*** Is there any large debris visible in the carbon-film image?**

*** Is there any unusual pattern in the Thon ring?**

*** If any abnormality is observed, move the stage slightly and try again.**

*** If the Thon ring cannot be detected, turn the Focus dial and apply a defocus of –800 nm.**

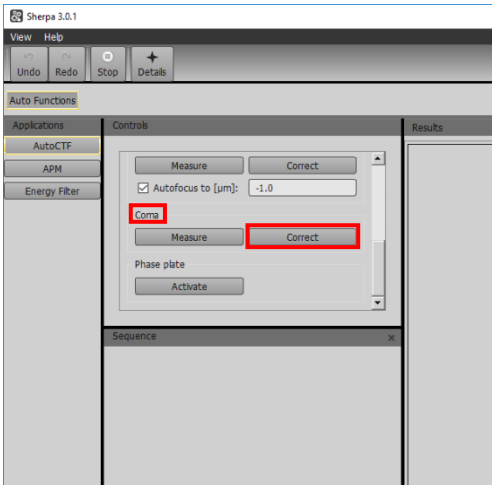
If the Thon ring is detected, click "**Correct**."



* It corrects the objective-lens astigmatism. If the image is too dark, it may fail to detect it.

* Do not stop the process midway. If you stop it, the system may remain in an abnormal intermediate state.

When "Completed" appears in the lower right corner, click "**Correct**" for **Coma** (to correct coma aberration).



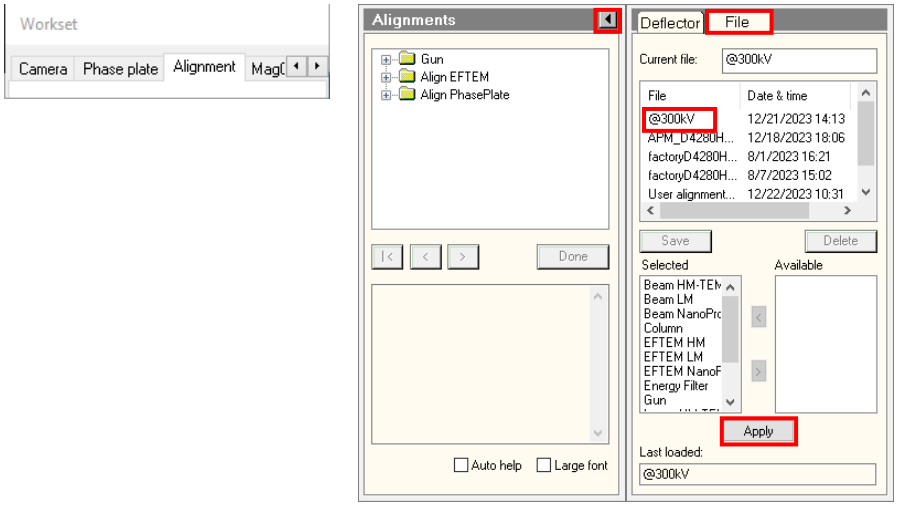
When "Completed" appears in the lower right corner, click "**Correct**" for **Objective Stigmation** once again.

* If AutoCTF is not working at all, something is wrong. Start over from the beginning.

5. Recovery from Large Beam Shifts

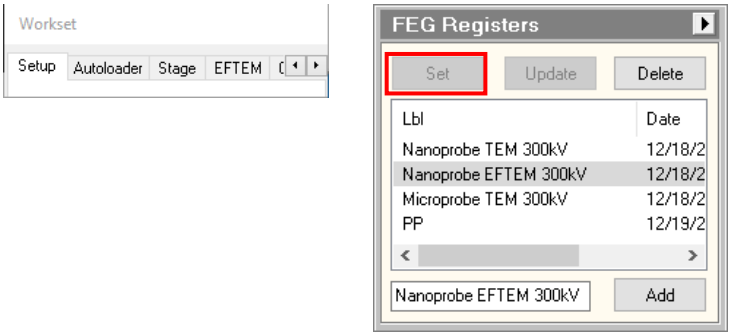
TUI > Alignment tab > Alignments > Option > File tab, select the item 300 kV (the latest one created on 11/27/2024),

move all items in **Available** to **Selected**, and click **Apply**.



TUI > Setup tab > FEG registers, select Nanoprobe EFTEM 300 kV (the latest version updated on 11/29/2024) and click **Set**.

* This is also effective in cases of beam loss. However, once you perform this operation, all adjustments must be redone from the beginning.



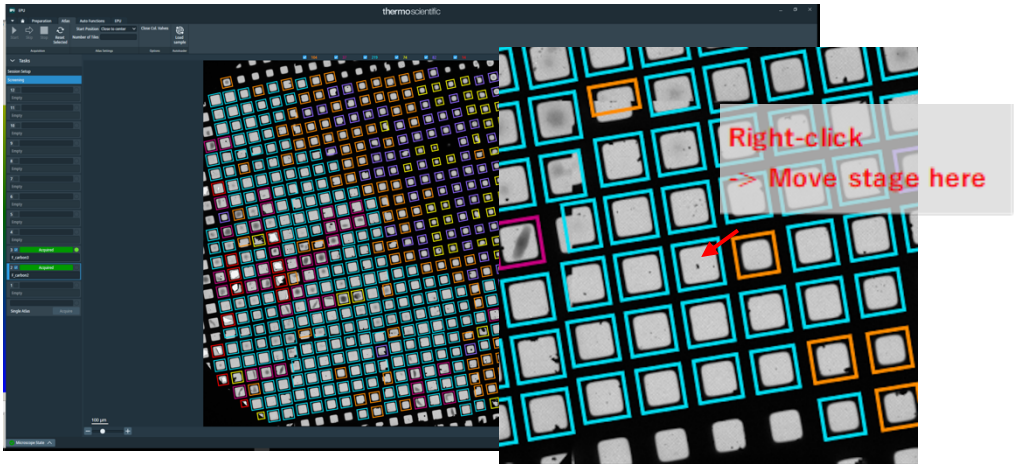
6. Rotation Center (passing the current axis through the center of the objective lens)

*This adjustment is not necessary when performing AutoCTF
(the coma correction in AutoCTF is more accurate).

*Perform this adjustment? only when, for some reason, AutoCTF is not performed.

EPU > Atlas tab

Right-click on a location with noticeable debris > **Move stage here**

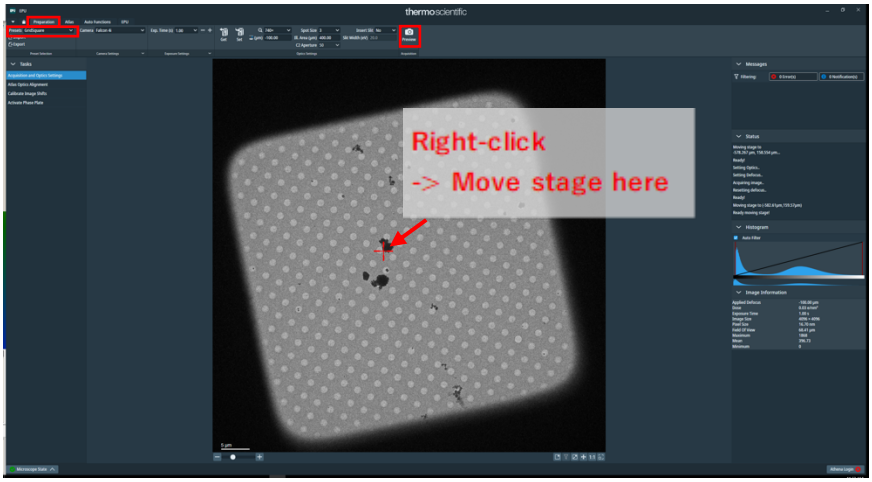


*Choose debris that is not too large. You can zoom in/out of the image using the mouse wheel. A relatively clean square makes the following adjustments easier.

It is good to place the center on the boundary between the debris and the hole.

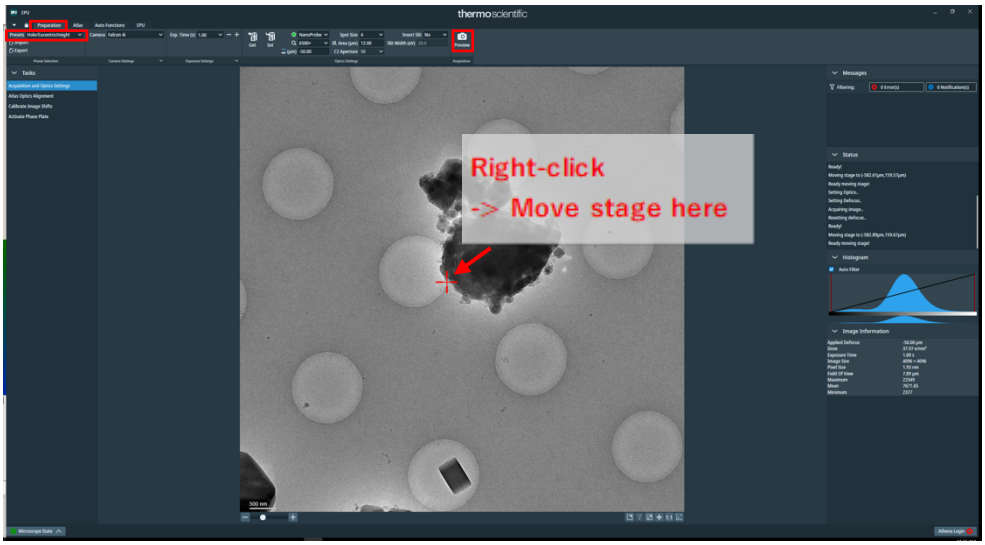
EPU > Preparation tab > Presets, select Grid Square and click **Preview**.

Right-click on a spot with noticeable debris > **Move stage here**.



EPU > Preparation tab > Presets, select Hole/Eucentric and click **Preview**.

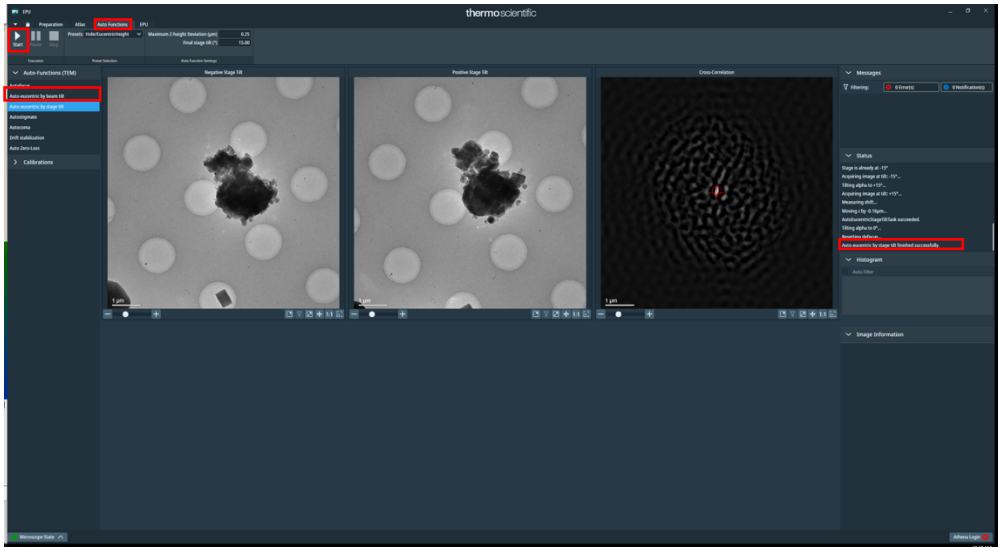
Right-click on a spot with noticeable debris > **Move stage here**.



EPU > Auto Functions tab, select **Auto-eucentric by beam tilt**.

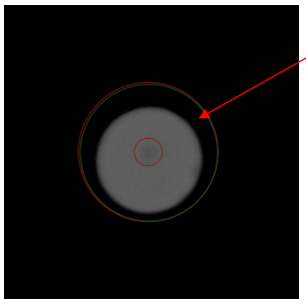
Set Presets to Hole/Eucentric and click **Start**.

In Status, confirm that the message "Auto-eucentric by stage tilt finished successfully" is displayed.



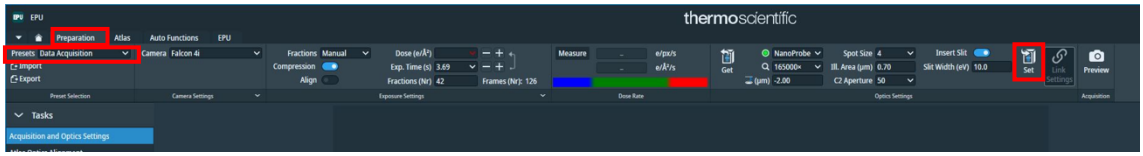
*The tilt axis runs horizontally relative to the screen.

Press **R1** to lower the fluorescent screen. (If the debris is not at the center of the green circle, move it to the center using the joystick.)



At this magnification, it's hard to see on the fluorescent screen, but you can use the mouse wheel to adjust the contrast. As long as you can faintly see a dark shadow, that's enough.

EPU > Preparation tab > Presets, select Data Acquisition and click **Set**.

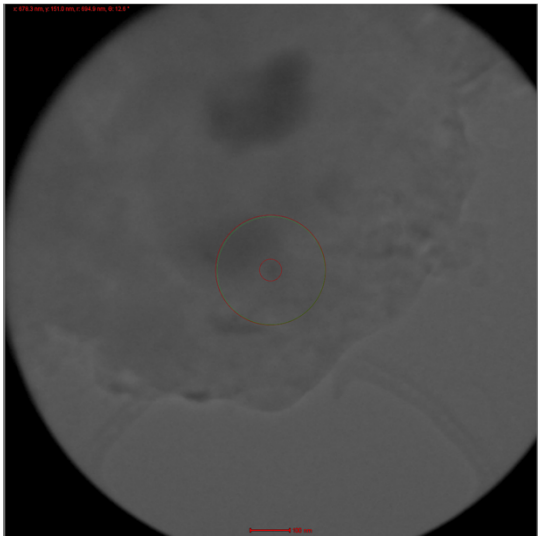


TUI > Stage tab > Apertures: set **C2 = 150** (to widen the field of view) and **Obj = none**.

*Since the image orientation differs between EPU and the fluorescent screen, keep that in mind (is it rotated by 90°?).

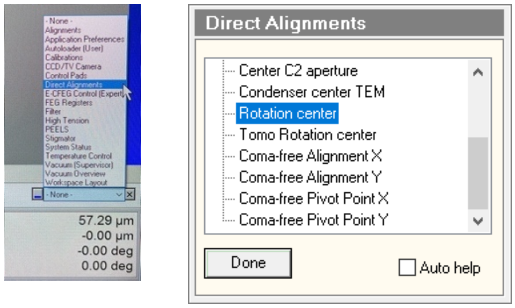
Turn the **Magnification** dial several clicks to the right to increase the magnification to x350k.

