EPU Software User’s Guide

PN 1025707-H

Automated Data Collection for Single Particle 3D Reconstruction

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1 Introduction

Overview

EPU™ is an FEI® software product for automated data acquisition for single particle analysis. Single particle analysis is an approach to 3D image creation, during which a large number of vitrified, low-contrast complexes are imaged at low electron dose conditions. This ultimately, after conformational classification and particle averaging, results in a high-resolution 3D representation.

Figure 1 Part of Interior of Cowpea Mosaic Virus 3D SPR Reconstruction with Atomic Coordinates Fitted

The acronym EPU is from the Latin *E Pluribus Unum* — “out of many, one” — and reflects the actual pathway of collecting numerous particle 2D images and converting them into one 3D structure. EPU takes care of the most time-consuming step: the acquisition of images containing useful particles for further reconstruction. The actual reconstruction is performed by various open source software applications.

FEI’s EPU facilitates the process of optimal area selection, the first crucial step of the total single particle analysis workflow. It also facilitates high-throughput data collection from the microscope, which is a primary prerequisite for high-resolution 3D imaging.
Cryo-electron microscopy (cryo-EM) for high resolution three-dimensional single particle reconstruction (3D-SPR) is approaching near atomic resolution for structures of biological complexes of all kinds. Since biological material under the electron beam is highly electron-radiation-sensitive, an extremely low dose of electrons is utilized to prevent specimen damage. Due to the low signal-to-noise ratio in the recorded images, high-resolution information must be built up by averaging large amounts of well-aligned data. Collecting large numbers of images is a demanding task for an operator; therefore, the acquisition software for data collection greatly improves workflow throughput.

**Benefits**

- Microscope-embedded solution for single particle acquisition
- Optimized for high-throughput particle collection
- Recording schemes defined for commonly used grid types
- Intuitive, graphic display of experimental setup
- Compatible with film, CCD cameras, and direct electron detectors
- Designed for Tecnai™, Talos™, and Titan™ TEM platforms
2 Preconditions

Overview

This chapter describes the preconditions that generally must be fulfilled for a successful run of the EPU software. Most are related to expectations about the microscope alignments and available calibrations.

Specimen Holder

Side entry holders should be pumped accordingly before use. This is also true for the Polara MSC (multiple specimen cartridge) transfer unit.

Specimen

After insertion into the microscope:

1. Manually adjust the eucentric height of the specimen (preferably at the center of the specimen).
2. Roughly focus the specimen.

Microscope (General)

The microscope must meet the following conditions:

- The column must be properly aligned.
- HT is stable.
- The CCD is at constant temperature.
- Gain and dark correction images should be available and well averaged.
- An alternate shutter should be chosen for CCD exposures or the use of both shutters should be enabled in case a pre-exposure is desired. (See the CCD control panel in the microscope UI.)
- Enough time should be allowed for settling after any actions that may have introduced exceptionally strong drift (cooling, inserting holder).
- Alignments, FEG registers, and necessary calibrations should form a consistent set. It is best to always use the same set for EPU.
- Calibrations must have been done for LM and SA ranges (Magnification Calibrations control panel in microscope UI).
Preconditions

- For non-FEG instruments, the gun saturation (heating) must be optimized and the emission chosen. Gun settings may need to be optimized via the direct alignments gun-tilt and gun shift.
- Apertures should be centered correctly.
- Focus calibration must have been done (currently a task that is performed in EPU).

Direct alignments should be checked/adjusted in the modes that are used for EPU (LM range and HM range nanoprobe/ Microprobe).

Alignments

The alignments are generally very stable and do not need frequent adjustment.

Gun and Condenser

During EPU data acquisition, conditions will be set up at different magnifications and with different illuminations (size and intensity of spot). When switching between EPU states, the beam should ideally not change in position. This is achieved by the Spot Size Dependent Gun Shift alignment (gun part) and the Condenser (zoom) alignments.

Verify that the C2 aperture is centered before performing the following tests:

Test Spot Size Dependent Gun Shift

1. Switch spot sizes.
2. Does the spot stay centered?

Test Condenser Alignment

1. Enable Auto zoom (Titan) or Intensity zoom (Tecnai and Talos).
2. Switch magnifications and normalize lenses.
3. Does the spot stay centered and of the same size (Titan) or intensity (Tecnai and Talos)?
Preconditions ■ Alignments

Test Titan Condenser Zoom

1. When changing the illuminated area, does the spot stay centered? This will hardly be the case over the whole available range. For EPU, it is best when the spot stays centered in the parallel illumination range, especially at the lower end of it.

