

# Arctica data acquisition using EPU

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## Starting your session

1. Consider which imaging conditions you wish to use for your experiment (magnification, spot size, etc). Review the [list of imaging conditions](#) (last page here).
2. Enable the microscope in [FOM](#).
3. Fill out the [logbook](#) linked here or bookmarked on the data analysis computer.
4. Make sure the microscope is in EFTEM mode (under “EFTEM” tab click “EFTEM” button in the top panel).
5. Open EPU from the start bar of the microscope PC. Click the “Import” button at top left, under “Preparation” tab and import settings for the Record mag you’ve chosen and grid type (if known). Default files can be found under “Documents\EPU Settings”. You can also save your own settings files here in the “User settings” folder if you’d like them saved locally.
6. If you’ve just loaded samples, wait for all temperatures  $<-165^{\circ}\text{C}$ , then do the inventory.

## Preparing the K3 and GIF

1. **Find a hole** in the sample, or do the gain ref and tuning without a grid loaded on the stage.
2. **Set up the beam:** Switch to the Data Acquisition preset in EPU and click “Set”. Insert the 150 um C2 aperture. On the fluscreen, center the beam with the trackball or beam shift direct alignment. Lift the screen (R1 button on the right hand panel).
3. **Set up the software:** Open GMS. In the “Technique Manager” window at the far right of the UI select TEM Imaging if it isn’t already up. Click “1x” binning, then click “View”. You may have to wait for the K3 to insert. Remove the slit in the “Filter Control” window at the right of the GMS UI by unchecking the box labeled “Slit in”.
4. **Take a new gain reference:** Click “Prepare gain reference” under “Camera” menu. **Skip Linear** mode, but collect for Counted. Use all the default settings for the gain reference (leave boxes unchecked). Set the beam intensity to the value requested by GMS (20 electrons per pixel per second). Wait for gain reference to complete (~5 minutes).
  - a. If “Camera” is not visible, go to Help -> User Mode and select “Power User”.
5. **Quick Tune the GIF:** Stop any camera view in GMS. Select the “Linear” button in the Technique manager window and switch to 1x binning. Reduce the spot size to 2 (L3 button on hand panel). Turn dose to **150-200 electrons/pixel/sec** using the intensity knob. Click “Tune GIF” under “Tune GIF” window at the left of the UI. Select “Quick Tune”. Wait for tuning to finish. (We switched to quick tune only at the start of 2021 - Katie does a “Full Tune” once a week.)
6. Click “Center ZLP” under “Tune GIF” menu on the left.
7. Switch back to Counted, 1x binned mode in the camera controls at right.

# Beam Alignments

1. Find an intact square of film on the cross-grating.
2. In EPU, switch to "GridSquare" preset. Take a preview image and verify that the square is centered. You may have to center the beam with beam shift direct alignment. Click the "Auto Functions" tab, click "Auto-eucentric by stage tilt" and press "Start" at the top left.
  - a. This task will fail a fair amount of the time - as it's running, check the Z-height at the bottom right of the microscope UI (on the main monitor). If it fails, the scope will set the Z-height back to zero.
    - If the task fails, you can manually set the height in the scope UI: under "Search" tab -> in the Stage flap-out -> under the "Set" tab, type this Z height in the Z box and click "Go To".
    - Fine-tune the eucentric height using "Auto-eucentric by beam tilt" at the "Hole/Eucentric" preset
3. Click the "Eucentric Focus" button on the right hand panel.
4. Lower the fluscreen and change to Data Acquisition preset. Click "Set". In the microscope UI, go to the "Align" tab. Condense the beam using the intensity knob to the smallest spot. Do the following alignments with the beam condensed:
  - a. Center C2 aperture
  - b. nP Beam tilt pp X and Y
  - c. Beam shift
5. Set parallel illumination conditions. Click "Set" again in EPU. Click into diffraction mode using "Diffraction" button on the right hand panel. EPU will pop up a warning, ignore it for now.
  - a. Roughly focus the diffraction pattern using the intensity knob. The diffraction pattern usually looks best in "HDR" mode (option at the bottom of the flucam viewer).
  - b. Insert an objective aperture. Adjust the focus knob until the edges of the aperture look sharp. Some tricks:
    - It can help to use a focus step ~4 to easily view changes in the aperture focus.
    - Overlap the edge of the aperture with the central spot of the diffraction pattern. Look for a small bit of intensity "bleed" outside the aperture from the central spot.
  - c. When you are satisfied, retract the objective aperture and finely focus the diffraction pattern using the intensity knob. It can help to look at the FFT (click "FFT" button below the center of the FluCam image).
  - d. Insert and center the objective aperture you wish to use for imaging (usually 100 um).
6. Click out of diffraction mode. In EPU, click "Yes" in the pop up notification. Click "Get" to save the new C2%.
7. Use beam shift direct alignment without condensing beam to center the record-mode beam on the GIF entrance aperture.

