S²C² | Stanford-SLAC Cryo-EM Center

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S²C² Cryo-EM Data Collection SOP- Krios (Beta) with K3 (EPU)

TEMUI version:3.17.1
EPU version: 3.9.1
DM version: 3.53

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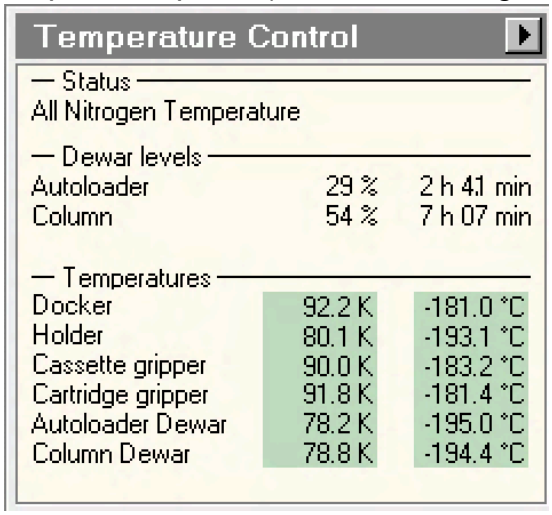
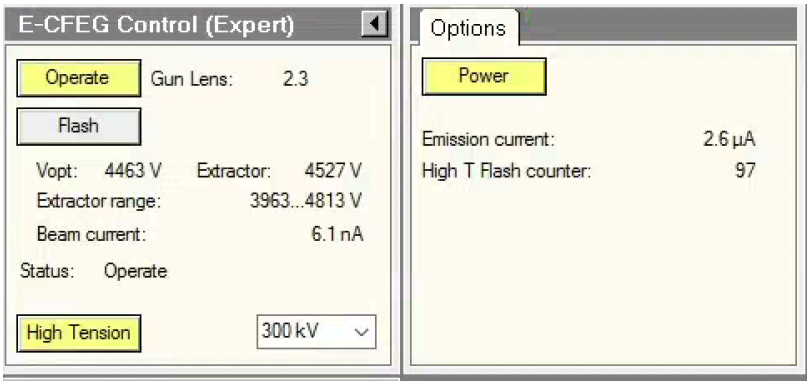
1. **<u>On the day of data collection</u>:**
   a. Clip grids for autoloader, if needed. See **Grid Clipping SOP** for details.
   b. Check microscope status.
      i. Is the autogrid cassette in or out of the microscope?
         1. If in, then prepare an cold, empty nanocab to undock the cassette
         2. If out, locate an empty cassette and prepare loading in the relative humidity control lab
   c. Check the temperature panel (all should be in green. If not, let the staff know)



   d. Check the vacuum panel (all should be in green. If not, let the staff know)
   e. **CFEG ONLY**: Check the status of CFEG. The “Operate” box should be in yellow. If not, click “Operate” to turn on the CFEG.



f. Check if any error messages on TEMUI
g. Print out sample safety form
h. Stop **Data Acquisition** of the previous user session via the EPU
i. Close column valves via the TEMUI.

2. Loading grids:

a. See the **Loading SOP**

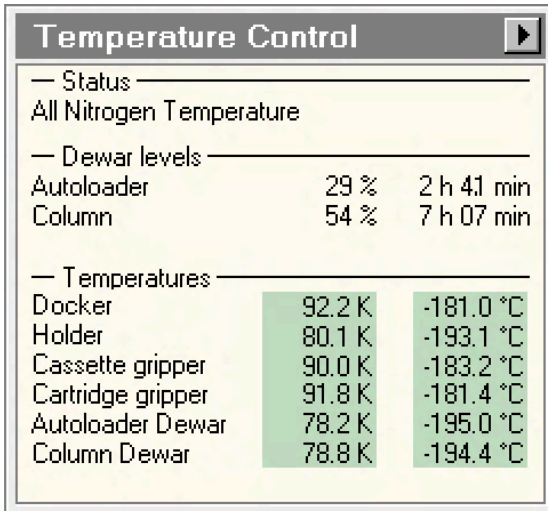
3. On eLogbook:

- Put the instrument to **Standby**
- Create an Experiment with the name “YYYYMMDD-XXXX” where XXXX is the proposal number.
- Add collaborators to the experiment

