

**Okayama University**  
**Cryo-electron Microscope Manual**

**Titan Krios G4 and Falcon 4i**

**For User**

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](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.

**International Center for Structural Biology,  
Research Institute for Interdisciplinary Science,**

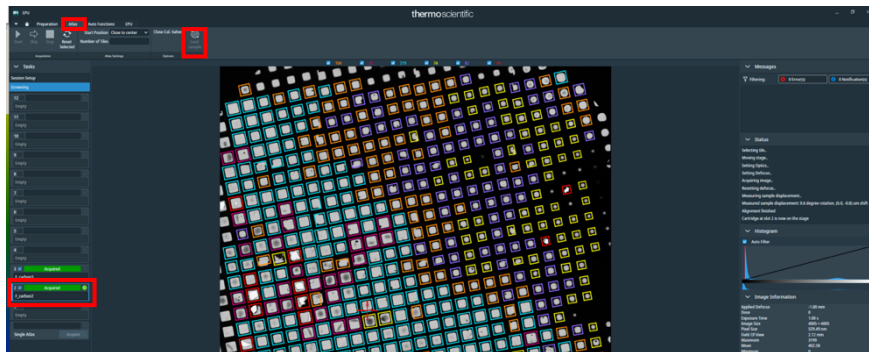
**Okayama University**

**Tomoe Iio, Nobutaka Numoto**

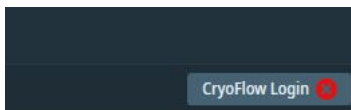
# Data Acquisition

## 1. Grid Screening

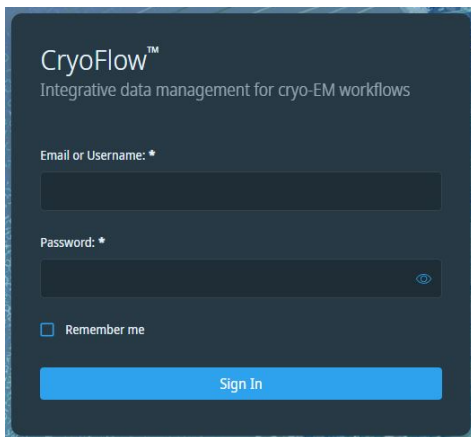
EPU > Atlas tab, select the target grid and click **Load Sample**.



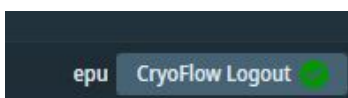
EPU at the bottom right > Click **CryoFlow Login**



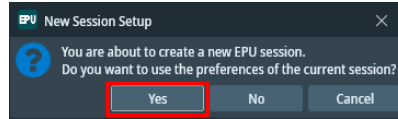
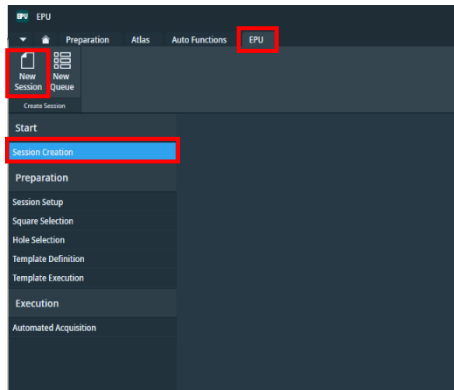
Enter the User ID (epu) and password, then click **Sign In**.



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



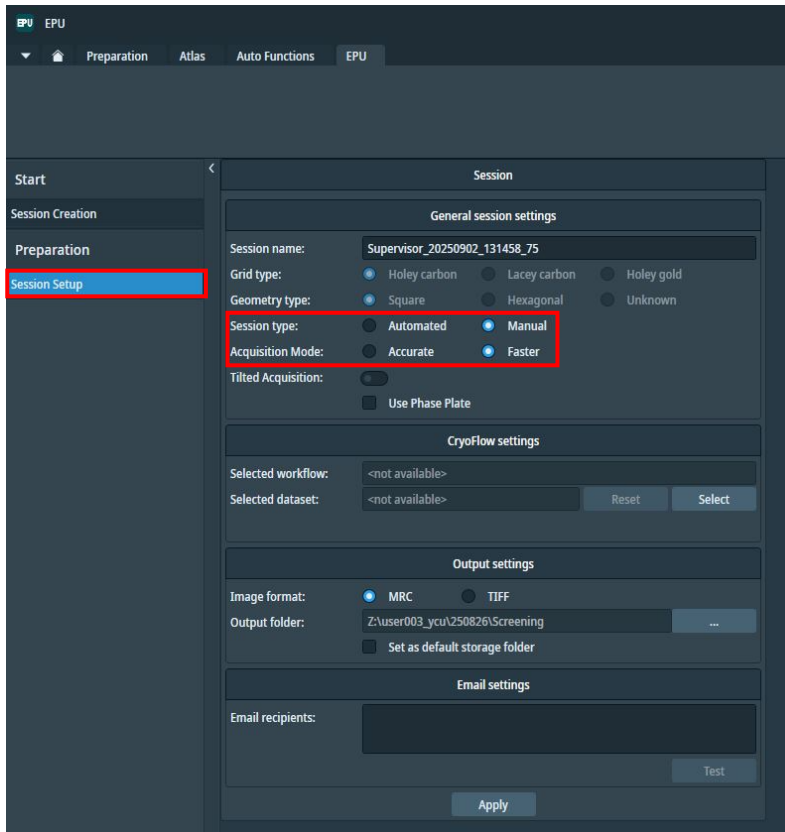
EPU > EPU tab, select **Session Creation** > **New Session** > **Yes**.



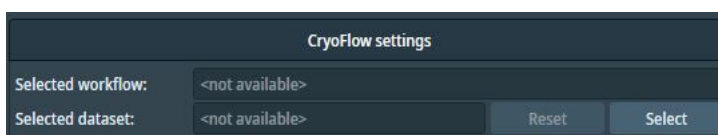
Click **Session Setup**.

Session name: **Do not change**

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

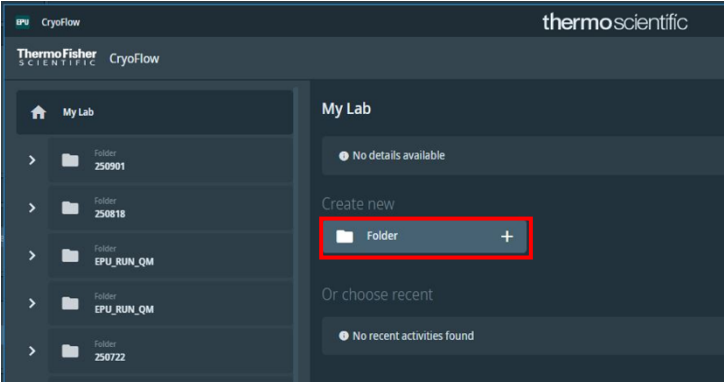


CryoFlow settings > **Select**

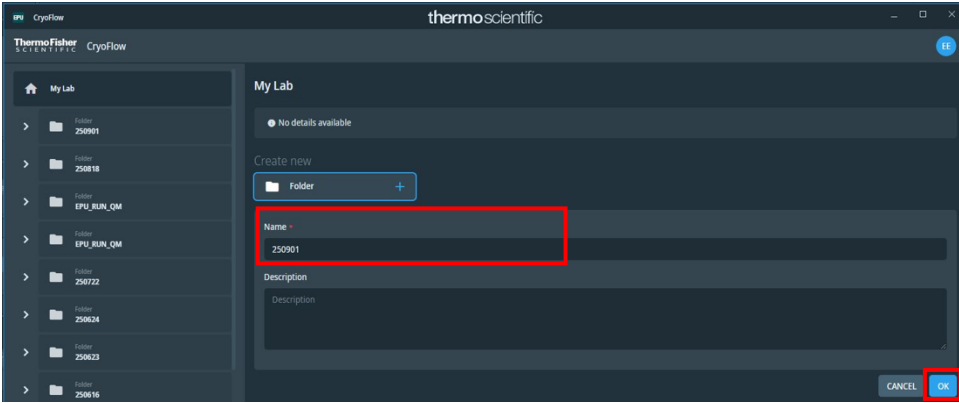




Create newFolder



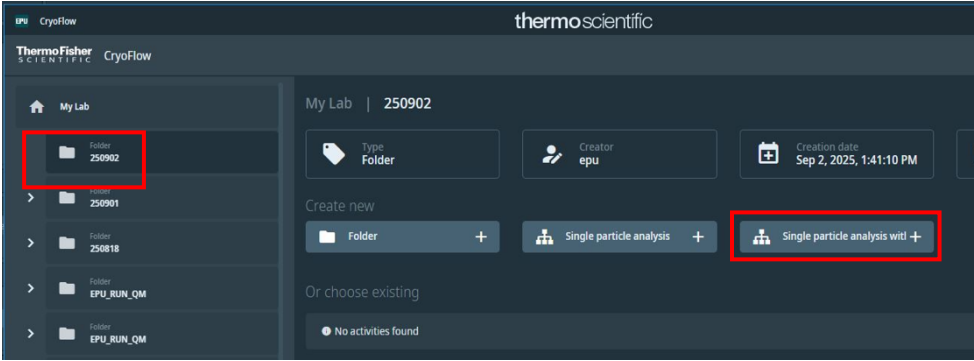
Enter the date of the day (yymmdd) in **Name** > **OK**



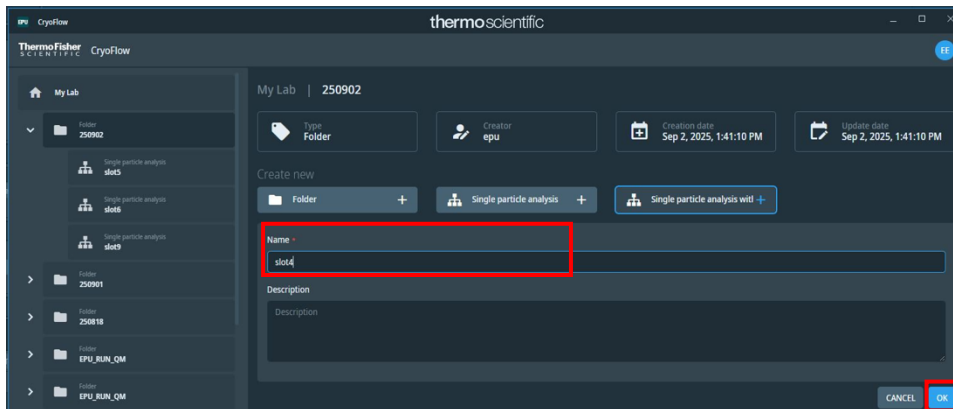
Confirm that a new folder has been created.