8. Check focus: Click “View” in GMS. Open the live FFT (if not already open) under Process->Live->Life FFT. Focus the image using the focus knob, then go slightly under focus to see the first few dark fringes/Thon rings in the FFT.
9. Set Objective stig: Use the Objective stigmator (scope UI, Align tab, Stigmator box, Objective button) to make Thon rings of the FFT perfectly round. To very carefully check the astigmatism, you can take a Capture image and look at its FFT to see if further adjustments should be made in View mode.
10. Coma-Free alignment: Start View in GMS and toggle the “Coma-free Alignment X” in microscope UI. Look at the FFT, and change MF X knob slowly until the defocus at both sides of the beam wobble appears the same. An apparent change in the stigmatism between the wobbles is ok. Repeat for “Coma-free Alignment Y”. You can change the defocus during these alignments to better see the changes in the FFT.
11. In EPU, copy Record settings to Autofocus by selecting that preset in the dropdown and clicking “Get”.
12. If you plan to use Drift Measurement, copy the beam settings from Data Acquisition by using “Get”. Make sure the exposure time is short (around 0.2 s).
13. For the Zero Loss preset, use the same intensity and magnification as Data Acquisition, but drop the spot size by 1 (usually 3 for 69kx and 2 for 79kx). Try an acquisition time of 0.5 seconds. **New:** Test the Zero Loss settings under Auto Functions -> Auto Zero-Loss. If the procedure is unsuccessful, ask Katie for help.
14. Click “Export” and save your EPU settings to your data folder.

## Setting up data acquisition in EPU

Load your specimen in the Autoloader menu of the Microscope UI. The microscope will automatically retract the objective aperture and close the column valves. Wait for the loading process to complete (a few minutes). The microscope UI will display “Cartridge loaded” when loading is finished.

Go to the lowest mag in EPU (Atlas preset), “Set”, and open the column valves. Check that you can see thin grid squares on the fluscreen. It can help to switch to the 150 um C2 aperture if you don't see much. If you drive the stage around and don't see many thin squares, change to a different grid. Proceed once you've found a grid with good-looking ice.

## Check imaging presets

Under the “Preparation” tab, check that the magnifications for Atlas, GridSquare and Hole/Eucentric are good for this grid.

1. Center a thin grid square using the stage (want one of the largest-looking squares on your sample).
2. Switch to “Atlas” preset in EPU and click “Preview”. Make sure the C2 aperture selected is 150 um. A magnification of 110x works well. Check that the image looks ok, and adjust exposure time if desired (recommended 0.5-1s).
3. Switch to “GridSquare” preset in EPU and click “Preview”.
4. Switch to AutoFunctions tab and run “Auto-eucentric by stage tilt”.

5. Check that the square fills most of the image, but usable holes are not cut off at the top and bottom. If this is the case, reduce the magnification by one step in the dropdown and check again. If there are holes you can't see in the square image, you won't be able to collect on them. The optimal mag here will depend on both the grid mesh and whether thick ice has accumulated around the edges of the holes.
6. Switch to "Hole/Eucentric" preset and click "Preview". Right click in the center of a hole and select "Move stage here and acquire". Repeat until the hole is well-centered.
7. The Hole/Eucentric image should show all of one Quantifoil hole and parts of the nearest neighbors. Increase the mag if more than one full hole is visible; decrease if not much of the nearest neighbors can be seen. The optimal mag should be the same each time you use the same Quantifoil spacing (eg 1.2/1.3 or 2/2). For this reason, the hole spacings are sometimes saved in the name of the image settings files (though it may take us some time to populate the whole list).
8. Check defocus settings for the Hole/Eucentric preset: If you are using gold foil, change the defocus to 0. If using carbon, try -15 um. This can be changed later if it seems like hole centering is not working well.
9. Click "Export" and update the saved EPU settings in your data folder.