Confirm that it is set to 350 kx

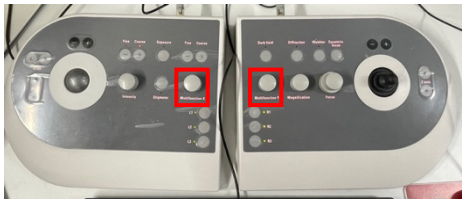


* If the debris is hard to see, click 'Natural'

TUI Bottom-right > Direct Alignment > Rotation Center

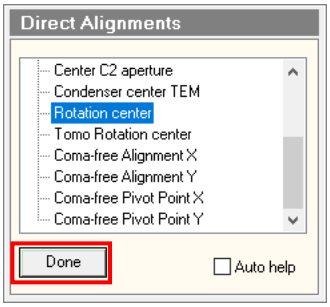


Use the **Multifunction dial** to make fine adjustments so that the debris no longer moves up, down, left, or right.



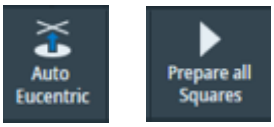
* Since coma aberration will be corrected later with AutoCTF, it only needs to be reasonably aligned at this stage.

Click **Done**.



IV. Other notes / Additional cautions

1. When Auto-eucentric fails in the EPU tab (Hole selection)



Auto Functions tab > **Auto-eucentric by beam tilt** > **Start**

If it still fails, select **Auto-eucentric by stage tilt** > **Start**

Either one succeeding is sufficient.

Once the Eucentric height has been determined, return to **Hole selection** and click **Acquire**.

*If you do not do this, the Eucentric height will not be applied.

*However, with this method, you must perform the operation manually each time it fails.

*This cannot be handled when using Multi-grid.

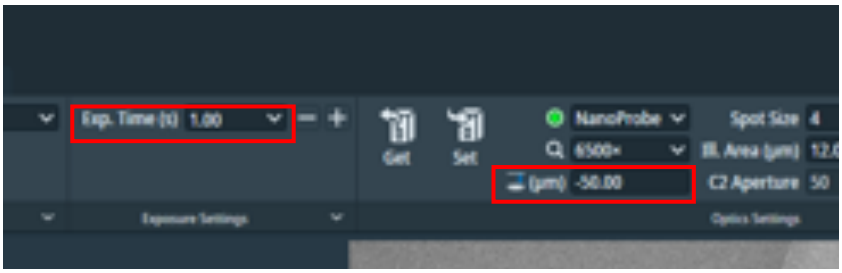
Eucentric failure generally occurs because the image is dark (poor contrast) and proper correlation cannot be achieved.

*It often fails when the ice is thick.

> **Increase exposure time (increase beam intensity) or increase defocus to compensate.**

EPU > Preparation tab > Presets > Hole/Eucentric, adjust the Exp. Time and defocus values.

*Increasing exposure causes more damage. With Spot Size = 4, up to around 4 seconds?



However, the defocus value set in the Preparation tab under Hole/Eucentric is not reflected in Auto-eucentric in the EPU tab.

*It seems to be fixed at 5 μm.

Therefore, the only option is to compensate by increasing the Exp. Time.

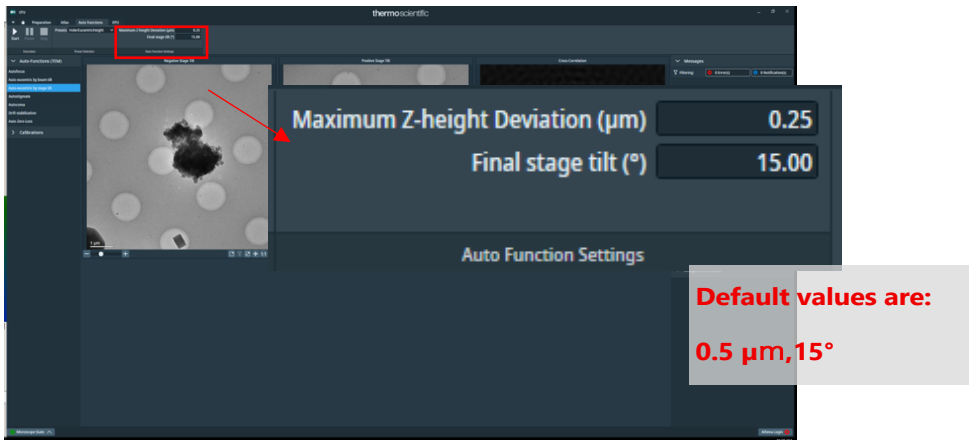
2. Determining Eucentric Height at a Specific Location

EPU > Preparation tab > Presets > Hole/Eucentric:
Set the Exp. Time and defocus values to larger values.

EPU > Auto Functions tab > **Auto-eucentric by beam tilt** > click **Start**

Or:

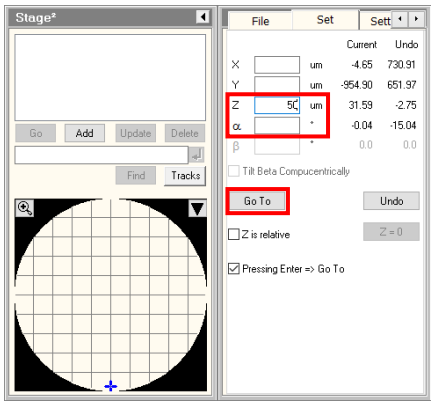
EPU > Auto Functions tab > **Auto-eucentric by stage tilt**:
Check the values in Auto Function Settings.

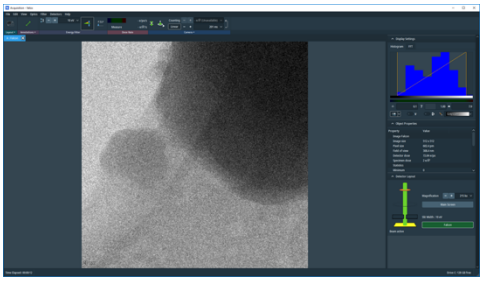


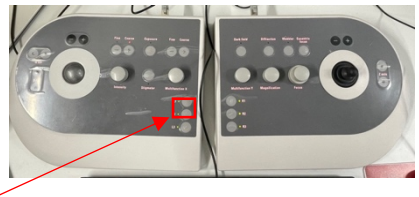
*If you increase the value of Maximum Z-height Deviation, the tolerance becomes looser, making it more likely to be judged as "success."
*You may also try setting the Final stage tilt to around 30

There is also the option of manually determining the tilt angle (α) and the Z-axis position using Stage2 while checking the image in Velox.

*It seems that this is how it used to be done in the past.







The α wobbler on control panel L1 is also enabled

3. EER Data Collection Notes

EPU > Preparation tab > Presets, select Data Acquisition

Set Fractions = **EER** and Compression = **No**.

*The number of frames in an EER-format movie will be the value of Frames (Nr) found under

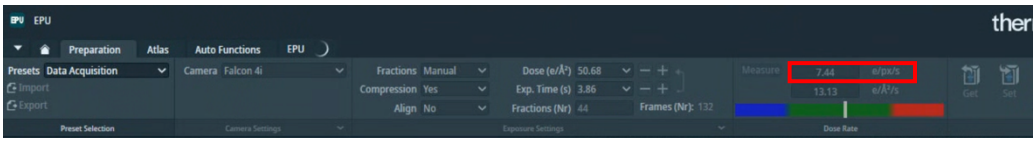
EPU > Preparation tab > Data Acquisition > Exposure Settings.

* When you divide this value by the Dose (e/Å^2) and enter the result into EER fractionation in RELION's Motion Correction, the Dose per frame becomes 1 (e/Å^2). (For example: if EPU displays 50 e/Å^2 and 1220 frames, divide 1220 by 50 to obtain 24, and enter this value in EER fractionation.)

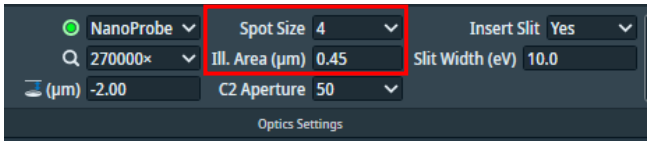
4. Dose Rate, Spot Size, Illuminated Area

When the magnification is increased, the dose rate also increases.

-> If you want to keep the total dose constant, the exposure time becomes shorter.



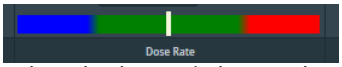
In addition to magnification, **spot size** and **illuminated area** also affect the dose rate.



*When you decrease the spot size number or narrow the illuminated area, electrons are concentrated into a smaller region, increasing the dose rate (i.e., the image becomes brighter).

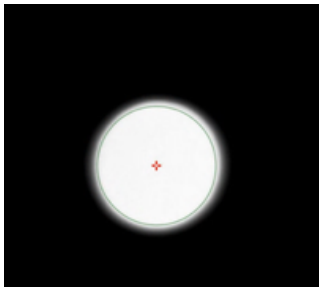
*Lowering the spot size number by one step roughly doubles the brightness.

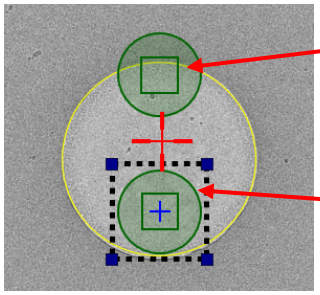
*If the dose rate is too high, it results in lower DQE and increased damage to the sample; therefore, settings should be adjusted so that the indicator bar does not reach the red zone.



*For the illuminated area, adjust it so that the beam is larger than the green edge of the fluorescent screen, and set an appropriate value while comparing it with the detector size in the Template Definition.

If it is too narrow, the beam may easily shift out of view during focus adjustment or near the edges of AFIS.





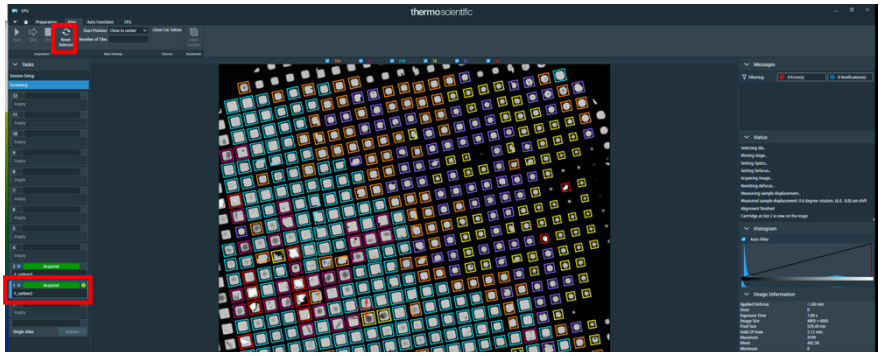
The square indicates the detector's field of view.

The circle represents the illuminated area. It should be taken sufficiently wide relative to the field of view.

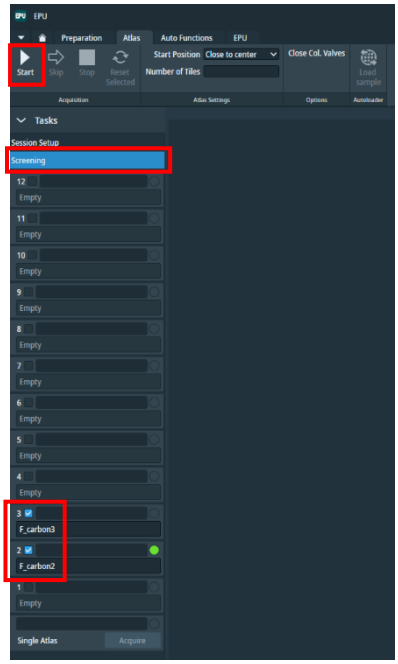
* As a standard, at a magnification of 165k, use Spot Size = 4 and Illuminated Area = 0.70.

V. Atlas Acquisition

EPU > Atlas tab, select the atlas acquired for adjustment and click **Reset Selected**.



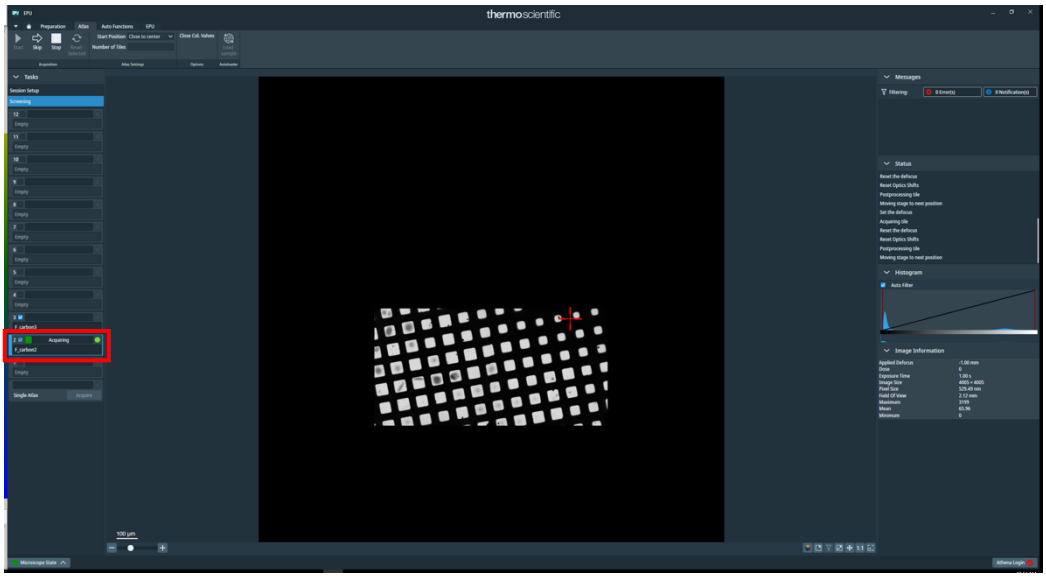
Then, check all grids again and click **Start**.



* It is better to rewrite (reacquire) the Atlas after performing Calibrate Image Shift.

Click **Acquiring**, and the image will be displayed.

