BU Glacios-2 automated cryo-EM data acquisition

by Chad Hicks

Obtaining a publication-quality cryoEM structure often requires both high quality sample and a high quality dataset. Obtaining a high-quality dataset requires a precisely aligned microscope adjusted with an optimal set of imaging parameters. This protocol describes a minimal set of steps to obtain a high quality dataset using the BU cryogenic EM core Glacios 2 microscope. Depending on your sample type and imaging requirements, you may need to adjust specific steps or add to this protocol.

1. **Prepare for the session.**
   1. Checkthe Cryogenic EM Core Microsoft Teams group for any updates about the core facility.

* *BU cryogenic EM core staff use the Teams group to provide urgent updates about the state of the facility including instruments errors/malfunctions, and unfavorable room conditions.*
  1. Bring the necessary materials: pre-screened clipped grids, hard drive *(optional if screening grids and not collecting data)*, laptop *(optional)*, jacket *(optional)*.
* *You should screen at least one grid in the same grid freezing session before reserving a data collection time slot to make sure that you are satisfied with the sample quality.*
* *A laptop is useful for taking notes during the session.*
* *Your data must be transferred to a hard drive within 1 week of the data collection. It is best to start the data transfer immediately after starting the automated data collection.*
* *The cryogenic EM core is a bit chilly, so it may be a good idea to bring a jacket.*
  1. Open a blank microscope Microscope Daily Log libreoffice file on the microscope PC (MPC) “C:/Desktop/Microscope \_Daily\_Log/Template.docx”, perform the pre-session checks, annotate the file, and save it to the folder “C:/Desktop/Microscope\_Daily\_Log” using the filename format “[YYYYMMDD]\_MDL\_[FirstName]\_[LastName].odt” (ex. “20240714\_MDL\_Chad\_Hicks.odt”). If there is more than one user in a single day, they should also add their names to the end of the file name.
  2. Continue to write in the Microscope Daily Log to record any errors or problems that you encountered during your data collection session. Make sure to take and attach screenshots of these errors or problems to the Microscope Daily Log document.

1. **Unload grids from the microscope**

**\*Skip this section if there is no cassette in the microscope.**

* 1. Coordinate with core facility staff and the previous user and make a plan for unloading the grids currently on the microscope and loading your grids.
* *You may need to stop a previous user’s data collection if it is ongoing, their microscope session has ended, and you do not get a response to them after messaging them.*
* *Some users may like to unload grids themselves while others may be okay with you unloading their grids and storing them.*
* *Some users may not want to save their grids. If unclear, always err on the side of caution and save the grids.*
  1. Make sure that the cross-grating (XG) grid is on the microscope stage. If it is not then load the cross-grating grid to the stage using EPU.
* *As a core facility policy, the XG grid should remain on the stage during grid unloading/loading and you should always leave slot 1 empty in the cassette for the cross grating grid.* 
  1. Fill a dry dewer with LN2 and use it to cool down transfer station and nanocab.
  2. Make sure column valves are closed (the microscope on-screen display should say AVAILABLE in green) and insert the nanocab into the microscope autoloader, click ”Undock”, and wait for the message “nanonab can be removed”.
  3. Remove the nanocab, attach it to the transfer station, and use the cassette gripper to move the cassette to the transfer station.
  4. Store unloaded clipped grids in the appropriate slots in the correct grid boxes and transfer to the core facility grid storage, as indicated on the previous Microscope Daily Log.

1. **Load grids into the microscope.**
   1. Cool down the transfer station and nanocab if you haven’t already, and transfer the clipped grids inside their grid boxes to the transfer station.
   2. Load clipped grids into the cassette. Remember to leave slot 1 empty for the cross-grating grid.

Detailed instructions:

* + 1. Place the clipped grid into the blue-colored groove and rock it back and forth gently to ensure that it is properly clipped.
  + *Improperly clipped grids could fall apart in the microscope which would require opening the column and removing the clipped grid pieces leading to extensive microscope down-time.* 
    1. Grasp the whole clipped grid with the clipped grid tweezers and insert it into the appropriate cassette slot with tweezers vertical.
  + *The clipped grid will not sit correctly in the cassette slot if the clipped grid is inserted into the cassette slot at an angle.*
  + *Orienting the clipped grid so that the clip ring faces the cassette gripper handle will ensure that the microscope can properly grip each clipped grid.*
    1. Transfer remaining clipped grids to the cassette.
  1. Attach the nanocab to the transfer station and transfer the cassette to the nanocab using the cassette gripper handle.
  2. Remove the nanocab from the transfer station, ensure that the cassette is at the bottom of the nanocab by pulling up on the silver pin, insert the nanocab into the microscope, click “Dock”, and wait for the message “Nanocab can be removed”.
  3. Remove the nanocab, dump the remaining LN2, and dry the tools using the dehydrating oven.
* *The dehydrating oven should be set to 104°F or less to make sure that the tools and foam do not get too hot.*
* *You can also continue on to the next few steps of this protocol and clean up the station as soon as you reach a good waiting point in the microscope setup.*

