Data Collection with EPU Standard Operating Procedure (K3)

version 1.1

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1. Purpose

1.1. Collecting data on cryo-EM grids on a K3 detector with EPU software (ThermoFisher Scientific)

2. Definitions:

2.1. EPU is an automated data collection software from ThermoFisher Scientific (TFS).
2.2. TEMUI is the user interface of the microscope
2.3. TEM Image Analysis (TIA) must be running to ensure EPU is communicating with TEMUI
2.4. Imaging Presets in EPU:

2.4.1 Atlas
a. Create a grid map with stitching image tiles at low dose
b. Optic and camera settings are relatively fixed for each microscope

2.4.2 Grid Square
a. Depends on mesh size of the grid (can see one grid square)
b. Illumination covers the entire grid at a small dose rate.
c. Linear mode or counting mode (camera setting) with binning x2.

2.4.3 Hole/Eccentric Height
a. Field of view covers 3~ 4 holes.
b. Illumination covers ~ 15 um at a small dose rate.
c. Counting mode (camera setting) with binning x2.

2.4.4 Data Acquisition
a. Beam diameter meets a parallel illumination and fringe free conditions.
b. Dose rate is within camera sensitivity range.
c. Beam edge doesn't reach adjacent targets.
d. Exposure time is not too long to achieve the required dose (~ 50 e/A^2, depends on sample).
e. Electron counting mode with dose fractionations.

2.4.5 Auto Focus
a. Same as Data Acquisition with shorter exposure and 2x binning.

2.4.6 Thon Ring
a. Same as Data Acquisition with 2 s exposure and 2x binning.

2.4.7 Drift Measurement
a. Same as Data Acquisition except with shorter exposure and 2x binning.

2.4.8 Zero Loss
a. Same as Data Acquisition except with shorter exposure and 2x binning.

3 Supplies & Equipment

❏ Microscope with a K3 detector and BioQuantum Energy Filter

❏ Software: TEMUI, TIA, EPU, GMS

4 Procedure:

4.4 Microscope and Software Checks

4.4.1 Microscope's states are within specifications such as vacuums, HT, emission and temperatures.
4.4.2 Stop Data Acquisition of the previous session on **EPU** and close column valves.
4.4.3 Cassette with grids have been loaded (see **Loading SOP**)

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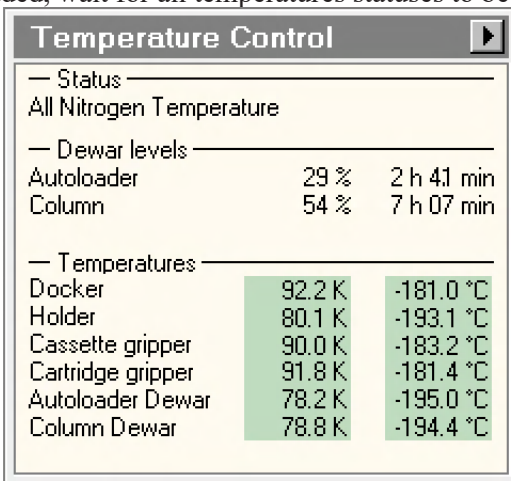
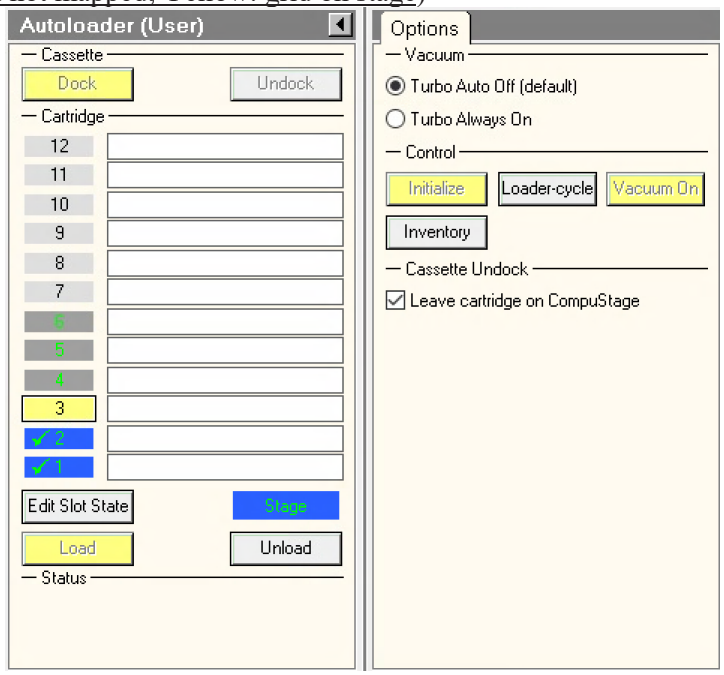
4.5 Making Grid Atlases with EPU

