

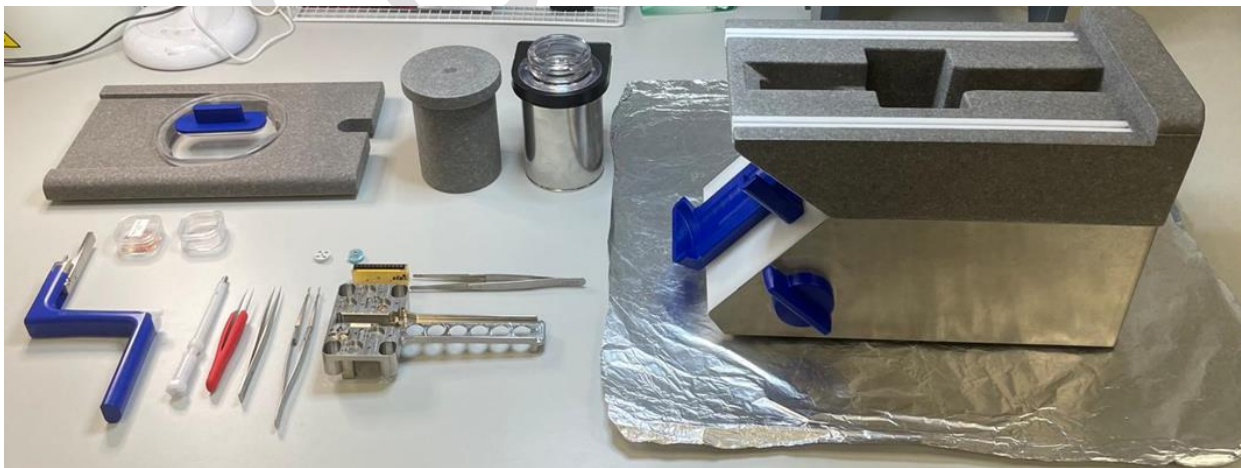
Krios Cryo-TEM (300kV) Standard Operation Protocol

Contents

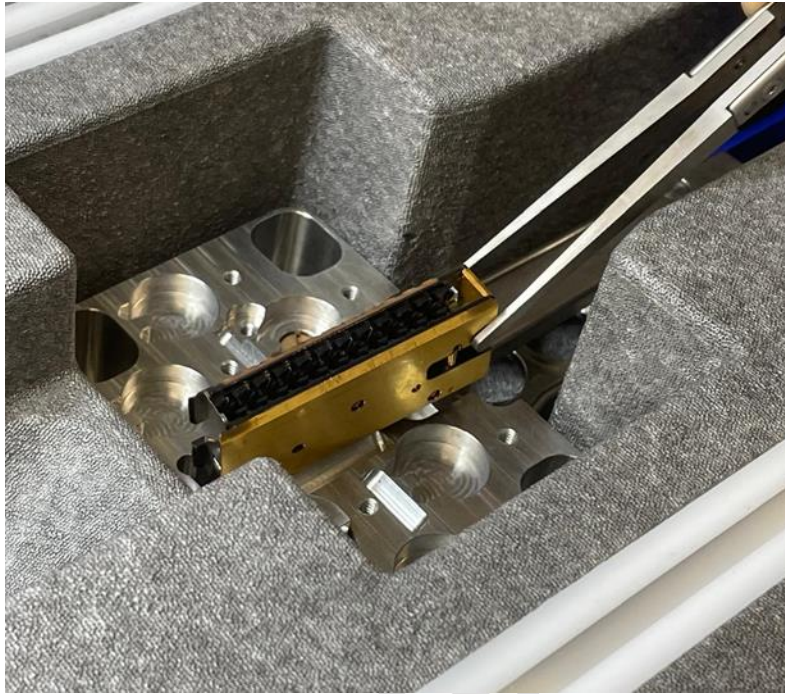
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Cassette Loading

1. Prepare the grid clipping station materials as shown below.



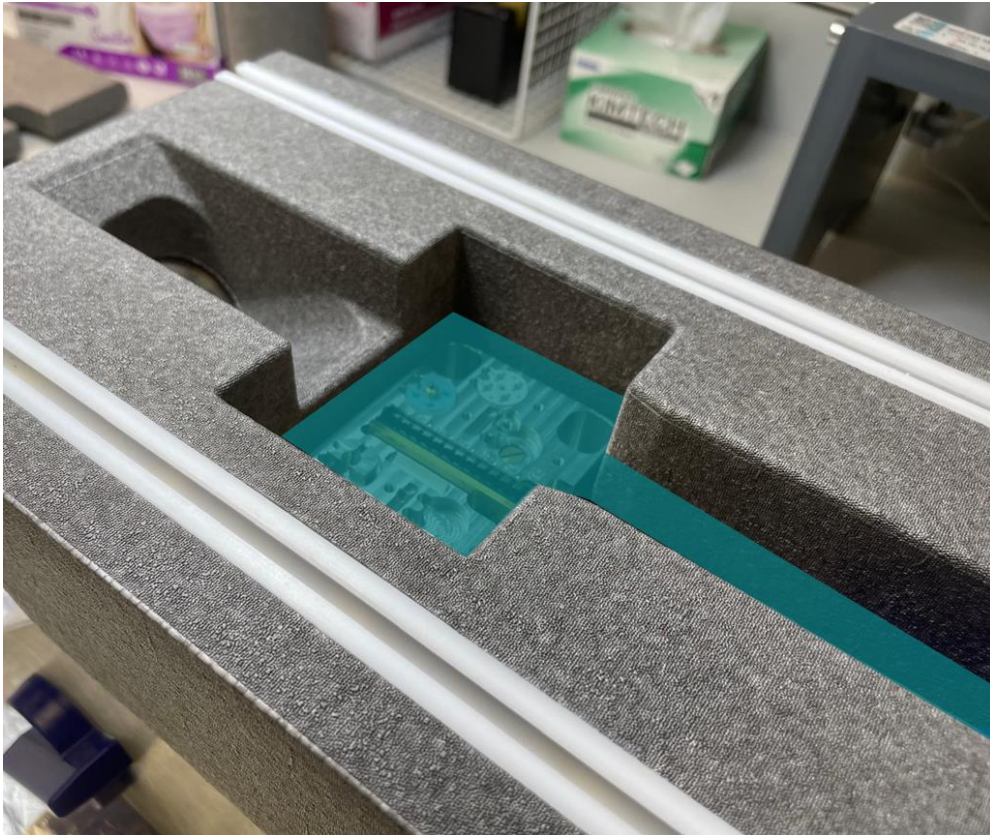
2. Put the cassette into the station with gloves or cassette forceps. Use the designated cassette for 300kV.



3. Put grid boxes, autogrid boxes and cassette holder into the clipping station.



4. Add adequate liquid nitrogen to the station. Do not exceed the level indicated in the below photo.

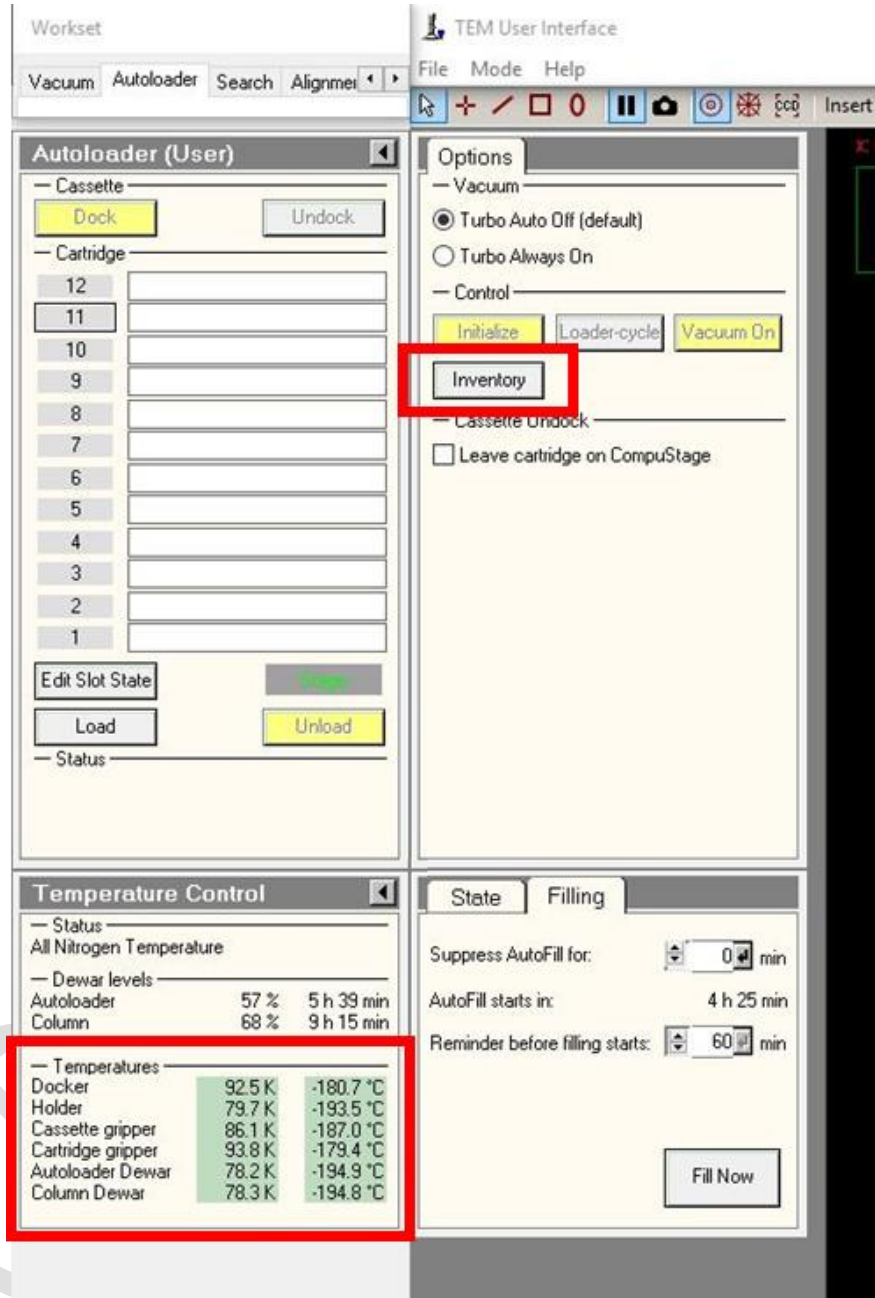


5. Fill the cab with liquid nitrogen and cover it with plastic cover.

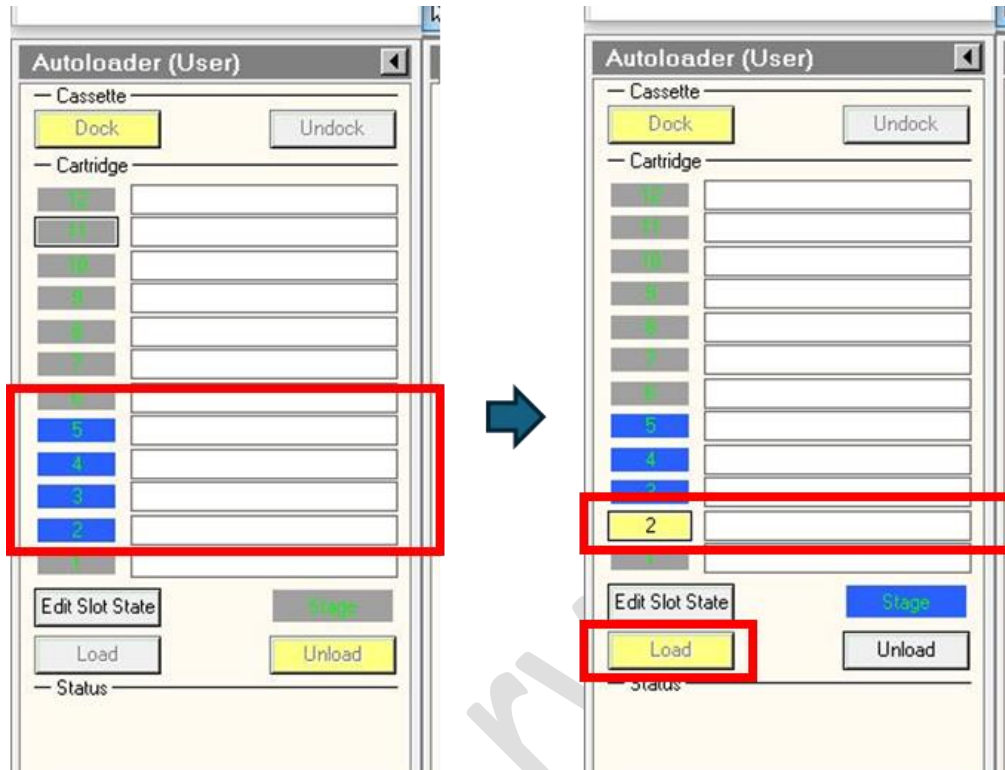


6. Contact our staff for grid clipping and cassette loading.

7. Click **Inventory** when the temperature reaches -170°C or below as shown in the software “TEM User Interface”.

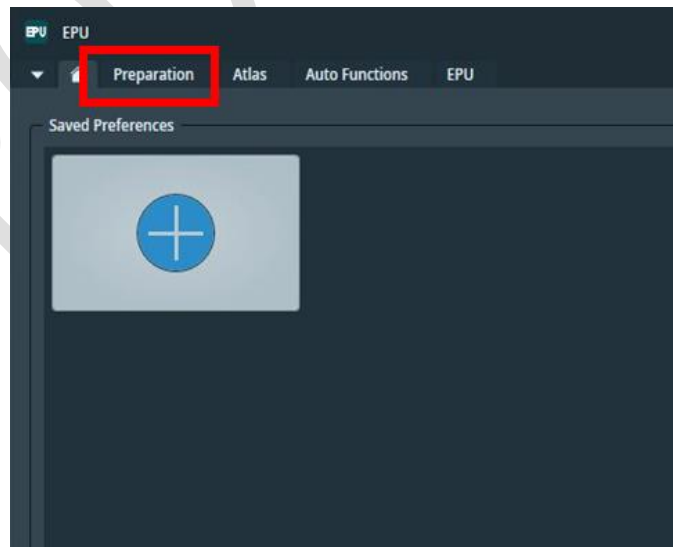


- After finishing inventory, the slots with grid are indicated in blue. Select the slot and click “Load”. When the selected grid is loaded into TEM, the slot becomes yellow.

