UTSW CEMF EPU protocol for Krios

All icons and menus in EPU appear in Blue Font, Dan's Tips and Notes appear in Red font

Main steps for quick reference:

1.Make sure microscope is properly aligned and energy filter is tuned
2. Open EPU, center all beams, double check settings
3. Do image shift calibration
4. Set up an Atlas
5. Set up EPU session
6. Square selection
7. Hole selection
8. Define the Template
9. Test the template
10. Final alignments
11. Automated data collection

1.Make sure microscope is properly aligned and energy filter is tuned
-In EFTEM mode
-EFCCD (K2/3) camera selected
-column valves open
-standard grid still on the stage

2. Open EPU, center all beams, double check settings

(Beams will be centered on the standard grid to avoid dose damage to samples of interest)

- In the Preparation tab, under the Tasks menu (bottom left) click on the Acquisition and optics settings button tab (this should appear by default when opening EPU). Select each of the individual beams from the presets dropdown and use the Set button to set the microscope to the preset conditions corresponding to the desired beam
-Observe the beam on the FLU screen and make necessary changes i.e. Center with beam shift, spread or condense to desired illumination area, stigmate etc.
-Once the beam is in the desired state, use the Get button to save the current beam settings to EPU

-If desired, a preview acquisition can be taken with the Preview button to ensure that the image will be good
-Repeat this process for each beam, and check/double check!

*Note: You may need to use the track ball to assign a user shift instead of an Align shift (using beam shift MF XY). This will be done to add a shift to one beam, condition individually. DO NOT add any extra user shift to your Data Acquisition beam

Example settings for each beam:
All will use K2/3 camera with full readout

Beam	Mag.	Exp. time	Dose Frac.	Camera Mode	Probe Mode	Binning	Ill. Area	Spot Size	EF Slit
Atlas	220X	1s	No	Either	Micro	2	640µm	9	No
GridSquare	940X	1s	No	Either	Micro	1	324µm	9	No
Hole/Euc	15000X	1s	No	Counted	Nano	1	16.7µm	9	No
Data Acq.	130kX	10s	.4/s	Super	Nano	0.5	0.75µm	9	30
Autofocus	130kx	1s	No	counted	Nano	1	0.75µm	9	30
Drift	130kx	1s	No	counted	Nano	1	0.75µm	9	30
Zero Loss	130kx	1s	No	Linear	Nano	1	1.00	5	30

3. Do image shift calibration

*Note: It can be easier to find a feature on the standard grid at lower mag, then increase mag until the data acquisition mag (130kx) is reached. Then Set the microscope to the data acquisition settings and begin the image shift calibration with a feature already centered. Corners and points work best as the feature will scale with the mag and always be observable

-Center a feature at 130kx

-Click on Calibrate Image Shifts under Tasks. Click Start Calibration. (you should your feature, if image is black, check camera blanking, screen, ZLP). If not centered, double-click on the feature to center a second crosshair and click Re-acquire

-Once feature is centered, click proceed

-Double click to center feature, scroll to zoom in

-Proceed until calibration is complete

*Note: the procedure will go through all of your mags, in our case 130kx, 15kx, 940x, 220x. Your crosshair centering should be extremely accurate between 130kx and 15kx, but being off by a few nm between 940x and 220x will be negligible

4. Set up an Atlas

-Click the Atlas tab
-Click New Sample (User name appears in the Name of session)
-Choose output folder by clicking on Output Folder (save in your KEEP folder on Dose fractions)
-Click Apply
-Click Atlas acquisition, then check settings. If number of tiles is not selected the default is to image the entire grid and can be stopped at any time by clicking Stop. Close to center starts Atlas collection close to the center of the grid, Close to current starts Atlas acquisition close to the current stage position
-Click Acquire (10-25 mins collection time depending on settings)

*Note: It is often desirable to quickly pre-screen the grid for ice thickness/quality and particle distribution using Low Dose Mode in the UI prior to setting up the Atlas