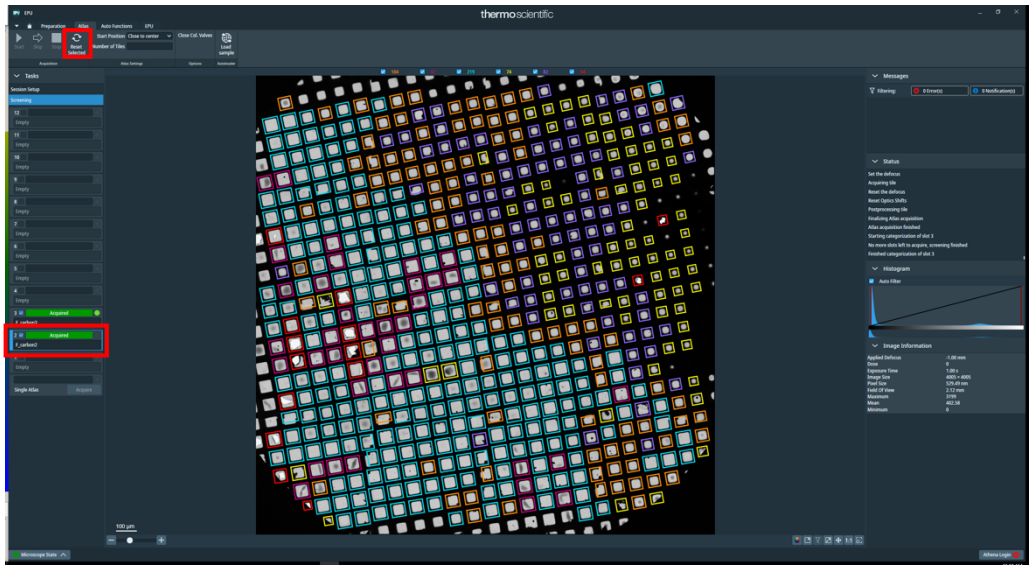
* Since a 4 × 4 overview image is acquired, wait for a while. Including the time required for grid exchange, it takes about 8 minutes per grid.



If, for some reason, you want to re-acquire the Atlas for only a specific grid:

Select that grid in the Atlas tab, click the **Reset Selected** button in the upper left, and the data will be cleared.

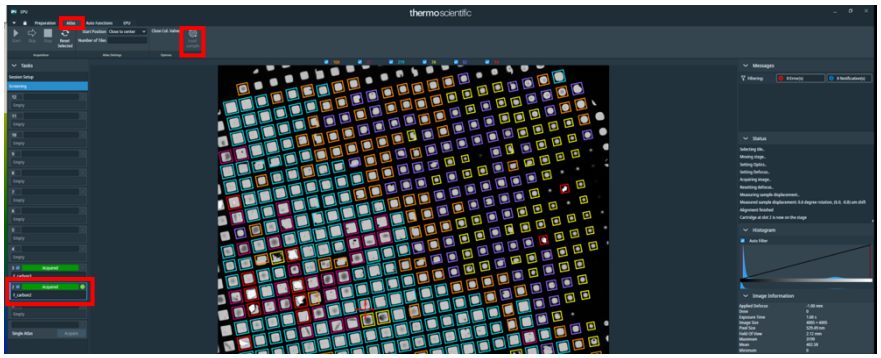
Then, check the grid again and click **Start**.



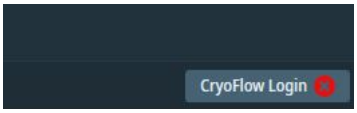
VI. Data Acquisition

1. Grid Screening

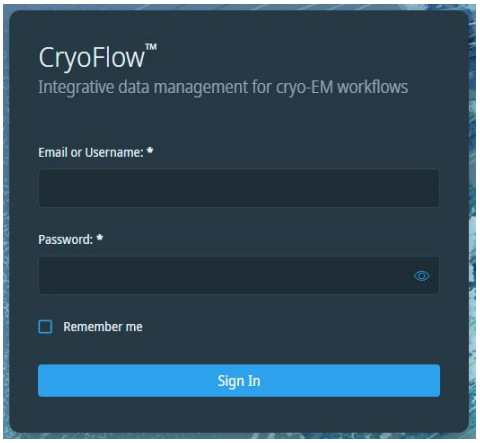
EPU > Atlas tab, select the target grid and click **Load Sample**.



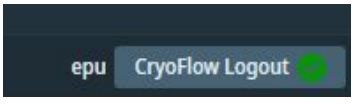
EPU at the bottom right > Click **CryoFlow Login**



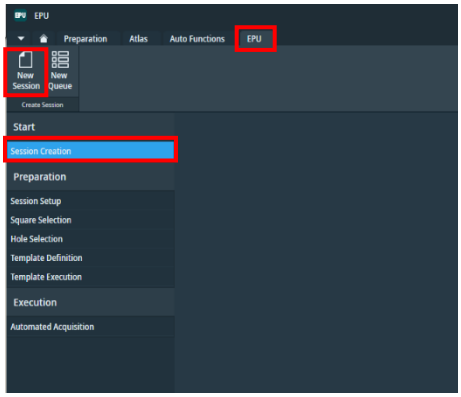
Enter the User ID (epu) and password, then click **Sign In**.

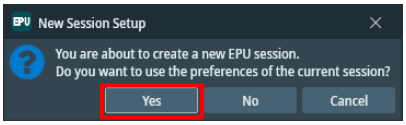


Once you are logged into CryoFlow, the display at the bottom right of the EPU screen will change.



EPU > EPU tab, select **Session Creation** > **New Session** > **Yes**.

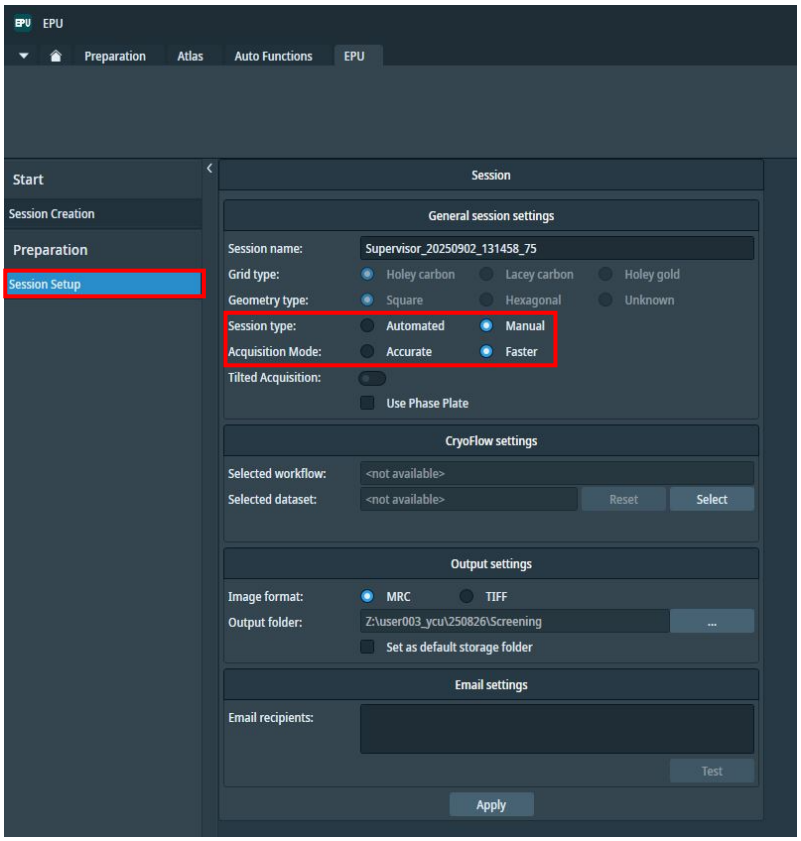




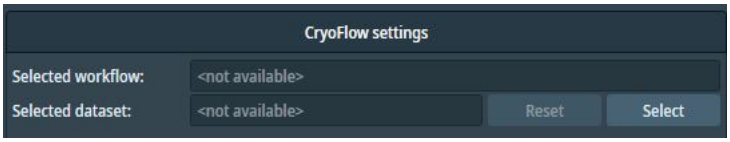
Click **Session Setup**.

Session name: **Do not change**

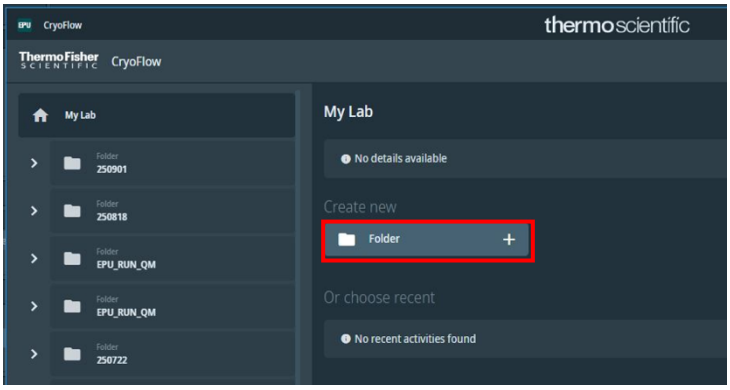
Select Session type: **Manual** and Acquisition mode: **Faster**.



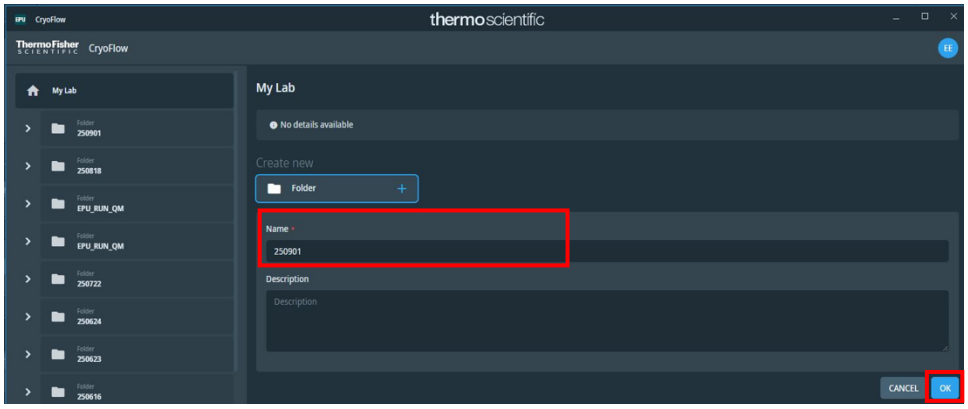
CryoFlow settings > **Select**



Create new**Folder**



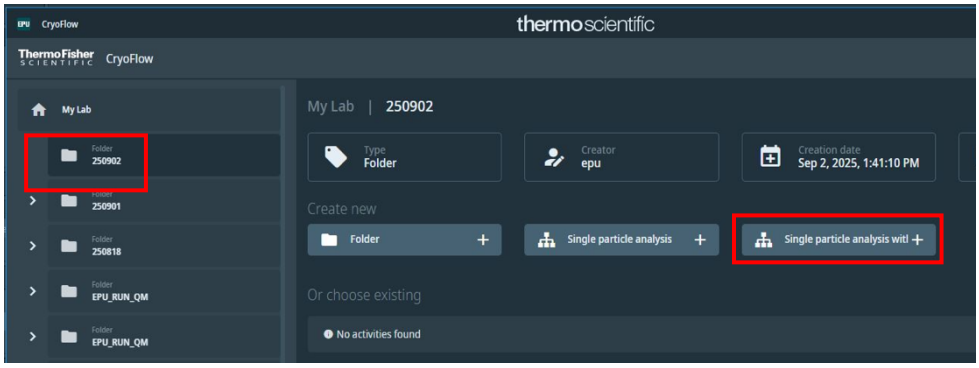
Enter the date of the day (yymmdd) in **Name** > **OK**



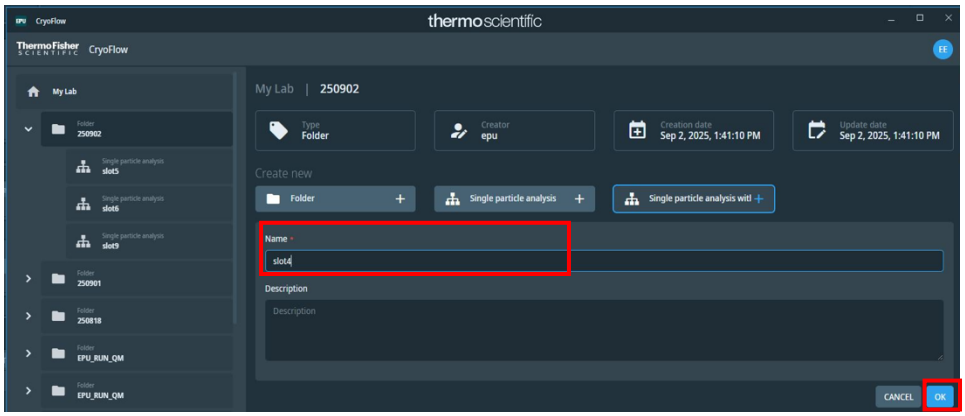
Confirm that a new folder has been created.

* When creating a new Session after changing the grid, select this folder.