1. **Prepare the microscope for imaging.**
   1. Wait for the microscope cartridge gripper to cool below -175degC, then take an inventory of the loaded cassette in the TEM User Interface (TEM UI) by clicking “Autoloader 🡪 Autoloader (User) 🡪 Pop-up side panel 🡪 Inventory”.
   2. Label the grids with their appropriate identifying information in the text field adjacent to their respective slot number in the TEM UI in “Autoloader 🡪 Autoloader (User)”.
   3. ***A table with black text and black text

      Description automatically generated***Import the appropriate EPU preset in EPU by clicking “Preparation 🡪 Import” and selecting from the list in “C:/Users/Supervisor/Desktop/Presets”. The core facility default preset “YYYYMMDD\_130kx-data\_11,5kx-hole\_690x-square\_20umC2.sxml” will work well for most single particle imaging needs.

* *If you need to adjust the EPU preset parameters outside of what is described in the protocol please let the core facility staff know so that they can provide assistance.*
* *The actual pixel size of the images differs from nominal pixel size in EPU. Please use the table on the right when trying to determine the actual pixel size for a specific microscope magnification.*
  1. Center the beam over the energy filter aperture using beam shift direct alignments at the Grid Square magnification preset optics.
* ***Do not attempt*** *to adjust the beam shift alignment for the atlas magnification. The beam will never look perfectly centered on the flu screen because the physical hardware positions of the differential pumping apertures block edges of the beam at the atlas preset magnification. If you are seeing beam edges at the atlas preset images, please ask core facility staff to see if they can correct it.*
* *If you accidentally move the beam using the multifunction knobs or trackball when you are not in direct alignments adjustment mode, you can clear the user defined change by clicking “Column🡪 Beam Settings 🡪 Reset Beam” in the TEM UI.*

Detailed instructions:

* + 1. Set the microscope optics to grid square preset optics in EPU by clicking “Preparation 🡪 Presets (Grid Square) 🡪 Set”.
    2. Click “Insert screen” in the TEM UI.
    3. Open column valves by clicking “Autoloader 🡪 Vacuum (User) 🡪 Col. Valves Closed”. You should see a beam.
* *The button will be yellow if the column valves are closed and gray if the column valves are open.*
  + 1. Enter the beam shift direct alignment mode in the TEM UI by clicking “Align 🡪 Direct Alignments 🡪 Beam shift”.
    2. Use the Multifunction X and Multifunction Y knobs on the microscope control pads to center the beam over the energy filter aperture (green circle), then click “Done”.
* *You may need to click the “EF” icon on the top bar of the Flucam Viewer to make the energy filter aperture appear on the screen.*
* *You can use the joystick on the control pad to move the stage to better see the beam if the grid bars are in the way.*
  1. Repeat the beam shift direct alignments at the hole/eucentric height magnification preset optics in EPU by clicking “Preparation 🡪 Presets (Hole/EucentricHeight) 🡪 Set” and following the rest of the procedure that you performed to center the beam at the grid square magnification preset optics.
* *If you do not see a beam, you may be on a grid bar. You should use the joystick to move the stage so that you are not centered on a grid bar.*
  1. Repeat the beam shift direct alignments at the Data Acquisition magnification preset optics in EPU by clicking “Preparation 🡪 Presets (Data Acquisition) 🡪 Set” and following the rest of the procedure that you performed to center the beam at the grid square magnification and hole/eucentric height preset optics.

A table with numbers and percentages

Description automatically generated

* 1. Verify that the data acquisition EPU preset is set up to provide parallel illumination using the table on the right as a reference. Make sure that the C2 lens current on the microscope matches the corresponding spot size and probe mode in the EPU preset.
* *Parallel illumination is a microscope alignment state where the electron beam passes through the sample without converging or diverging. This ensures that there are no aberrations in the image.*
* *There is a specific C2 lens current value that provides parallel illumination for each spot size in microprobe (µP) mode and for each spot size in nanoprobe (nP) mode.*
  1. Center the energy filter zero loss peak (ZLP) in EPU using EPU auto-functions.