4. Screening grids using EPU

a. Load and Inventory grids

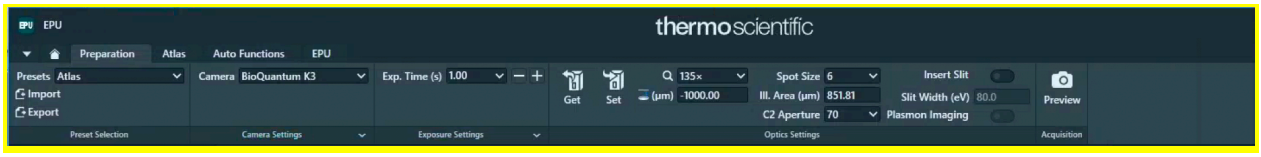
- After grids are loaded, wait for all temperatures statuses to be green AND <-160 C. The temperatures should recover to green like the image below



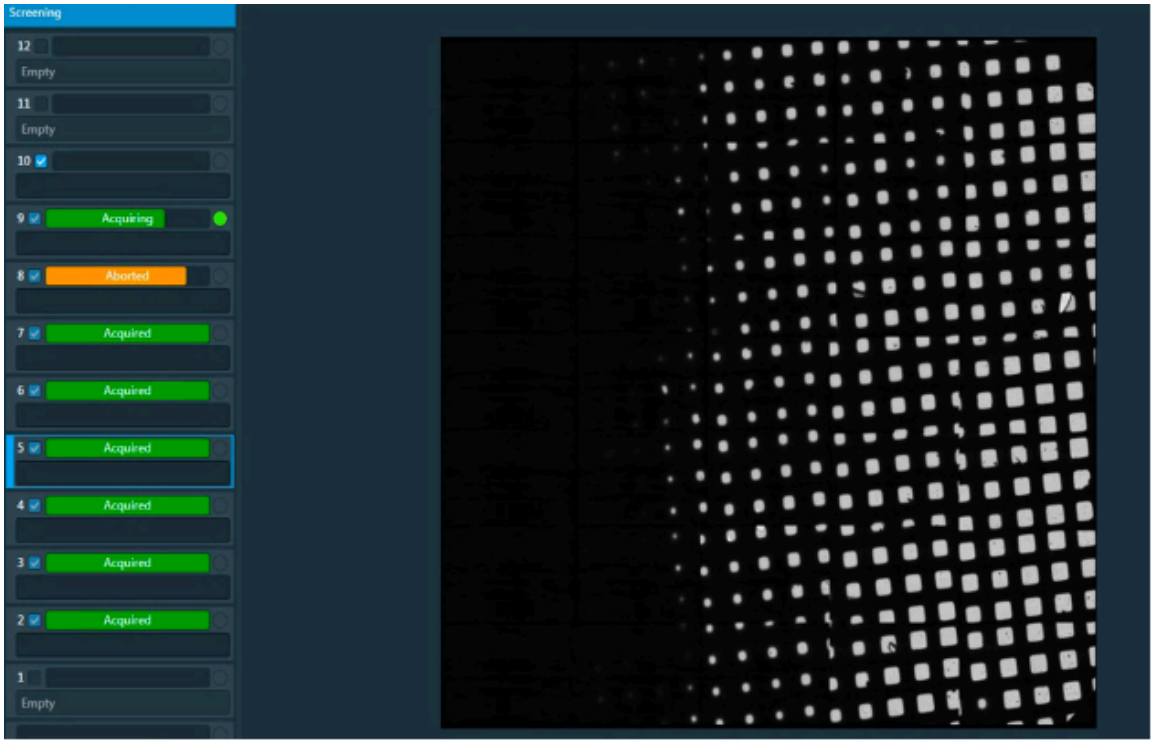
- Select **Inventory** to register occupied slots in the cassette with the TEMUI.
- Label grids with the user defined sample labels.

b. Creating a grid map or low magnification montage

- Open **EPU** software
- In **Preparation** tab, set up the magnification of **Atlas** preset
  i). **Camera setting**: EF-CCD, Binning = 2, Readout = Full, Exp Time = 1 s
  ii). **Optic setting:** magnification = 135x (any magnification around 100x would work, 135x is preferred), Defocus = -1000 um, Spot Size = 6 or more, insert slit (Yes, with 80 eV as the width)