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

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

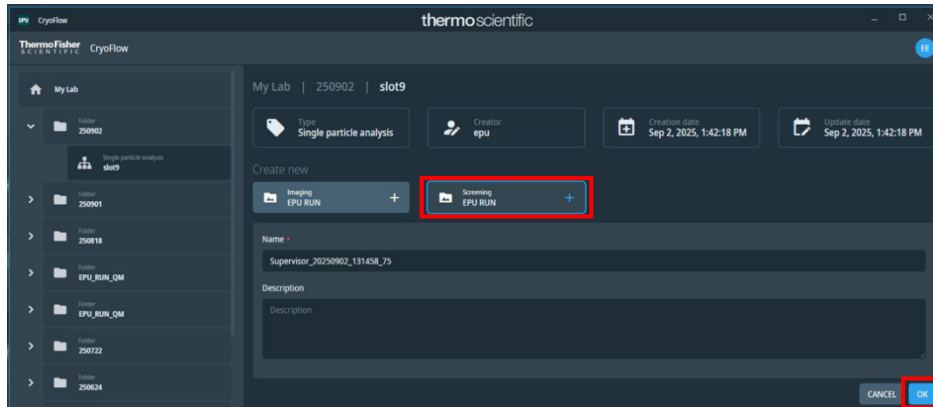


Enter the grid name, etc. in **Name** (e.g., slot\_x, etc.) > **OK**



Create new > **Screening**

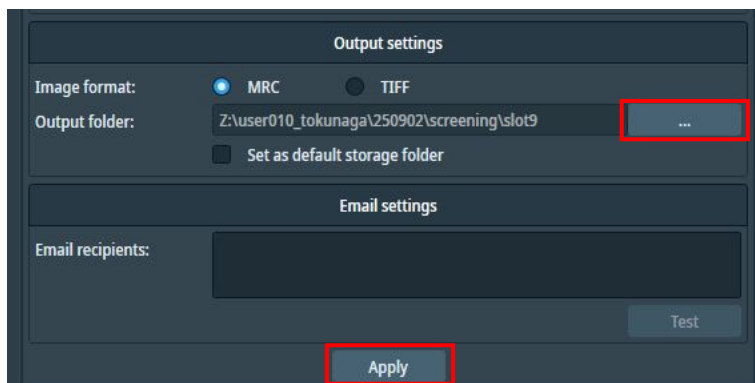
Leave Name as it is > **OK**



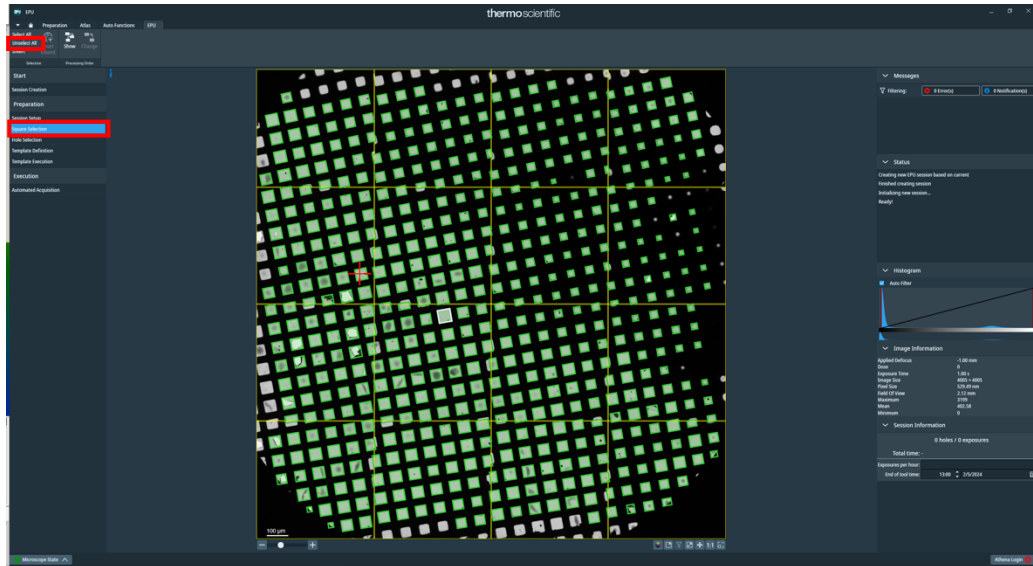
Output settings > ... and select the destination folder.

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

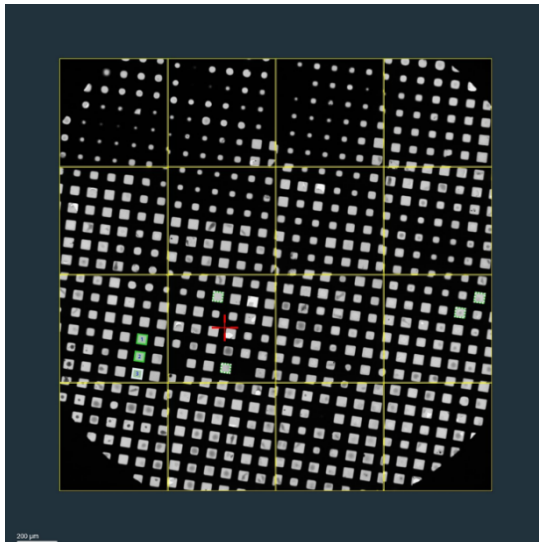
Click **Apply**.



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

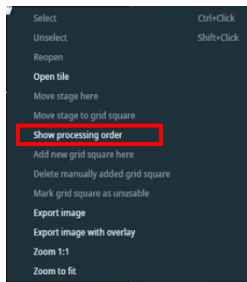


Select 2–3 representative squares (Ctrl + left-click).

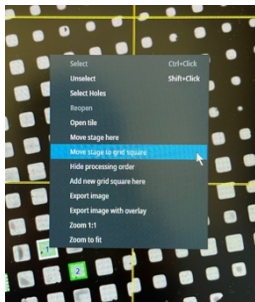


- \* It is good to include a variety such as thin ice, slightly thicker, and thick areas.
  - \* If you want to display the numbers of the selected squares, right-click and choose Show processing order.
  - \* Take a screenshot: right-click → Export image with overlay.
- Be careful: if you perform the above operations while zoomed in, the zoomed-in image will be saved.

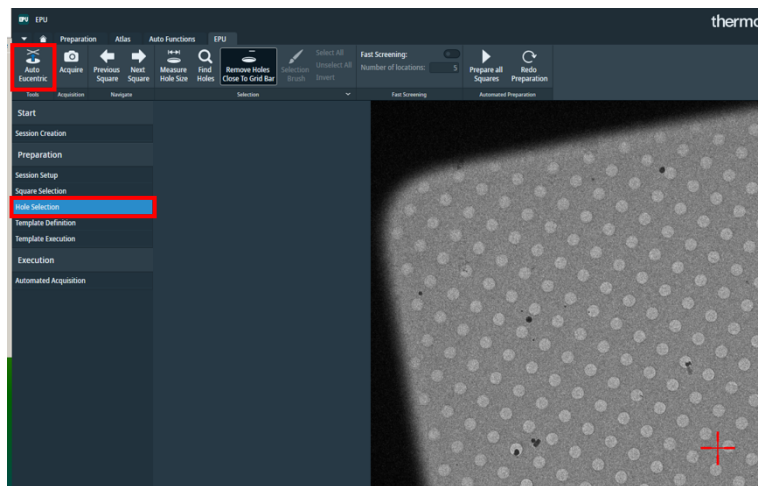
Right-click on the screen > Show processing order



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**.

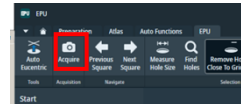
\* If it still fails, select **Auto-eucentric by stage tilt** and click **Start**.

\* It is fine as long as either one succeeds.