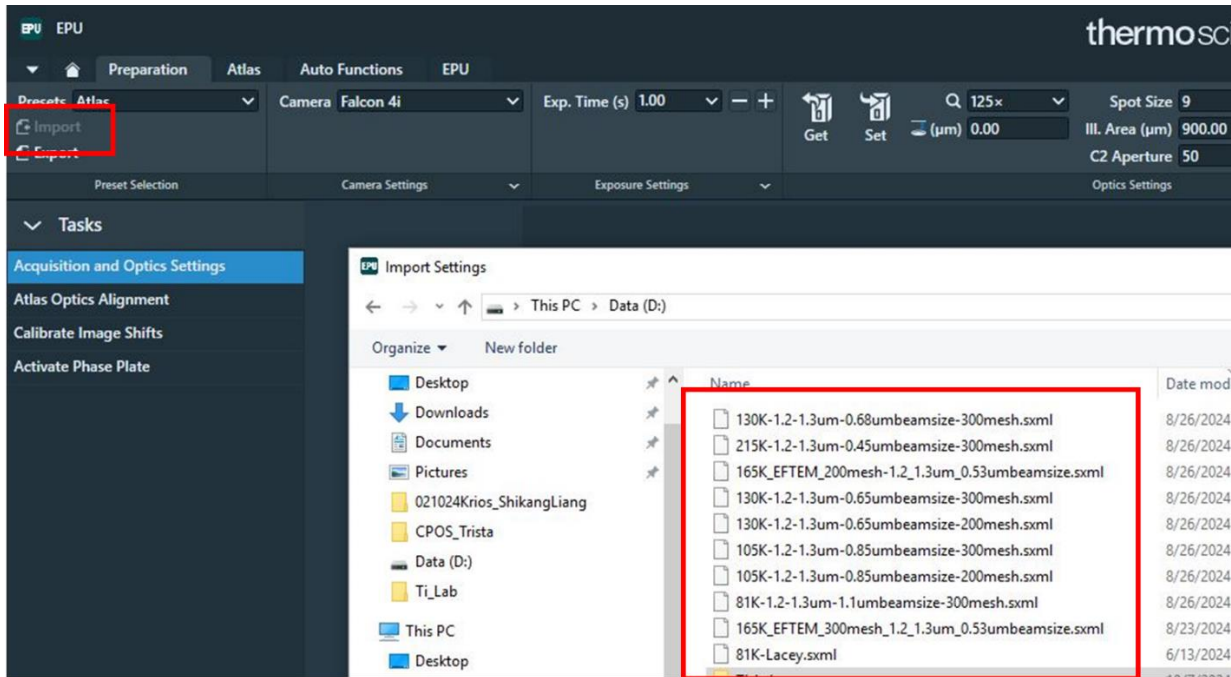


Parameter Setup

- Open the software EPU. Click “Preparation” to start.



2. Copy the pre-set file from **D:** to your folder and import.



**The magnification settings in the pre-set files are not recommended to change without adjusting the dose as it could damage the detector (camera).

For any adjustment of the pre-set files, please consult CPOS staff.

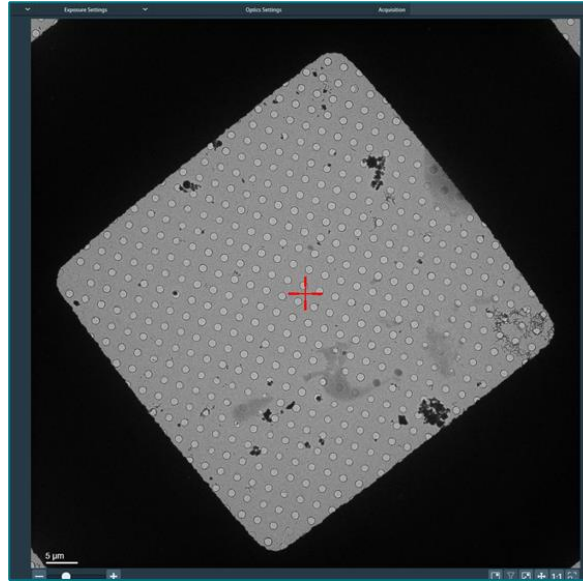
There are 4 magnifications settings in EPU:

1. Atlas (lowest magnification):

It is used for generating the map of the grid.

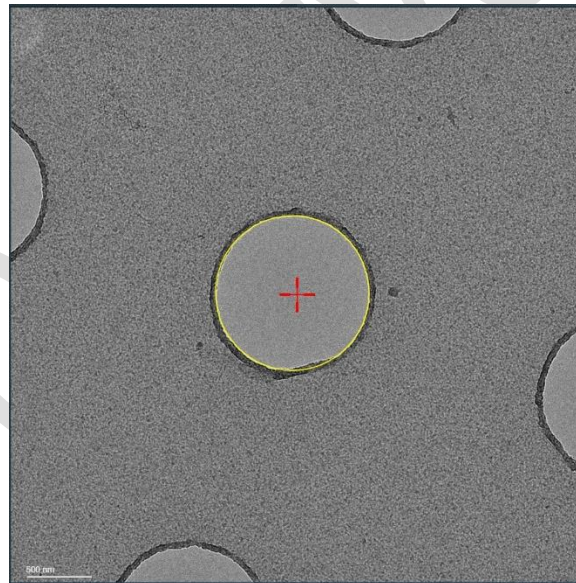
2. GridSquare

The optimal magnification is keeping 1 complete square in the field of view.



3. HoleEucentric Height

The optimal magnification is obtaining 1 complete hole with 4 partial holes in the field of view.



4. Data Acquisition (highest magnification)

The magnification is determined by the desired resolution. Generally, the target resolution is about 3 times the pixel size of the image acquired.

$$\text{Pixel size} = \text{Target resolution} / 3$$

Once the pixel size is determined, the corresponding magnification can be chosen according to the magnification calibration table.

Microscope		Krios--HKU	
Magnification Calibration Date		2023/08	
High Tension		300 kV	
BM-Falcon4i		EF-Falcon4i	
TEM Mag. (SA Mode)	Pixel Size(Å)	EF-TEM Mag. (SA Mode)	Pixel Size(Å)
1700	46.77	2250	56.57
2200	36.29	3600	35.01
2850	27.77	4800	26.66
3800	20.80	6500	19.41
5000	15.75	8700	14.55
6500	12.05	11500	11.16
8700	8.767	15000	8.571
11000	7.084	19500	6.255
14000	5.644	26000	4.735
18000	4.422	33000	3.747
22500	3.528	42000	3.057
29000	2.744	53000	2.417
37000	2.165	64000	1.972
47000	1.683	81000	1.571
59000	1.344*	105000	1.210*
75000	1.051*	130000	0.9557*
96000	0.8229*	165000	0.7393*
120000	0.6473*	215000	0.5835*
155000	0.5165*	270000	0.4656*
195000	0.4014*	350000	0.3613*
250000	0.3133*		

(*) Re-calibrated with gold sample.

For example, if the target resolution is 3Å, the pixel size should be around 1Å. Therefore, 130kX should be chosen as the magnification of data acquisition.

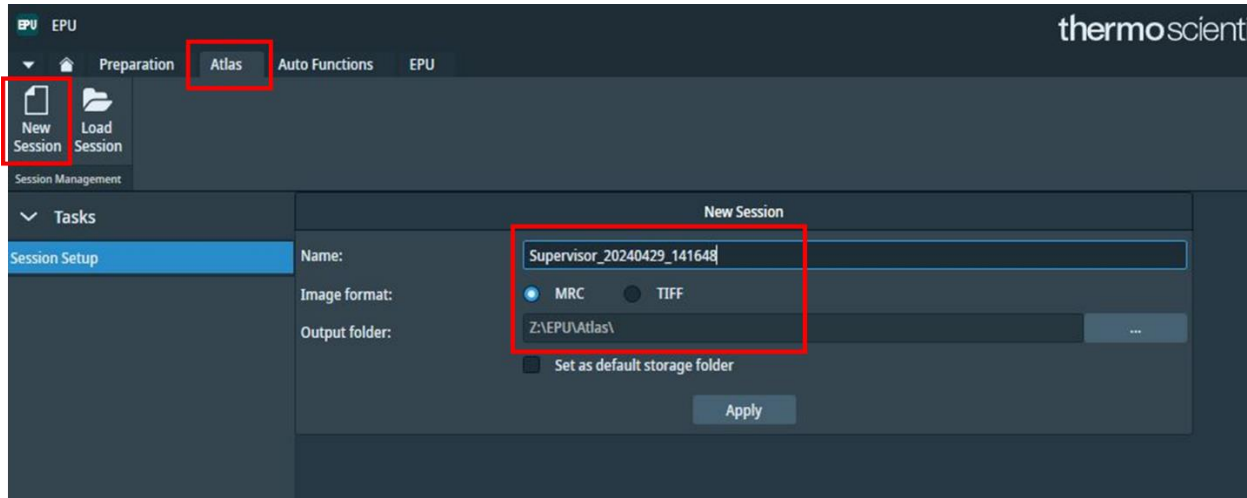
**If you want to increase the magnification in the existing pre-set file, the dose will decrease. Therefore, the intensity and spot size should be adjusted. The dose should be measured and confirmed at the green region.

**If you want to decrease the magnification in the existing pre-set file, the dose will increase. Before measuring the dose, it is highly recommended that to reduce the intensity for protection and adjusted with caution. If you are not certain in the procedure, please contact staff for assistance.

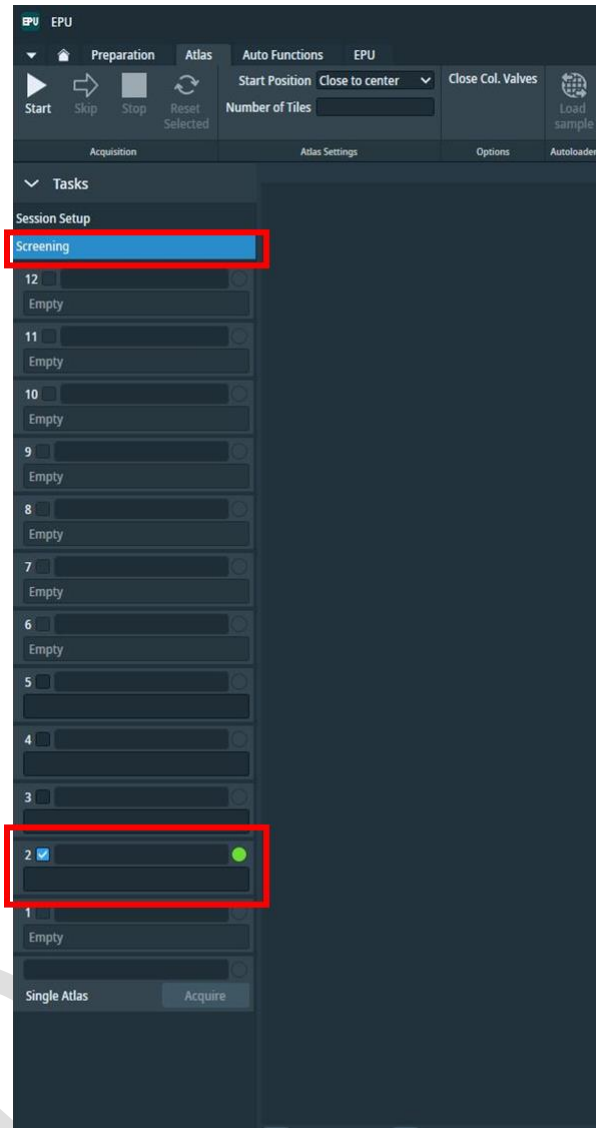
Image Shift Calibration

To align the images taken under different magnification, calibration of image shift is a necessary procedure before start of the experiment.

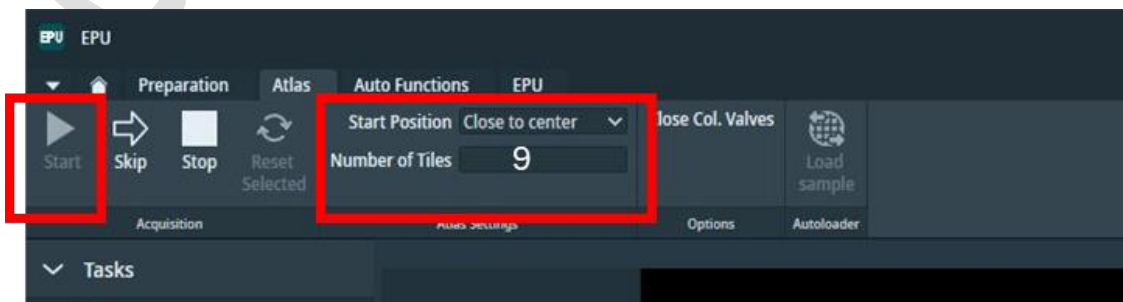
1. Create a new session at Atlas. Fill in the name and choose image format. Select Z:\EPU\Atlas\ as output folder.