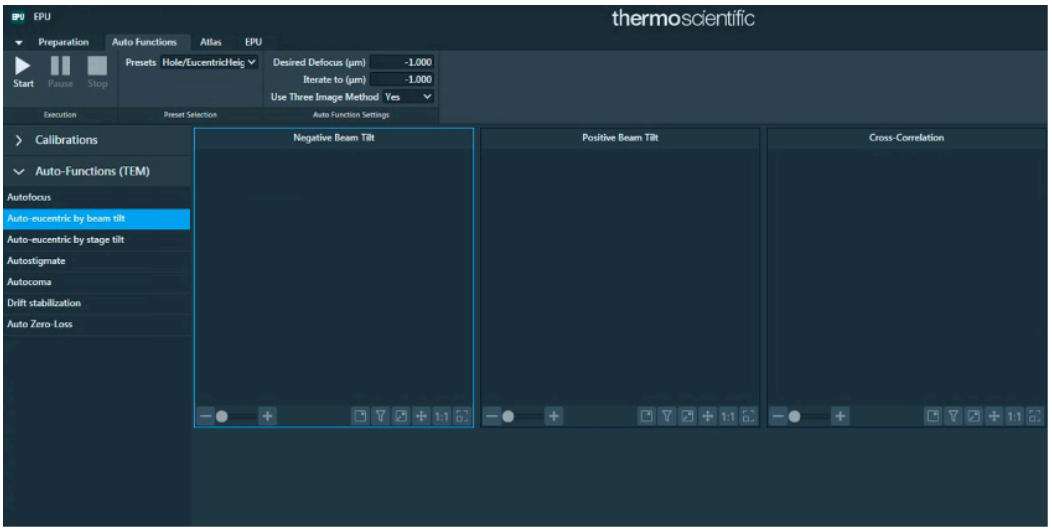


- **Set** the microscope at **Atlas** preset. Open column valves, and check beam on the FluScreen (press **R1** to put screen down). Beam should be centered over the green circle (i.e., GIF entry aperture).
  (*If there is a beam shift, in the **Tune** tab go to subsection **Direct Alignments** then select **Beam shift***
  *On the hand panel, use **MF-X** and **MF-Y** knobs to center beam over the green circle*
  *Click **Done** after adjusted*
  *Press **R1** again to retract the screen*)
- Take a **Preview** at the **Atlas** preset. Image should not be too bright nor too dim.
- Select the **Atlas** tab > **Session Setup** task.
- Select **New Session,** and enter a **Name** for the session
  i). **Session Name** should be yearmonthday-proposalnumber-atlas, for example 20210414-CT21-atlas
  ii). **Image format** = MRC, **Output folder** = X (or Z), click **Apply**
- In the **Screening** section, check the cartridges with grids and select **Start** to begin (the turbo pump is always on for atlasing)
- After Atlasing, evaluate ice thickness and grid quality (thick, damaged, level of transfer contamination). A good grid should have at least three quarters of thin ice area. An example atlas map is shown below.



c. Load grid of interest to screen particles

- Load the grids of interest by by clicking **Load from sample**
- When loading is done, choose a square (usually a thinner square), right click, then **Move stage here**
- **Screen and set up presets in Preparation tab**: set up the optics of each presets, such as DataAcquisition, Hole/EucentricHeight, and GridSquare, and screen sample
  i). Select **GridSquare** preset and take a preview. Adjust the magnification so that you only see one square without much cutoff;
  ii). Go to **AutoFunctions**, select Hole/EucentricHeight prest and run **Auto-eucentric by beam tilt** to find the eucentric height;



iii). Go to **Preparation** tab and take a preview at **Hole/EucentricHeight** preset;
iv). Autofocus: this is performed before capturing an image at **DataAquisitio**n preset. Move stage on carbon at **Hole/EucentricHeight** preset, and run **Autofocus** task with **Autofocus** preset under **AutoFunctions** tab.
v). At **Hole/EucentricHeight** preset, 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;
vi). Evaluate ice thickness, particle density, homogeneity etc.
vii). Screen at least one more hole on the same square and a couple more squares on the same grid. Load the next grid to screen if needed