5. Set up EPU session

-Click on the EPU Tab
-Click on New Session (Session name will show user name)
-make sure Manual Selection is selected
-Input person number (or desired user name under which the files will be saved) where it says User name
-Select output format MRC
-Select Unnormalized packed with gain reference files under Dose fractions output format. (Gain normalization increases image data size and should be done on another computer, may not be relevant for K3 supposed 10TB storage)
-Click Modify and select your KEEP folder
-Select Quantifoil, and Quantifoil Type: Quantifoil R1.2/1.3. Hole diameter should and spacing automatically change
-Click Apply

6. Square selection

-Click on the Square Selection tab under Tasks (Your Atlas should show up with all squares highlighted in green)
-Click Unselect All to deselect all squares
-Right click and click add to select squares of interest (They should turn green)

7. Hole selection

-Right click on the first grid square and select Move stage to gridsquare or right click on the square and select Move stage here

-Set the hole/eucentric beam (15kx) in the Acquisition and Optics Settings under the Preparation tab

-Quickly put the screen down and adjust the eucentric height, then put screen up

*Note You must do this for each square of interest before taking the hole selection picture for that particular square. The automated eccentricity function in EPU takes much longer. Alternatively, one can do the eucentric height and record the X,Y,Z information In the Stage tab of the UI prior to hole selection. This can double as an opportunity to screen particle distribution in UI Low Dose Mode

-Click on the Hole Selection tab under Tasks

-Click Acquire (Center image if need be by right clicking on the center of the square and selecting Move stage here, then click Acquire. If the square is very far from being centered, the image shift calibrations may need to be redone)

-Select/change hole size by clicking on Measure hole size and dragging the yellow circles, then place each circle on neighboring holes

- Deselect dark or undesirable holes by moving the vertical bounds of the Filter Ice Quality panel on the bottom right, and by clicking the Selection Brush icon. (hold the shift key and scroll to change the size of the selection brush)

-Repeat for every selected grid square. (number of selected holes will appear in the bottom right)

8. Define the Template

-Click on the Template Definition tab under Tasks

-Click Acquire

-Click Find and Center Hole

*Note: If yellow ring is not centered because it is too big or too small, go back to the Hole Selection tab and adjust the hole size by clicking on the Measure Hole Size icon

-Click Add Acquisition area and then click the hole image to add the area, move this area to the desired location. Multiple acquisition areas can be added, at 130K the standard for CEMF is 3/hole arranged in a triangle

-For each Acquisition area select the desired defocus targets (three are usually enough, an example would be -1µm, -1.5 µm, -2 µm)

*Note: The purpose of using the different defocus numbers is to ensure that the CTF does not zero out in the same place in every image, thus omitting data of certain frequencies

from the final reconstruction. It is also preferable to select the same defocus numbers in the same order, so focus will not need to be changed within a hole after autofocusing

*Note: when using the phase plate -100-300nm is optimal for all exposures

-Click Show/Hide Tilt Axis to reveal the tilt axis of the stage
-Click Add Autofocus area icon and then click the hole image to add an autofocus area on the carbon along the tilt axis
-Click Add Drift Measurement Area icon and then click the hole image to add a drift measurement area somewhere on the carbon, then drag the drift measurement area on top of the autofocus area. Having both Autofocus and drift in the same place just simplifies the process, but is not required

*Note: It is important to have the autofocus area somewhere on the tilt axis so changes in tilt do not change the height of the grid. This ensures that the autofocus measurements are valid for the hole, even though the autofocus operation are performed 1-3 microns away from the hole

9. Test the template
-Click on the Template Execution Tab and click Preview to go through one cycle of your template. All images should be in focus at the desired area, if there are any issues, go back to the Template Definition tab and fix them before proceeding

10. Final alignments
-Recheck the Objective Stigmation, and Coma free alignments using the K2 camera and Digital Micrograph
-Update the hardware dark reference (dark ref should be updated every 4hrs)
-Do a full tune of the energy filter
(This will automatically also center your Zero Loss Peak and should be repeated every 8-12 hours. ZLP centering can be automated in the Automated Acquisition tab in the Auto Zero Loss Panel)
-If using the phase plate, set the phase plate to move to the next area every 30-40 images. This is done under the Automated Acquisition tab in the Phase Plate Panel where it says Periodicity (Exposures)

11. Automated data collection
-Click on Start run
-Observe the first few holes to make sure data collection is progressing smoothly
-The data collection can be paused to update the hardware dark reference, and tune the energy filter