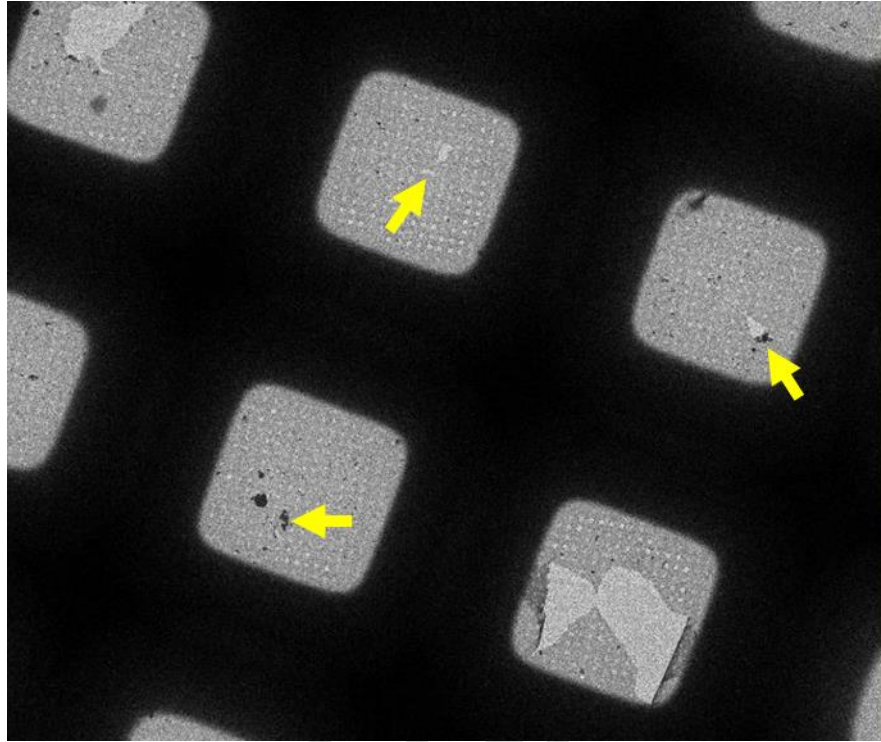
2. At the **Screening**, select one grid.



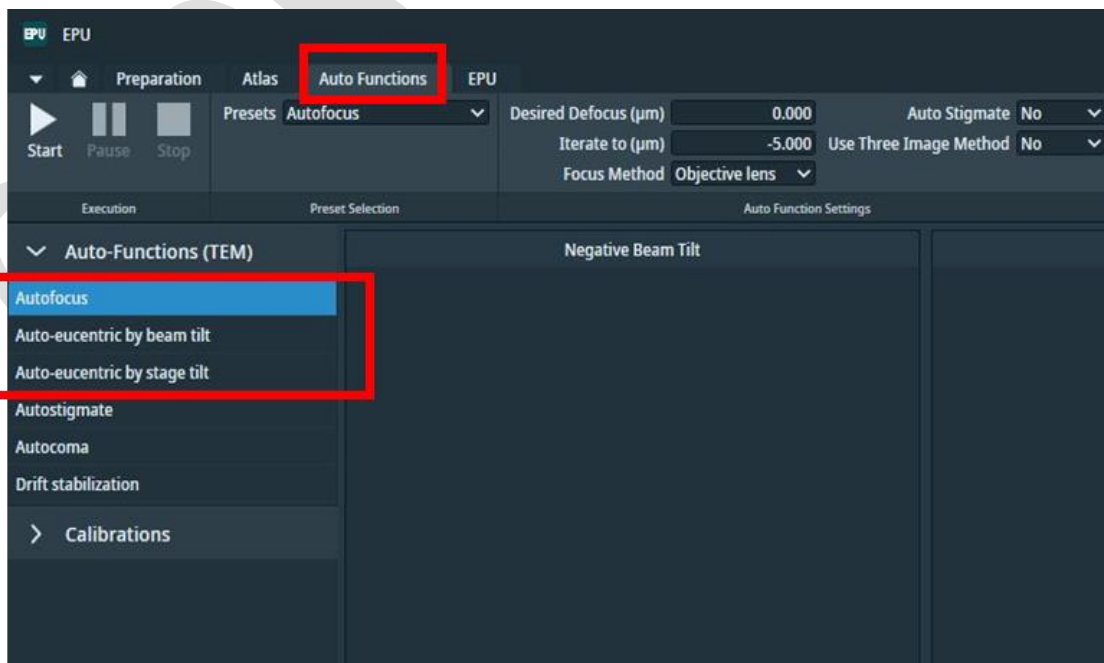
3. Enter “9” in the box of **Number of Tiles** and **Start**. The system will scan the center of the grid for 9 tiles. Find a feature (can be broken corner / ice) as reference for image calibration.



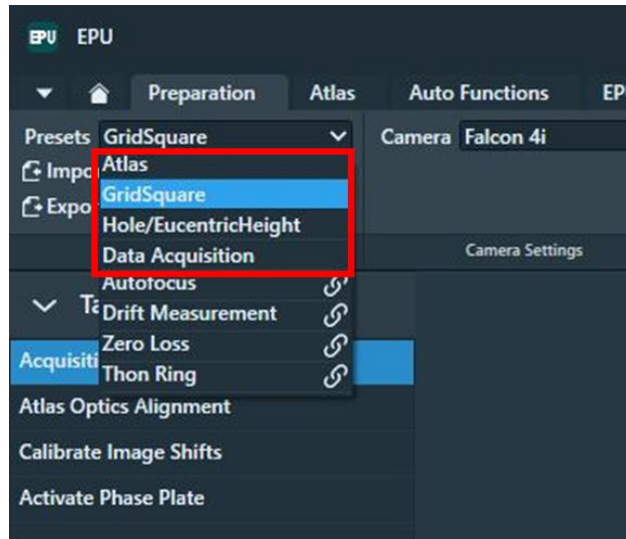
Here are some examples of the features that can be used for image shift calibration.



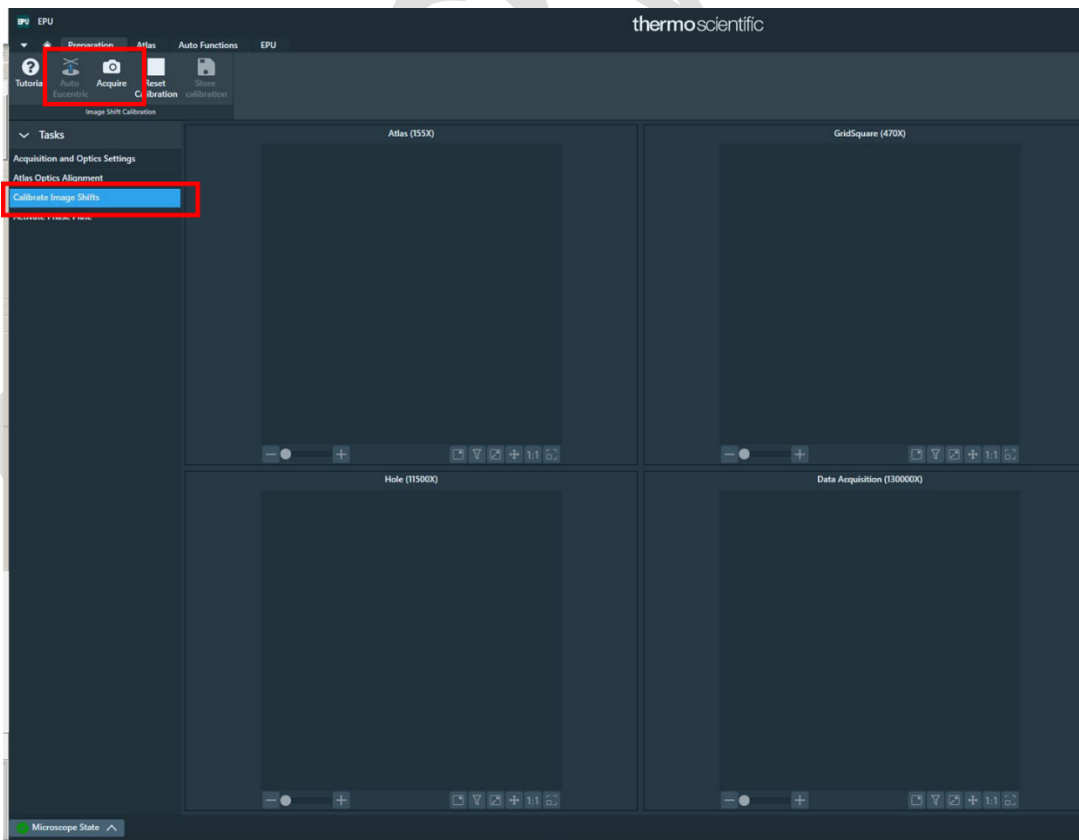
4. To get a focused image, auto-eucentric and auto-focus should be done. At the tab of **Auto Functions**, click either **Autoeucentric by beam tilt** or **Auto-eucentric by stage tilt** to move the sample stage to correct plane of eucentric height. Then, click **Autofocus** to adjust the beam current and lens to focus the sample automatically.



5. After identifying the feature, acquire images in different magnification. Check if the feature is located at the center of the images in all magnification. If the image of the feature shifted, click **Calibrate Image Shifts** for calibration.

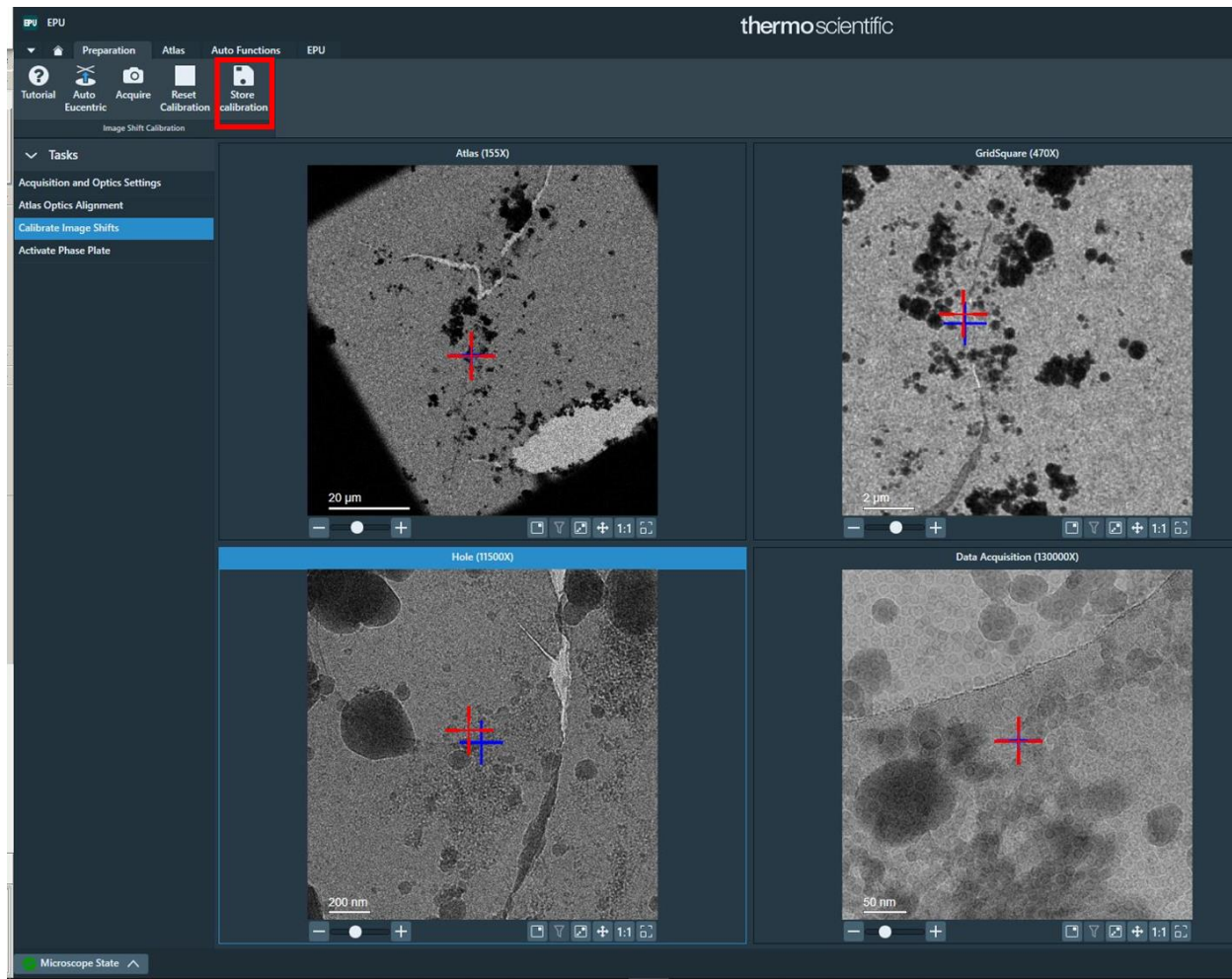


6. If the image of the feature shifted, click **Calibrate Image Shifts** for calibration. Select **AutoEucentric** to adjust the stage height to appropriate position. Then, click **Acquire** to collect the images at different magnifications.