4.5.1 After grids are loaded, wait for all temperatures statuses to be green AND <-160C



4.5.2 Select **Inventory** on the **Autoloader** panel and wait till the loaded grids have been detected correctly in the cassette (Blue: slot occupied with grid; Dark grey: slot detected empty; Light grey: slot not mapped; Yellow: grid on stage)



4.5.3 Open **EPU** software, click **Preview** to take an image using **Atlas** preset (Column valve should be open)

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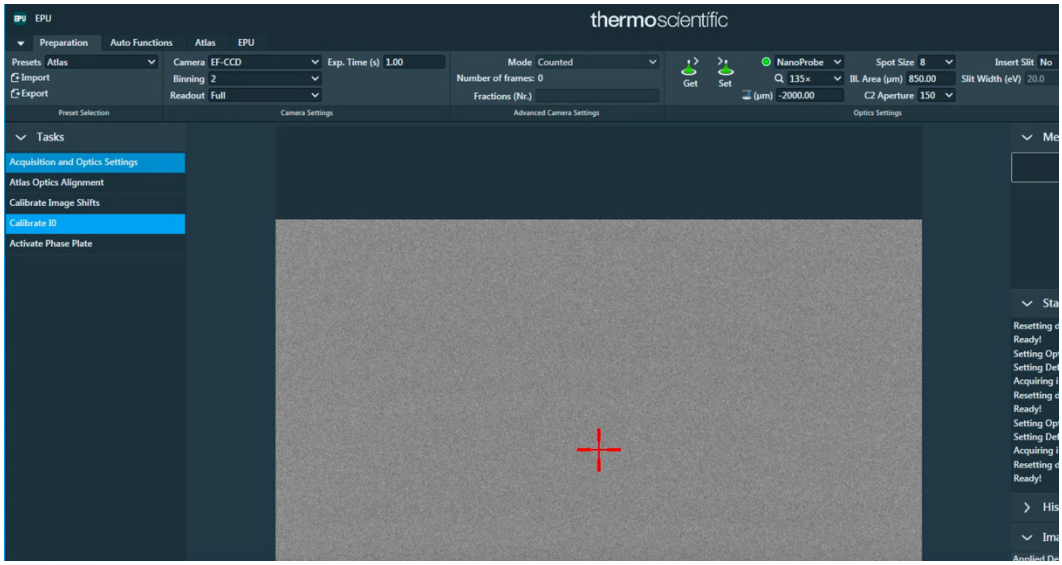
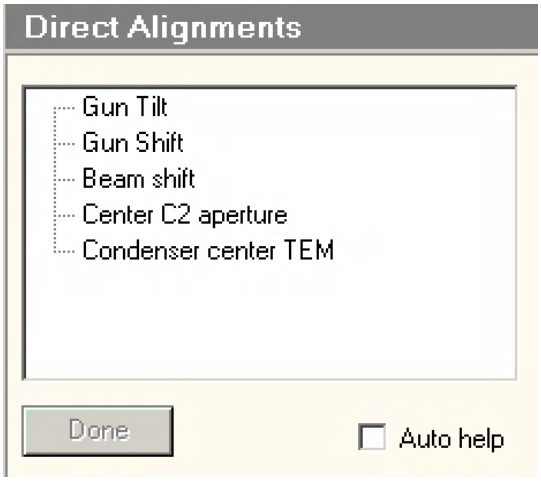