## Calibrate Image Shifts

This step can be done before or after taking an Atlas. If you are fairly certain you want to collect data on this grid, do it before the Atlas. Otherwise the Atlas can be done first.

1. Find a grid square with some large schmutz that will be visible at Atlas mag and also findable at Data Acquisition mag. This can be in the same grid square you used in the last section.
2. Go to eucentric height using "Auto eucentric by stage tilt" (as above, if you aren't already at eucentric for that square)
3. Move the stage to the schmutz, switch to the "Hole/Eucentric" preset and take a Preview image. Re-center the schmutz using "Move stage and Acquire" until it is centered.
4. Switch to "Data Acquisition" preset and repeat step 3.
5. Click on "Calibrate Image Shifts" at the right. At the top, click "Start Calibration".
6. Follow the directions at right to run the procedure.

## Take an Atlas

1. Click on the Atlas tab. Select "New Session". Give the session an appropriate name, and save it to your user folder in the "Users" directory. Any file type you like is fine.
2. Click on the "Screening" button at left. Check the box next to each grid you would like to Atlas. If you only want to map the current grid, only check its box. If multiple grids are selected, EPU will load each grid and take an atlas.
3. Leave the "Number of tiles" box empty to atlas the whole grid.
4. Click "Start". EPU should automatically retract any objective aperture and reinsert it when acquisition is completed. Wait for atlas acquisition to finish. If you want to view the atlas as it's being collected, click on the slot position.

5. **New:** EPU should automatically detect and categorize grid squares by intensity (this may take a few seconds). At the top of the window, check the box next to each category that you would like to use in your EPU session. These squares will be available to select in the next steps. If EPU does not detect any grid squares, ask Katie for help.

## Measure Dose and Set Data Acquisition Parameters

1. Put the 50 um C2 aperture and objective aperture back in if necessary.
2. Find a sizable hole in the specimen (a grid square with no film for example) using your Atlas, right click and move stage to that position. Go back to the Preparation tab and select the Data Acquisition preset. Take one preview image to make sure you're in a hole.
3. In Digital Micrograph, take a **1x binned counted mode** "Capture" image of at least one second. Look at the bottom of the program for the dose rate in electrons per pixel per second. Convert this to electrons per square angstrom per second by dividing by the pixel size squared (or use the spreadsheet).
4. Now that you know the dose rate, determine the total exposure time and number of frames you wish to use. Change the preset settings for "Data Acquisition" to your desired values. Click "Export" and save your imaging settings again.

## Start an EPU session

1. Click the EPU tab at the top and select "New Session".
2. Give your EPU session a name that **starts with your netID** for example: "kas466\_191203\_EPU\_sample". This is recommended due to the way that EPU saves the K3 movies - they are in the top level of the "Users" directory, so starting with your netID keeps things organized and lets you see which sessions are your own more easily.
3. Choose "Manual" and "Accurate hole centering".
4. Image format: choose "Tiff LZW". To save space, you can save the images without gain correction but **you must manually copy the current gain reference file from the Gatan PC to your data folder for the day**. EPU will try to copy the gain but it saves it with the wrong bit-depth. The gain reference is located on the Gatan computer under C:\Program Data\Gatan\Reference Images\K3-18030008 Gain Ref X1.m1.dm4 (it should be the largest file). You can use the program dm2mrc in sbgrid to convert the gain reference to mrc to feed to your motion correction software. The gain reference is flipped from left to right (flip in x or flip around y) relative to the data.
5. For the storage folder, navigate to your sub-folder in the "Users" directory and save it there.
6. Select the appropriate specimen carrier (usually Quantifoil) and choose the correct type if you know it. It's ok if the type is wrong, later when you do "measure hole size" you'll fix it.
7. If desired, enter your email address to receive a message when the automated acquisition run finishes or ends due to an error.
8. Click "Apply".