Detailed instructions:

* + 1. In EPU, click “Auto Functions 🡪 Auto Zero-Loss 🡪 Presets (Zero Loss) 🡪 Start” to center the energy filter aperture.
* *Make sure that you cannot see the beam edge in the preview images. If you can see the beam edge in the image, the energy filter centering will fail.*

1. **Select the appropriate exposure settings and measure the dose.**

**\*Skip this section if you are only interested in screening grids, not collecting a dataset.**

* 1. Move the stage to an empty area and Set Data Acquisition Preset Optics.
* *You can view the position of the optical axis on a simple grid overlay in the TEM UI by going to “Search 🡪 Stage”.*
* *If you move the stage so that the optical axis is too close to the edge of a grid, you will get an “End of Range” error in the TEM UI which indicates that you cannot move the stage any further.*
* *The following detailed instructions describe how to find an empty area using EPU. You can also use the TEM UI and the control pads joystick to find and center on an empty area. Make sure that the TEM UI Flu screen is in “High Contrast” mode so that you can better see the empty areas.*

Detailed instructions:

* + 1. Take a low magnification image in EPU by clicking “Preparation 🡪 Presets (Atlas) 🡪 Preview”.
    2. Right click in the middle of an empty area and click “Move Stage Here”.
    3. Go to Data Acquisition preset optics settings and verify that the image area is empty by clicking “Preparation 🡪 Presets (Data Acquisition) 🡪 Preview” in EPU.
  1. Select the appropriate exposure settings for data acquisition in EPU under “Preparation 🡪 Presets (Data Acquisition) 🡪 Exposure Settings.
* *The core facility default exposure settings for the data acquisition preset is: Fractions 🡪 EER, Align 🡪 Yes, Dose (e/Å2) 🡪 50.00, Exp. Time (s) 🡪 automatically adjusted by dose rate (typically ~4 seconds).*
* *If you expect to get very high-resolution data (<2.5 Å), I recommend collecting data in electron event representation (EER) format. The EER format data is larger in size than TIFF LZW, but it allows you extra flexibility about during data processing in deciding how to fractionate the data for motion correction.*
* *At the later steps in this protocol, if you find that EPU Automated Acquisition is struggling to accurately find holes and center on holes, you may want to increase the Grid Square and Hole/EucentricHeight preset exposure times to 3 seconds each.*
  1. In EPU click “Preparation 🡪 Presets (Data Acquisition) 🡪 Measure (Dose Rate)” and take a screenshot of the dose rate and data acquisition preset parameters for use when processing the dataset.
* *To get an accurate dose rate, it is important to perform this step in an empty area with beam completely covering the energy filter.*
* *The dose rate should be within the green portion of the blue-green-red scale.*

1. **Collect a gain reference.**

**\*Skip this section if you are only interested in screening grids, not collecting a dataset. You can also skip this section if a gain reference has already been collected in the past two weeks.**

* 1. Open the Falcon 4(i|iS) Reference Image Manager by clicking “Microscope Software Launcher 🡪 Tools 🡪 Camera and detector 🡪 Falcon 4(i|iS) Reference Image Manager”.
  2. In PostCounting Gain Mode, click “Measure Dose”. Make sure the dose rate is within the green range.
  3. With an Exposure time of “5” and Images to average “90”, click “Acquire” to begin taking a gain reference.
* *The gain reference acquisition will take 15-30min.*
* *To get an accurate gain reference, it is important to perform this step in an empty area with beam completely covering the energy filter.*

1. **Tune the energy filter.**

**\*Skip this section if you are only interested in screening grids, not collecting a dataset.**

* 1. Set the microscope optics to the data acquisition preset if it is not already at the data acquisition preset optics.
  2. Tune energy filter isochromaticity, geometric distortions, and chromatic distortions in Sherpa.
* *To accurately tune the energy filter, it is important to perform this step in an empty area with beam completely covering the energy filter.*

Detailed instructions:

* + 1. Tune isochromaticity in Sherpa by clicking “Energy Filter 🡪 Isochromaticity 🡪 Tune”.
* *If Sherpa is not already open and accessible from the bottom applications bar, you can open it by clicking “Tools 🡪 Sherpa” in the Microscope Software Launcher.*
  + 1. Tune geometric distortions in Sherpa by clicking “Energy Filter 🡪 Geometric and Chromatic Distortions 🡪 Tune Magnification”.
* *If you see “Error in Distortion Analysis: Error #2853...”, and you see a single large circle in the preview image of Sherpa, this is a software bug. Restarting Sherpa should fix this issue allowing you to successfully tune geometric distortion.*
  + 1. Tune chromatic distortions in Sherpa by clicking “Energy Filter 🡪 Geometric and Chromatic Distortions 🡪 Tune Distortions”.
  1. Move the stage back over the cross-grating grid surface and insert the screen.

1. **Align the microscope.**

**\*Skip steps 1-2 of this section if you are only interested in screening grids, not collecting a dataset.**

* 1. (Stable alignment, rarely needs adjusting) Check the beam for condenser astigmatism. If there is astigmatism, correct it using the condenser stigmator, in diffraction imaging mode.