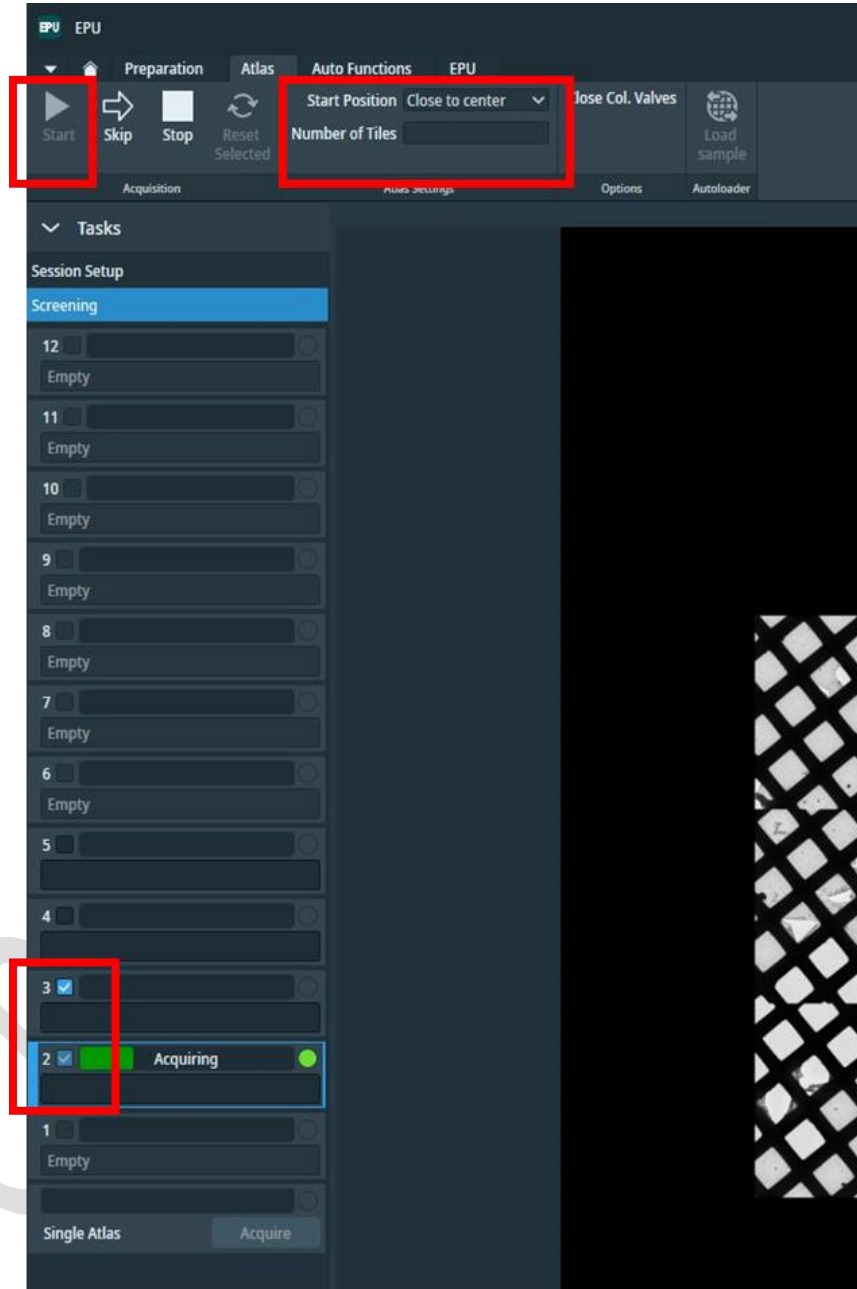
Krios Cryo-TEM (300kV) SOP – CPOS Cryo EM
V1.0 2024

7. If the image is not aligned, double click the correct region at the image of lower magnification. A blue cross indicates the correct position.
8. Click **Store calibration** when finished.



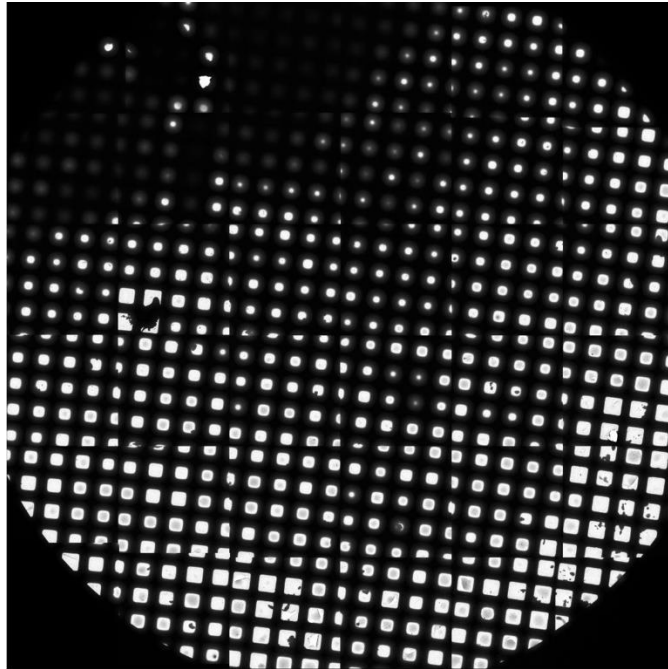
Atlas

After image shift calibration, acquire the maps of all grids in **Atlas**. Select all grids in the cassette, remove the number from the **Number of Tiles** and click **Start**.

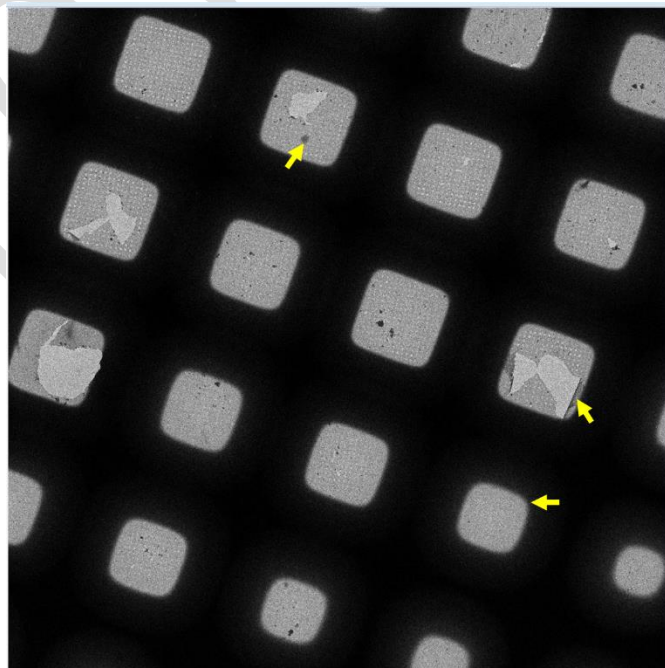


Grid Screening

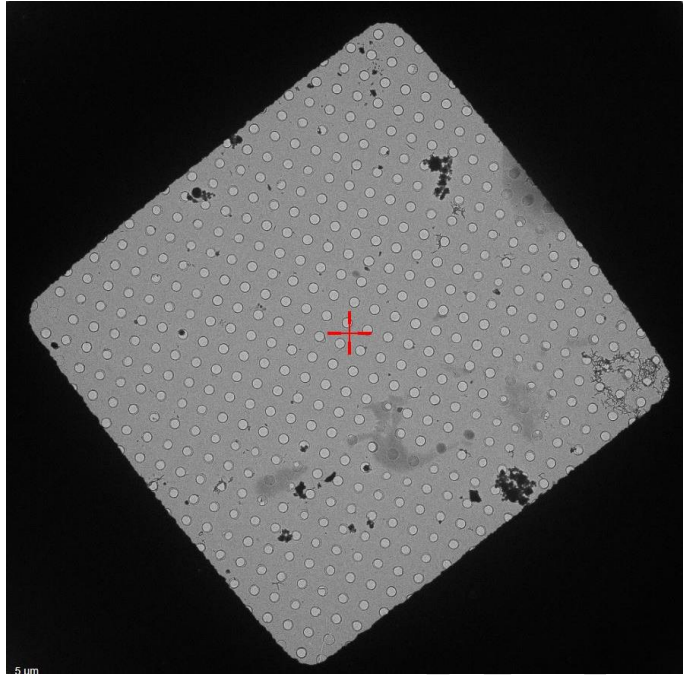
1. After acquiring the whole map, the condition of the grid can be investigated under different magnifications. Here are some examples:
 - a. At **Atlas** or low magnification, the evenness of the ice can be investigated. The darker parts implied thicker ice layer.



- b. At **Atlas**, the square with round corner, broken square, and dark spots can be observed.



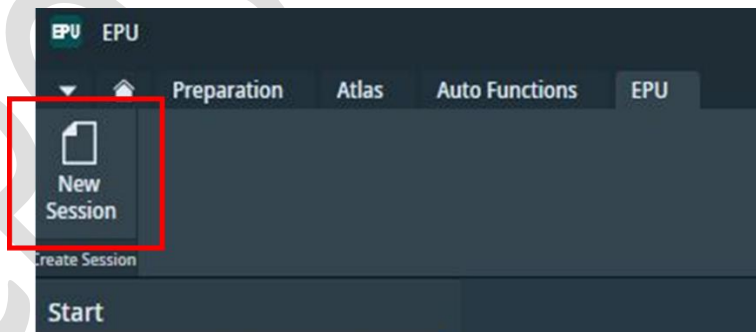
- c. At **GridSquare**, the aggregates and ice can be observed.



- d. At **HoleEucentric Height** and **Data Acquisition** (high magnification), the sample can be seen.

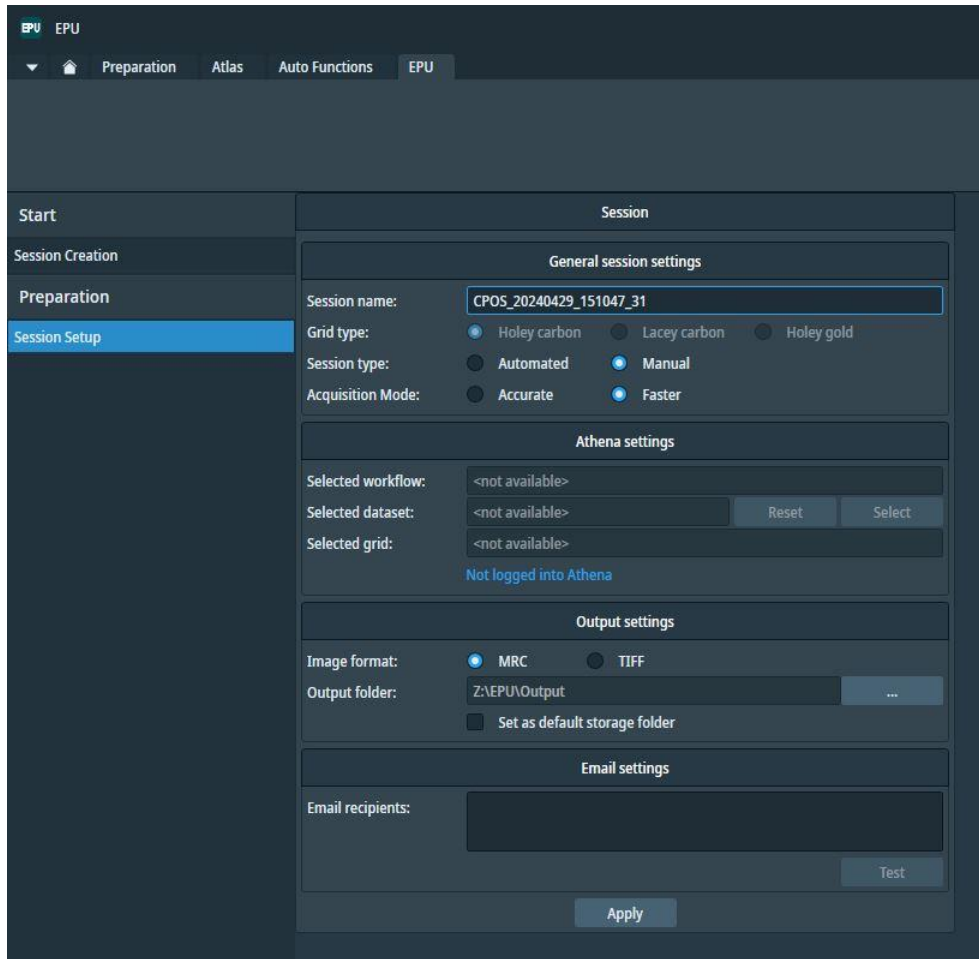
Set EPU for Data Acquisition

1. At the tab of **EPU**, create a new session.

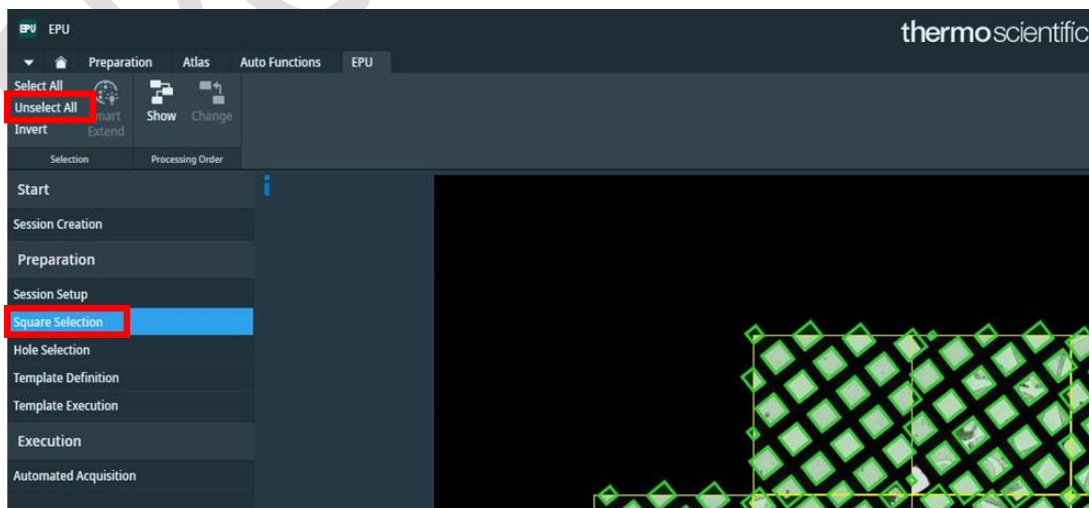


2. Setup the session.
- Session name: Username_date_time
 - Grid type: choose the appropriate grid type
 - Session type: select **Manual**.
 - Acquisition Mode: Select **Faster**.
 - Image format: Select either **MRC** or **TIFF**.
 - Output folder: **Z\EPU\Output**