## Square Selection

1. Click “Square Selection” at left. The newest Atlas is shown on the screen with all findable grid squares highlighted in green. Click “unselect all” at the top, then manually add the squares you want to collect on by right clicking and selecting “Add” or using control+click to add, shift+click to remove.
  - a. **New:** EPU categorizes the grid squares based on intensity. Once you have added some squares, you can click “Smart Extend” to add similar squares. You should also see suggested similar squares with a green-and-white dashed border.
  - b. To get a better view of the grid squares, you can right click and select “Open tile”. This opens the individual piece of the atlas containing that square and shows more detail than the full atlas overview. Right click and “Close tile” to return to the Atlas.
2. You can adjust the processing order by clicking “Show” and then “Change”. Click on the grid squares in the order you would like the scope to visit them.

## Hole Selection

1. In the Square Selection task, right click on a grid square and select “Select Holes”. The stage will move to the grid square and open the Hole Selection task.
2. At top, select “Acquire”. Center the grid square if necessary.
3. Click “Auto Eucentric”. Wait while the auto eucentric by beam tilt task runs. When it’s finished, it will take another grid square overview at the new Z-height. The Z-height should be displayed at the bottom left corner of the display. A green number means Auto Eucentric has been performed.
4. **First square only:** click “Measure hole size” at top. Place the two yellow circles on two **adjacent** quantifoil holes. Resize the circles to match the hole size if necessary.
5. Click “Find holes” at top. If your ice is darker than the surrounding film, select “Allow Dark Foil holes” and click “find holes” again.
6. Add or remove holes using the “Remove holes close to grid bar”, ice/histogram filter, brush tool, or mouse clicks until all the holes you want to collect are selected.
  - a. **New:** If you’d like to use the automated preparation function, use the ice filter to select your squares.
7. Repeat for all the squares you want to collect in, or (**new**) click “Prepare all squares” at the top. This will visit each square in order, determine Eucentric height, and select foil holes based on the ice quality filter set in the first square. You can still go back to each square and manually add/remove holes if desired.

## Template Definition

1. In a square overview map, click one of the holes and select “Move stage to location”. Wait for the stage move to finish.
2. Click “Template definition” task at left.
3. Select “Acquire” to collect a Hole/Eucentric image.

4. Select “Find and Center Hole”. Click Acquire again and verify that the hole is centered well. Otherwise, adjust the Hole/Eucentric imaging preset by changing exposure time, defocus (but not by more than 5  $\mu\text{m}$ , otherwise the mag changes) to find settings for which hole centering works well.
5. Work from left to right in the top bar to define the template for data acquisition.
6. Choose “Add Acquisition Area” and click in the image where you’d like to acquire data (usually the center of the hole, but you can offset it if your sample likes to hang out around the hole edge).
  - a. At right, the defocus list will appear. Add the amounts of defocus you’d like to use for data acquisition to the list. **New:** type them in, separated by a space. A good place to start is -0.8,-1, -1.2, -1.4, -1.6 .
  - b. Specify the maximum image shift and delay time. For data acquisition, recommend a maximum image shift of 2  $\mu\text{m}$ . Keep the Delay after Image Shift short. Specify the Delay after Stage Shift to whatever you like - if you’re using the drift measurement function, a short delay (2-5s) makes sense; otherwise using a longer delay (10-15s) gives the stage time to stabilize.
7. Choose “Add Autofocus Area” and click where you’d like to autofocus. This should be on the foil film next to the hole in question, **not overlapping any other grid holes**. EPU acquires points in a spiral shape, so there’s no way to make it autofocus in areas that have already been acquired. Remember that the beam size is  $\sim 1.7 \mu\text{m}$  with the 50  $\mu\text{m}$  C2 aperture, larger than it’s drawn in EPU.
8. When the autofocus area is selected, you can see options for autofocus at the top right. You can specify whether to autofocus at every hole or every “x” distance (default 10  $\mu\text{m}$ ) to save time. Always use Objective Lens to focus.
9. Choose “Add Drift Measurement Area” if desired, and enter your maximum tolerable drift rate (2  $\text{\AA}$  usually for EPU) and how often you’d like to measure drift. According to the EPU manual, if drift measurement fails it will take 10 minutes to time out and move to the next hole. We haven’t seen this often so far, but if you notice it often it may be better to do drift measurement only once per square or turn it off altogether and use a longer stage delay instead.