2. When the beam is centered on a feature of known dimensions, does the diameter have the right value? (See Beam Settings control panel in the microscope UI.) If not, the condenser calibration must be redone.

Deflectors

The image/beam shift calibration must have been done carefully in order to keep the illumination centered when some image shift is added, or in order to shift the image when you want to move the beam to another area of the specimen.

Both Image and Beam Shift pivot points must be aligned accurately in order not to change illumination conditions (coma, rotation center) and crossover shifts (GIF energy selection) when applying image (beam) shift.

Test Image/Beam Calibration

1. Apply image shift (use the Image Settings control panel in the microscope UI) in X and Y directions.

2. Does spot stay centered?

Projector

Lens Series Alignment

The lens series (LM and HM) must be aligned accurately to ensure that a centered feature stays centered when switching to another magnification (or EPU setting). The alignment should have been done accurately on the CCD that is used for EPU.

1. Select TIA.

2. Activate CCD Search.

3. Place marker in center of image display.

4. Make sure that there is no image shift applied (see Image Settings control panel)

5. Center a feature that would be recognizable on CCD over a wide magnification range.

6. Switch magnification and see that the feature stays centered.
7. Start at lower magnification and see whether at high magnification the detail is present that you wanted to zoom in the center of the CCD image.

| NOTE | Often this alignment is done on the flu screen, which might have a different magnification center. However, EPU’s image shift calibration routine is capable of accounting for the differences that emerge from this. |

**Eucentric Focus Preset**

The HM eucentric focus preset should not deviate more than a few microns from the true focus at stage eucentric height.

1. Adjust stage eucentric height (use the stage wobbler).
2. Switch to a higher SA magnification (such as that used for EPU data acquisition).
3. Press the Eucentric Focus button on the hand panel.
4. Make sure the defocus is shown in one of the status windows in the microscope UI.
5. Reset the defocus display value to zero (usually this function is bound to a user button, but also available on the Image Settings control panel).
6. Focus accurately (minimum contrast or use the FFT).
7. Read the new defocus value.

**Magnification-Dependent Focus Presets**

The lens series alignment should be parfocal as much as possible i.e., switching magnification should not switch focus much. In practice, this alignment is often badly done, especially for the lower magnifications of the HM series (and completely for LM).

- **LM Mode**: After switching magnifications, use the wobbler and flu screen to check whether the focus presets are adjusted correctly. This is accurate to fractions of a millimeter in defocus. Switching magnifications should not induce much shift in defocus (less than one millimeter).

- **HM Mode**: Use either the wobbler (for the lowest magnifications) or focus to minimum contrast. Switching magnifications should not induce much change in defocus (less than a few microns).
Preconditions  ■ Apertures

**HM/LM Alignment**

The LM lens series should be aligned against the HM lens series. This is one of the alignments that may change somewhat over time due to adjustment of direct alignments. This is no problem, as long as the shift stays inside a fraction of a grid square. Before each EPU run, this shift can be fine-aligned by use of the EPU image shift calibrations.

1. If EPU was already set up once, simply use the Set buttons on the EPU Selection view (*Grid Square Magnification*) and Template definition view (*foil hole magnification*). This is the recommended procedure.

2. You can also:
   - Switch between highest LM and lowest HM mode.
   - Normalize all lenses in between.

**Apertures**

**C2 Apertures**

C2 apertures should be centered.

Automated apertures’ positions are stored and reproducible. For EPU, typically smaller C2 apertures will be used. Check also in Nanoprobe.

**Test**

A centered beam should expand symmetrically without moving when the condenser is in two-lens condenser mode.

- **Talos and Tecnai:** Always the case, as there are only two condenser lenses.
- **Titan:** Switch off the C3 lens (in the Beam Setting control cluster, **Free Control**).

**Objective Lens Aperture**

Objective lens aperture should be centered. EPU will use large 100 or 70 micron apertures.

Check also in LM mode at lower magnification: is the area that is selected by the OL aperture centered? If so, in automatic runs of EPU, the aperture can be left stationary. Otherwise, the React At Mode Switch option must be used.