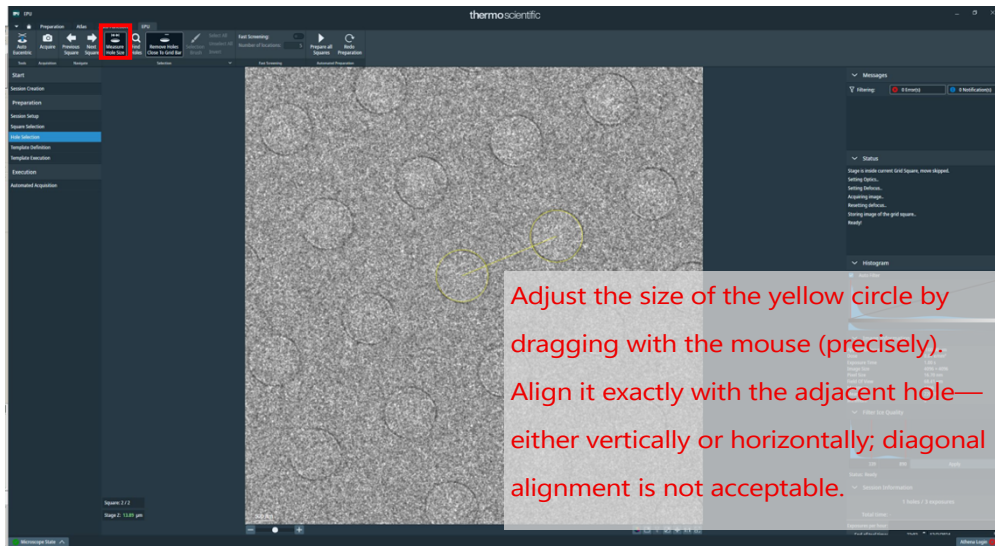
\* Once the Eucentric height has been determined, return to **Hole selection** and click

**Acquire**.

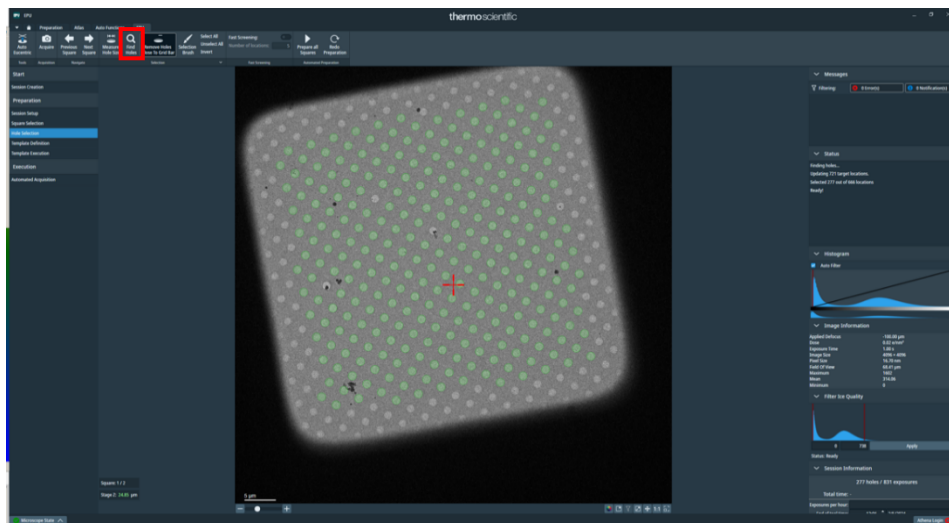
(If you do not do this, the determined Eucentric height will not be applied.)



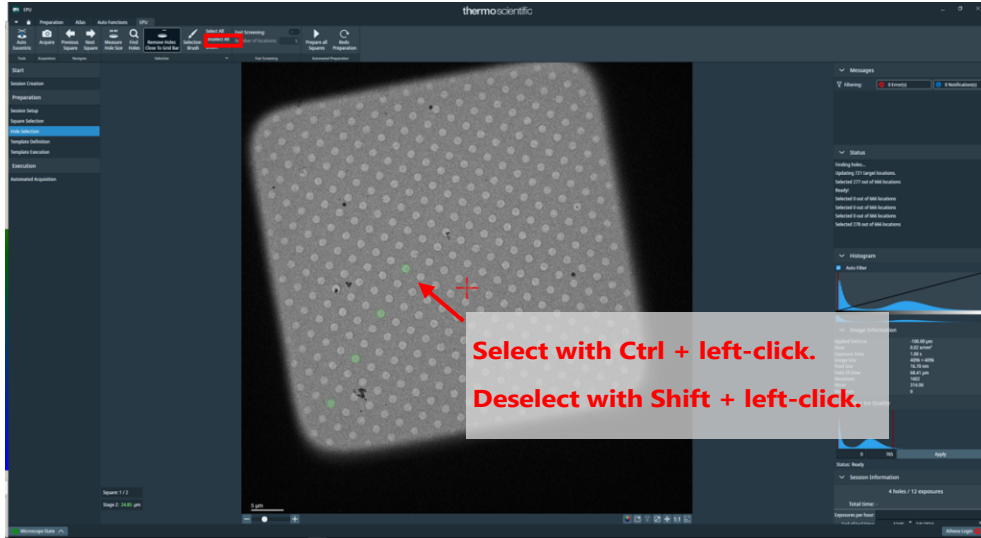
When the Eucentric height is successfully determined, click **Measure Hole Size** to define the size of the hole and the spacing between adjacent holes.



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

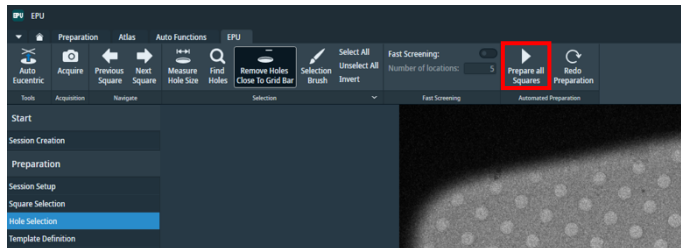


If the holes are successfully detected, click **Unselect All**, then select several representative holes (Ctrl + left-click).



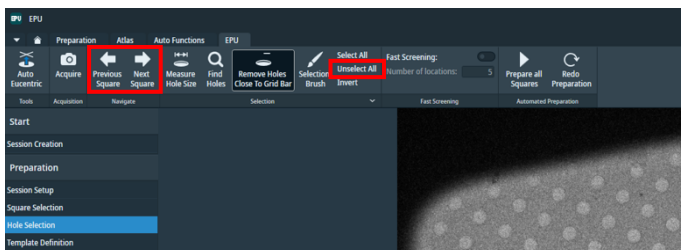
\* It is good to consider the apparent ice thickness—such as holes near the center or at the edges of the square—when choosing them.

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.

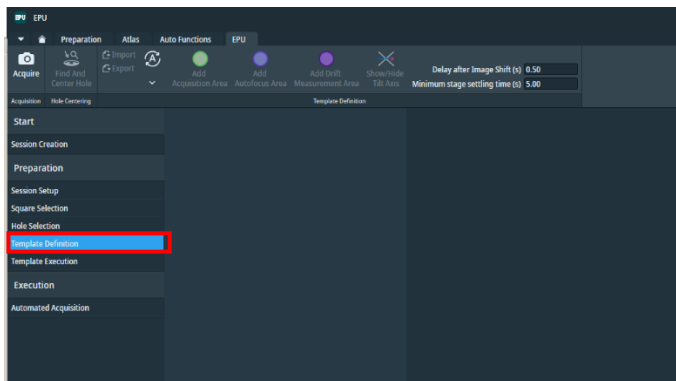
For each square, click **Unselect All**, then select several representative holes.



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

Right-click > Export image with overlay

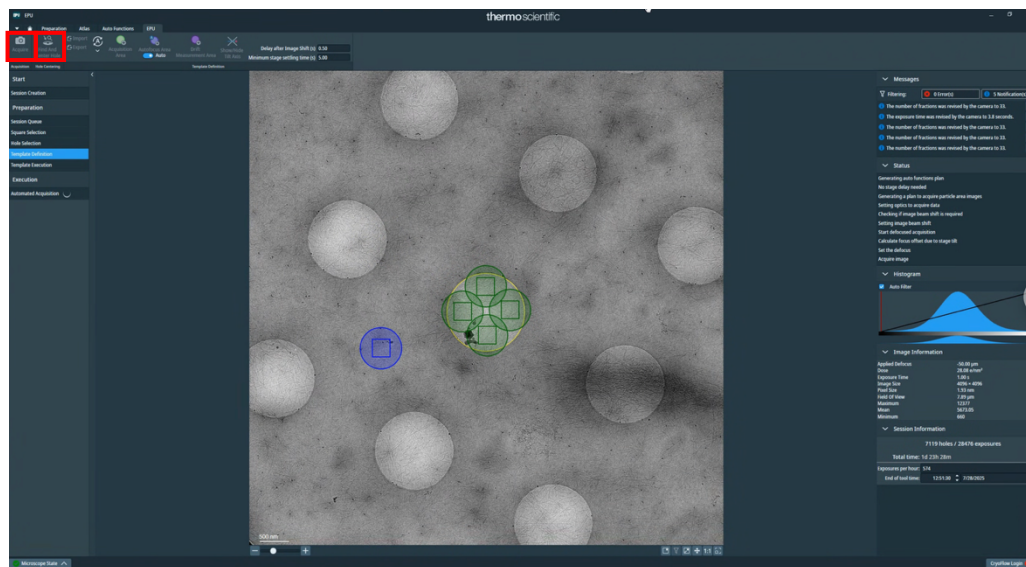
Click **Template Definition**.



Acquire > **Find and Center Hole**

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

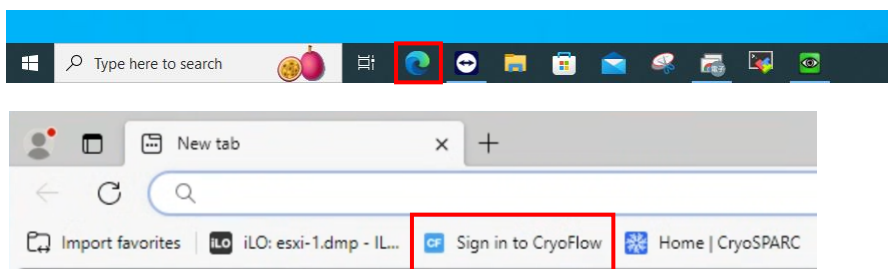
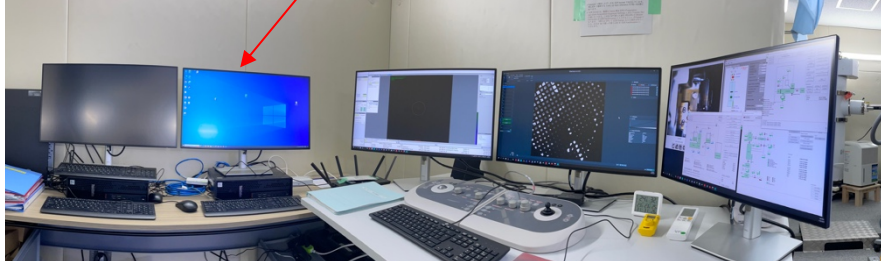
\* If this does not work properly, all subsequent acquisitions will fail.