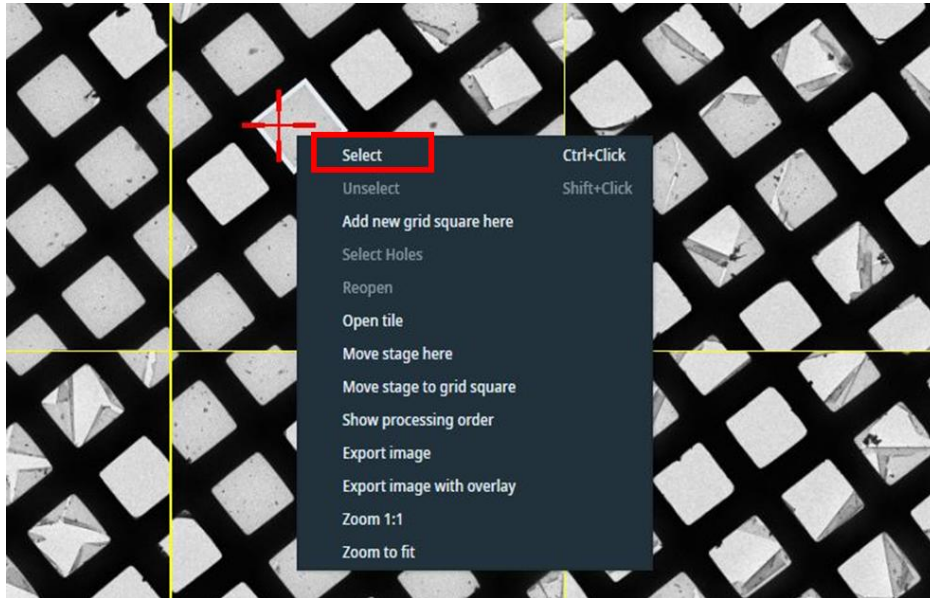
3. Click **Apply**.



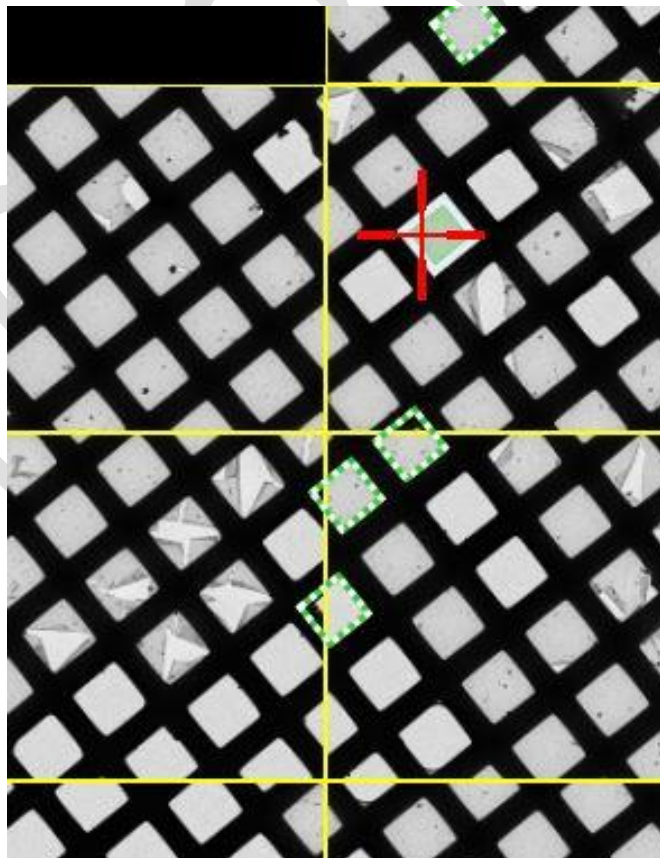
4. After setting up the session, more setups are shown in the list at the left panel.
5. In the session of **Square Selection**, all the squares are selected as default. Therefore, click **Unselect All** to remove all selections.



6. Select the target square and right-click. Click **Select**.

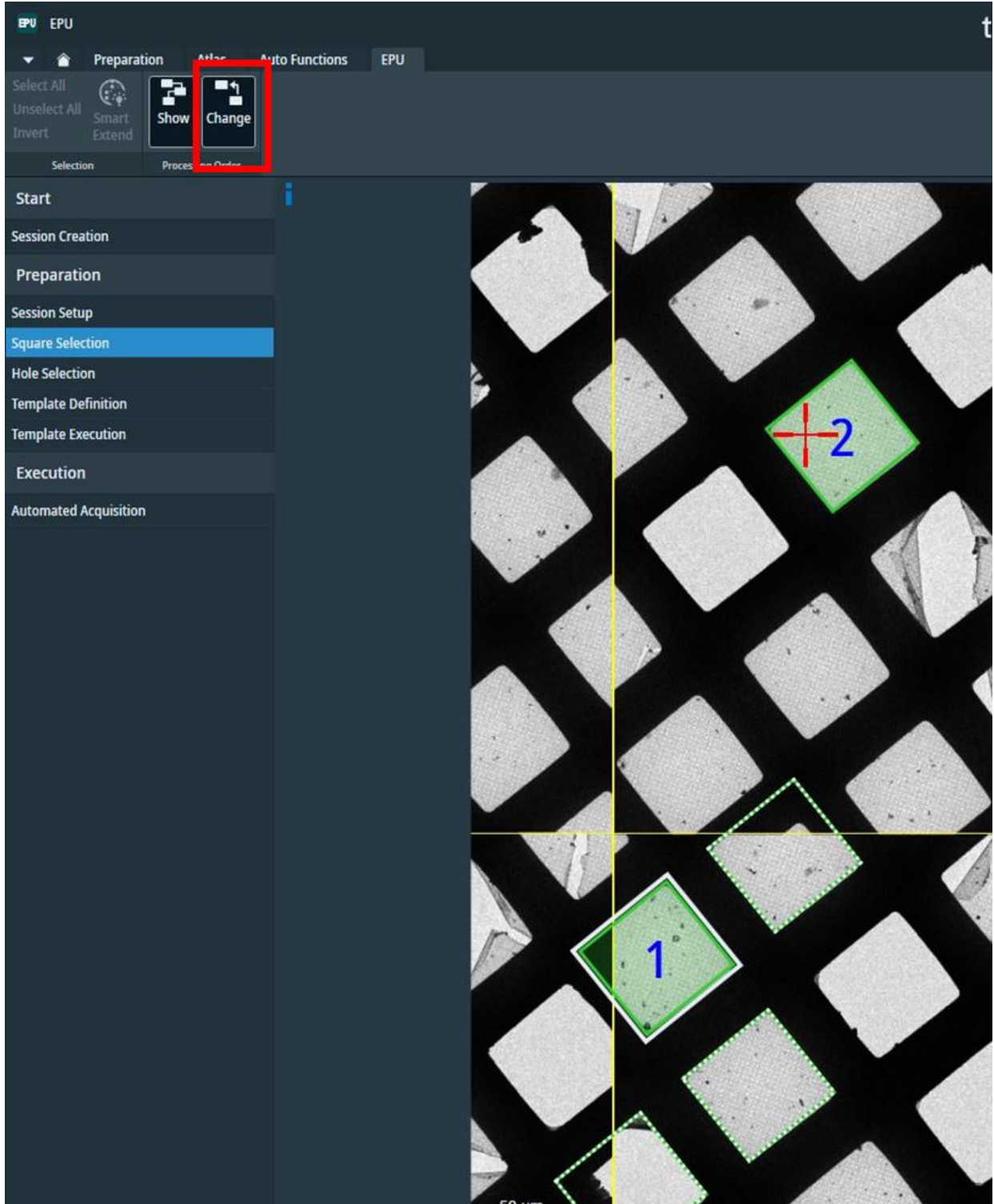


7. According to your selected square, the software suggests several squares to you and indicates as green dash squares. If you want to select the suggested square, right click and **Select**.