4.5.3.1 **IF** there is a beam shift, insert FluScreen. In **TEMUI Tune** tab, go to subsection **Direct Alignments** then select **Beam shift**. **DO NOT TOUCH GUN TILT AND GUN SHIFT.** Proceed to 4.2.4 if no beam shift adjustment is needed



4.5.3.2 On the hand panel, use **MF-X** and **MF-Y** knobs to center beam over the green circle.
4.5.3.3 Click **Done** after adjustment. Press **R1** to lift the screen
4.5.3.4 Take another **Preview** to ensure the beam is centered

4.5.4 Select the **Atlas** tab in **EPU** > **Session Setup** task
4.5.5 Select **New Session**, and enter a Name for the session: Name should be yearmonthday-projectnumber-atlas, for example 20210414-CT21-atlas
Image format = MRC, Output folder = X, click **Apply**
4.5.6 In the **Screening** section, check the cartridges with grids and select **Start** to begin. It'll take 10-15 minutes to map each grid

4.6 Screening Grid Squares
4.6.1 After Atlasing, evaluate ice thickness and grid quality. A good grid should have at least three quarters of thin area

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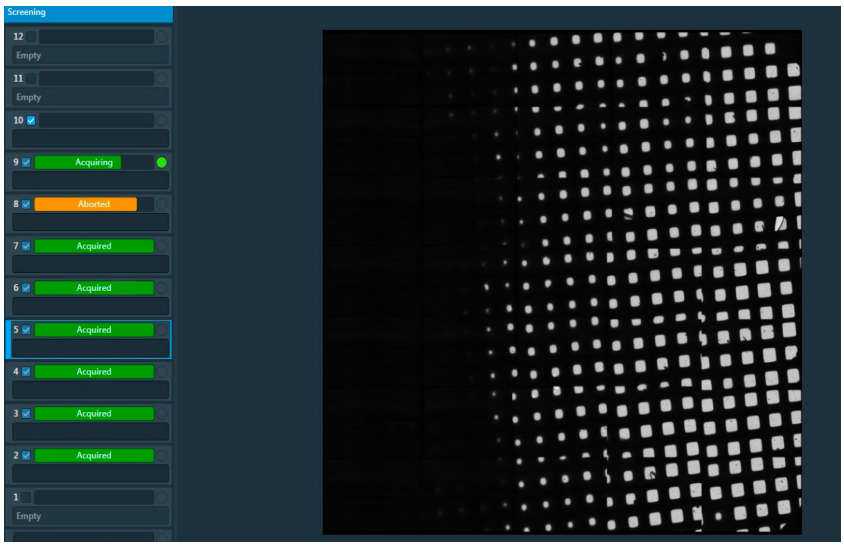
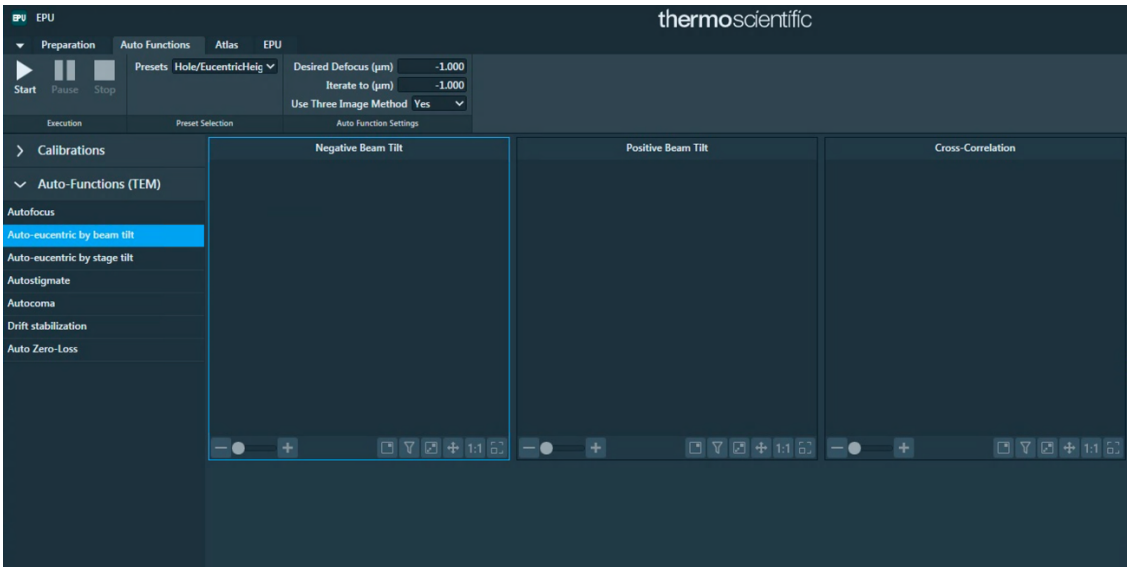