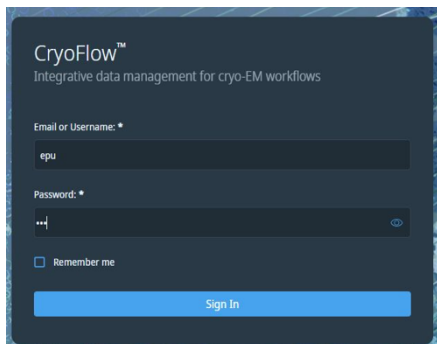


## 2. Confirming Results in CryoFlow

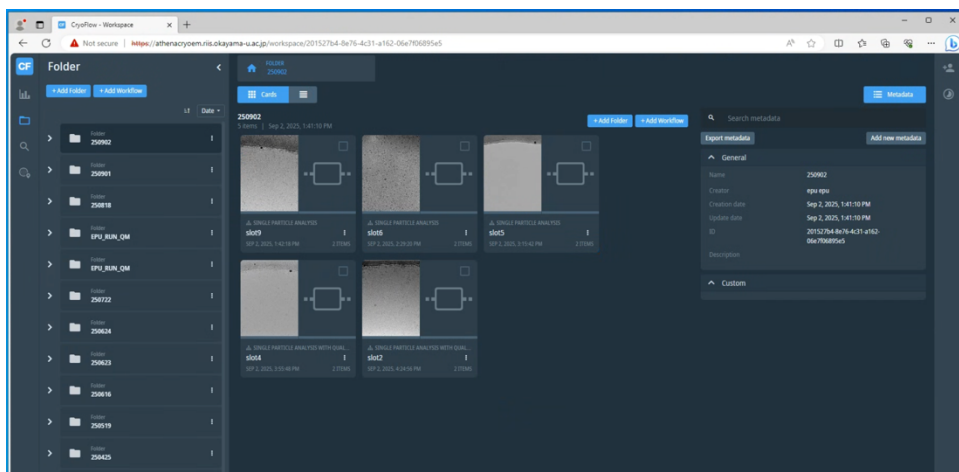
On the CryoFlow monitoring PC, Edge > Bookmark > Sign in to CryoFlow



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

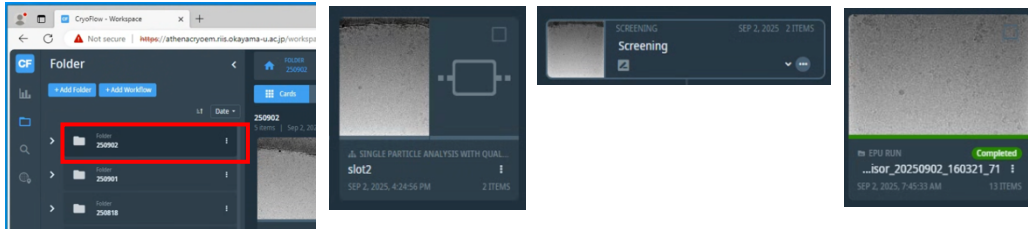


The CryoFlow Workspace will be displayed.

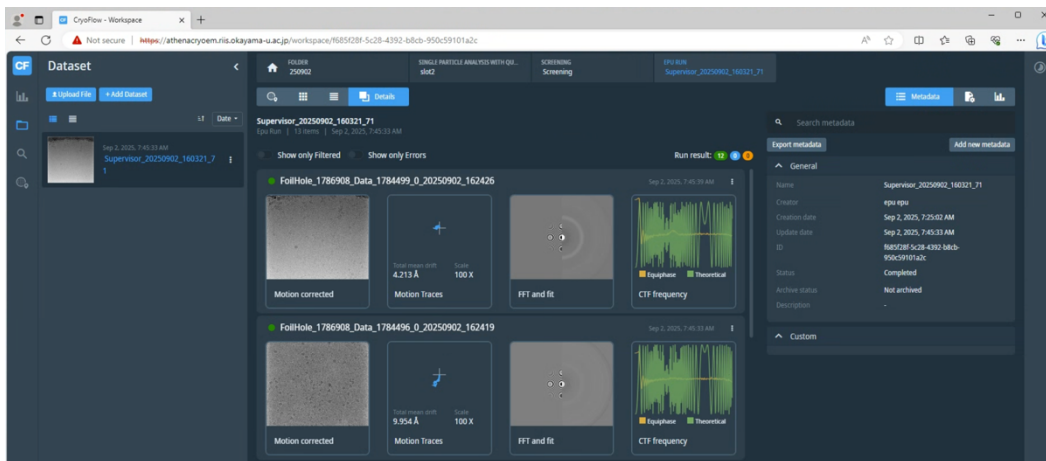


Click through the following in order:

Folder (date) > slot\_x > Screening > Supervisor\_xxx

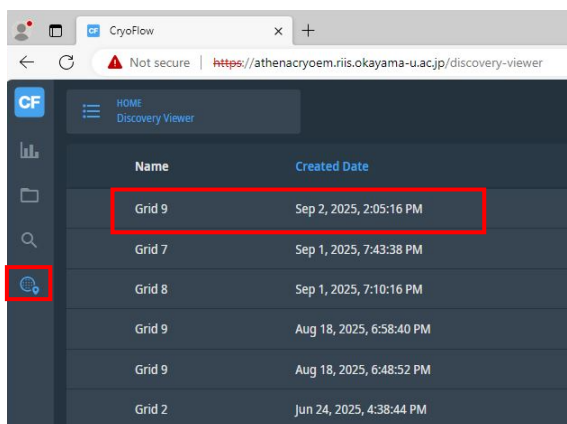


The results of Motion correction and CTF estimation will be displayed.

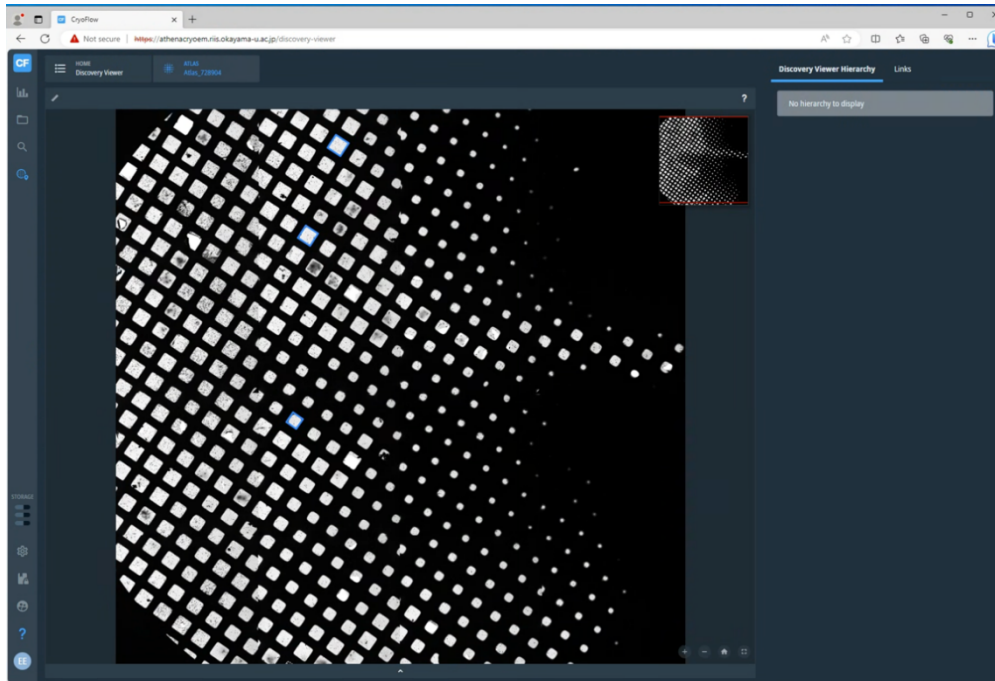


\* From the CTF, you can evaluate the resolution?

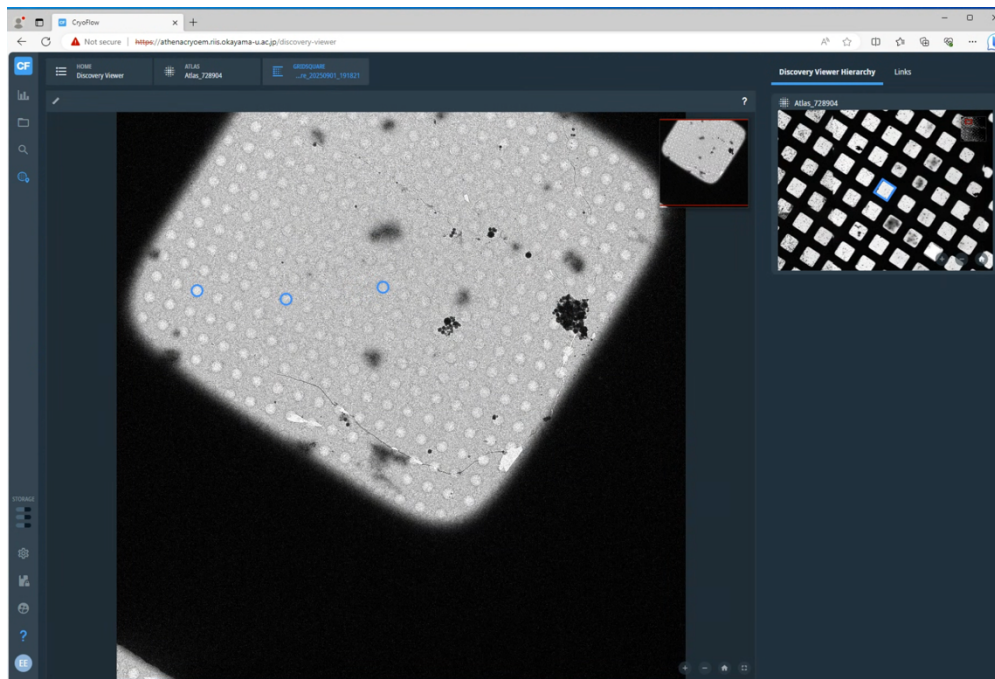
Click the Discovery viewer icon at the upper left of the screen, and select the grid you want to view.