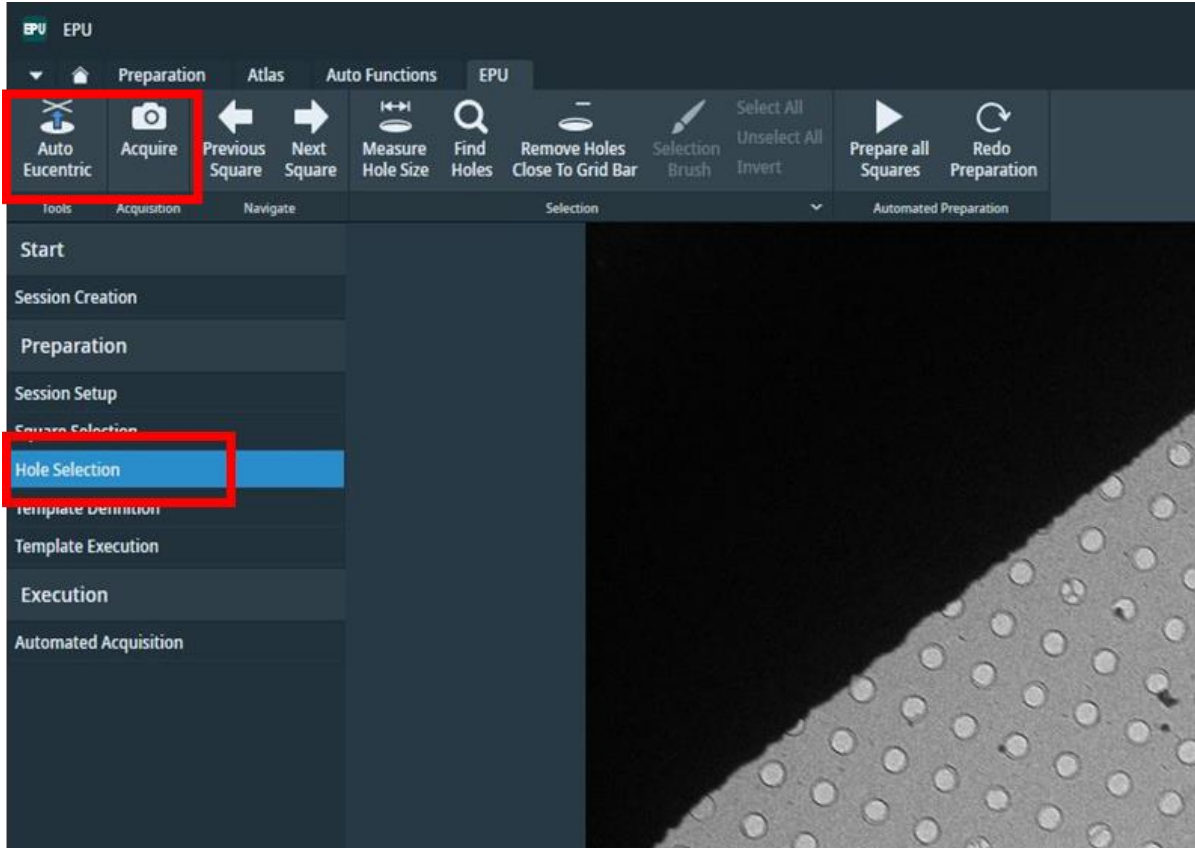


****Never select squares at the x / y position over 950µm.**

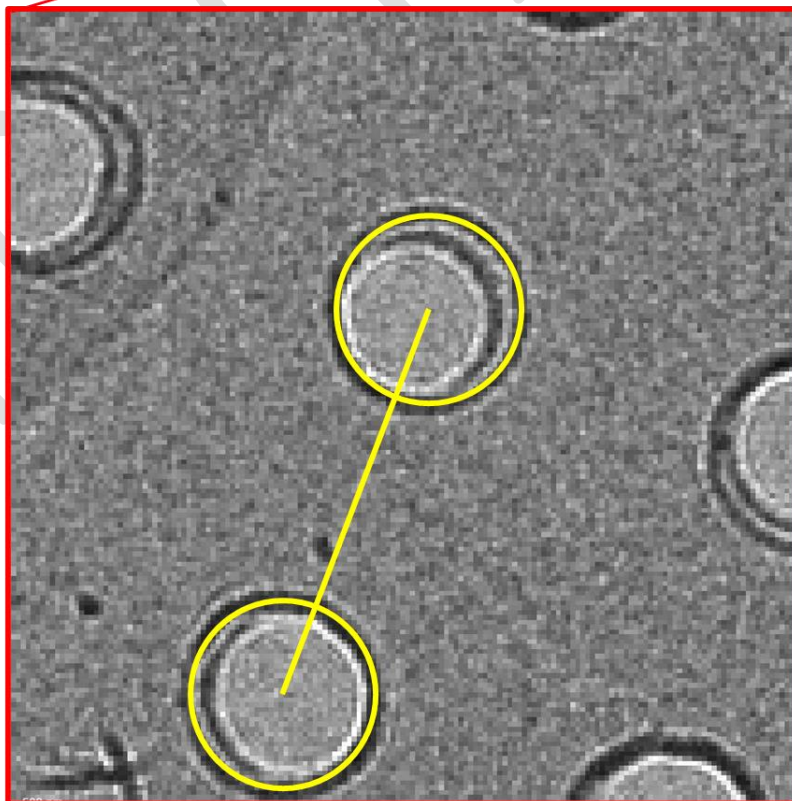
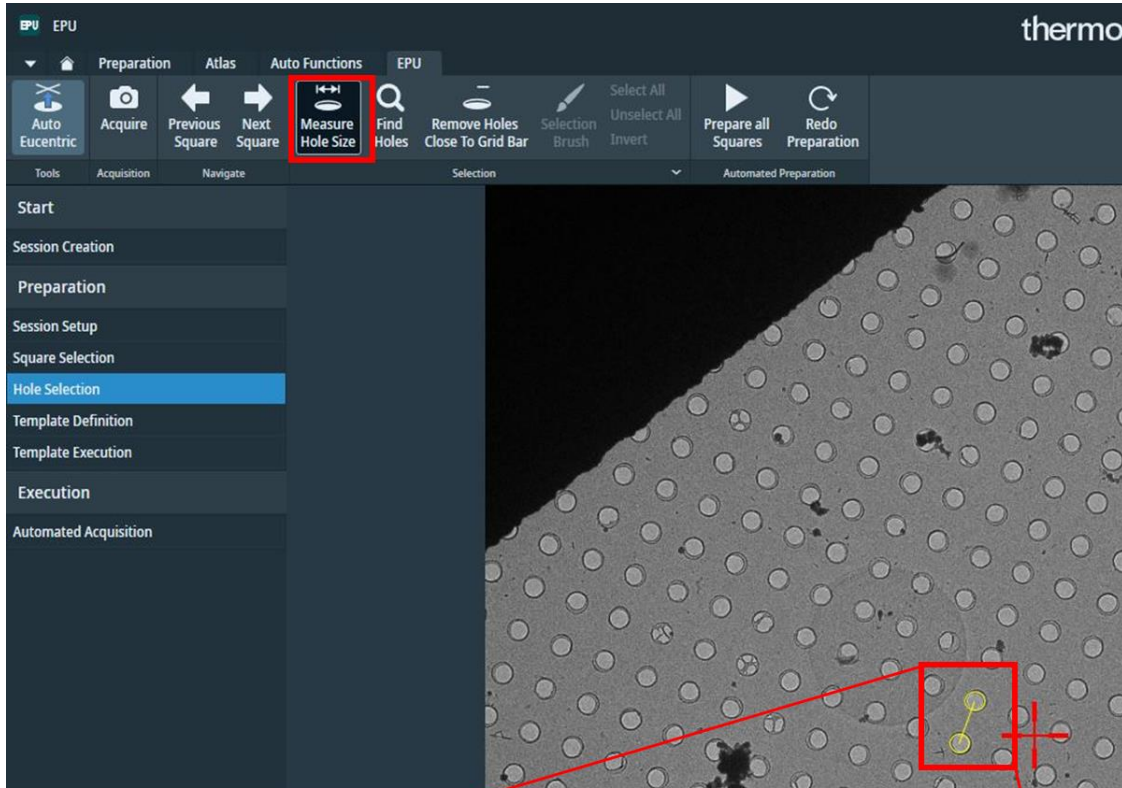
- When more than one square is selected, the squares will have blue numbers which indicate the sequence. The sequence can be changed when you click **Change**.



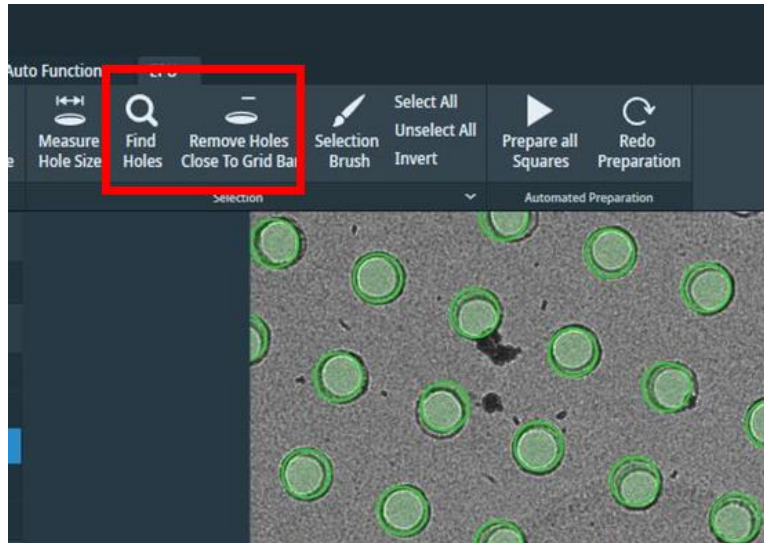
9. After selecting the squares, click **Hole Selection**. Click **Auto Eucentric** to ensure the sample stage moves to the correct height. When you click **Acquire**, the image of 1st square will be shown on the screen. Find a square with obvious holes by clicking **Previous Square** and **Next Square**, i.e. the boundary of the hole is obvious.



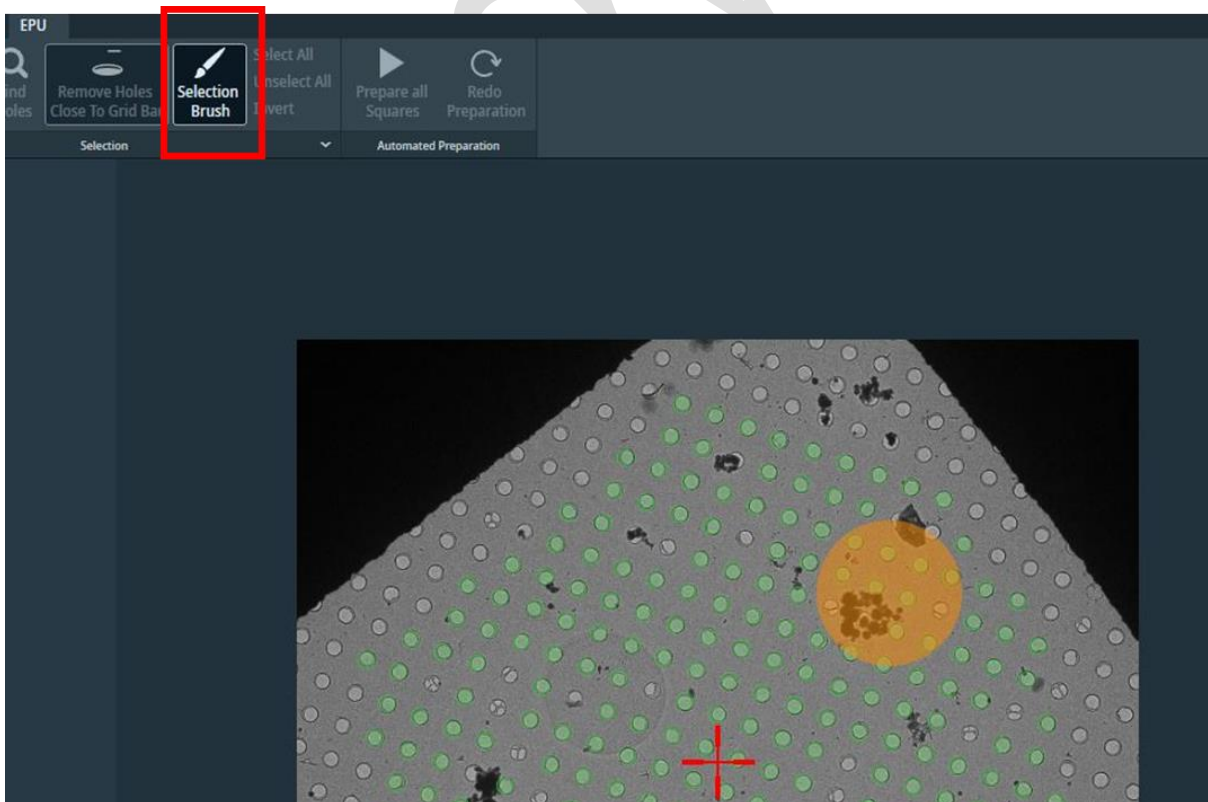
10. Click **Measure Hole Size**. Adjust the size of the hole and distance of two holes.



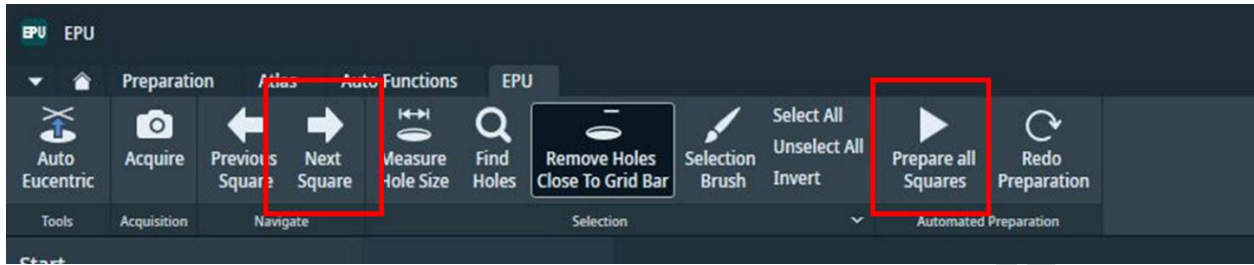
11. Click **Find Holes** and **Remove Holes Close to Grid Bar**. The green circles in the image indicate the holes selected.



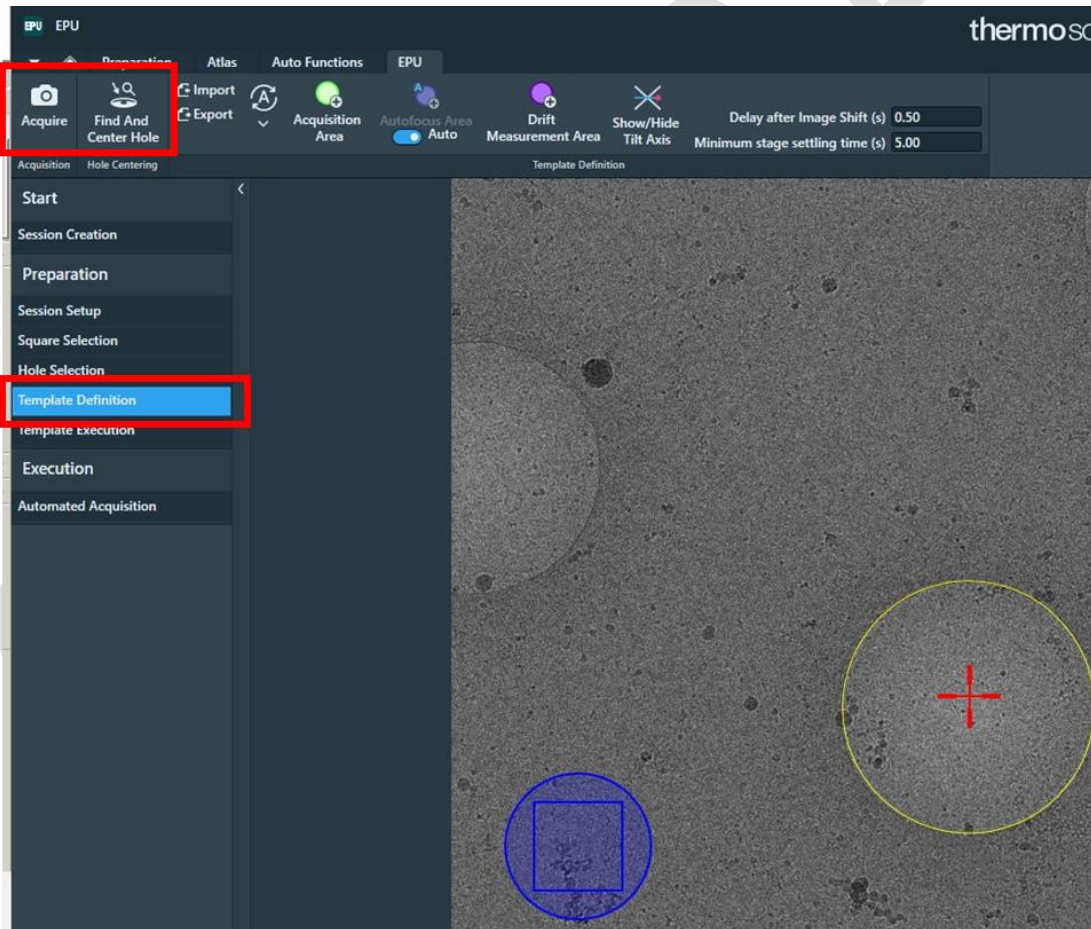
12. When **Selection Brush** is selected, the cursor becomes an orange circle. The unwanted holes can be removed by clicking. The size of the brush is adjusted with holding Shift at the keyboard and scrolling the mouse scroll wheel.