Create new > **Single particle analysis with...**

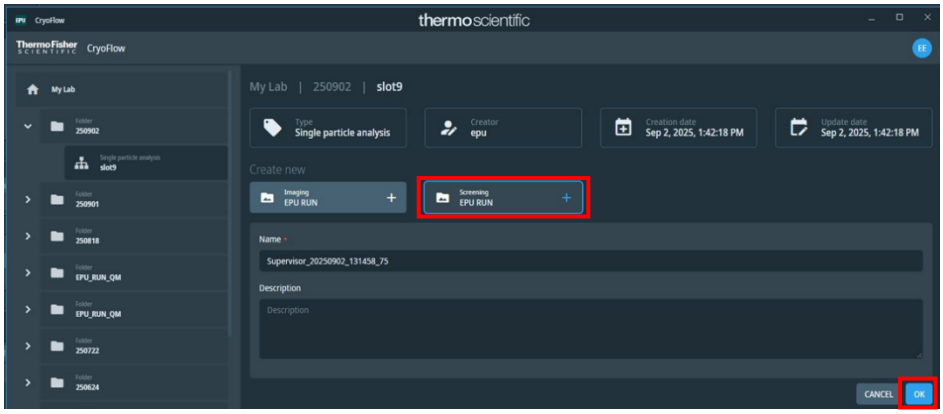


Enter the grid name, etc. in **Name** (e.g., slot_x, etc.)　> **OK**



Create new > **Screening**

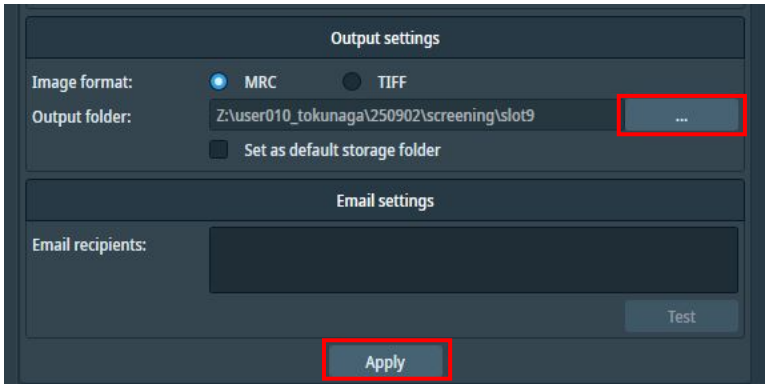
Leave Name as it is > **OK**



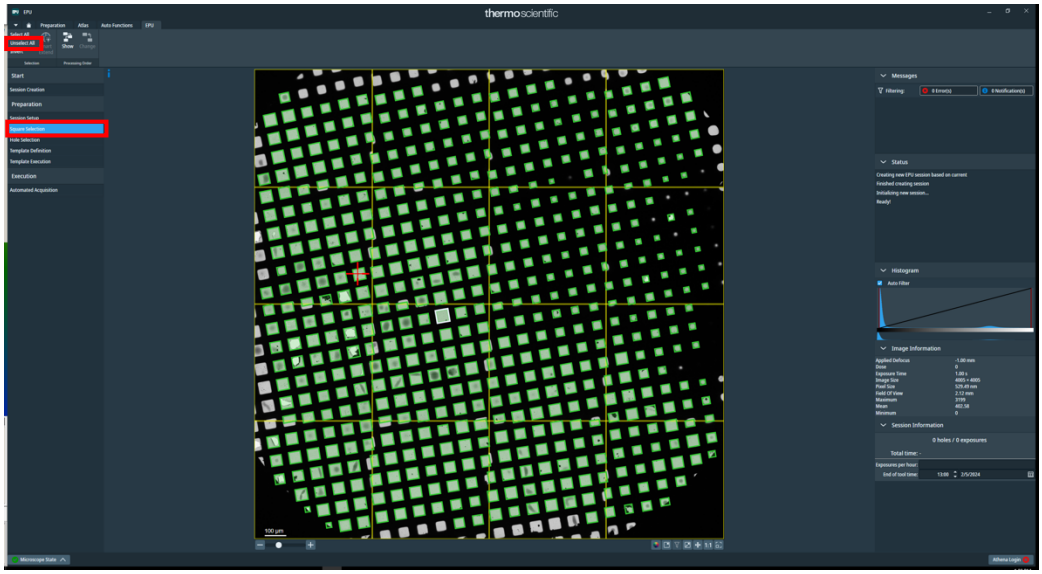
Output settings > … and select the destination folder.

Example of a save location：Z:/userxxx/yymmdd/screen/slotxx

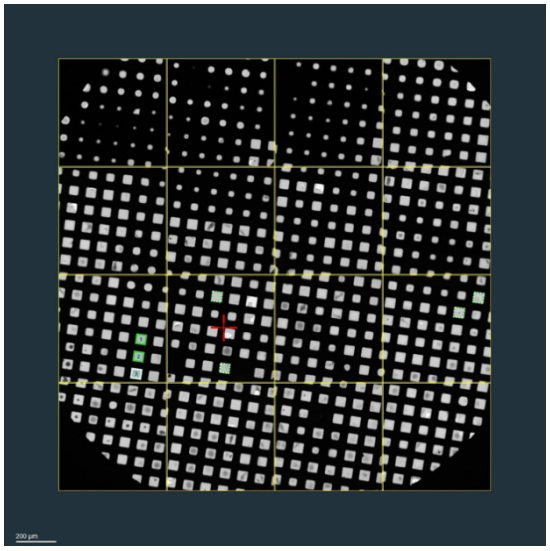
Click **Apply**.



Click **Square Selection** > Squares are automatically selected, but click **Unselect All**.



Select 2–3 representative squares (Ctrl + left-click).



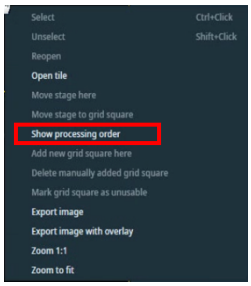
* It is good to include a variety such as thin ice, slightly thicker, and thick areas.

* If you want to display the numbers of the selected squares, right-click and choose Show processing order.

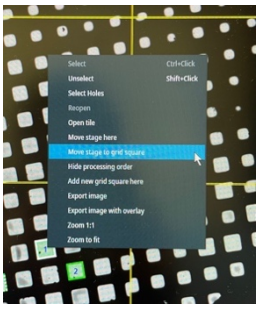
* Take a screenshot: right-click → Export image with overlay.

Be careful: if you perform the above operations while zoomed in, the zoomed-in image will be saved.

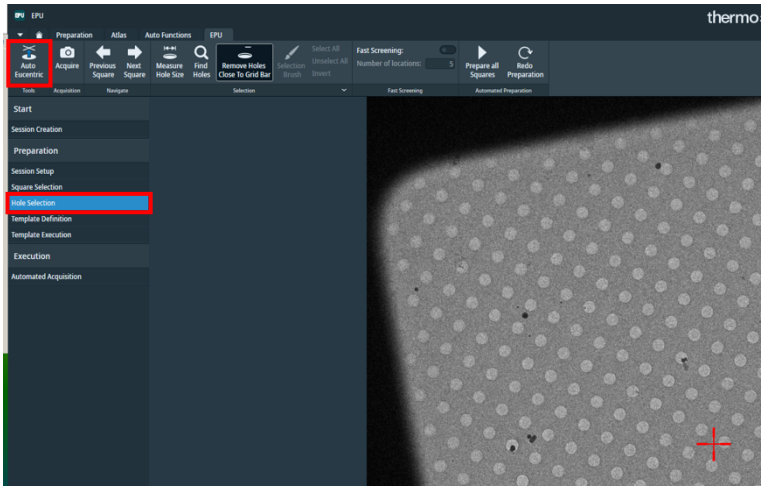
Right-click on the screen > Show processing order



Right-click the first square > Move stage to grid square



Hole selection > click **Auto Eucentric**



Wait for a while as the system automatically searches for the Eucentric height.

*If Auto Eucentric fails, go to the Auto Functions tab and confirm that **Auto-eucentric by beam tilt** is selected, then click **Start**.

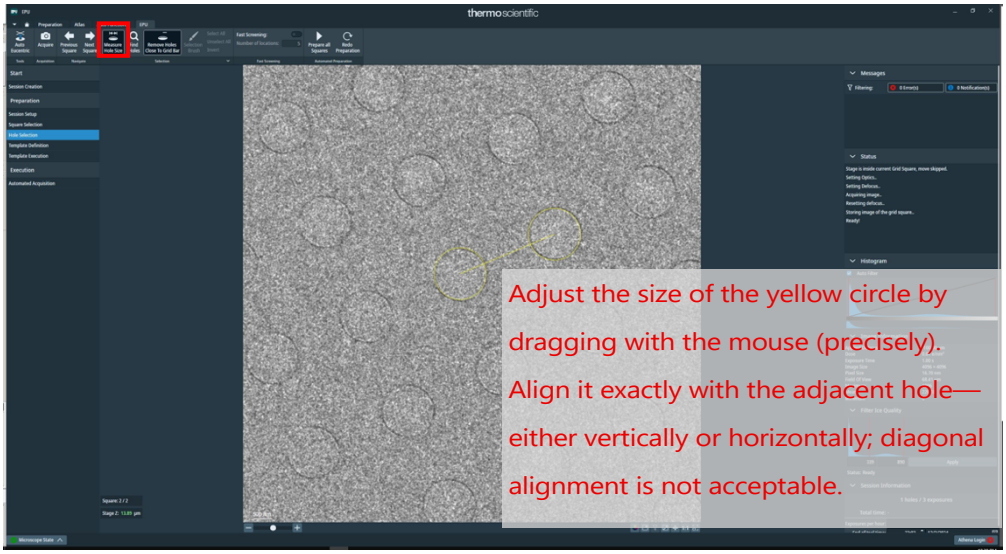
* If it still fails, select **Auto-eucentric by stage tilt** and click **Start**.

* It is fine as long as either one succeeds.

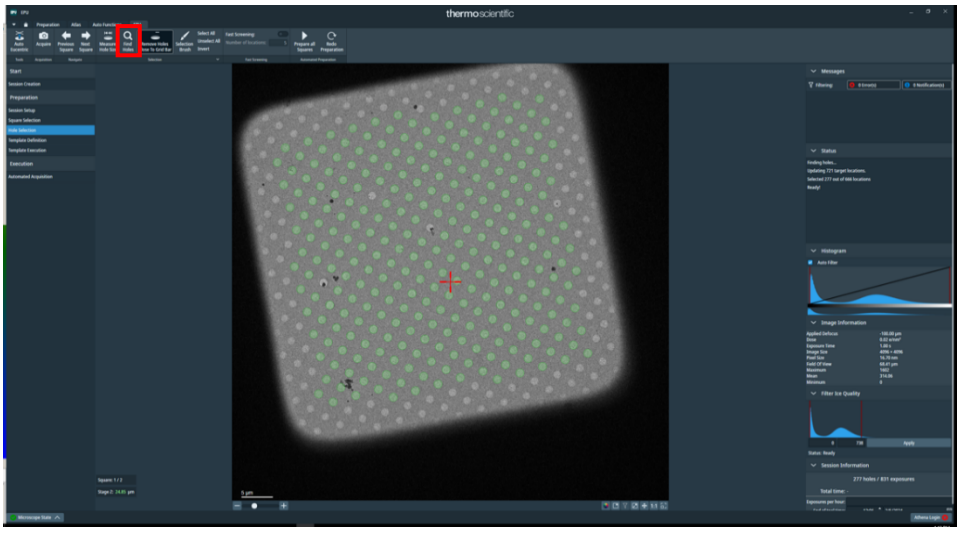
* Once the Eucentric height has been determined, return to **Hole selection** and click **Acquire**.

(If you do not do this, the determined Eucentric height will not be applied.)

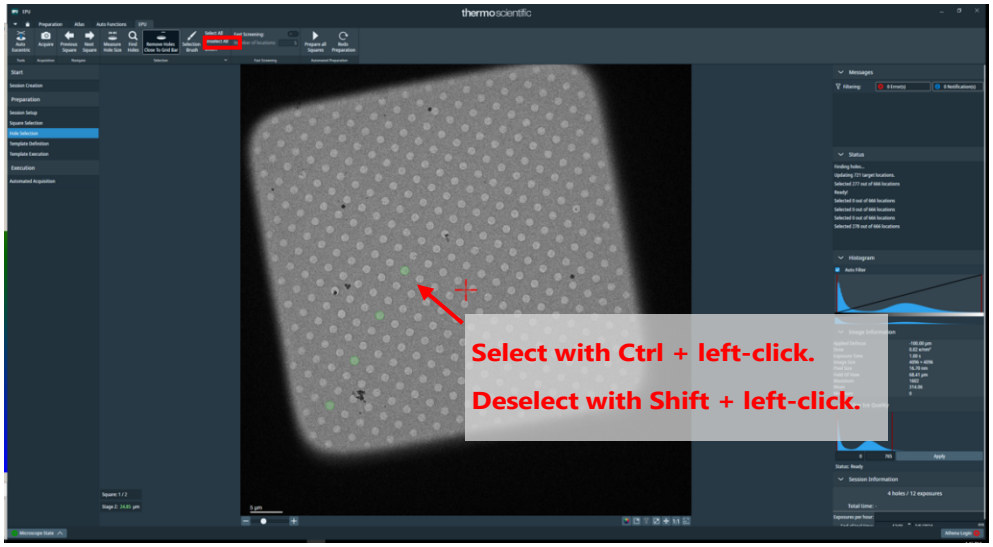
When the Eucentric height is successfully determined, click **Measure Hole Size** to define the size of the hole and the spacing between adjacent holes.



Use **Find Holes** to automatically detect the holes.

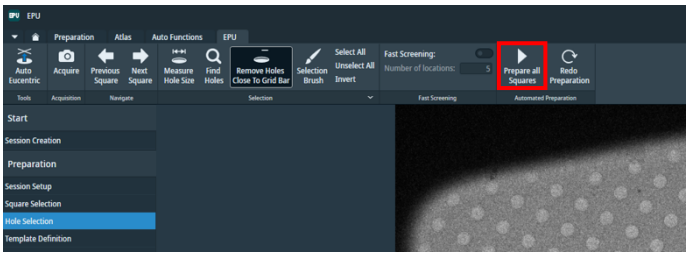


If the holes are successfully detected, click **Unselect All**, then select several representative holes (Ctrl + left-click).



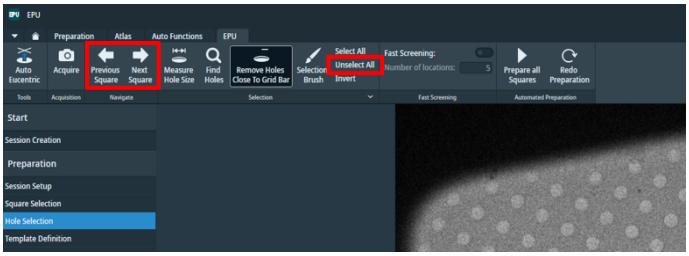
* It is good to consider the apparent ice thickness—such as holes near the center or at the edges of the square—when choosing them.

Click Prepare all Squares to apply the same procedure to all selected squares.



Use **Next Square / Previous Square** to move between squares and confirm hole detection.

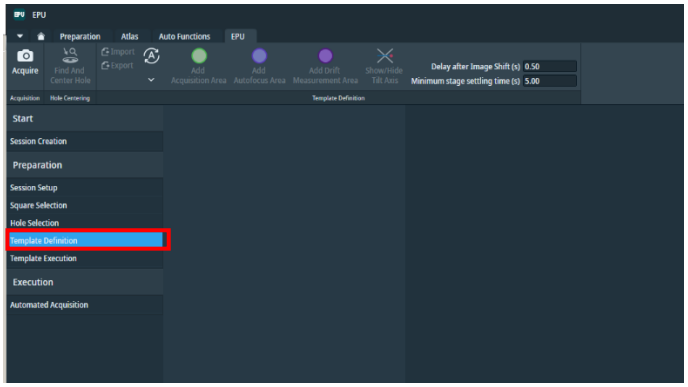
For each square, click **Unselect All**, then select several representative holes.



* For each square, take a screenshot of the selected holes.