d. Prepare for Image Shift Calibration

- After screening and all presets are determined, Image shift calibration is required for each session
- In the atlas map, find a feature, like a piece of ice contaminant
- Select **Preparation** tab > **Acquisition and Optics** task.
- In the **Preset Selection** section of the Ribbon Bar, set **Presets** as **GridSquare**, and **Preview**
- Move stage close to the feature and run **Auto-eucentric by beam tilt** with Hole/EucentricHeight preset under **Autofunctions**
- **Preview** at Hole/EucentricHeight and make sure you can find the feature and center it in the image
- **Preview** at DataAcquisition preset and make sure the feature is visible
- Select **Preparation** tab > **Calibrate Image Shifts** task, and click **Acquire.** Once four images have been taken, use the stage position of the image under the DataAcquisition magnification to calibrate the other mags. Double click to make a new crosshair to set the new position.

5. Data collection on the Krios using EPU

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*Before automated data collection, an Atlas or grid map must be created. The settings of each presets in the **Preparation** tab are completed.*

a. GIF Tune and Gain Reference

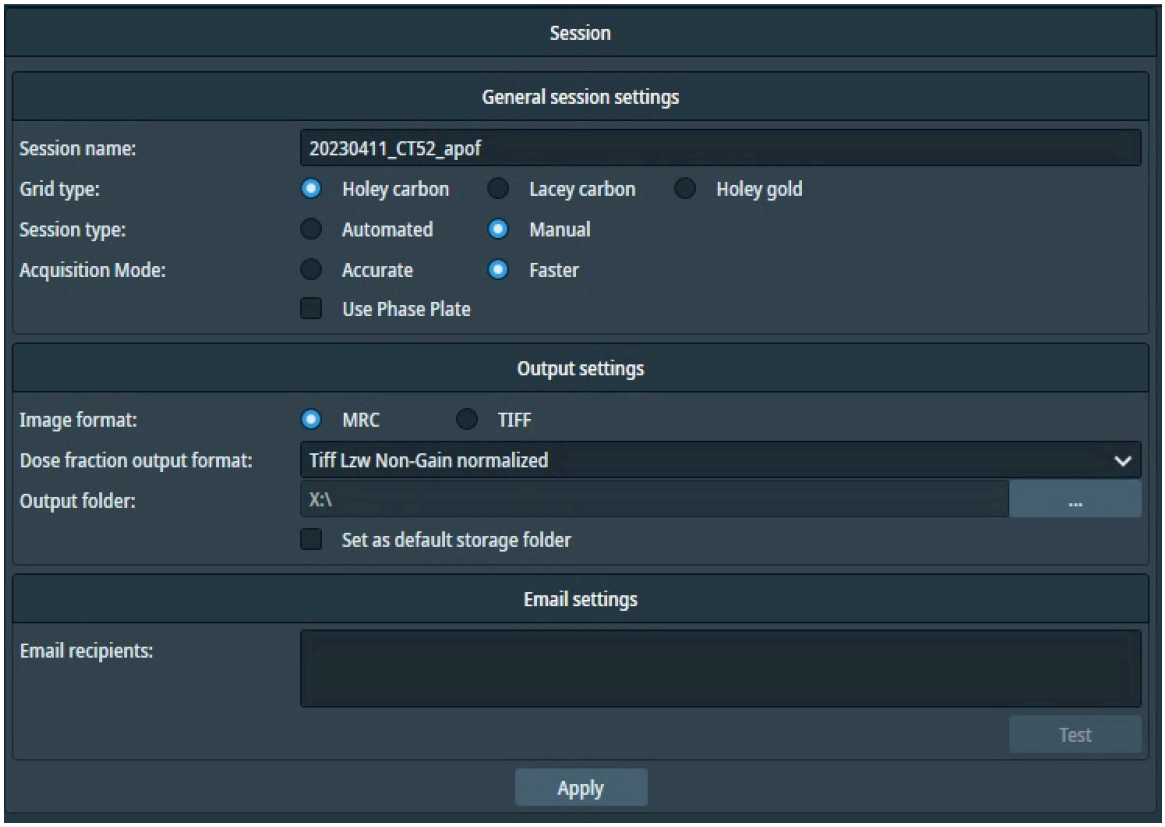
- Move the stage to an empty square and set the microscope to the same optic setting as in **Data Acquisition** preset
- Insert the screen, change Spot Size to 1, and C2 aperture to 150 μm
- In **Digital Micrograph**, select **Tune GIF** and click **Full Tune.** This will take about 15 minutes.
- After GIF Tune, under **Camera** tab select **Prepare Gain Reference**
- Follow instructions to collect linear first and then counting-mode gain reference. Put down FluScreen (R1), use **Spot Size** and **Intensity** knob to adjust the dose rate.