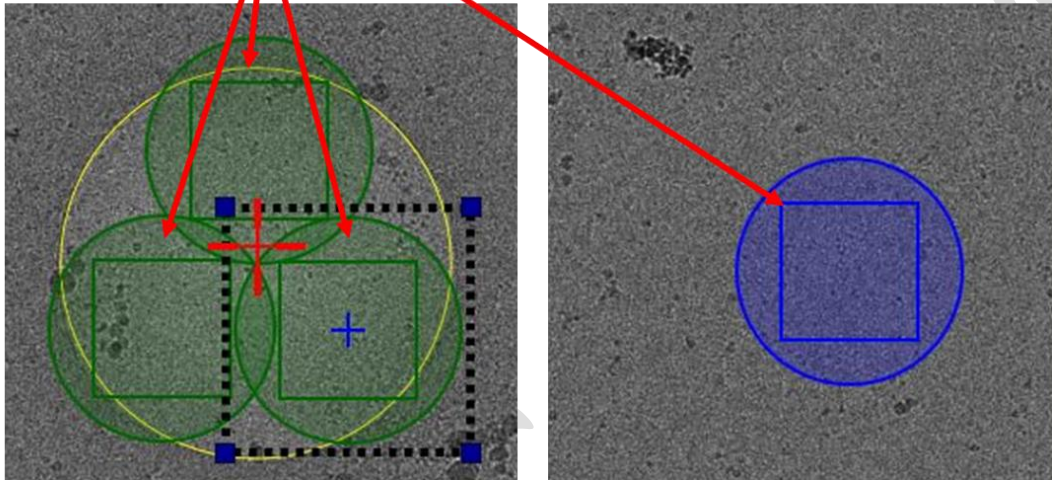
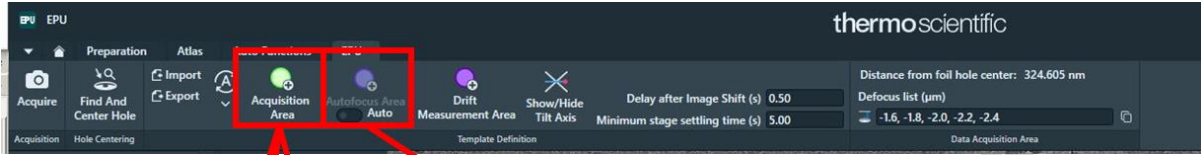
13. After selecting the holes in one square, click **Next Square** to process manually or click **Prepare all Squares** to process automatically.



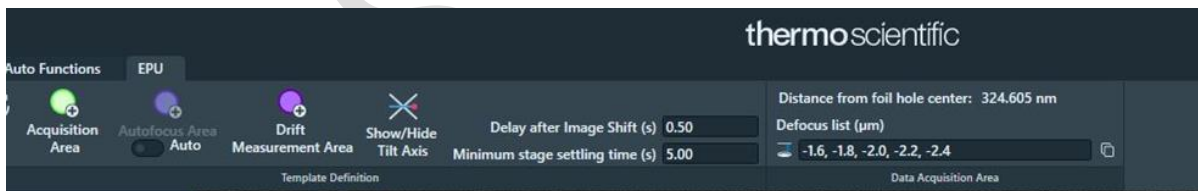
14. To define template, move stage to a hole and acquire an image at the magnification of **HoleEucentric Height**. In the view of field, there should be one complete hole at the center and four partial holes at the edges. If the magnification is not correct, adjust it in the **Preparation** tab. Click **Find And Center Hole**. The center hole is identified as yellow circle.



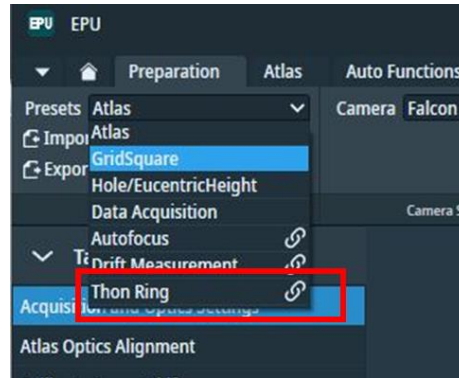
15. **Click Add Acquisition Area.** The green circle indicates the beam. It should not cover the other circles. The green square is the image taken by the camera. It should be located in the center hole. Click **Add Autofocus Area.** The purple circle should be put at the carbon film. Minimize the region of overlapping with holes.



16. Click the green circle and set the defocus list. For example, if you set **-1.6, -1.8, -2.0, -2.2, -2.4**, The images will be taken with these 5 defocus.



After determining the defocus list in template setting, set the middle value of the defocus list at **Defocus** in **Thon Ring** in the tab of **Preparation**. For the above example, the defocus set in **Thon Ring** should be -2.0.

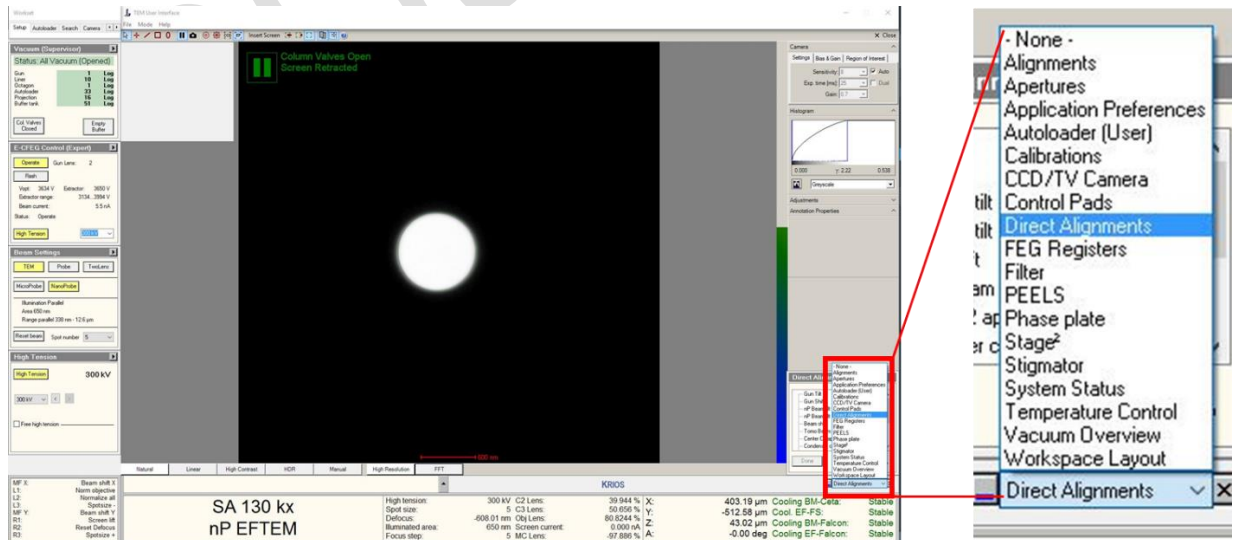


Alignments and Autocorrection

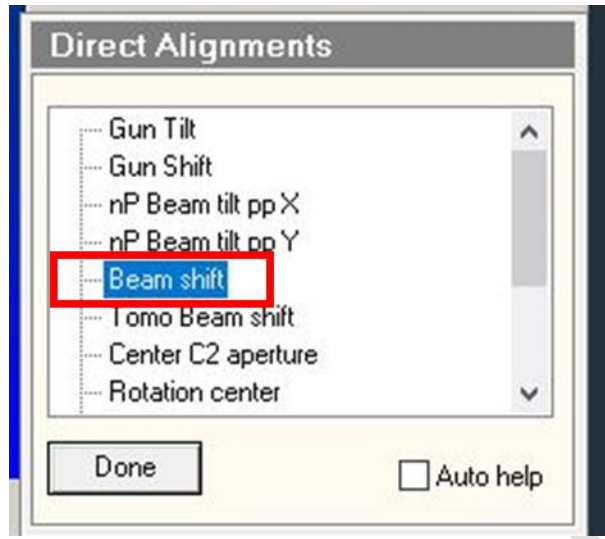
There are several items needed to be align / autocorrect before data acquisition:

- Align beam shift and beam tilt
- Measure dose rate and set dose
- Energy filter alignment
- Autostigmat
- Autocoma

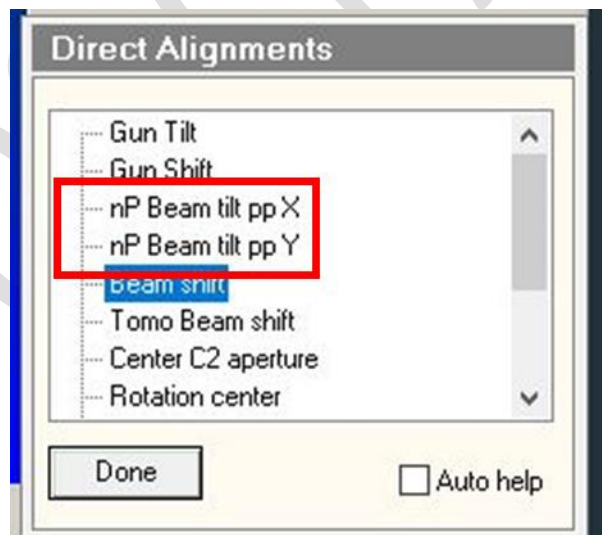
1. Move the stage to an empty space. Choose the magnification of **Data Acquisition**. In the software **TEM User Interface (UI)**, click **Direct Alignment**.



2. Press **Eucentric Focus** on the control panel. Select **Beam Shift**.



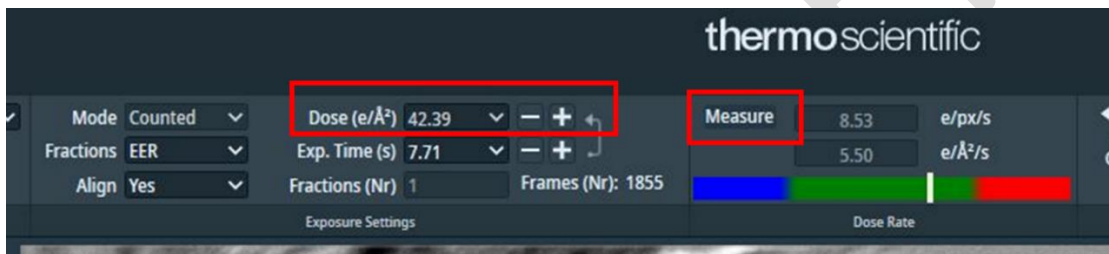
3. Adjust the knobs of X and Y on the control panel to move the beam to center. Increase the intensity / reduce magnification to center the beam. Click **Done** after adjustment.
4. Click either **nP Beam tilt ppX** or **nP Beam tilt ppY**. Adjust the knobs of X and Y on the control panel to achieve the least “shaking” of the spot. Click **Done** after adjustment.



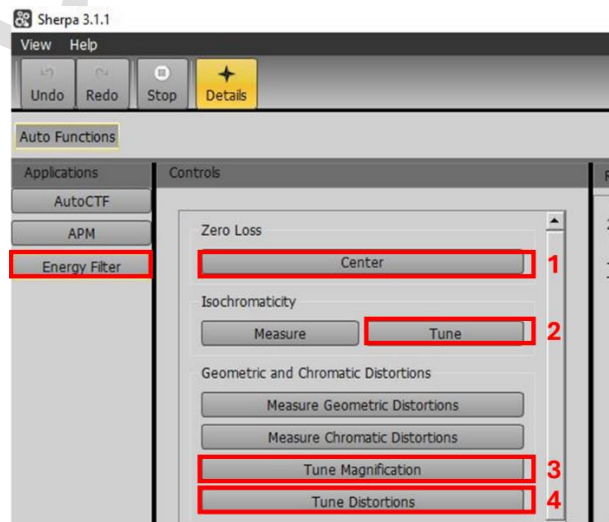
- Before measuring dose, check if the **beam current** at **UI**. If the value is smaller than 5 nA, click **Flash**.



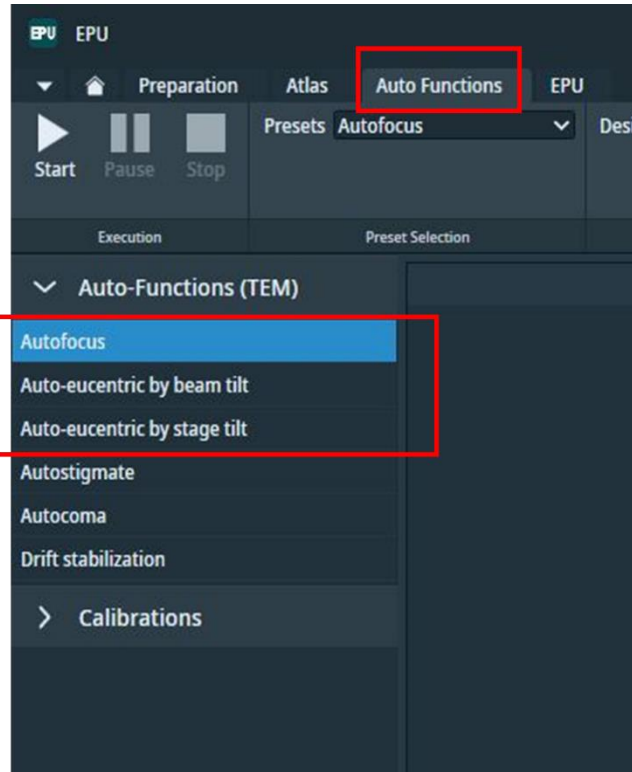
- In **EPU**, go to **Data Acquisition**, click **Measure**. Check the Dose Rate. The setting is appropriate if the white line is in the range of green. Otherwise, intensity and spot size should be adjusted accordingly.