4.6.2 Load a grid of interest by clicking on the slot number and selecting **Load from sample**

4.6.3 When loading is done, pick a square (usually a thinner square). Right click on the square, then select **Move stage here**

4.6.4 Go back to **Preparation** tab, select **GridSquare** preset and take a **Preview**. Adjust the magnification so that you only see one square without much cutoff

4.6.5 Go to **AutoFunctions**, select **Hole/EucentricHeight** preset and run **Auto-eucentric by beam tilt** to find the eucentric height



4.6.6 Go back to **Preparation** tab and take a preview at **Hole/EucentricHeight** preset

4.6.7 Move stage over a hole and capture at **DataAcquisition** preset (**DataAcquisition** preset allows you to set up optics for data collection); Make sure the pixel size is adjusted to your sample

4.6.8 **Autofocus (optional)**: this is performed before capturing an image at **DataAquisition** preset. Move stage on carbon at **Hole/EucentricHeight**, and run **Autofocus** task with **Autofocus** preset under **AutoFunctions** tab

4.6.9 Evaluate ice thickness, particle density, homogeneity etc. Below is a good image:

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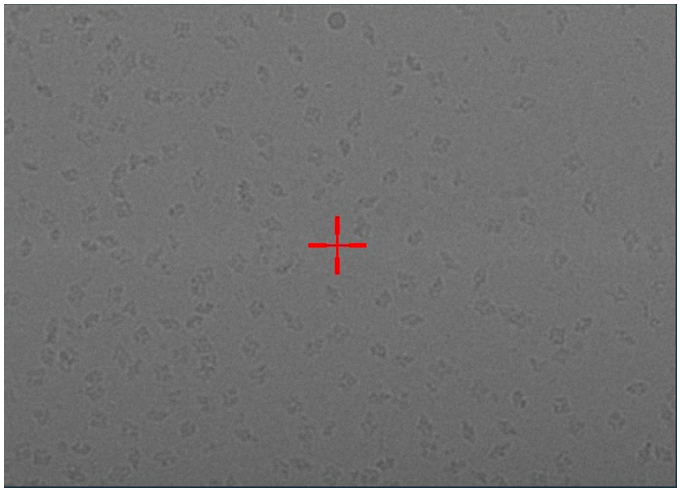


4.6.10 Screen at least one more hole on the same square and repeat 4.6.3-4.6.9 to screen other squares on the same grid. Load the next grid to screen if needed

4.6.10 Image Shift Calibration

4.6.10.1 After screening and all presets are determined, Image Shift Calibration is required for each session

4.6.10.2 On the atlas map, find a feature, such as a piece of ice contaminant on a square. Right click and select **Move stage here** to move the stage onto the square