b. Measure Dose rate and Dose fractionation

- In an empty square, 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 dose rate using **Intensity** knob. For the K3 camera, 15-25 e^-/pixel/s is desired.
- Based on the dose rate, calculate exposure time to yield a cumulative exposure of ~40-50 $e^-/Å^2$. Determine the number of frames. 1-1.5 electrons/A^2/frame is commonly used.

c. Square and hole selection

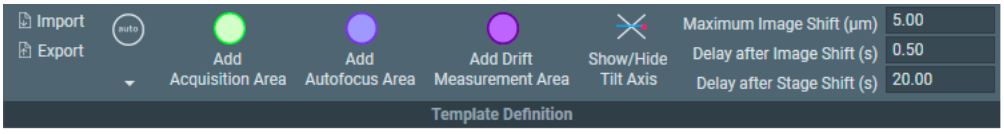
- Select the **EPU** tab > **Session Creation** task. Select **New Session**
- In the **Session Settings**, input names and select appropriate parameters and click **Apply**



- Under **EPU** tab, select **Square Selection** task. **Unselect All** if needed.
- Right click on a good grid square, select **Select**. Add at least 10-20 squares and select **Move to Grid Square** to Square 1
- Under **Hole Selection** task, click **Auto Eucentric**
- Select **Measure Hole Size,** adjust circles to match the actual holes
- Select **Find Holes**
- Use the **Filter Ice Quality** histogram to adjust the boundary of ice
- Select **Remove Areas Close To Grid Bar**
- To remove multiple foil holes, select **Selection Brush**
- Remove holes with contaminations or cracks

d. <u>Template Definition</u>

- Select the **EPU** tab > **Template Definition** task.
- Select **Acquire** to acquire an image
- Select **Find and Center Hole**
- Create a template by using the functions in the ribbon bar



- Add **Add Acquisition Area** in the image display

- Define a **Defocus List** and ensure each acquisition area has the same defocus range
- Add **Add Autofocus Area** in the image display
- In the **Autofocus Area Settings**, set **Recurrence** to **After Distance** and input 8 µm
- Optional: Add **Add Drift Measurement Area** in the image display and specify **Recurrence** as **Every Square**
- Doublecheck all the settings to make sure there is no mistake
- Under **Session Information**, click the calendar button to set up the time.
- Go back to **Hole Selection** and prepare the rest of squares
- Clean up hole selection in each square removing unwanted holes
- If more exposures are needed, go back to **Square Selection** task to continue selecting squares and holes

6. Microscope Alignment

Use Direct Beam Alignment in TEMUI and AutoFunctions in EPU

- Navigate the stage to a square that can be used for alignment.
- In **EPU**, under **AutoFuncitons** tab, select **AutoEucentric by beam tilt**
- **Preview** at **Hole/EucentricHeight,** and move stage to carbon
- In **EPU**, under **AutoFuncitons** tab, select **AutoFocus** using **AutoFocus** preset
- In **TEMUI**, Under **Tune** tab, align Beam Shift, pivot points and C2 aperture if needed
- Under **AutoFuncitons** tab, perform **AutoStigmitism** using **Thon Ring** preset
- Under **AutoFuncitons** tab, perform **AutoComa** using **Thon Ring** preset
- Insert **Objective** aperture, put FluScreen down, and press **Diffraction**
- Use Multifunction X and Y to center the aperture. Exit **Diffraction** mode
- Perform **AutoStigmitism** using **Thon Ring** preset again

7. eLogbook:

- Add sample with imaging conditions
- Switch the experiment on and activate the sample

8. Start Data Acquisition:

- Select the **EPU** tab > **Automated Acquisition** task
- Enable “collecting dark reference every hour”
- Select **Start Run**