Detailed instructions:

* + 1. Draw a perfect circle around the beam by selecting the red oval tool in the upper bar of the Flucam Viewer, holding shift, and expanding a red circle marker. Drag the circle over the beam. If the beam is circular then there is no condenser astigmatism. If the beam is ovular, then it shows astigmatism and you should proceed to the next step to correct for the astigmatism.
    2. Remove the red circle marker that you created by right-clicking on the marker and clicking “delete”.
    3. Click “Natural”, “High Resolution”, and “FFT” in the Flucam Viewer, if they are not already on (yellow).
    4. Enter diffraction imaging mode by pressing “Diffraction” on the control pads, then spread the beam a bit (2-3 times larger) by rotating the “Intensity” knob clockwise on the control pad.
    5. Enter Condensor Stigmator adjustment mode by clicking “Column 🡪 Stigmator 🡪 Condensor” in the TEM UI.
* *Yellow indicates that the setting is selected while gray indicates that the setting is not selected.*
  + 1. Use the Multifunction X and Multifunction Y knobs on the control pads to make the pattern in the FFT window of the Flucam Viewer more circular.
    2. Leave Condensor Stigmator adjustment mode by clicking “Column 🡪 Stigmator 🡪 None” in the TEM UI.
  1. Check if the C2 aperture is properly centered. If the C2 aperture is not properly centered, correct it using the C2 aperture adjustment mode, in diffraction imaging mode.
* *There are 4 different sized C2 apertures on the microscope (20, 50, 70, and 150 µm). Each aperture that you intend to use for your imaging session must be properly centered. Different core facility default preset files will use different C2 apertures. The 150 µm aperture does not typically need to be centered as it is only used for the atlas montage acquisition, but it is important that the C2 aperture used at data acquisition EPU preset optics settings is properly centered.*

Detailed instructions:

* + 1. Enter diffraction imaging mode by pressing “Diffraction” on the control pads if you aren’t in diffraction mode already.
    2. Condense the beam to a point by rotating the “Intensity” knob clockwise on the control pad.
    3. Mark the point of the beam by using the red (+) marker tool in the upper bar of the Flucam Viewer.
    4. Spread the beam to ~50% of the size of the energy filter aperture (green circle) by turning the “Intensity” knob clockwise. If the beam spreads concentrically (the center of the beam doesn’t move after spreading), the C2 aperture is properly centered. If the beam doesn’t spread concentrically, then you should proceed to the next step to center the C2 aperture.
    5. Enter C2 aperture adjustment mode by clicking “Column 🡪 Apertures 🡪 (Condenser 2) (20/50/70/150) Adjust” in the TEM UI.
* *Yellow indicates that the setting is selected while gray indicates that the setting is not selected.*
  + 1. Use the Multifunction X and Multifunction Y knobs on the control pads to center the expanded beam on the red (+) mark that you had created earlier.
    2. Remove the red (+) mark by right-clicking on it and clicking “Delete”.
    3. Condense the beam to a point and place a new red (+) mark.
    4. Spread the beam to check for concentric spreading. If it is spreads concentrically then the C2 aperture is properly aligned. If it does not spread concentrically, then repeat the C2 aperture centering using the aperture adjustment mode until the C2 aperture is properly centered.
    5. Leave C2 aperture adjustment mode by clicking “Column 🡪 Apertures 🡪 (Condenser 2) (20/50/70/150) Adjust” in the TEM UI.
    6. Leave diffraction imaging mode if you haven’t already by pressing the diffraction button
  1. Move the stage over the cross-grating grid carbon surface if it isn’t already over the carbon surface.
  2. Bring the cross-grating grid to the eucentric height using EPU auto-functions.
* *The eucentric height is the Z-height at which the sample does not shift laterally away from the optical axis when the grid is tilted. Several alignments must be performed at eucentric height to be accurate.*
* *This step may fail if the eucentric height is very far from Z: 0 µm, which may happen if the grid was inserted into the cassette in the wrong orientation. Adjusting the Z-height to the approximate eucentric height using the Wobbler should allow the auto-eucentric height adjustment functions to succeed.*

Detailed instructions:

* + 1. Perform an auto-eucentric height adjustment by beam tilt in EPU by clicking “Auto Functions 🡪 Auto-eucentric by beam tilt 🡪 Presets (Hole/EucentricHeight) 🡪 Start”.
  + *If you are on a grid bar, you will need to move the stage until you no longer see a grid bar in the image.*
    1. (If previous step failed) Perform an auto-eucentric height adjustment by stage tilt at the GridSquare preset magnification by clicking “Auto Functions 🡪 Auto-eucentric by stage tilt 🡪 Presets (GridSquare) 🡪 Start” in EPU. Then repeat the auto-eucentric height adjustment by beam tilt at the Hole/EucentricHeight preset magnification.
    2. (If previous step failed) Move the Z-height closer to eucentric height using the wobbler in the TEM UI and repeat euto-eucentric height adjustment by beam tilt in EPU.