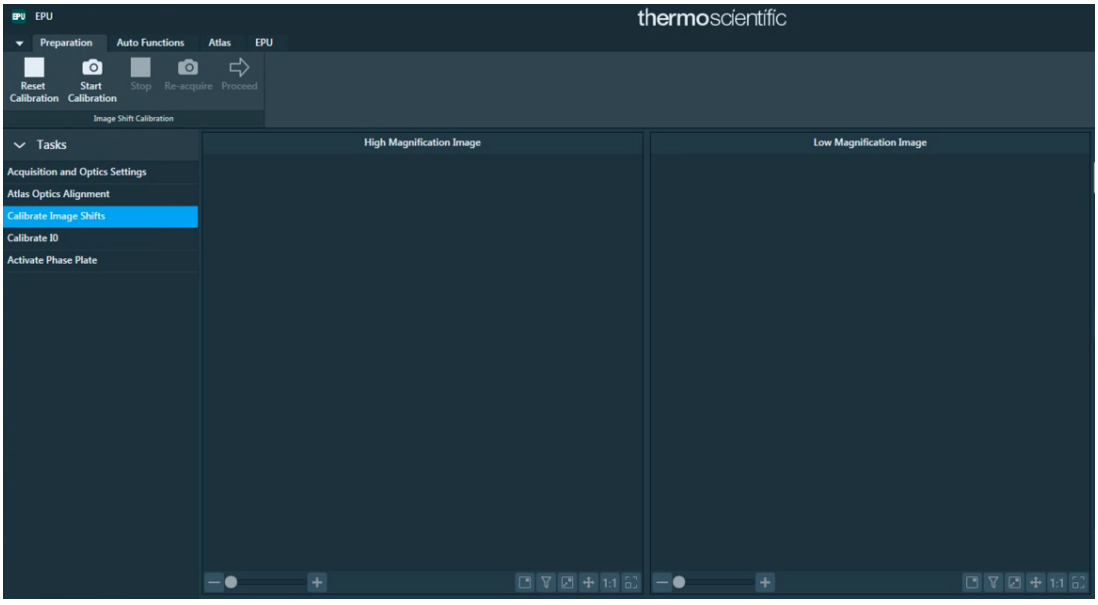
4.6.10.3 Set Presets as **GridSquare**, and **Preview**

4.6.10.4 Move stage close to the feature and run **Auto-eucentric by beam tilt** with **Hole/EucentricHeight** preset under **Autofunctions**

4.6.10.5 **Preview** at **Hole/EucentricHeight** and make sure you can find the feature and center it in the image. If the feature isn't visible, you may need to insert the FluScreen and move the stage to find the feature

4.6.10.6 **Preview** at **DataAcquisition** preset and make sure the feature is visible. If not, you may need to insert the FluScreen and move the stage to find the feature

4.6.10.7 Select **Preparation** tab > **Calibrate Image Shifts** task, and click **Start Calibration**, and follow the instructions. Use **Proceed** to continue and double click on the feature to center it



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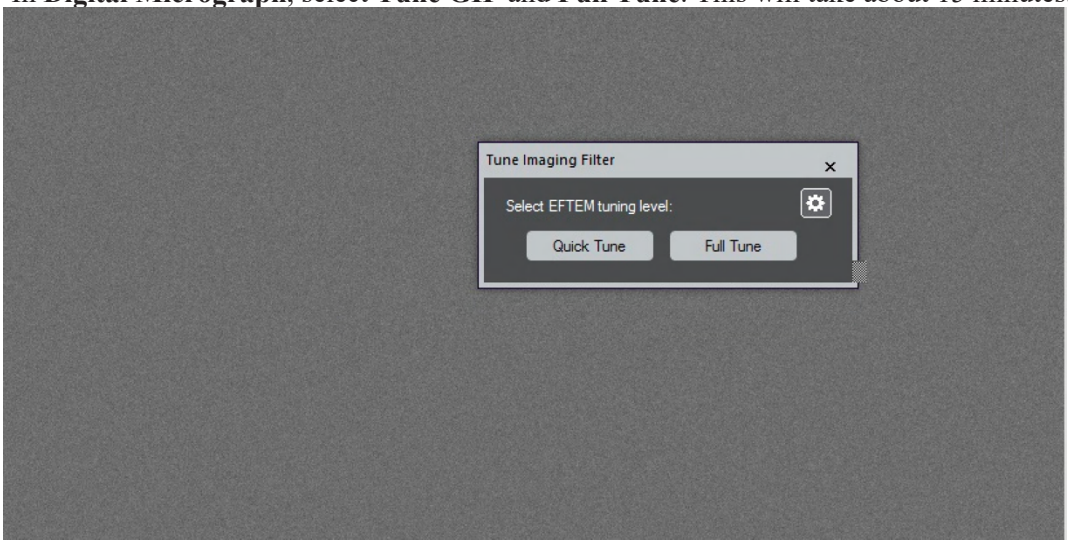
4.7 BioQuantum Energy Filter Tuning