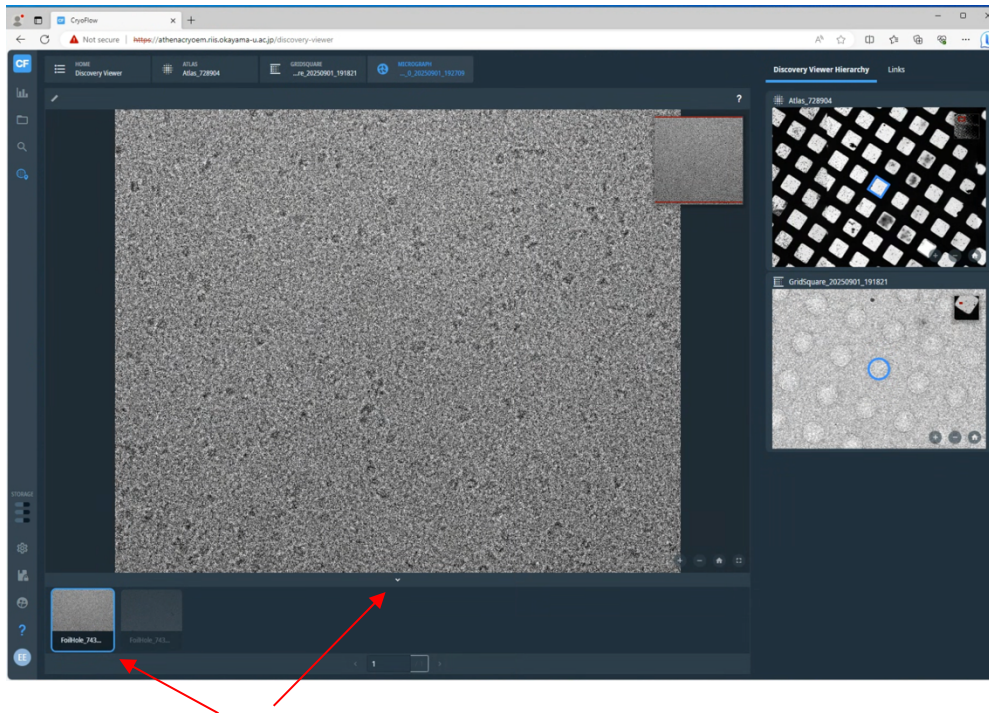
The atlas is displayed, and the squares where data were acquired are highlighted. Click the square you want to check.



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.



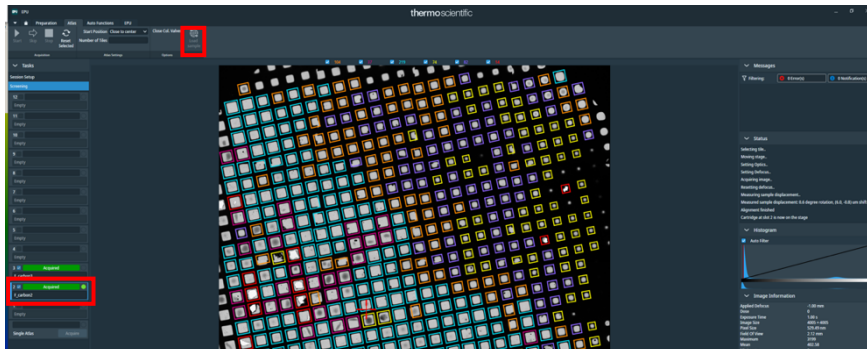
\*When you click the bar, thumbnails of the images taken at that hole are shown.

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

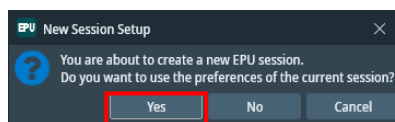
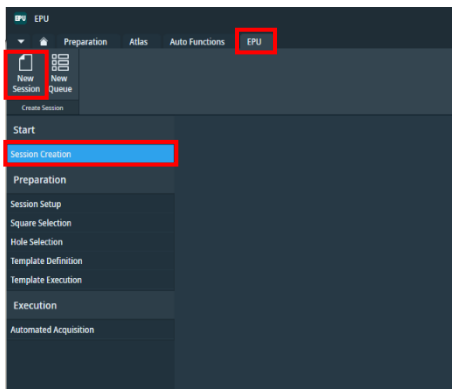
Once the screening of one grid is completed, replace the grid and repeat the same procedure.

### 3. Continuous Data Acquisition

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



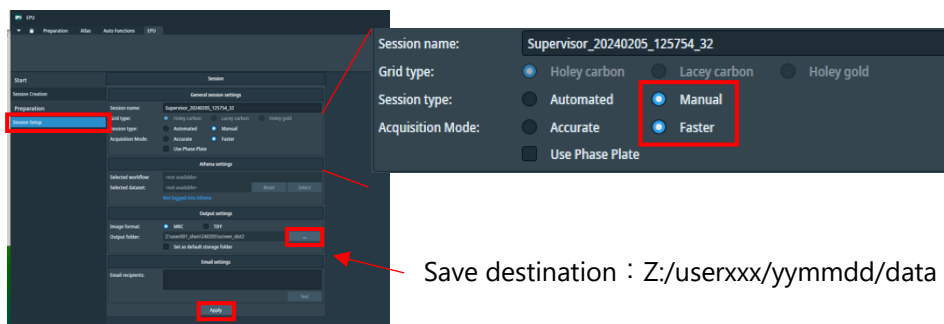
EPU > EPU tab, select **Session Creation** > **New Session** > **Yes**



Click **Session Setup**.

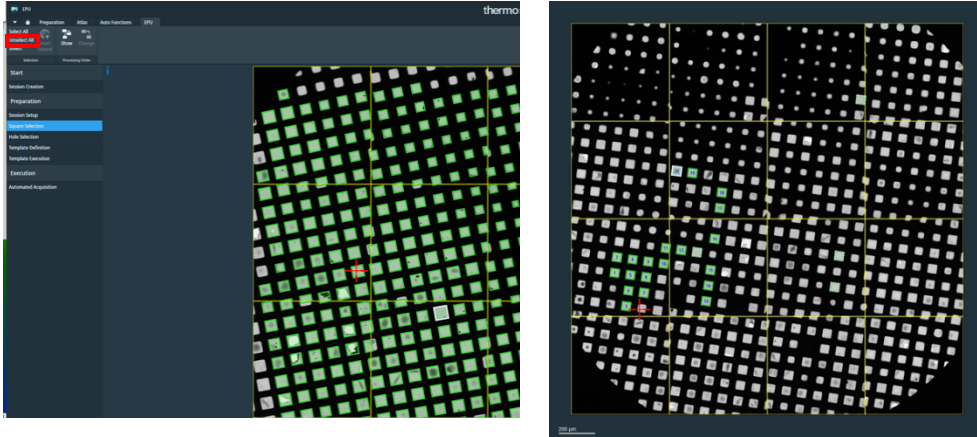
Select Session type: **Manual** and Acquisition mode: **Faster**.

\*CryoFlow is not used.



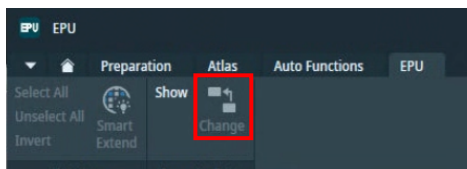
Click **Apply**.

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

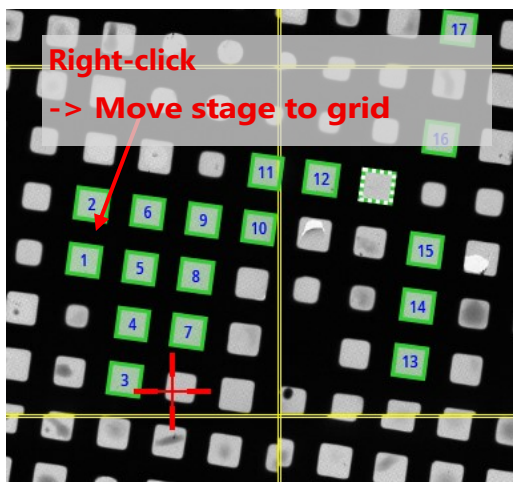


\*If the movement distance between squares is too large, measurement time will be lost.  
\* It is better to select squares so that the system moves sequentially through nearby squares.

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:

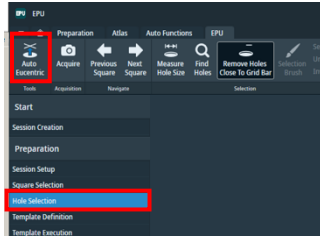
Move to the clicked location

Move stage to grid square:

Move to the center of the clicked square

Click **Hole selection**.