Detailed instructions:

* + - 1. Insert the screen.
      2. Turn on the wobbler to oscillate the stage tilt in the TEM UI by clicking ”Search 🡪 Stage2 🡪 Control 🡪 Wobbler”.
      3. Adjust the Z-height by pressing the Z-axis buttons on the control pads until the image is stationary.
      4. Turn off the wobbler to stop the stage tilt oscillations in the TEM UI by clicking ”Search 🡪 Stage2 🡪 Control 🡪 Wobbler”.
      5. Repeat auto-eucentric height adjustment by beam tilt in EPU by clicking “Auto Functions 🡪 Auto-eucentric by beam tilt 🡪 Presets (Hole/EucentricHeight) 🡪 Start”.
  1. Bring the grid to focus by clicking in EPU “Auto Functions 🡪 Autofocus”
  2. Specify in the TEM UI that the grid is at focus by clicking on the control pads R2 (Reset Defocus).
  3. Align the beam tilt pivot points using direct alignments.
* *Beam tilt pivot point alignments are only valid when the image is in focus.*

Detailed instructions:

* + 1. Insert screen if it is not already inserted
    2. Set microscope optics to Data Acquisition magnification preset optics if they are not already set.
    3. Enter beam tilt pivot point X direct alignment mode in the TEM UI by clicking “Align 🡪 Direct Alignments 🡪 Beam tilt pp X”.
    4. Use the Multifunction X & Y knobs to superimpose the two beam circle areas and click “Done”.
    5. Enter beam tilt pivot point Y direct alignment mode in the TEM UI by clicking “Align 🡪 Direct Alignments 🡪 Beam tilt pp Y”.
    6. Use the Multifunction X & Y knobs to superimpose the two beam circle areas and click “Done”.
  1. (Stable alignment, rarely needs adjusting) Align the rotation center using direct alignments.

Detailed instructions:

* + 1. Enter rotation center alignment mode in the TEM UI by clicking “Align 🡪 Direct Alignments 🡪 Rotation Center”.
    2. Use the Multifunction X and Multifunction Y knobs on the control pads to minimize the directional movement of the beam circle and the movement of the underlying image, then click “Done”.
  1. Center the beam over the energy filter aperture using beam shift direct alignments.
  2. Correct for objective astigmatism and coma using EPU auto-functions.

Detailed instructions:

* + 1. In EPU click “Auto Functions 🡪 Autostigmate 🡪 Presets (Thon Ring) 🡪 Start” to correct for objective astigmatism.
* *There will be a white X across the power spectrum image if the image isn’t successful.* 
  + 1. In EPU click “Auto Functions 🡪 Autocoma 🡪 Presets (Thon Ring) 🡪 Start” to correct for coma.
    2. In EPU click “Auto Functions 🡪 Autostigmate 🡪 Presets (Thon Ring) 🡪 Start” to correct for objective astigmatism again.
  1. Center the beam over the energy filter aperture using beam shift direct alignments.

1. **Calibrate image shifts.**
   1. (Optional) You can view the instruction to calibrate images shifts in EPU by clicking “Preparation 🡪 Calibrate Image Shifts 🡪 Tutorial”.
   2. Find and center the stage on a unique piece of ice contaminant at the highest magnification preset.

Detailed instructions:

* + 1. Take an atlas magnification image in EPU by clicking “Preparation 🡪 Presets (Atlas) 🡪 Preview”.
    2. Center the stage on a unique piece of ice contaminant in EPU by right-clicking on the ice contaminant and clicking “Move Stage Here”.
* *I like to use the small piece of ice contaminant near the center of the cross-grating grid just to the left of the fiducial in the shape of the letter “A”.*
  + 1. Take a grid square magnification image in EPU by clicking “Preparation 🡪 Presets (GridSquare) 🡪 Preview”.
    2. Center the stage on the same unique piece of ice contaminant in EPU by right-clicking on the ice contaminant and clicking “Move Stage Here”.
    3. Take a hole/eucentric height magnification image in EPU by clicking “Preparation 🡪 Presets (Hole/EucentricHeight) 🡪 Preview”.
    4. Center the stage on the same unique piece of ice contaminant in EPU by right-clicking on the ice contaminant and clicking “Move Stage Here”.
    5. Take a data acquisition magnification image in EPU by clicking “Preparation 🡪 Presets (Data Acquisition) 🡪 Preview”. You should see the ice contaminant in the field of view.
    6. If you cannot see the ice contaminant in the field of view of the data acquisition height magnification preview image, you can insert the screen and move the stage until you find the ice contaminant.
    7. Once you find and center the stage on the ice contaminant at the highest magnification preset, you can proceed to the next step.
  1. Bring the cross-grating grid to the eucentric height using EPU auto-functions.