4.7.1 Move the stage to an empty square so that the electron beam is over vacuum. Click the **Set** button on the **Data Acquisition** preset in EPU and set the microscope to the same optic setting as in **Data Acquisition** preset

4.7.2 Insert the Flu screen, change Spot Size to 1, and C2 aperture to 150 µm

4.7.3 In **Digital Micrograph**, select **Tune GIF** and **Full Tune**. This will take about 15 minutes.



4.8 Gain reference collection

4.8.1 After GIF Tuning, select **Prepare Gain Reference** under the **Camera** menu.

4.8.2 Follow instructions: the linear gain reference will be collected first followed by the counting gain reference. The linear gain reference requires a dose rate of 1500 while the counting mode gain reference uses 15 e/pixel/second. Put down the Flu Screen, use Spot Size and Intensity knob to adjust the dose rate.

4.9 Dose Fractionation

4.9.1 This is still done over vacuum. **Set** the microscope to the **Data Acquisition** imaging condition. In **Digital Micrograph**, capture an image with 1 second exposure, binning = 1X. The dose rate (e-/pixel/s) will show up under the image window. Adjust the dose rate using **Intensity** knob. For K3 camera, 15-25 e-/pixel/s is desired.

4.9.2 Using the determined dose rate, calculate exposure time to yield a cumulative exposure of ~40-50 e-/Å2. Determine the number of frames. 1-1.5 electrons/A^2/frame is commonly used.

4.10 Microscope Alignment

4.10.1 Navigate the stage to a square that can be used for alignment.

4.10.2 In EPU, under **AutoFuncitons** tab, select **AutoEucentric by beam tilt**

4.10.3 Preview at the preset of **Hole/EucentricHeight**, and move stage to carbon

4.10.4 Under **AutoFuncitons** tab, select **AutoFocus** using **AutoFocus** preset

4.10.5 In **TEMUI**, under **Tune** tab, align **Beam Shift**, **pivot points** and **C2 aperture**

4.10.6 Under **AutoFuncitons** tab, perform **AutoStigmitism** using **Thon Ring** preset

4.10.7 Under **AutoFuncitons** tab, perform **AutoComa** using **Thon Ring** preset

4.10.8 Insert **Objective aperture**, put **FluScreen** down, and press **Diffraction**

4.10.9 If the aperture is not centered, click **Adjust** next to the objective aperture box. Use **Multifunction X** and **Y** to center the aperture. Exit Diffraction mode