Right-click > Export image with overlay

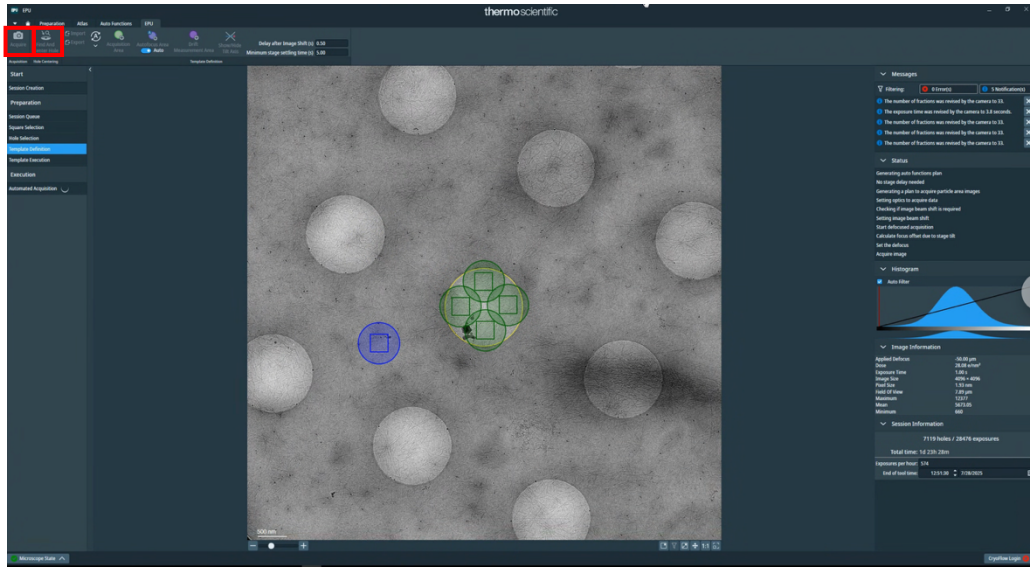
Click **Template Definition**.



Acquire > **Find and Center Hole**

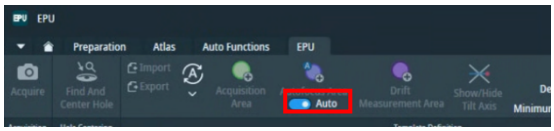
Confirm that the hole is correctly recognized and positioned almost at the center of the screen.

* If this does not work properly, all subsequent acquisitions will fail.

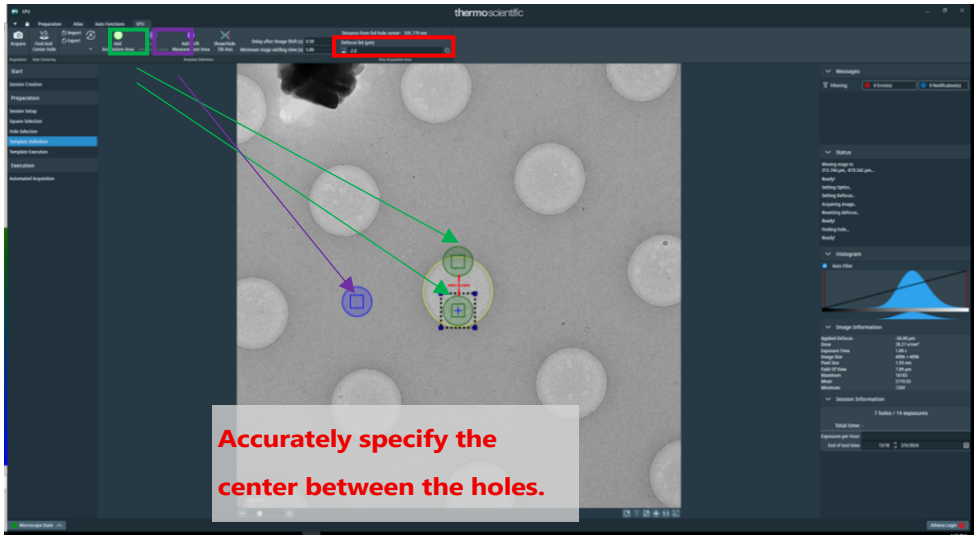


In **Add Acquisition Area**, specify which part of the hole to capture. You may select multiple locations, such as the center and edge.

Under **Add Autofocus Area**, turn **Auto** ON.



Alternatively, turn Auto OFF and manually specify the irradiation position used for focusing.

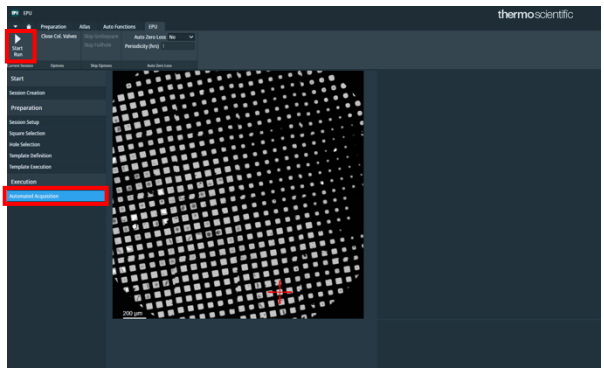


Specify the defocus value. For screening, it is better to use a slightly larger value, such as –2.0 µm.

If multiple capture points are specified, each can be configured individually

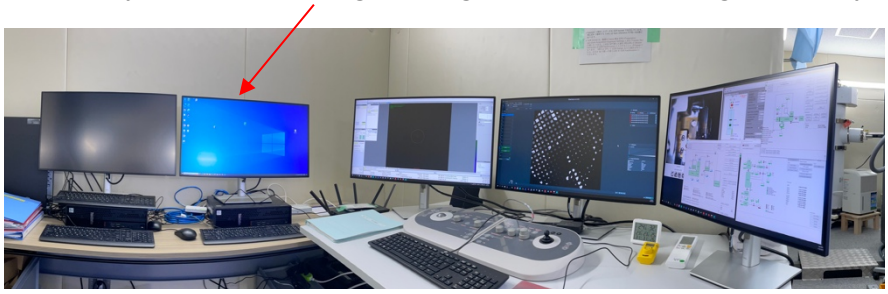
* Take a screenshot (use an app to capture the entire screen including the defocus value).

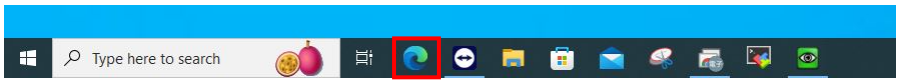
Automated Acquisition > **Start**

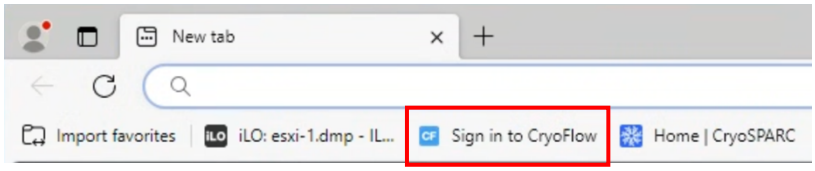


2. Confirming Results in CryoFlow

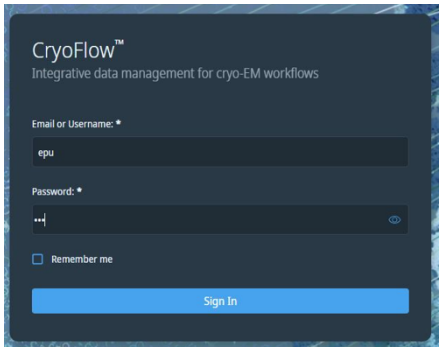
On the CryoFlow monitoring PC, Edge > Bookmark > Sign in to CryoFlow



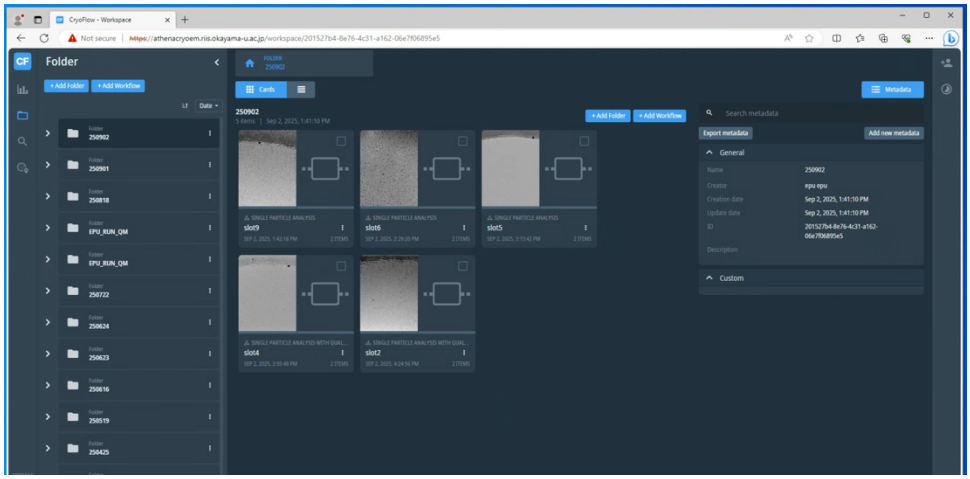




On the login screen, enter the ID (epu) and password, then click **Sign In**.

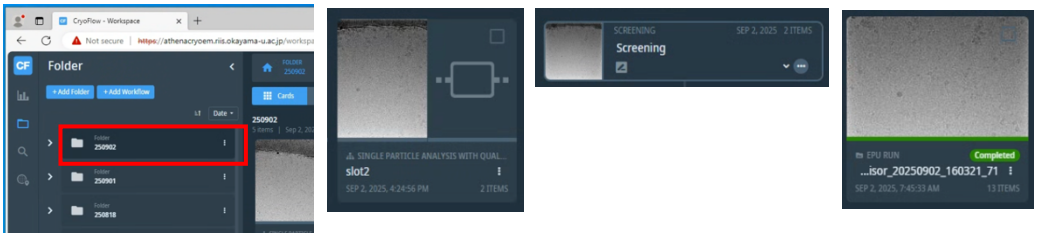


The CryoFlow Workspace will be displayed.

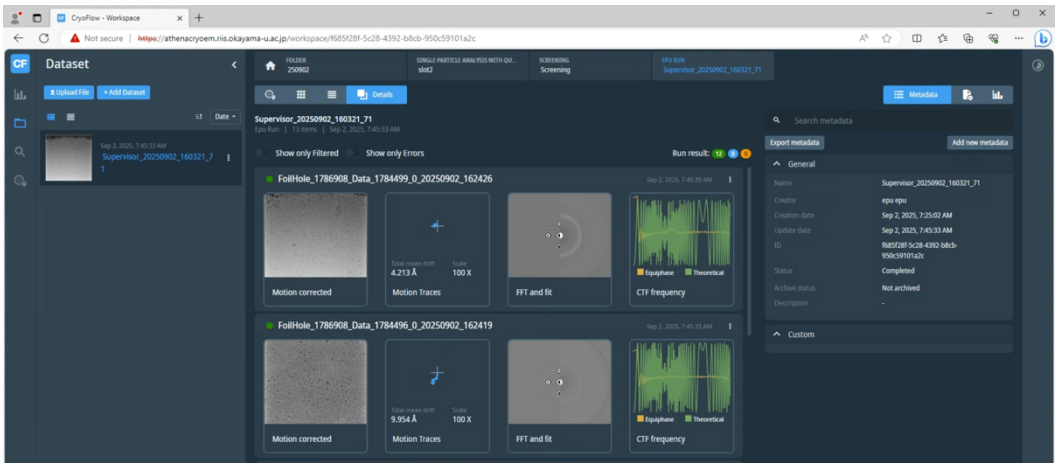


Click through the following in order:

Folder (date) > slot_x > Screening > Supervisor_xxx

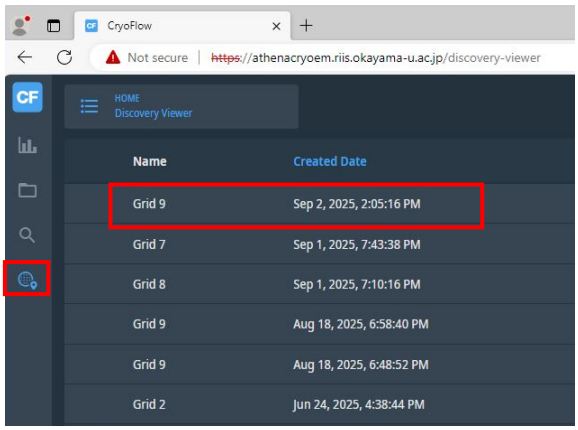


The results of Motion correction and CTF estimation will be displayed.

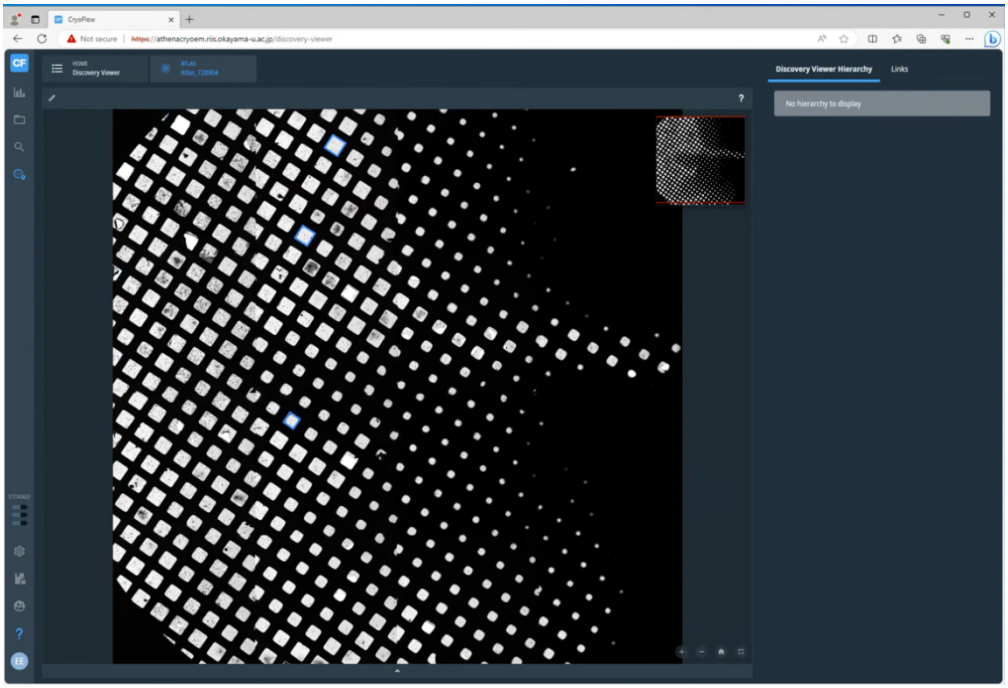


* From the CTF, you can evaluate the resolution?