- *Calibrating image shifts close to the area where you calculated the eucentric height will produce the most accurate image shift calibration.*

Detailed instructions:

* + 1. Perform an auto-eucentric height adjustment by beam tilt in EPU by clicking “Auto Functions 🡪 Auto-eucentric by beam tilt 🡪 Presets (Hole/EucentricHeight) 🡪 Start”.
    2. (If previous step failed) Perform an auto-eucentric height adjustment by stage tilt at the GridSquare preset magnification by clicking “Auto Functions 🡪 Auto-eucentric by stage tilt 🡪 Presets (GridSquare) 🡪 Start” in EPU. Then repeat the auto-eucentric height adjustment by beam tilt at the Hole/EucentricHeight preset magnification.
    3. (If previous step failed) Move the Z-height closer to eucentric height using the wobbler in the TEM UI and repeat euto-eucentric height adjustment by beam tilt in EPU.

Detailed instructions:

* + - 1. Insert the screen.
      2. Turn on the wobbler to oscillate the stage tilt in the TEM UI by clicking ”Search 🡪 Stage2 🡪 Control 🡪 Wobbler”.
      3. Adjust the Z-height by pressing the Z-axis buttons on the control pads until the image is stationary.
      4. Turn off the wobbler to stop the stage tilt oscillations in the TEM UI by clicking ”Search 🡪 Stage2 🡪 Control 🡪 Wobbler”.
      5. Repeat auto-eucentric height adjustment by beam tilt in EPU by clicking “Auto Functions 🡪 Auto-eucentric by beam tilt 🡪 Presets (Hole/EucentricHeight) 🡪 Start”.
  1. Take images at all four preset magnifications using the calibrate image shifts utility in EPU by clicking “Preparation 🡪 Calibrate Image Shifts 🡪 Acquire”.
  2. Double left-click on the same spot of the ice contaminant feature in all four images to place a blue (+) mark there.
  3. Store the image shifts calibration by clicking “Preparation 🡪 Store calibration”
  4. Validate that proper calibration of image shifts by re-acquiring images at the four different preset magnification in EPU by clicking “Preparation 🡪 Calibrate Image Shifts 🡪 Acquire”. If they are still not well-aligned, repeat image shift calibration by double left-clicking on the same spot of the ice contaminant feature in all four images and storing the new image shifts by clicking “Preparation 🡪 Store calibration”.

1. **Obtain a grid atlas montage.**
   1. Create an atlas session in EPU by clicking “Atlas 🡪 Session Setup 🡪 New Session”.
   2. Append the Session file name using the following filename format “Supervisor\_XXXXXXXX\_XXXXXX\_[LabLastName]\_[YourInitials]\_[gridIDsOfAllLoadedGrids]” (ex. “Supervisor\_20240718\_140541\_Bullitt\_CWH\_gridCWH1-4”).

* *The atlas session stores the atlas montage files of all grid atlases. You will later create another set of session files for each automated data acquisition session.* 
  1. Set the Output folder to the “Z:\[LabLastName]” drive
  2. Click “Apply”.
  3. In EPU click “Atlas 🡪 Tasks 🡪 Screening” and check the box next to the grid you would like to load onto the stage and image.
  4. In EPU click “Atlas 🡪 Start” to start the atlas acquisition.
* *If you would like to obtain atlas montage images of multiple grids you can check the boxes of multiple grids before clicking “Start”. This will take atlas montage images of all selected grids. However, taking atlas montage images of multiple grids will require unloading the current grid from the stage and loading a new grid onto the stage for each selected grid, which will take some additional time.*

1. **Perform automated data acquisition.**
   1. Create a new EPU automated data acquisition session in EPU by clicking “EPU 🡪 Session Creation 🡪 New Session”.
   2. Append the Session file name using the following filename format: “Supervisor\_XXXXXXXX\_XXXXXX\_[LabLastName]\_[YourInitials]\_[gridIDOfGridOnStage]” (ex. “Supervisor\_20240718\_150221\_Bullitt\_CWH\_gridCWH4”).