4.10.10 Perform **AutoStigmitism** using **Thon Ring** preset again

4.11 Setting up EPU Session

4.10.1 Select the **EPU** tab > **Session Creation** task. Select **New Session**

4.10.2 In the **Session Settings**, input names and select appropriate parameters and click **Apply** (see

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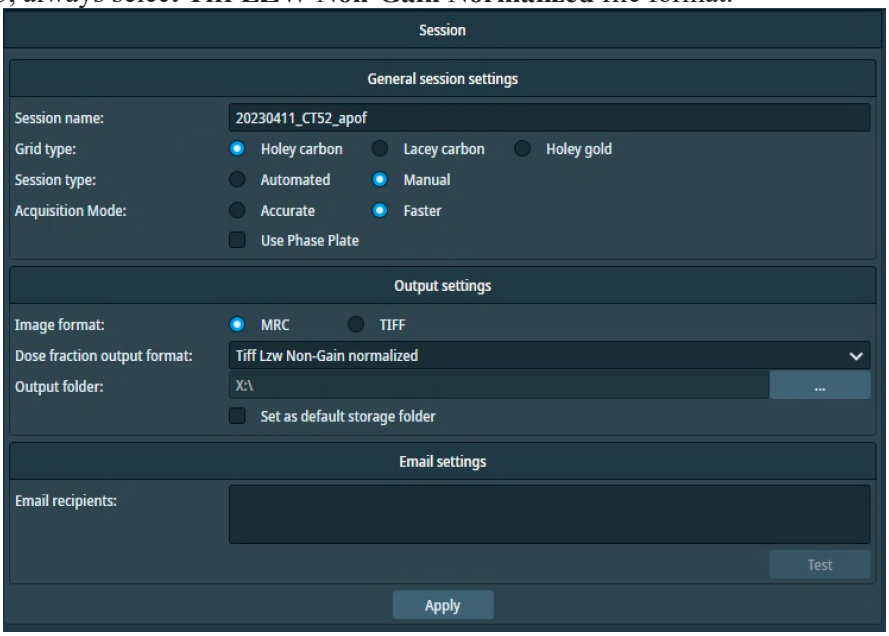
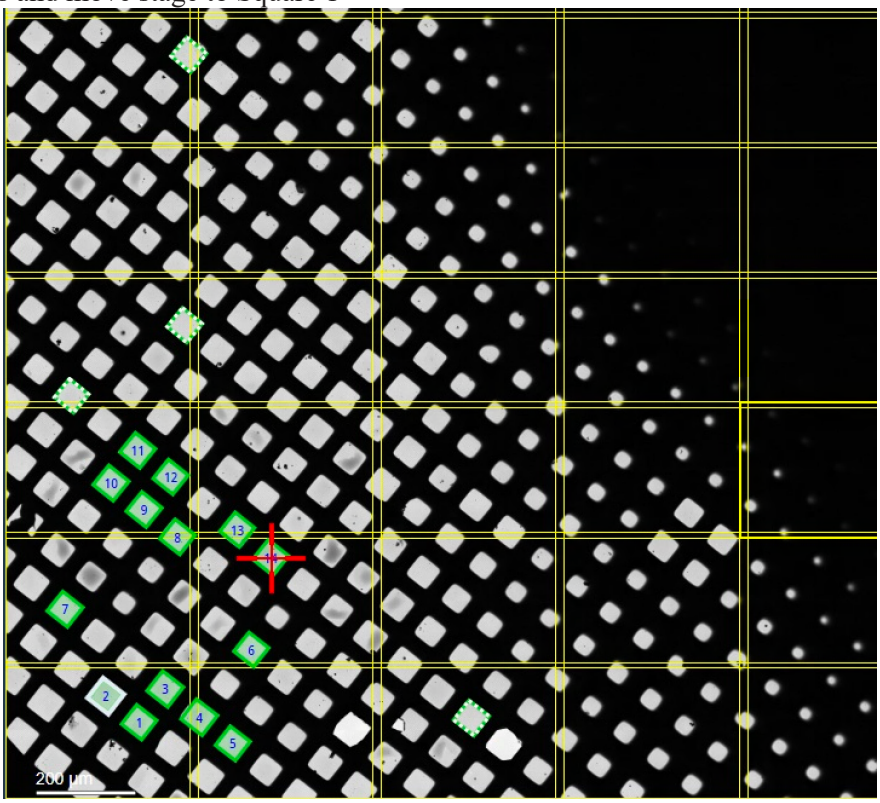
below). For K3, always select **Tiff LZW Non-Gain Normalized** file format.



4.10.3 On the left, select **Square Selection** task. **Unselect All** the pre-selected squares if needed. Click **Show**.

4.10.4 Zoom in the Atlas map on the EPU session, right click on a good grid square, and **Select**. Add at least 10 squares and move stage to Square 1



4.10.5 Under **Hole Selection** task, click **Auto Eucentric**

4.10.6 Select **Measure Hole Size**, adjust circles to match the actual holes and select **Find Holes**

4.10.7 Select **Remove Areas Close To Grid Bar**, and use the **Filter Ice Quality** histogram to adjust the boundary of ice