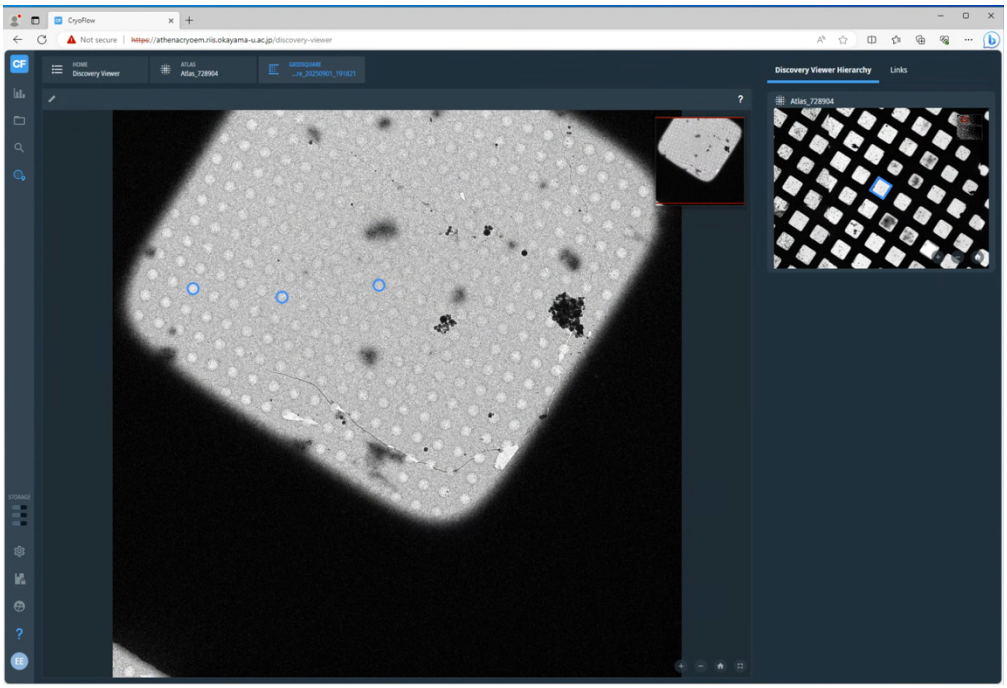
Click the Discovery viewer icon at the upper left of the screen, and select the grid you want to view.



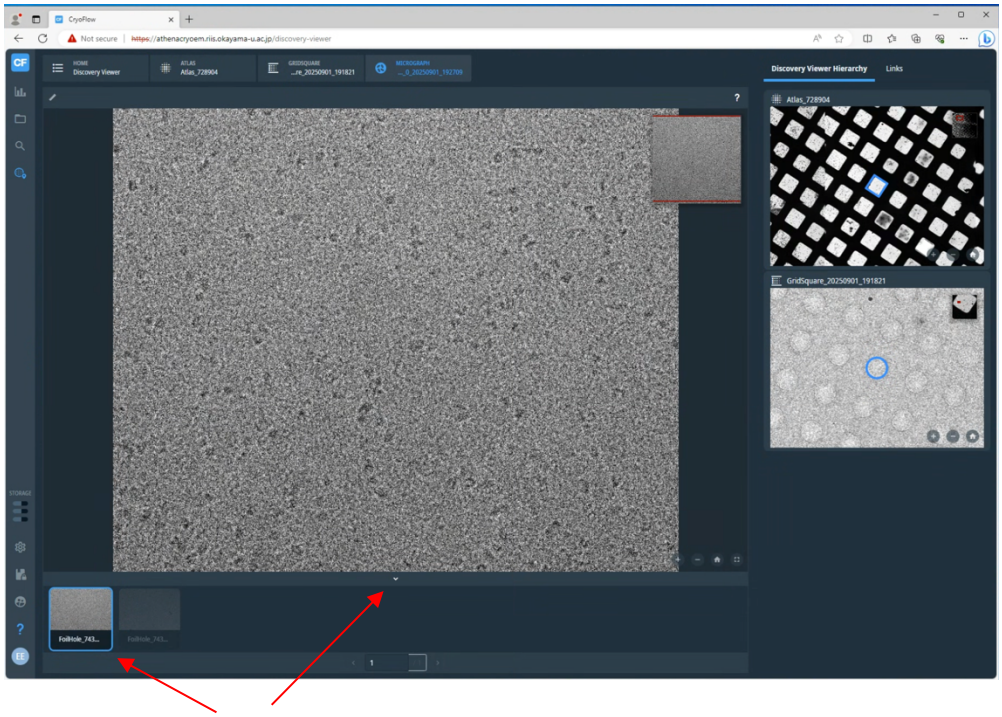
The atlas is displayed, and the squares where data were acquired are highlighted. Click the square you want to check.



The square is displayed, and the holes where data were acquired are highlighted. Click the hole you want to check.



The image captured from that hole is displayed.



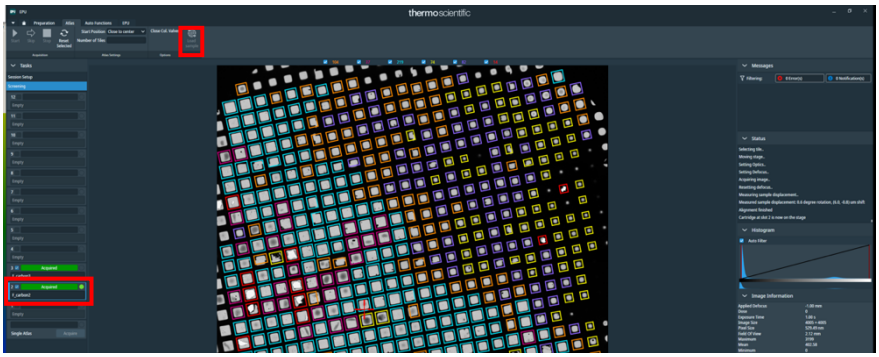
*When you click the bar, thumbnails of the images taken at that hole are shown.

Compare the acquired images from various locations on the grid and identify the characteristics of good squares and good holes.

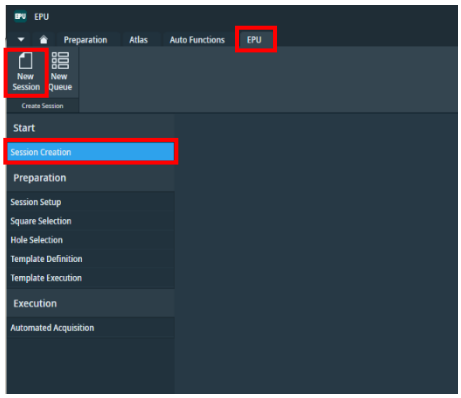
Once the screening of one grid is completed, replace the grid and repeat the same procedure.

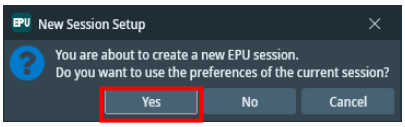
3. Continuous Data Acquisition

EPU > Atlas tab, select the grid for data acquisition and click **Load Sample**.



EPU > EPU tab, select **Session Creation** > **New Session** > **Yes**

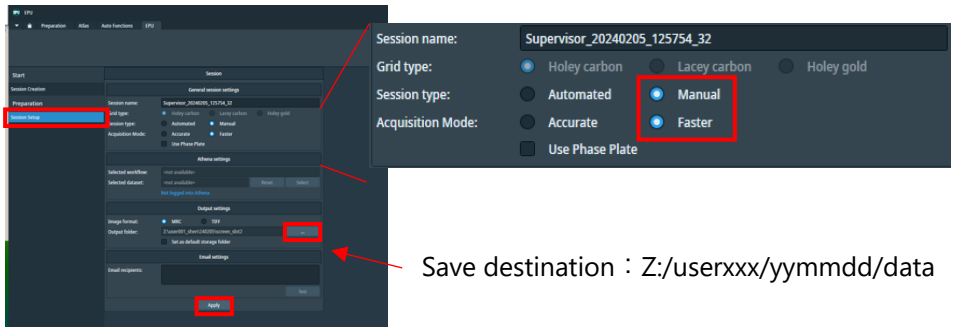




Click **Session Setup**.

Select Session type: Manual and Acquisition mode: Faster.

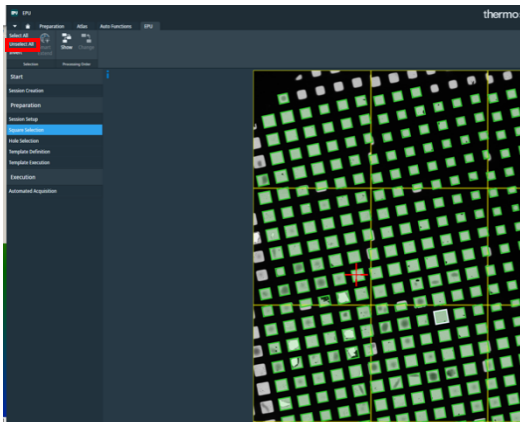
*CryoFlow is not used.

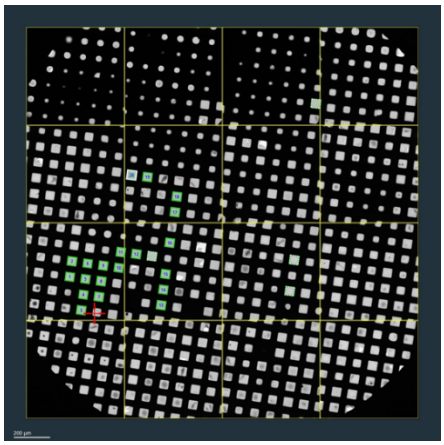


Save destination：Z:/userxxx/yymmdd/data

Click **Apply**.

Click **Square Selection** > Squares are automatically selected, but click **Unselect All**, then select 20–30 squares.

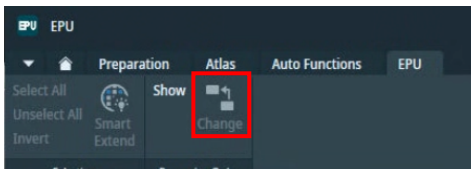




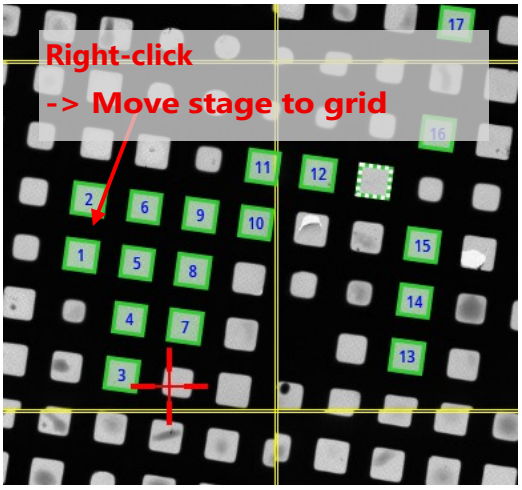
*If the movement distance between squares is too large, measurement time will be lost.

* It is better to select squares so that the system moves sequentially through nearby squares.

Click the **Change** button, then click the squares in the desired order to renumber them accordingly.



Right-click the first square (No. 1) > **Move stage to grid square**

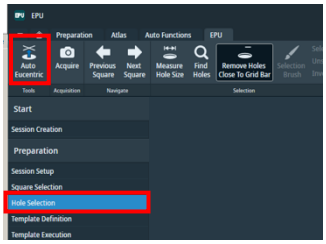


Move stage here:
Move to the clicked location

Move stage to grid square:
Move to the center of the clicked square

Click **Hole selection**.

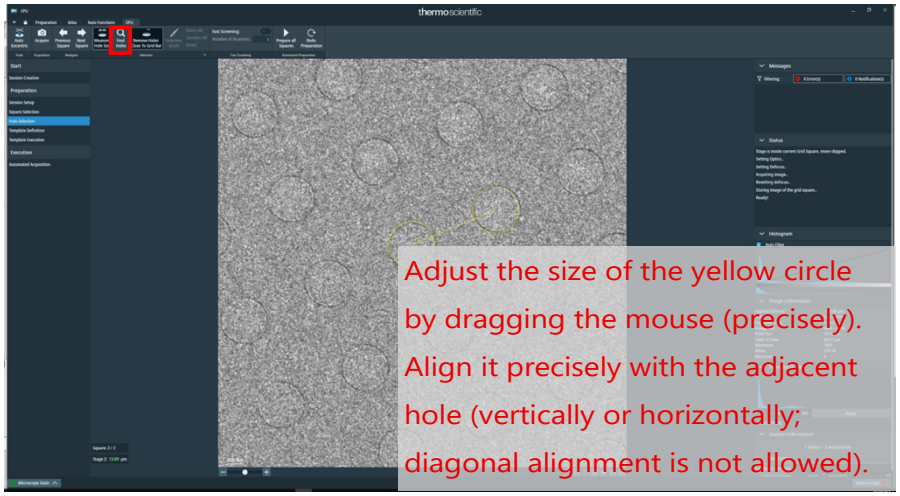
Click the **Auto Eucentric** button at the upper left.



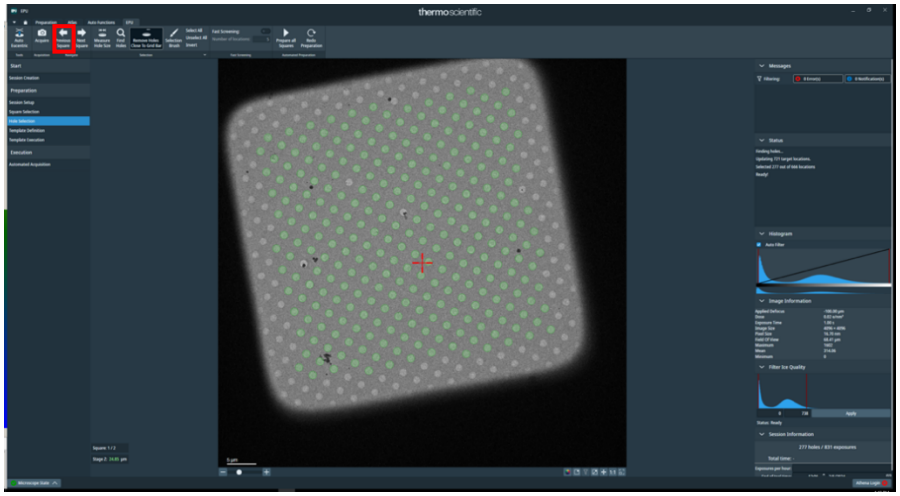
*If Auto Eucentric fails, go to the Auto Functions tab and run **Auto-eucentric by beam tilt** or **Auto-eucentric by stage tilt** → **Start**.