* *The EPU session store data from an automated data collection on a single grid. If you would like to collect data from multiple grids in a single automated data collection, you will need to click “EPU 🡪 New Queue”.*
  1. Select the appropriate grid type and geometry type for your imaging needs.
* *Select “holey carbon” for carbon mesh grids with regularly spaced circular holes, “holey gold” for gold mesh grids with regularly spaced circular holes, or “lacey carbon” for carbon mesh grids with irregularly shaped lacey holes.*
* *Select “square” for a square hole pattern, “hexagonal” for a hexagonal hole pattern or “unkown” is the geometry type is unknown.*
  1. Select “Session type 🡪 Manual”, “Acquisition Mode 🡪 Faster”, “Image format 🡪 MRC”, and “Output folder 🡪 Z:\[LabLastName]”.
  2. (optional smartEPU with Quality Monitor) Login to Athena by clicking on the Athena button in the lower-right hand corner of EPU. Click “Athena Settings 🡪 Select”. You can receive the login information by requesting it from core facility staff after being properly trained.
  3. Click “EPU 🡪 Square Selection” and use the Selection options to select the grid squares that you would like to image.
* *You can use the histogram slider on the righthand side of the EPU software to help you evaluate which holes are empty and which holes have ice.*
* *If you are screening grids, I would recommend that you select 2 to 3 grid squares at varying ice-thicknesses for imaging.*
  1. Click “EPU 🡪 Hole Selection 🡪 Auto-eucentric”. This will move the stage to one of the selected squares, perform an auto-eucentric by beam tilt procedure, store the corresponding z-height in the metadata of the selected square, and take a preview image of the square.
* *The auto-eucentric procedure may fail if the eucentric height is far away from the starting z-height or if the procedure can’t identify a good cross-correlation comparison t between the different beam tilt images for the auto-eucentric procedure. The procedure may fail when the sample side of the grid is not oriented towards the clip ring within the cartridge, when the grid is not sufficiently flat, or when there is not enough contrast between the images being compared at different beam tilt angles.*
* *If the auto-eucentric procedure fails you can try first bring the z-height close to eucentric height in EPU by clicking “Auto-Functions 🡪 Auto-eucentric by beam tilt 🡪 Presets (Grid Square) 🡪 Start”. Then redo the EPU session auto-eucentric procedure at the hole-eucentric height mag in EPU by clicking “EPU 🡪 Hole Selection 🡪 Auto-eucentric”. If the auto-eucentric procedure fails again skip the grid square and move on to the next one in EPU by clicking “EPU 🡪 Unusable Square”.*
  1. In EPU click “EPU 🡪 Hole Selection 🡪 Measure Hole Size”, adjust the position and size of the yellow circles so that they are the same size as the holes in the grid square, adjacent to one another, and superimposed over the holes.
  2. In EPU click “EPU 🡪 Hole Selection 🡪 Find Holes” to command EPU to find the positions of all of the holes in the square.
  3. Adjust the hole selection manually with the Selection Brush or semi-automatically with the Filter Ice Quality slider on the righthand side of the EPU software.
* *I would recommend that you do not select any holes within a two-hole distance of the grid bar because these holes typically have thick ice and will produce low quality data.*
* *If you are screening grids, I would recommend that you select 3-5 holes at varying ice thicknesses per grid square.*
  1. Prepare all square individually in EPU by clicking “EPU 🡪 Hole Selection 🡪 Next Square” and repeating the steps to select holes starting from the auto-eucentric procedure or prepare all squares automatically using the Filter Ice Quality slider and clicking “EPU 🡪 Hole Selection 🡪 Prepare all Squares”.
* *If you use the “Prepare all Squares” procedure, make sure to watch to make sure it successfully prepares all of the squares. This procedure will often fail to obtain the auto-eucentric height for one or more squares which will stop the automated preparation of all squares.* 
  1. Right click in the center of a hole and select “Move stage here”.
  2. In EPU click “EPU 🡪 Template Definition 🡪 Acquire”.
* *If the hole is not well-centered, you can click “EPU 🡪 Template Definition 🡪 Find and Center Hole” to recenter the stage on the hole and re-acquire the image.*
  1. In EPU click “EPU 🡪 Template Definition 🡪 Acquisition Area” to enter acquisition area edit mode and click inside the hole to add one or more acquisition areas.
* *The green circle is the area of the beam and the green square is the area of the detector. Since the sample quality is irreversibly damaged after imaging, the same area cannot be imaged twice. Therefore, an acquisition area circle cannot overlap with an adjacent acquisition area square, but it can overlap an adjacent acquisition area circle.*
* *If you are looking for the highest quality data and highest resolution reconstruction, you can add one acquisition area per hole, but this will reduce the number of movies per hour. However, if you are not expecting to achieve very high resolution reconstructions with your sample, you can add multiple acquisition areas per hole which will reduce individual movie quality but significantly boost the number of movies per hour.* 
  1. Click on an acquisition area and adjust the defocus list to a range that is best for your imaging needs.
* *Since cryoEM samples are not stained, movies must be collected with a slight defocus in order to see the particles against the background. Higher defocus means higher contrast but lower achievable resolution. Lower defocus means lower contrast but higher achievable resolution.*
* *A good defocus list (µm) starting point is: -0.8, -0.9. -1.0, -1.1, -1.2, -1.3, -1.4, -1.5, -1.7, -1.9, -2.2, -2.5*