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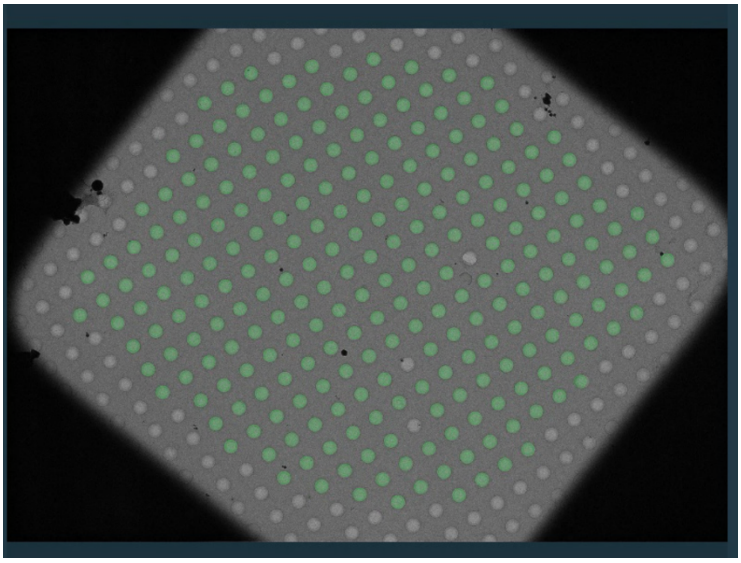
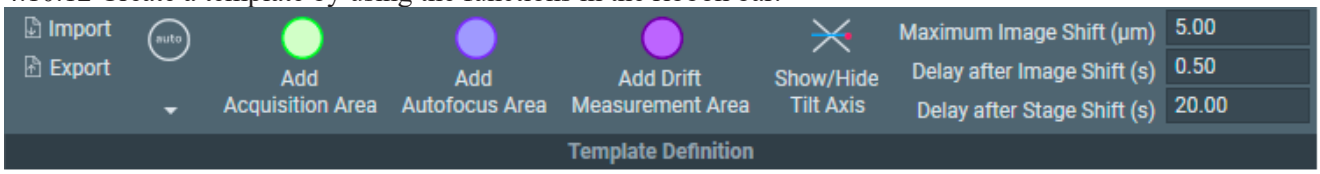
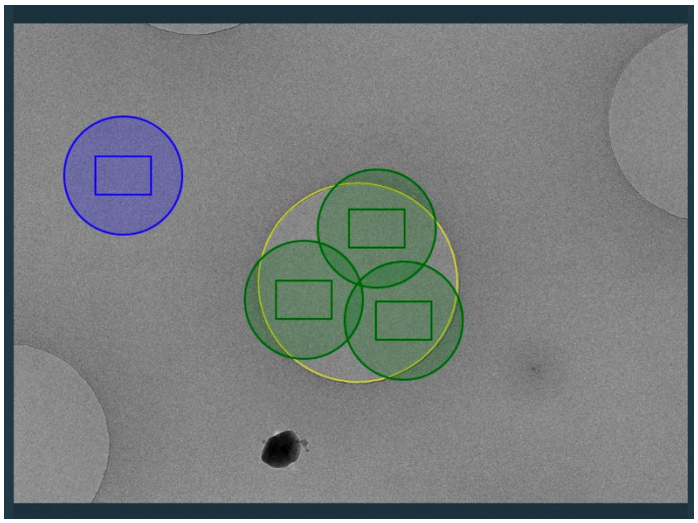


4.10.8 To remove multiple foil holes, select **Selection Brush**. Remove holes with contaminations or cracks. To add holes, hold "CTL" and click on the hole while **Selection Brush** is highlighted**.**
4.10.9 Click **Prepare All Squares** button to prepare the rest of the squares.
4.10.10 Select the **EPU** tab > **Template Definition** task.
4.10.11 Select **Acquire** to acquire an image and select **Find and Center Hole**.
4.10.12 Create a template by using the functions in the ribbon bar.



4.10.13 Add **Add Acquisition Area** on the hole.
4.10.14 Define a **Defocus List** and ensure each acquisition area has the same defocus range
4.10.15 Add **Add Autofocus Area** in the image display. **Autofocus area** should be placed on carbon film.



4.10.16 In the **Autofocus Area Settings**, set **Recurrence** to **After Distance** and input 8 µm.
4.10.17 Optional: Add **Add Drift Measurement Area** in the image display and specify **Recurrence** as **Always**
4.10.18 Doublecheck all the settings to make sure there is no mistakes. Go back to **Hole Selection** and

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use **Selection Brush** to touch up the hole selection on the rest of squares.
4.10.19 Select enough squares/holes for an overnight data collection.
4.10.20 Select the **Automated Acquisition** task and select **Start Run**

5 Chemicals: N/A

6 Waste Disposal: N/A

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