## Template Execution

Optional: select “Preview” under this tab, make sure hole centering, autofocus, and data acquisition all working as expected. You can also just start the automated acquisition and watch the first few holes closely.

## Automated Acquisition

1. Before starting an automated run, go to the Data Acquisition preset and check that the objective aperture is centered and run “Center ZLP” from Digital Micrograph.
2. Choose the “Automated Acquisition” task in the EPU tab. At the top, specify “Close column valves” to close the valves when the run finishes.
3. Specify the time interval at which the Auto Zero-Loss procedure should run ( $\sim 4$  hours is usually good).

4. Click “Start Run”. Watch to verify that automated acquisition is running as expected. Stop the collection and adjust parameters if necessary.

## Ending a session

1. Click “Stop” in the Automated Acquisition run.
2. Remove your grid and insert the cross grating grid (slot 1 of the cassette).
3. Disable the microscope in FOM. If you’re not watching when data collection finishes and need Katie to adjust your time, send her an email.
4. Let the next user know if you want to save any of your grids. Make a note in the “Arctica Loading” logbook, and leave labeled grid boxes and tube. You can unload your own grids if there is at least a 4 hour window before the next user (to allow loading stations to dry in the oven).
5. If no other user is scheduled directly after you, start a K3 anneal. Go to “Camera” -> “Temperature” in GMS. Type in 50. The settings for the anneal cycle will pop up. Change the time to 10 hours (or the number of hours until the next user minus 2), and click “Start”. The camera will automatically cool back to -20 when the cycle is complete.

## Image Acquisition Parameters

EFTEM Magnification	minimum C2 Aperture	1x pixel size (Å)	0.5x pixel size	Field of view (nm)	Spot size
100 kx	50 um	0.832	0.416	479.232	2
79 kx	50 um	1.07	0.535	616.32	3
63 kx	50 um	1.34	0.67	771.84	4
49 kx	70 um	1.71	0.855	984.96	5
39kx	70 um	2.16	1.08	1244.16	5

## Appendix: Settings that work well at 63kx

### Data acquisition:

Binning: 1  
 Readout: Full  
 Exp Time: whatever gives desired dose  
 Mode: Counted Super-Resolution (recommended, can also use Counted)  
 Fractions: 50+  
 Imaging mode: Nanoprobe  
 Spot size: 4 for 63kx, 3 for 79kx  
 Intensity: parallel beam

Defocus: usually -2 or -2.5, not used for data collection, only used when you click “preview” in this mode.

### Autofocus:

Binning: 2  
 Readout: half  
 Exp time: as fast as possible with good result, ~0.2 s  
 Mode: Counted  
 Mag: same as data acq  
 Imaging mode: Nanoprobe  
 Spot size: 4  
 Intensity: same as Data acq



Defocus: -5 (?)  
Drift Measurement:  
Mag: same as data acq or higher  
Binning: 1 (or 2)  
Readout: half  
Exp time: As fast as possible with  
good result (0.2s?)  
Mode: counted  
Imaging mode: nP  
Spot Size: 4 or 3  
Thon Ring:  
Not used  
Hole/Eucentric:  
Mag: usually around 9600 (R1.2/1.3)  
Spot size: 5  
Intensity: 100  
Grid Square:  
Mag: usually 540x or 690x  
Spot size: 7  
Atlas:  
Mag: 110x  
Spot Size: 8

Intensity: same as Data acq  
Defocus: -2  
Zero Loss:  
Mag: same as data acq  
Binning: 2  
Readout: full  
Exp time: 1 s  
Mode: counted  
Spot size: 3 or 2