* *If you are screening grids, a good defocus list (µm) is: -1.5, -2.5, -5.0.*
  1. Click the small icon to the right of the defocus list (looks like two small squares) to copy the defocus list to the other acquisition areas if you selected multiple acquisition areas.
  2. Click “EPU 🡪 Template Definition 🡪 Autofocus Area” to enter autofocus area edit mode and click outside of the hole to add one autofocus area. Make sure the Recurrence of the Autofocus area is set to “After Centering”. This means that it will perform a single autofocus measurement for every cluster of holes that EPU images with AFIS.
* *You need to perform the autofocus procedure on an area that you are not imaging so that you do not image your sample twice. This is why the autofocus acquisition area is outside of the hole.*
* *An autofocus area may have been automatically added to the template image area, and you may need to use the mouse scroll-wheel to zoom out to see it.*
  1. (optional if screening only) Click “EPU 🡪 Template Definition 🡪 Drift Measurement Area” to enter Drift Measurement area edit mode and add one drift measurement area on the surface outside of the hole overtop of the autofocus area. Make sure that the Recurrence of the Drift Measurement is set to “Once per Gridsquare”.
* *You can perform the autofocus procedure and drift measurement in the same area.*
* *If you are screening and not collecting data you do not have to add a drift measurement area.*
  1. (optional) Click “EPU 🡪 Template Execution 🡪 Start” to see execute a single template of the automated acquisition.
  2. (optional if screening only) Click “EPU 🡪 Automated Acquisition 🡪 Close Col. Valves” to activate the close column valves after run option
  3. (optional if screening only) Set “EPU 🡪 Automated Acquisition 🡪 Auto Zero Loss” to Yes with a periodicity of 1 hour.
  4. (optional) Calibrate image shifts again but this time on the grid that you intend to use for data collection.
* *Repeating image shift calibrations will make the automated data acquisition more accurate which will result in fewer skipped holes and less mesh surface in the images.*
  1. Bring the image to focus using the EPU Autofocus auto-function.
  2. Realign pivot points X and Y at the data acquisition preset magnification optics using the TEM UI direct alignments.
* *The grid square preparation steps will bring the beam off center and pivot points out of alignment. To collect high quality data, it is important to realign pivot points recenter the beam and immediately before starting the automated data acquisition. If you add additional squares to the automated acquisition after starting the run make sure to recenter the beam and realign the pivot points. These realign steps are especially important if working at lower data acquisition magnifications and when using smaller C2 apertures.*
  1. Recenter the beam while at the data acquisition preset and hole-eucentric height magnification optics using TEM UI direct alignments.
  2. Start the run in EPU by clicking “EPU 🡪 Automated Acquisition 🡪 Start Run”.
  3. Monitor the run for 30 min to make sure everything is going as expected. You may need to briefly stop the run and recenter the beam because it will continue to drift for up to 30 min after performing the grid square preparation steps.
  4. (Optional smartEPU with Quality Monitor) If you enabled Athena to run smartEPU with Quality Monitor, you can track the ongoing automated data acquisition by going to <https://glacios01-athena.bumc.bu.edu/>. You can receive the login information by requesting it from core facility staff after being training.
* *The athena website can be accessed from any browser while connected to the BU network. You can connect to the BU network by using BU wifi while on campus or by using the BU VPN while off-campus.*
  1. (Optional cryoSPARC LIVE) You can set up an on-the-fly data processing pipeline using cryoSPARC LIVE by going to <http://callisto.bumc.bu.edu:39000/>. You can receive login information by requesting it from core facility staff with proper training.
* *The microscope PC Offload Data “Z:/” drive is mounted on the Callisto workstation under the path “/nfs/OffloadData”. You can import the movies from this directory into cryoSPARC LIVE on the Callisto workstation.*
  1. (Optional Remote Microscope Access) You can remotely control the microscope PC and Enceladus PC using noMachine and RealVNC. You can receive the login information by requesting it from core facility staff after being trained.

1. **Wrap up the microscope session.**
   1. Clean up the space.
   2. Save your Microscope Daily Log file on the Microscope PC at “C:/Desktop/Microscope\_Daily\_Log”.
   3. Leave the Microscope Daily Log file open and visible on the Microscope PC so that the next user can see the status of your session and see which grids are loaded in the microscope.
   4. If you are unloading grids at the end of your session, make sure to leave the cross-grating grid loaded on the stage of the microscope.
   5. Make sure that the microscope column valves are closed.
   6. At the end of your session, make sure to copy your data onto your hard drive using the Callisto workstation. You must do this within 1 week of collecting your data. You can use the rsync command with flags aP. The command should like like this: rsync -aP /nfs/OffloadData/[MyData] /run/media/spuser/[MyHardDrive]
   7. Make sure that you have taken screenshots of the data acquisition microscope paramaters for your paper methods section (magnification, total dose, dose rate, defocus list, one-shot or multi-shot per hole imaging strategy, energy filter slit width, px size, etc.)
   8. Make sure that you have taken a screenshot of the grid squares that you have imaged in case you need to collect additional data and reimage the same grid.

* *You cannot image the same grid squares that you have previously imaged.*