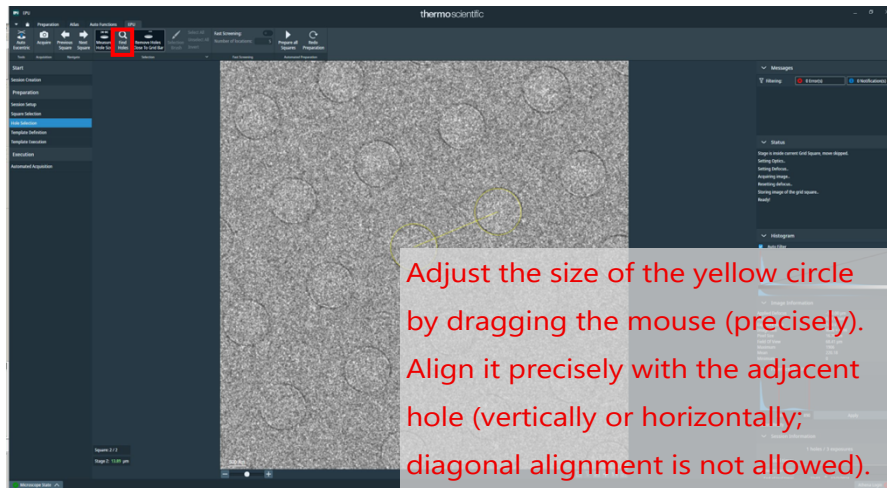
Click the **Auto Eucentric** button at the upper left.



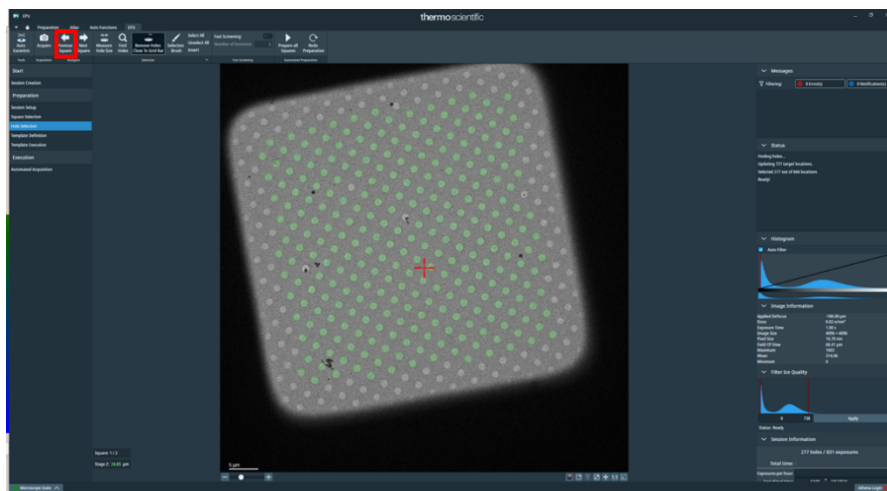
\*If Auto Eucentric fails, go to the Auto Functions tab and run **Auto-eucentric by beam tilt** or **Auto-eucentric by stage tilt** → **Start**.

\*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**.

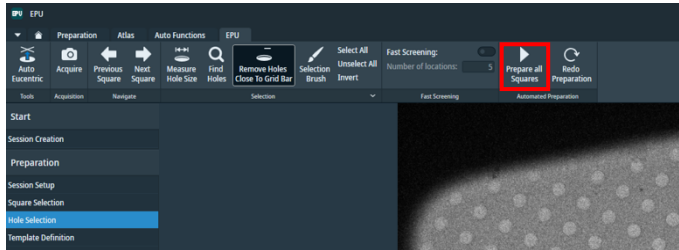


Use **Find Holes** to automatically detect the holes.



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.

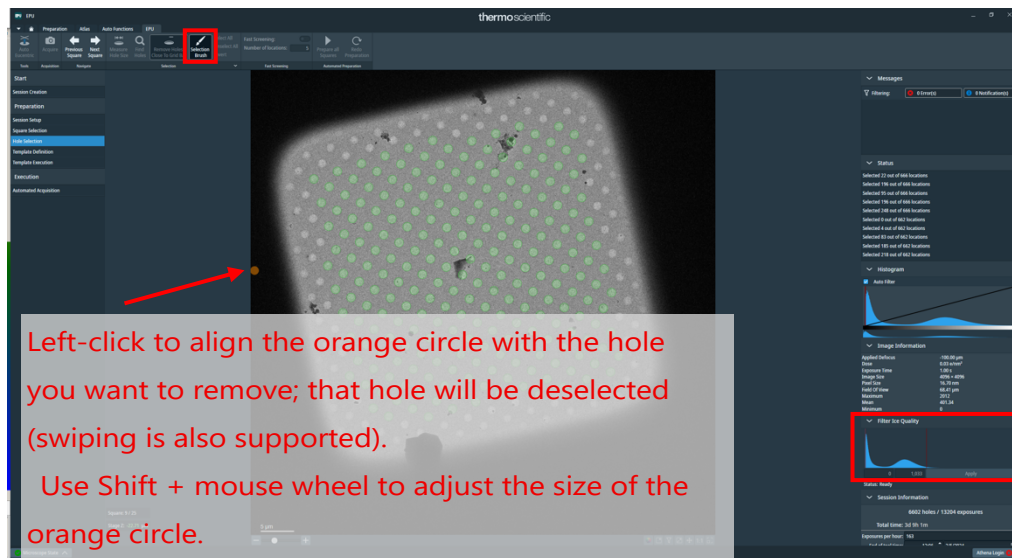
\* If Eucentric fails, that square will be skipped.

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

Check the automatically detected holes.

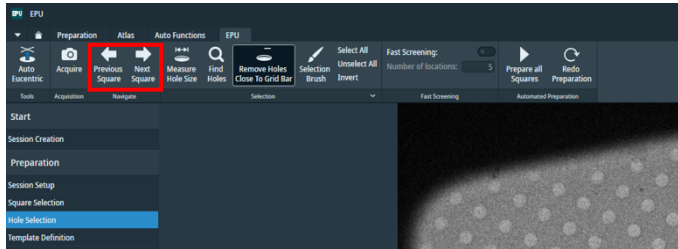
For holes containing debris or cracks, remove them manually using the selection brush.

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

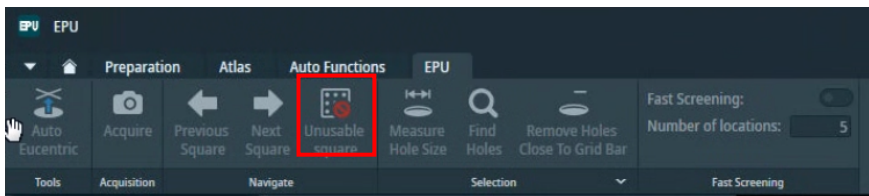


\* It is safer not to select squares with cracks, as they tend to shake when irradiated with electrons.

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**.

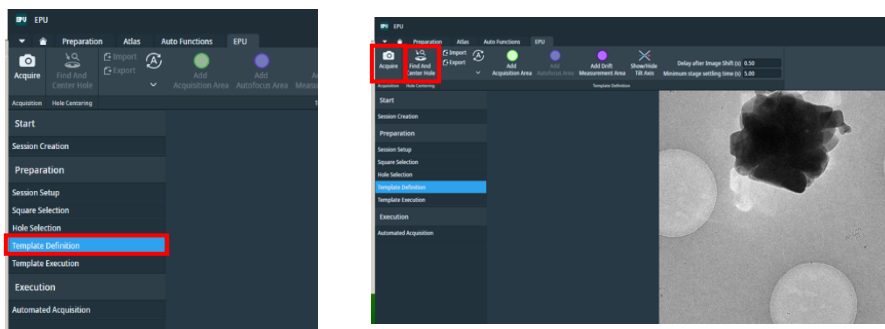


\* In **Square selection**, the square will be shown in red.

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

Click **Template Definition**.

**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.

\* 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).

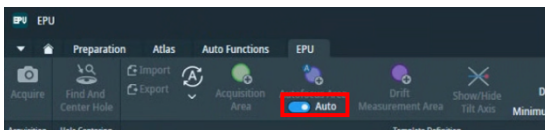
\* Multiple areas can be set (e.g., center and edge).

Specify the **defocus value**:

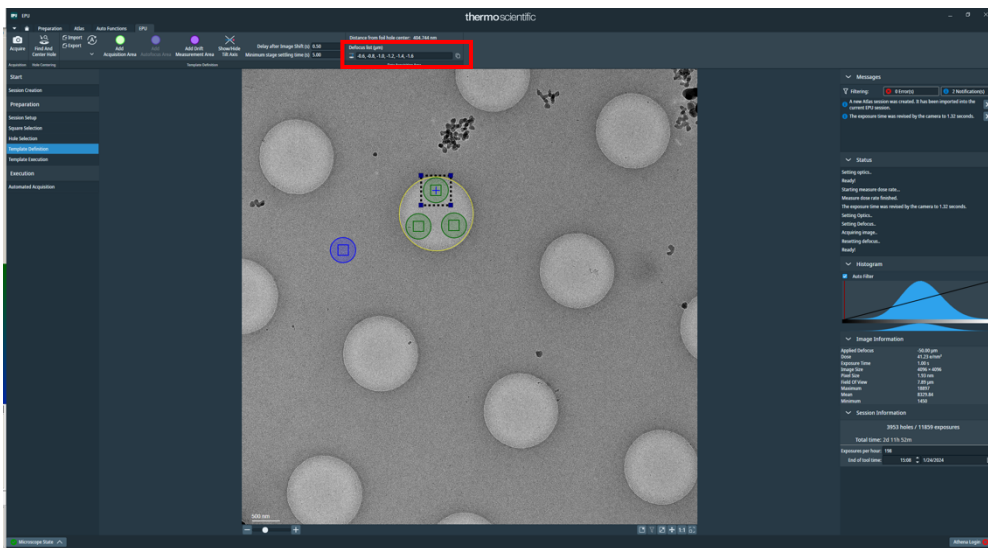
-0.6, -0.8, -1.0, -1.2, -1.4, -1.6, -1.8  $\mu\text{m}$ , etc.