- Set target dose. 40 or 50 e/Å² is commonly used. The exposure time will be adjusted accordingly.
- In **Sherpa**, align the energy filter with the following buttons:
 - Zero Loss: **Center**
 - Isochromaticity: **Tune**
 - Geometric and Chromatic Distortions: **Tune Magnification** and **Tune Distortions**

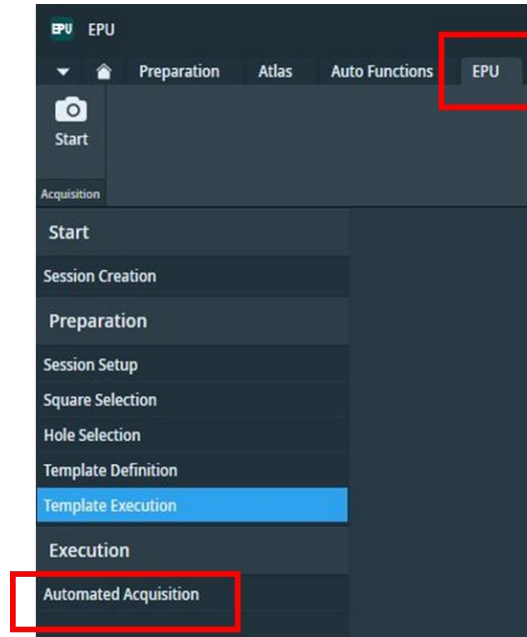


9. Move the stage to carbon film. Click **Auto Functions** and adjust the stage height by clicking either **Auto-eucentric by beam tilt** or **Auto-eucentric by stage tilt**.

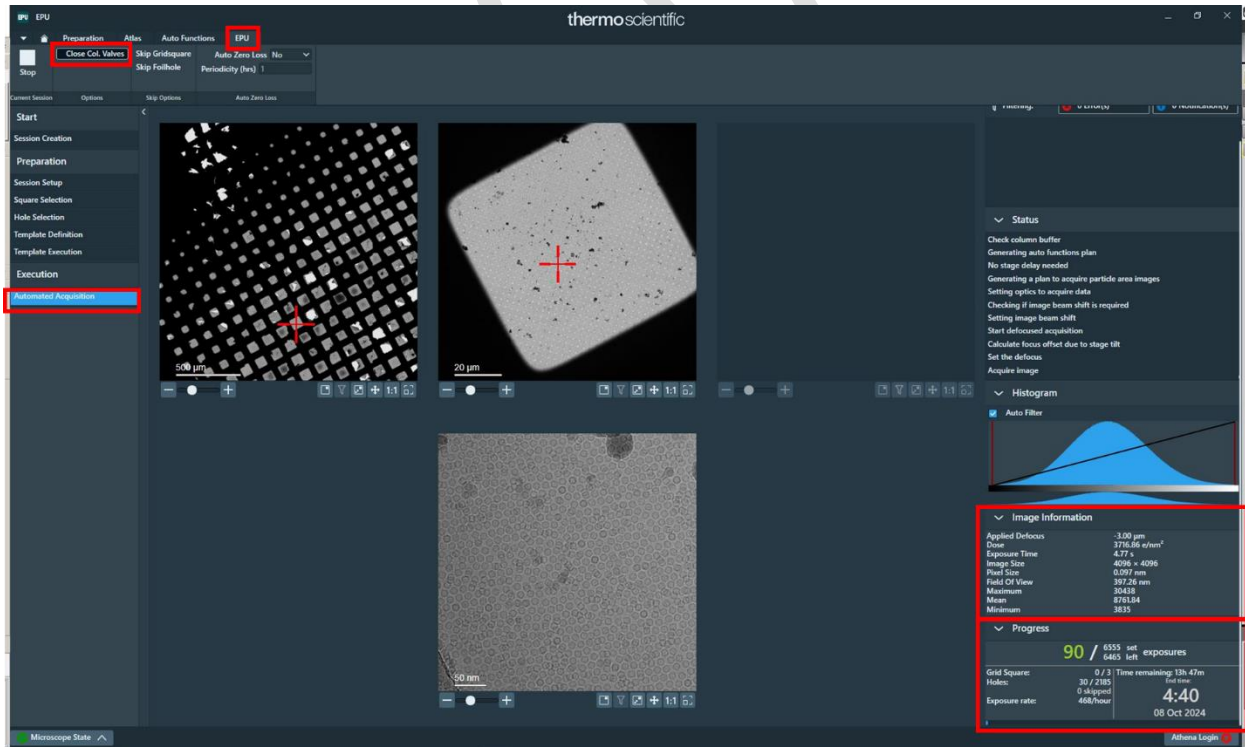


10. Click **Autofocus** and start.
11. Click **Autostigmatate** and start.
12. Click **Autocoma** and start.
13. Change the magnification in preparation and back to high magnification. Click **Autocoma** again. Repeat this step for several times to stabilize coma.

14. After alignment, start the experiment by clicking **Automated Acquisition** in the tab of **EPU**.



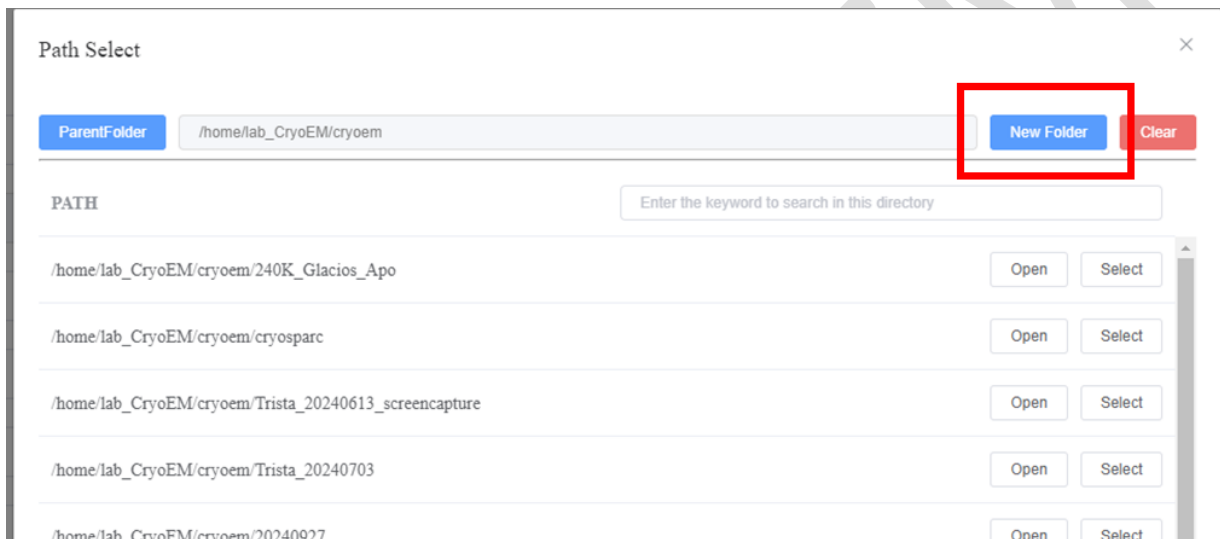
15. When the images are acquired, select **Close Col. Valve** so that the valve will be closed automatically when the experiment finished.



16. When the first image is acquired, you can check if the dose and exposure time is closed to your setting. You can also check the progress and the estimated time for the whole acquisition. The time required for the experiment depends on several factors, including exposure time, dose, focus recurrence, drift measurements, etc.

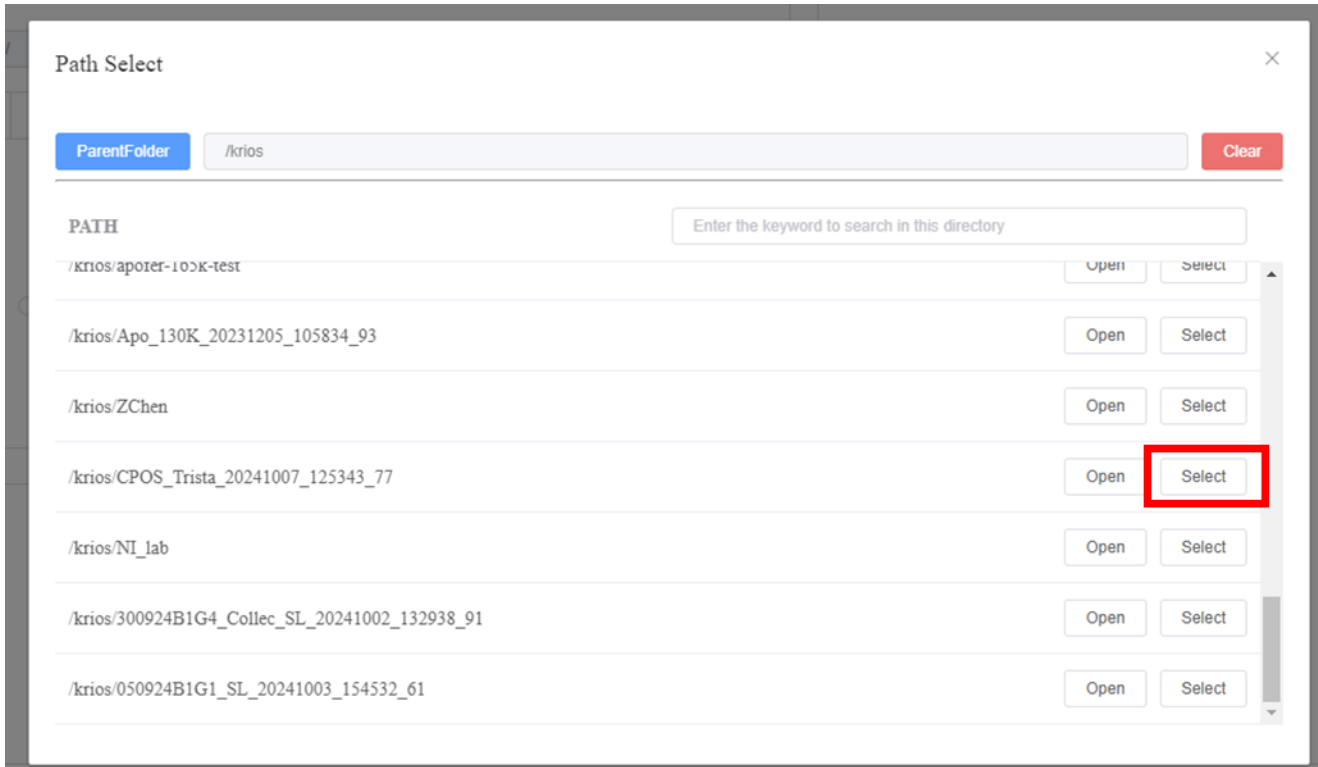
Data Transfer to HPC Server

1. Log in HPC server in EMShark. Username and password are provided by IT team.
2. Select Project path from the folder: `/home/lab_(your lab)\(username)`
3. Click **New Folder**.



4. Enter the new project.

5. Select raw data path: **/krios/(the name set in EPU session created)**, refer to step 2 at the session **Set EPU for Data Acquisition**, page 16). Click **Select**.

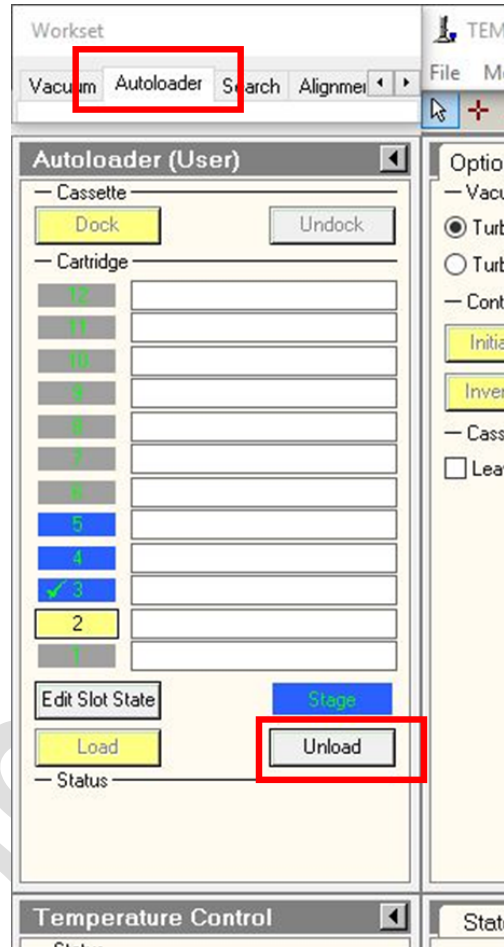


6. Select the other options as below:
 - Data format: all
 - Data sources: EPU
 - K2 Camera Data: off
 - Apply for All
 - Transmission: Copy
 - Auto stop time 24 hours after.
7. Click **Start**.
8. When data transfer finishes, click **Stop**. Otherwise, other users cannot transfer data.
9. Log out EMShark.

Checklist before leaving the lab

1. In UI, click **Unload** to unload sample. Close valves at **Vacuum**.

**If you will leave for more than 10 minutes, please close the valves.



2. Prepare and cool down the loading station. Request staff assistance to unload sample.
3. Sign the log book.
4. Close the **EPU** software.
5. Stop the transfer in **EMShark**.