*Once the Eucentric height is determined, return to Hole selection and click **Acquire**.

If the Eucentric height is successfully determined, define the hole using **Measure Hole Size**.

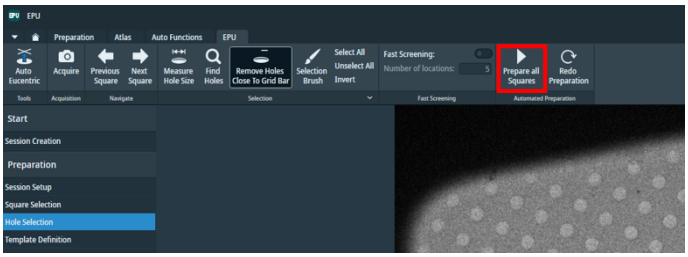


Use **Find Holes** to automatically detect the holes.



If the holes are successfully detected, click **Prepare all Squares**.

* The same procedure will be applied to all selected squares.



* It takes about one minute per square.

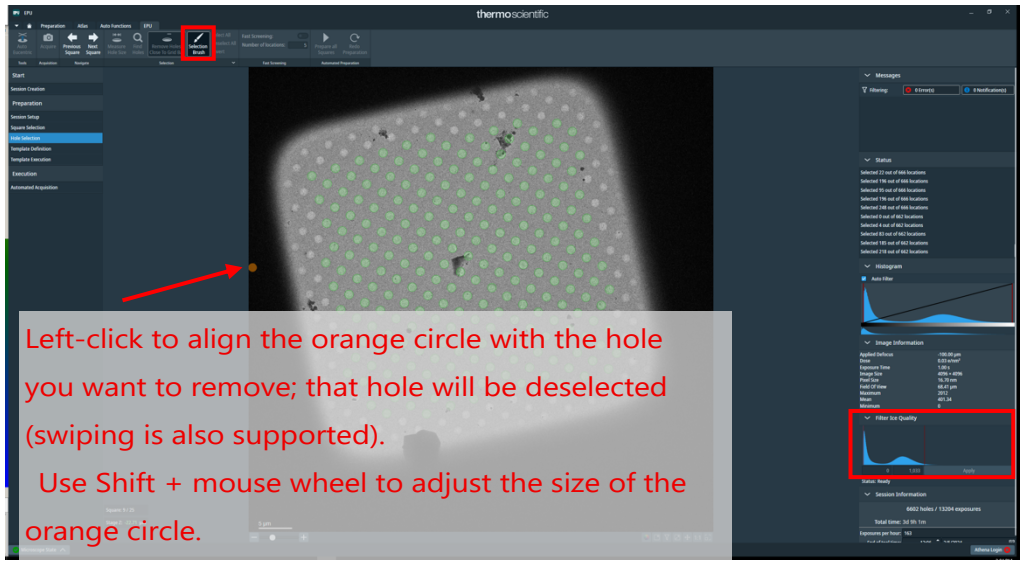
* If Eucentric fails, that square will be skipped.

If too many squares are skipped, adjust the Hole/Eucentric settings.

Check the automatically detected holes.

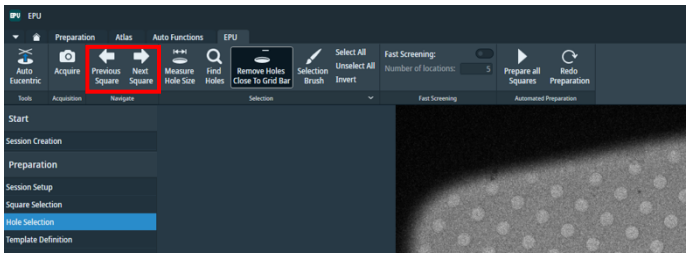
For holes containing debris or cracks, remove them manually using the selection brush.

You may also adjust the two bars under “Filter Ice Quality” at the bottom right.

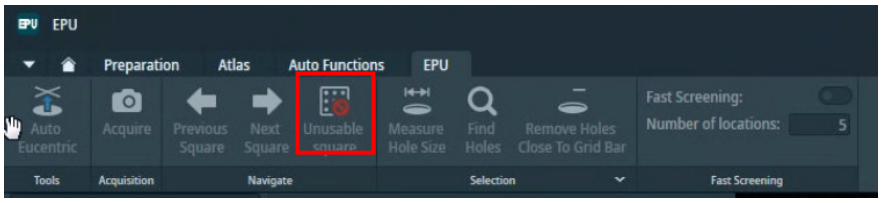


* It is safer not to select squares with cracks, as they tend to shake when irradiated with electrons.

Use **Next Square / Previous Square** to move between squares and confirm or correct the hole selection described above.



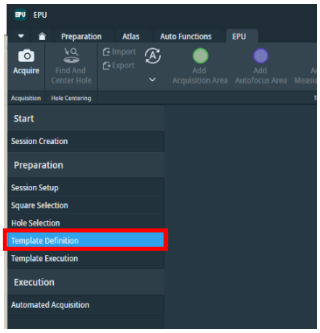
If you want to delete an entire square, display that square and select **Unusable square**.

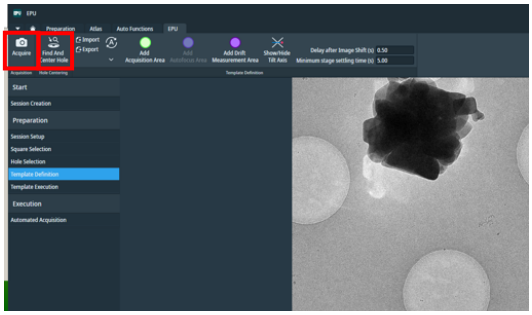


* In **Square selection**, the square will be shown in red.

Move to the first square (**Square Selection** > **Move stage to grid square**).
Click **Template Definition**.

Aquire > **Find and Center Hole**





Confirm that the hole is correctly recognized and positioned almost at the center of the screen.

* If this does not work properly, all subsequent acquisitions will fail.

* If it does not work well, redo **Measure Hole Size** in Hole Selection.

Use **Add Acquisition Area** to specify where in the hole the image will be acquired (shown in green).

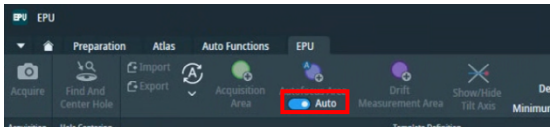
* Multiple areas can be set (e.g., center and edge).

Specify the **defocus value**:

–0.6, –0.8, –1.0, –1.2, –1.4, –1.6, –1.8 µm, etc.

When you check the button on the right, the value is applied to all areas.

Under **Add Autofocus Area**, turn **Auto** ON.

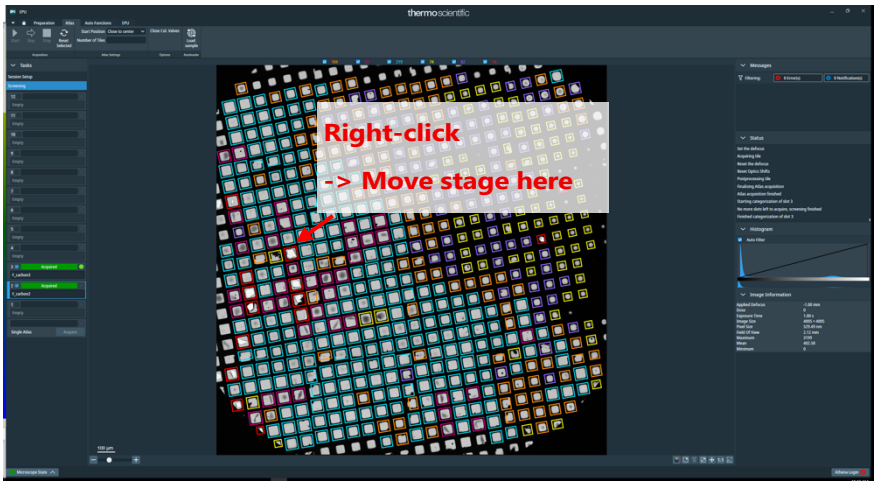


Alternatively, turn Auto OFF and manually specify the irradiation position used for focusing.

Take a screenshot (use an app to capture the entire screen including the defocus value).

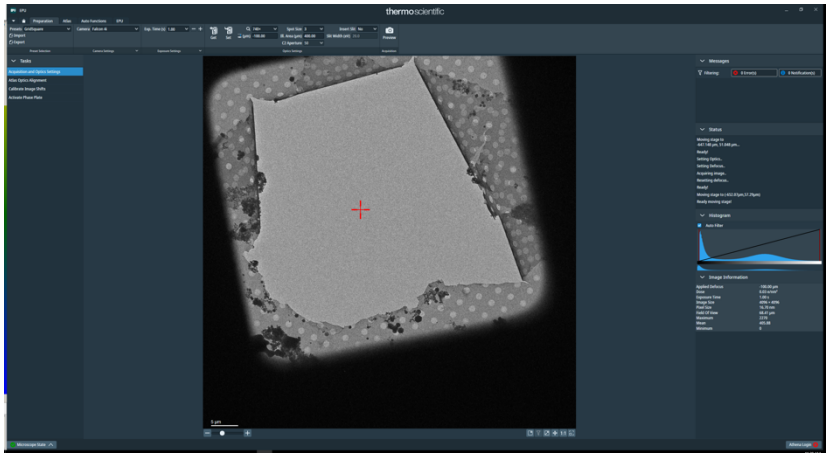
To estimate the dose, move to a hole in the grid.

EPU > Atlas tab, right-click on a hole in the grid > **Move stage here**



EPU > Preparation tab > Presets, select Grid Square and click **Preview**.

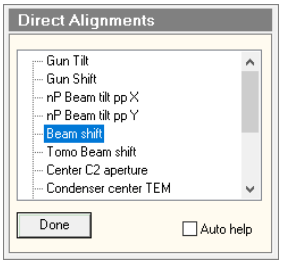
* Confirm that you are positioned over a hole.

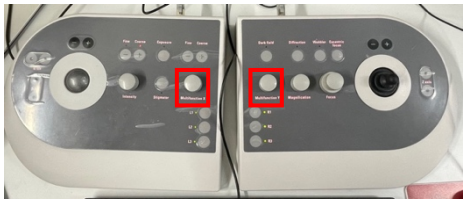


EPU > Preparation tab > Presets, select Data Acquisition and click **Set**.

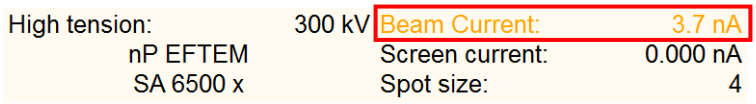
Press R1 to lower the fluorescent screen.

If the beam is not centered, use **Direct Alignment** > **Beam Shift** to center it, then click **Done**.





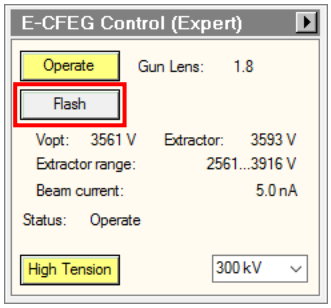
Check the Beam Current in the TUI.



If it appears to be below 5, perform a Flash.

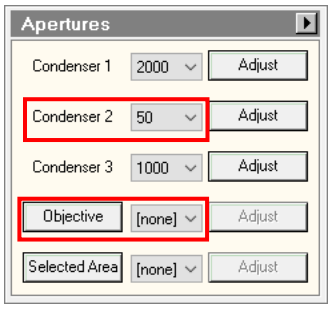
* TUI > Set up tab > E-CFEG Control

* When performing a flush, close the column valve.

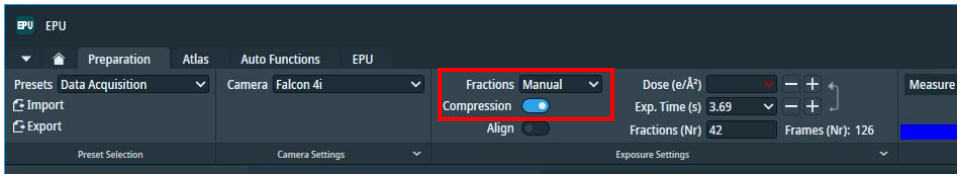


EPU > Preparation tab > Presets, select Data Acquisition and click Set.

TUI > Autoloader tab > Apertures, confirm that **C2 = 50** and **Obj = none**.



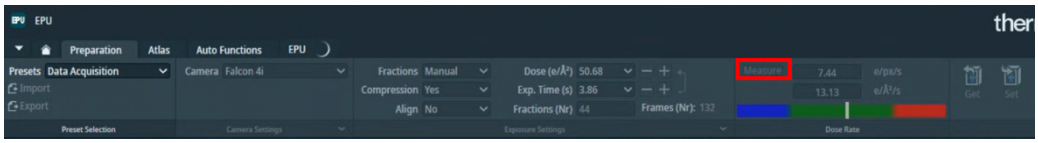
Confirm that Fractions = Manual and Compression = Yes.



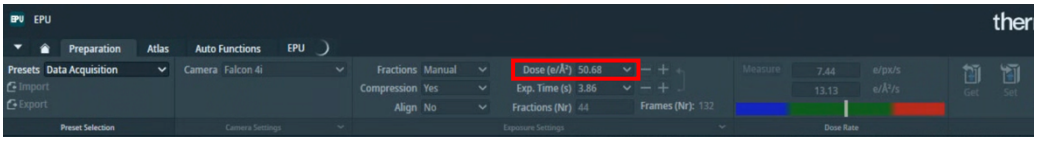
* Settings for TIFF format

* If you want to record in EER format, please refer to the separate description.

Click **“Measure”.**



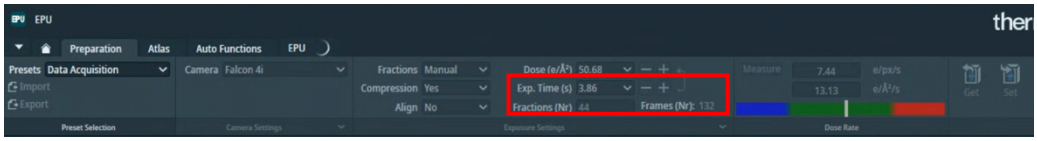
Select 50 from the Dose pulldown menu (this value is often appropriate).