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.

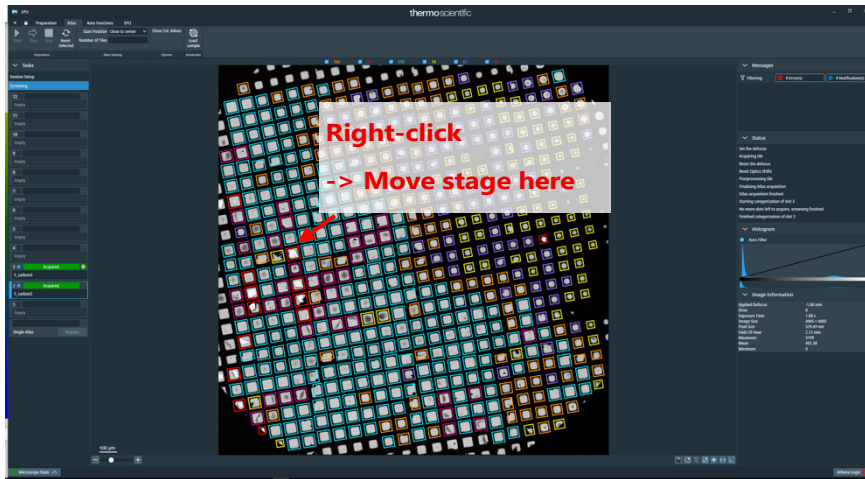


Take a screenshot (use an app to capture the entire screen including the defocus value).



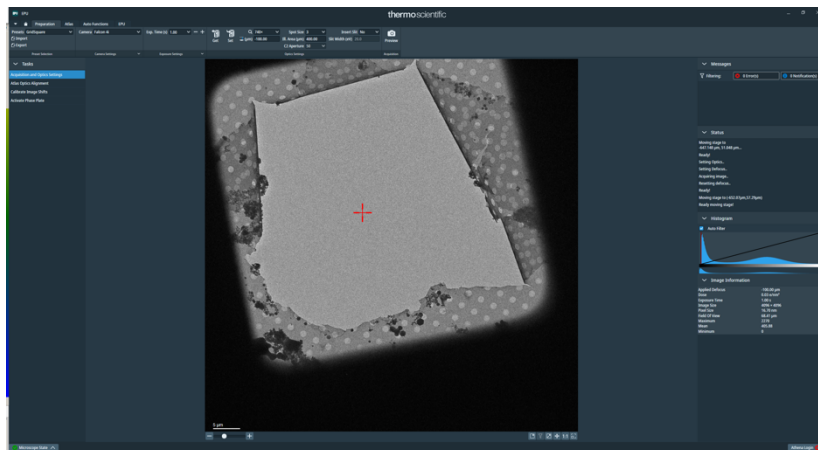
To estimate the dose, move to a hole in the grid.

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



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

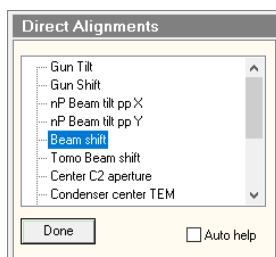
\* Confirm that you are positioned over a hole.



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**.



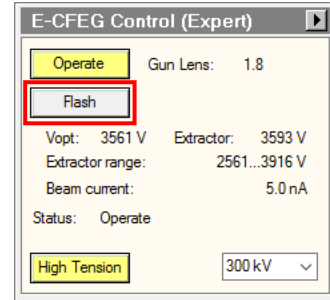
Check the Beam Current in the TUI.

High tension:	300 kV	Beam Current:	3.7 nA
nP EFTEM		Screen current:	0.000 nA
SA 6500 x		Spot size:	4

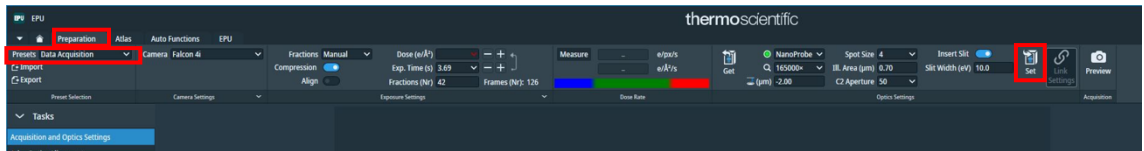
If it appears to be below 5, perform a Flash.

\* TUI > Set up tab > E-CFEG Control

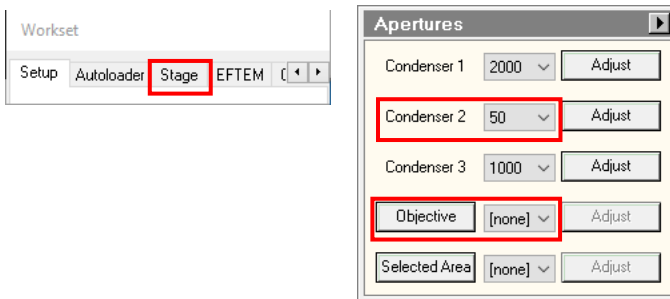
\* When performing a flush, close the column valve.



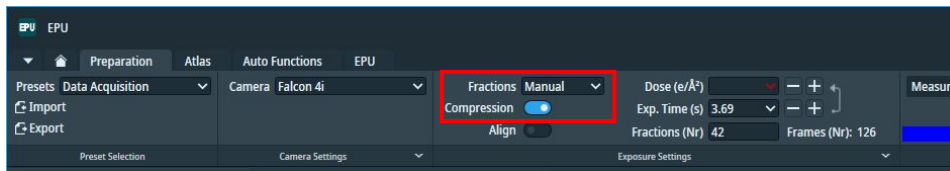
EPU > Preparation tab > Presets, select Data Acquisition and click Set.



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



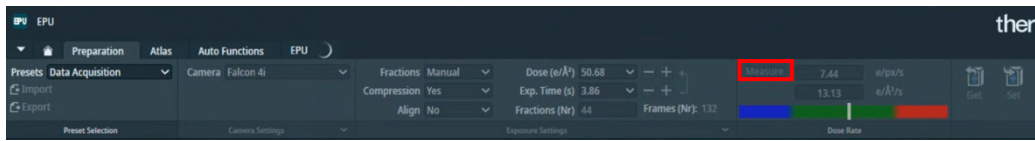
Confirm that Fractions = **Manual** and Compression = **Yes**.



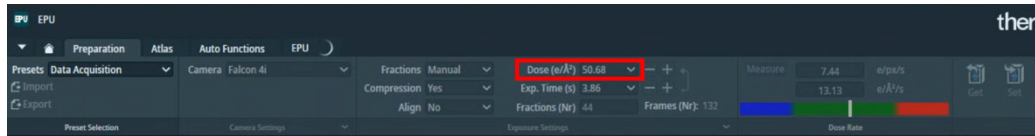
\* Settings for TIFF format

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

Click “Measure”.



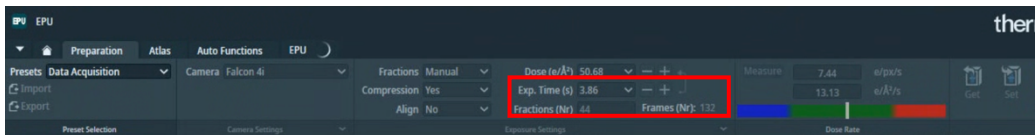
Select 50 from the Dose pulldown menu (this value is often appropriate).



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

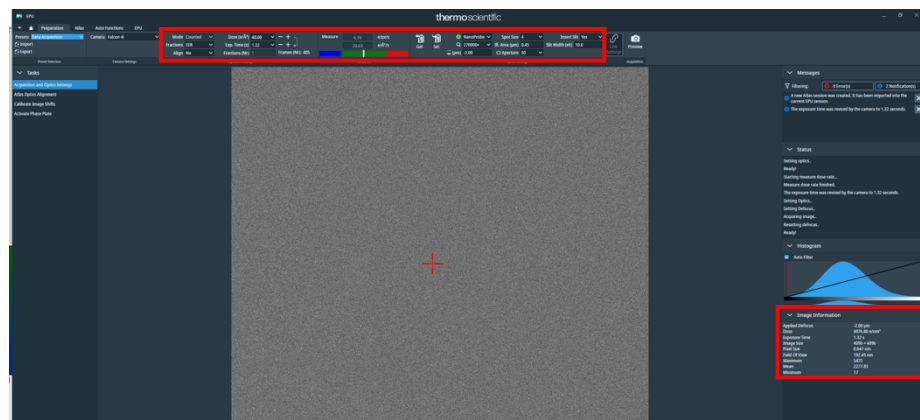
**Fractions (preferably 40–50) × (integer) = Frames.**

\*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.

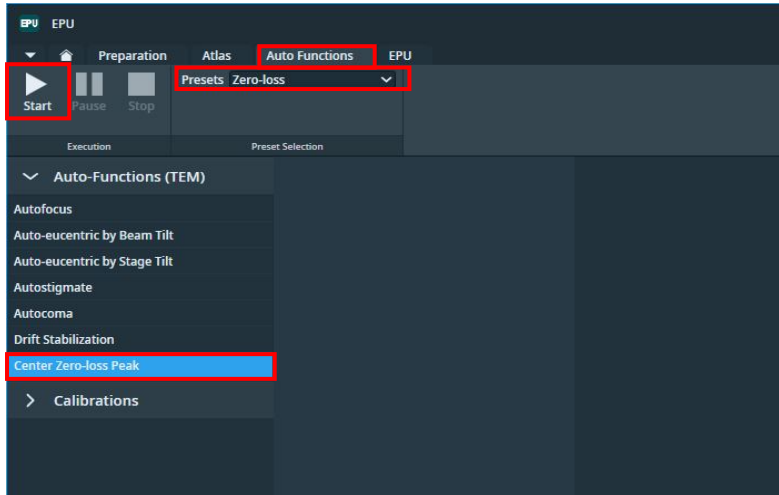


(Capture the entire screen using an app.)



EPU > Auto Functions tab > Center Zero-loss Peak

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

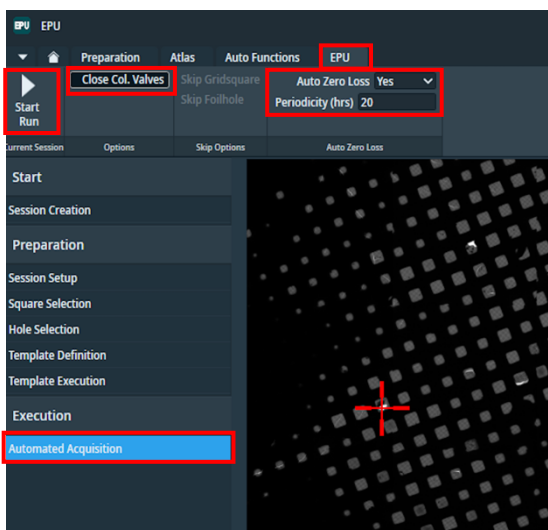


EPU > EPU tab > **Automated Acquisition**

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.

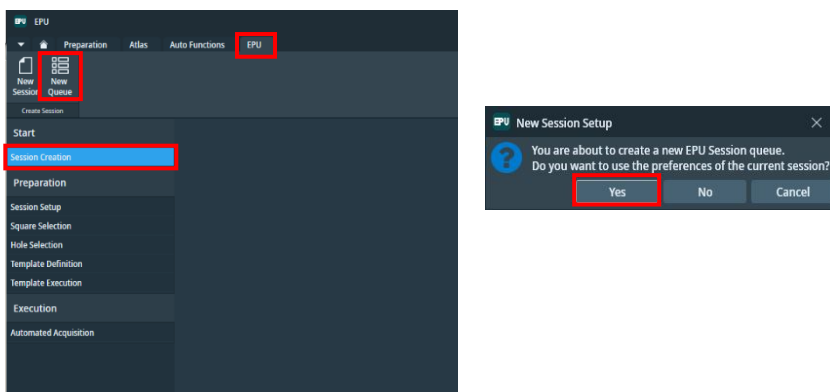


#### 4. Data Acquisition Using Multi-Grid Session

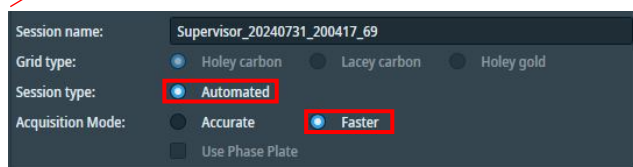
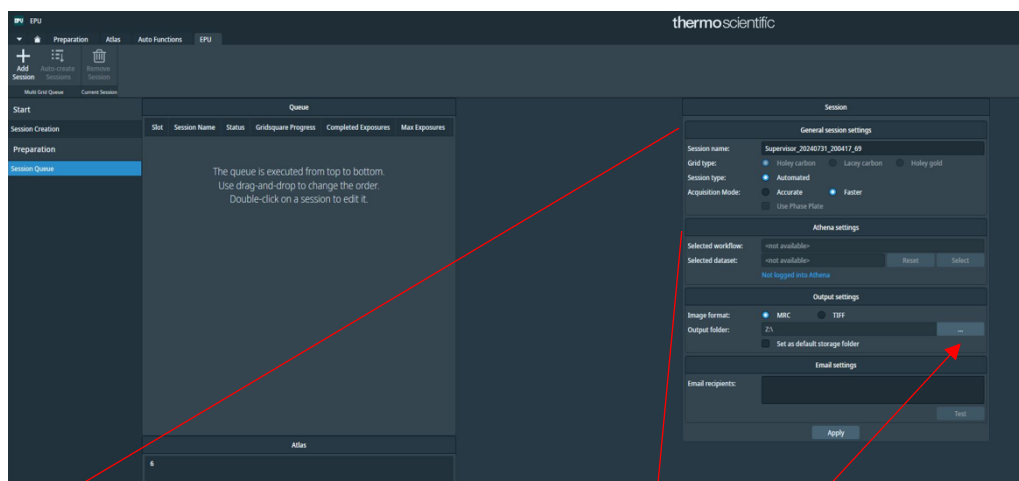
EPU > Atlas tab, select the first grid and click Load Sample.

**Following pp. 22–24, 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.



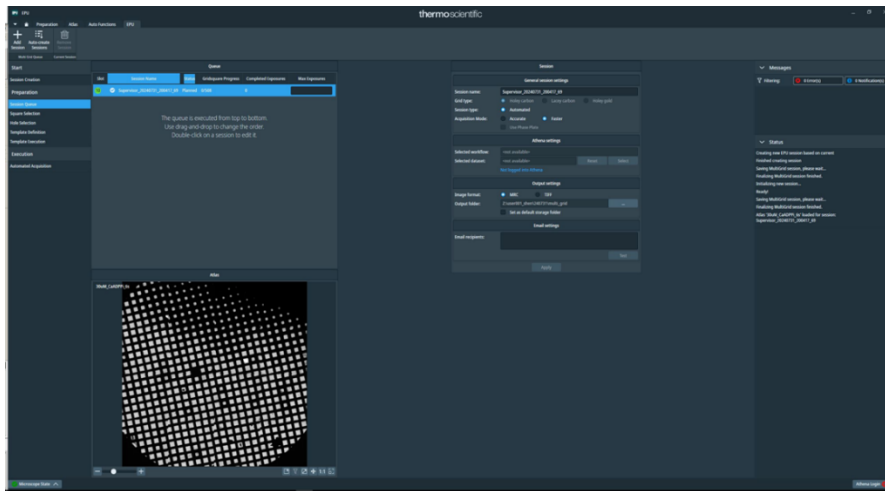
(Save destination :  
Z:/userxxx/yymmdd/multi)

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.



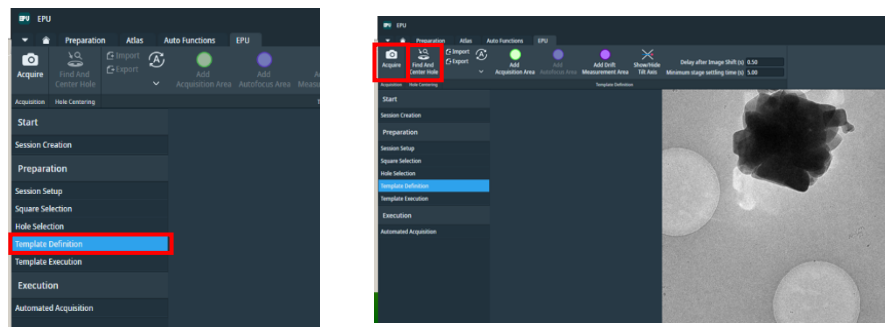
Following pp. 17–18, select the squares and select the holes.

\* 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.

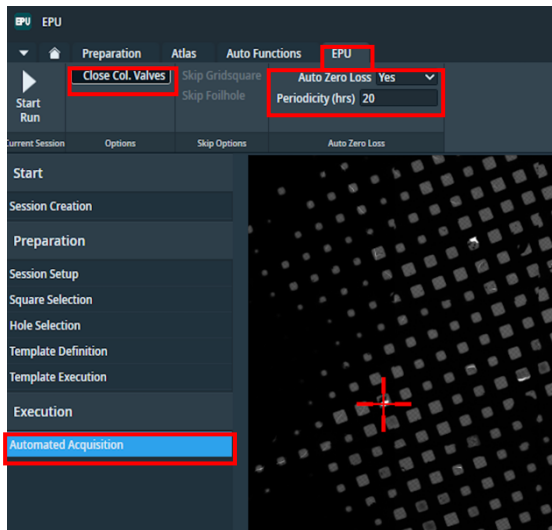
Following pp. 21, set the autofocus position, irradiation position, defocus values, etc.

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).

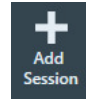
Enable “Close Col. Valves” to ensure the column valves close after measurement.



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

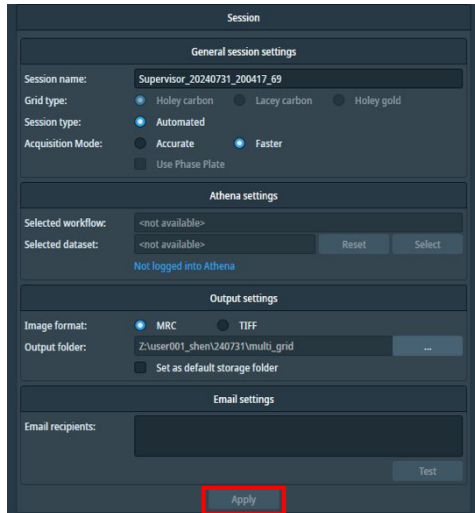
Replace the grid (**Load Sample** in the Atlas tab).

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



> **Yes**

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

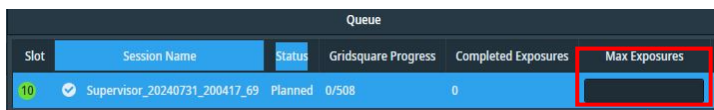


The current grid is added to the queue.

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**



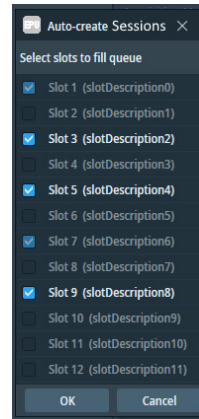
**\* Batch setup using Auto-create Sessions**

**Complete the dose estimation (pp. 22–24) 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. 25, configure the **Auto Zero-loss** settings.

**Automated Acquisition > Start Queue**



That's all.