Adjust **Exp. Time** and the **Fractions** value so that:

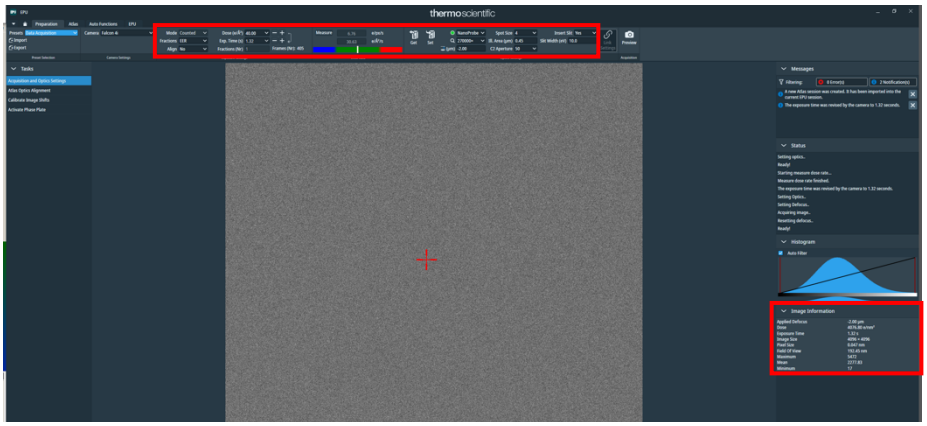
Fractions (preferably 40–50) × (integer) = Frames.

*The Dose value changes in conjunction with Exp. Time.



After that, perform a **Preview** and then take a screenshot.

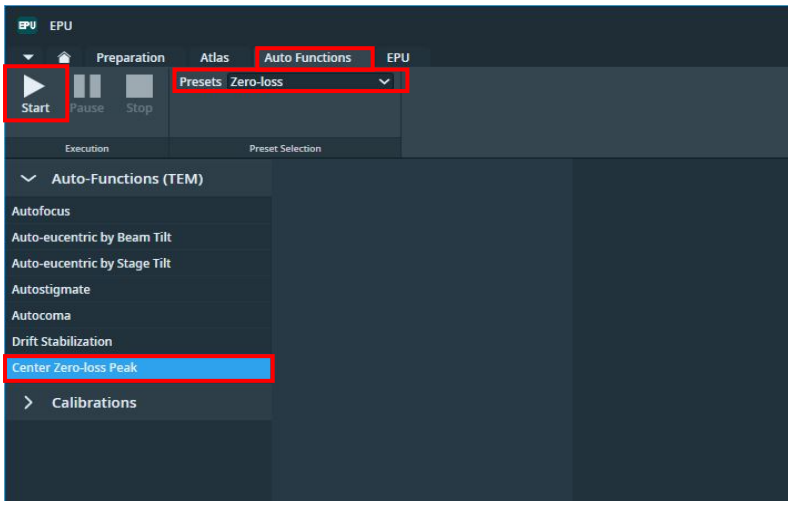
*When you preview, information such as pixel size appears in the lower-right corner of the screen.



(Capture the entire screen using an app.)

EPU > Auto Functions tab > Center Zero-loss Peak

Change Presets to Zero-loss and click **Start**.

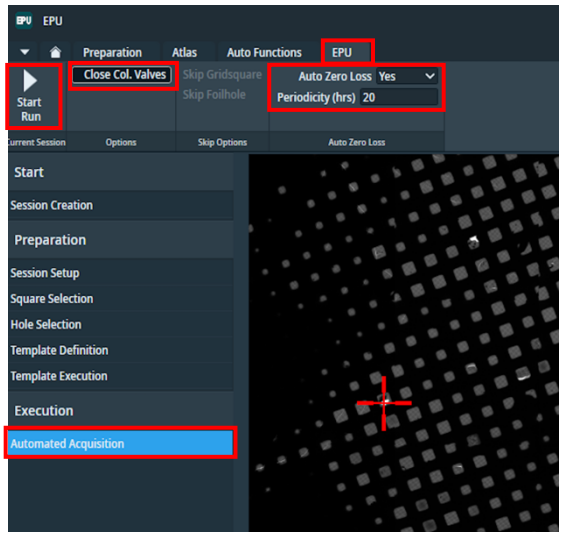


EPU > EPU tab > **Automated Acquisition**

Set Auto Zero loss = Yes and Periodicity (hrs) = 12 hrs

Enable Close Col. Valves so that the column valve will close after measurement.

Click **Start** to begin the measurement.

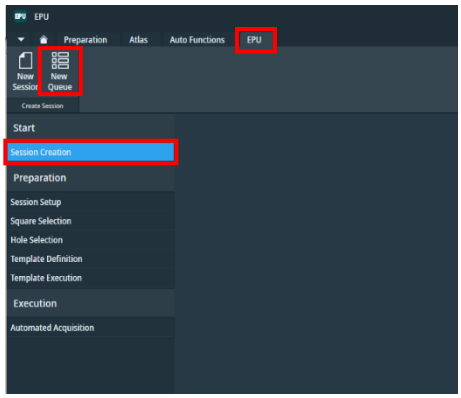


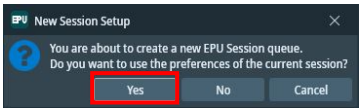
4. Data Acquisition Using Multi-Grid Session

EPU > Atlas tab, select the first grid and click Load Sample.

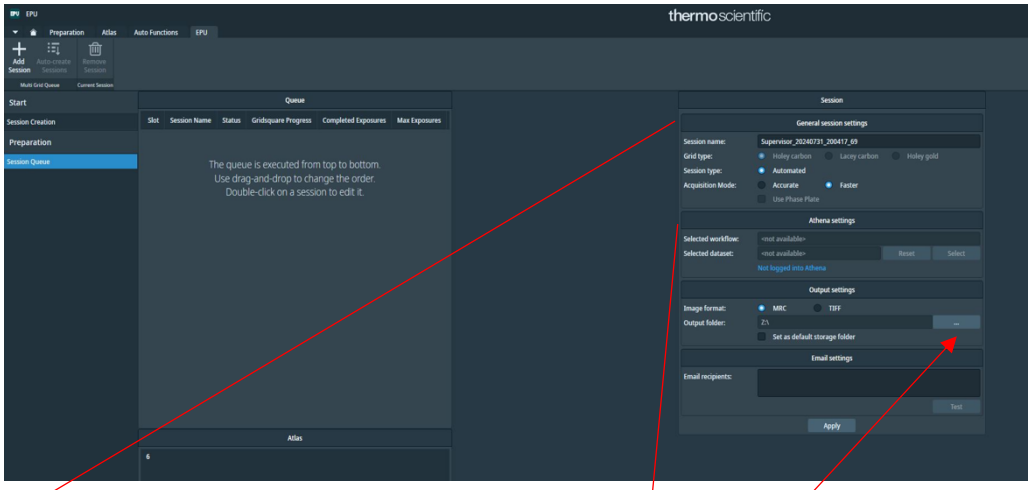
Following pp. 85–87, estimate the dose.

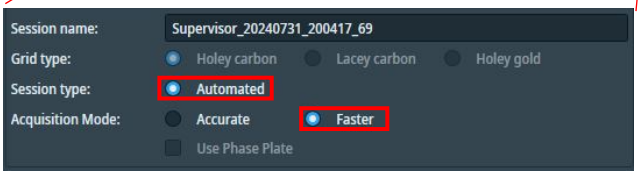
EPU > EPU tab, go to Session Creation > **New Queue** > Yes





Enter the required information in the **Session Queue** window on the right.





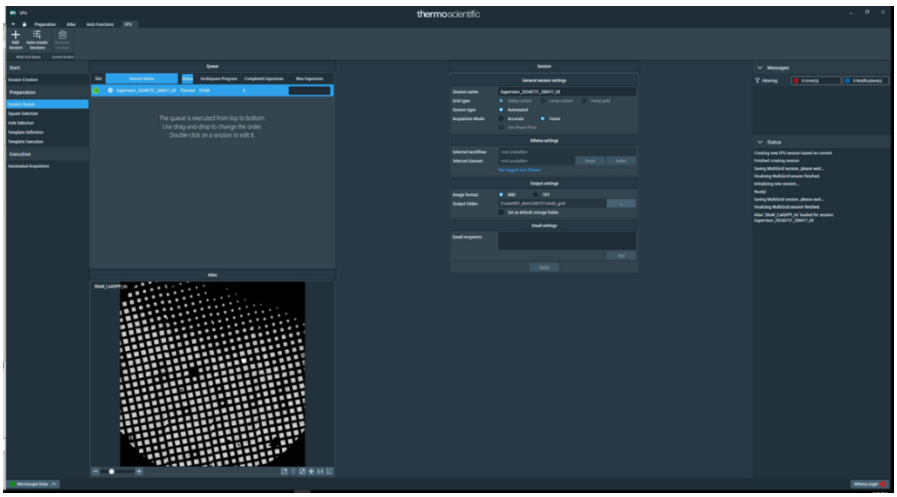
(Save destination：
Z:/userxxx/yymmdd/multi)

Session type: Automated（can only be selected）

Acquisition mode: Select Faster

Click **Apply**.

The first grid is added to the Queue, and the Atlas is displayed.



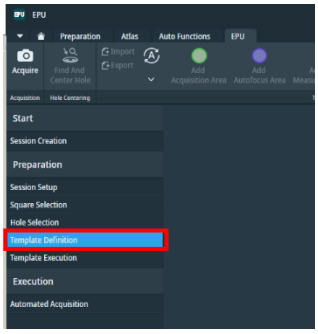
Following pp. 80–81, select the squares and select the holes.

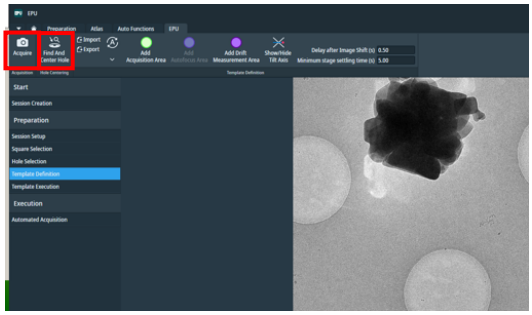
* Since this is Automated, hole-selection operations are performed only for the first square.

* You also cannot manually include/exclude holes (selection by Ice Filter settings is possible).

Click **Template Definition**.

Acquire > **Find and Center Hole**





Confirm that the hole is correctly recognized and positioned approximately at the center of the screen.

* If this does not work properly, all subsequent acquisitions will fail.

* If it does not work well, redo **Measure Hole Size** in Hole Selection.

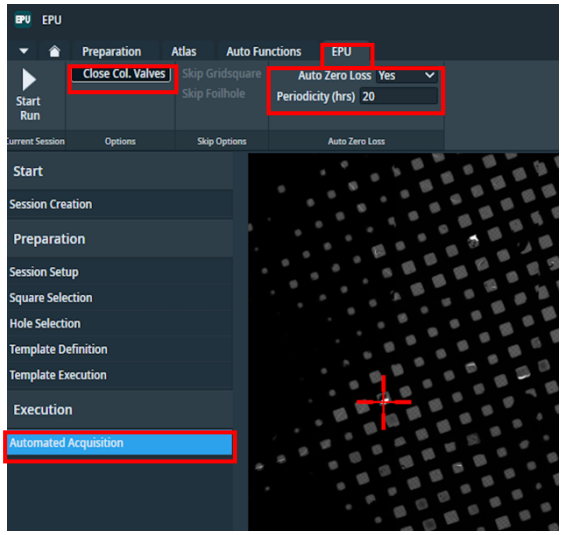
Following pp. 84, set the autofocus position, irradiation position, defocus values, etc.

EPU > EPU tab > **Automated Acquisition**

Auto Zero loss = Yes、Periodicity (hrs) = 12 hrs

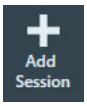
(Adjust the timing so that it runs at least once per grid).

Enable “Close Col. Valves” to ensure the column valves close after measurement.



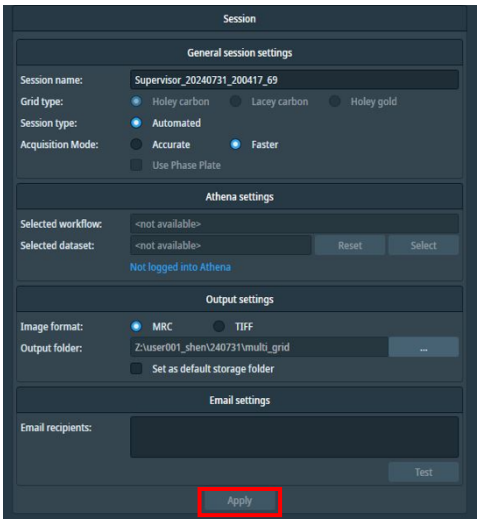
(With this, the setup for the first image is complete.)

Replace the grid (**Load Sample** in the Atlas tab).

EPU tab > Session Queue, **Add Session** 

> **Yes**

Enter the save destination in the new Session window and click **Apply**.

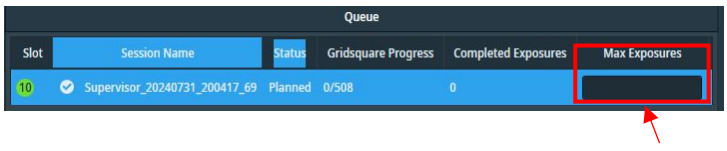


The current grid is added to the queue.

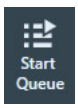
As with the first grid, perform square selection, hole selection, and Template Definition.

Repeat this for all target grids.

Set Max Exposures as needed.



Once the set number of images is reached, it moves on to the next grid.

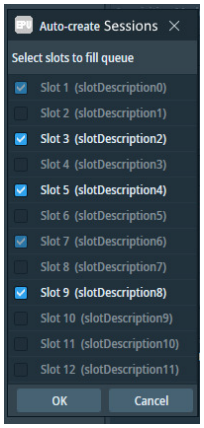
Automated Acquisition > **Start Queue** 

*** Batch setup using Auto-create Sessions**

Complete the dose estimation (pp. 85–87) in advance.

After finishing the setup for the first grid, select that queue in **Session Queue** and click **Auto-create Sessions**.

Select the grids you want to add to the queue.



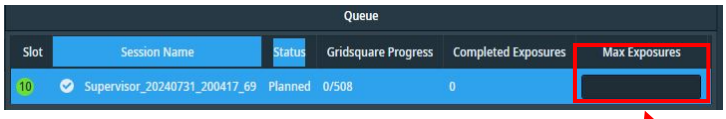
Click **OK**, and the selected grids will be added to the Queue.

The newly created queue has its save destination set directly under the Z drive, so enter the desired save destination in the Session window on the right and click **Apply**.

From **Square Selection**, select the squares.

* Since the grid is not on the stage, hole settings and Template Definition cannot be performed (the same settings as for the first grid will be used?).

Set Max Exposures as needed.



Once the set number of images is reached,
it moves on to the next grid.

Following p. 91, configure the **Auto Zero-loss** settings.

Automated Acquisition > **Start Queue**

That's all.