**Test**

In HM diffraction mode, the aperture should be centered around the transmitted beam.
Direct Alignments

Direct alignments are repeated regularly. Fine-tuning of the corrector eliminates coma, astigmatism, and other aberrations. The following paragraphs describe the direct alignments for uncorrected systems.

| NOTE | Systems with image correctors are not discussed here. |

Beam Tilt Pivot Points

These should be checked at the accurate eucentric height and focus. When the beam is tilted, the spot should not move (important for auto-focus/stigmator). Enter direct alignment, focus spot. Is spot movement minimized?

Beam Shift

To center beam when no user beam shift is applied. Perform at highest magnification that will be used in EPU.

Rotation Center

When focusing, the central image features should stay on the optical axis, not move. Perform at highest magnification that will be used in EPU.

Coma-free Alignment

For recording high resolution images, it is important that the illumination is coma-free. This procedure can only be done properly with the help of a thin carbon film and a CCD camera running with live FFT at the same time. Contrast and FFT should not change when the beam is tilted.

| NOTE | Coma free overwrites rotation center, but the difference should not be critical. |
Astigmatism
Use the objective stigmator and adjust on a piece of the carbon film while a CCD camera is running. Use the help of a live FFT.

| NOTE | In a simple FFT, astigmatism cannot be distinguished from coma induced by a beam tilt. Therefore, make sure that you are reasonably coma-free. |

Calibrations

Magnification Calibrations
These calibrations are very stable and only need to be redone when the alignments of the microscope have changed significantly. (The calibrations are accessible via the Magnification Calibration control panel in the microscope UI.)

EPU requires the calibration for LM and SA ranges to be present for the CCD in use for EPU (refer to the help for magnification calibrations for instructions). The calibrations do not only contain information about the magnification, but also about image rotation and how to transform shifts seen in the image to corresponding shift with stage and image/beam deflectors.

Focus Calibration
This calibration currently must be done via the Auto Functions tab in EPU. Start from a specimen that is at eucentric height and well focused, with astigmatism corrected. This calibration may need to be redone more frequently, as it may depend on changes in the direct alignments. (Redo this calibrations if the Auto Focus routine does not converge nicely any more.)

Eucentric Focus Calibration (Optional)
This calibration must be done via the Auto Functions tab in EPU. It is important when tilting of the stage is required.

Often there is a difference between the eucentric focus that is preset for every magnification and the real focus that has to be adjusted after a specimen is brought to eucentric height by wobbling the stage because the method to define the eucentric focus may have been different. This calibration captures the difference.
EPU will adjust the eucentric height by focus measurements. That means it adjusts the Z-position of the stage such that the specimen is in focus when the eucentric focus is set (do this with the button on the hand panel). When the eucentric focus calibration is available, EPU will set the Z-position of the stage such that it would be equal to the eucentric height adjusted by wobbling the stage. It is best to do this calibration at the magnification used for eucentric height adjustment in the automated run (usually the magnification at which the foil hole is imaged and centered).

**Focus**

EPU image acquisitions assume a focused specimen. It will apply the desired amount of defocus for each acquisition as specified in the various optical settings for atlas, grid square, foil hole, or data acquisition).

During the automated data acquisition phase, EPU will adjust eucentric height and focus automatically. For the setup phase, it is usually helpful if the following is done as preparation:

- Bring specimen to eucentric height
- Focus both in HM and LM mode (minimum contrast focus)

Alternatively, you can:

- Click **Eucentric Focus**
- Focus by changing the stage Z position.

What EPU will do during an automatic run depends on the presence of the eucentric focus calibration. If the calibration is available, the result will equal the result of the first method. Otherwise, it will be equivalent to the second method.
3 Getting Started

Overview

This chapter describes how to start the EPU session.

Prerequisites

The EPU software package requires one of the following with up-to-date software:

- Titan Krios™ with Titan software
- Titan Themis™ with Titan software
- Tecnai with Tecnai software
- Talos with Talos software

The correct alignments and FEG registers should have been loaded for the high tension and extractor settings of the instrument. Eucentric height, focus, and rotation center for LM and SA magnifications should have been adjusted (Direct Alignments). Perform CCD calibrations for magnification using the FEI magnification calibration software. Make sure the Low Dose option is turned off in the microscope’s user interface.

For more detailed instructions, see the online microscope help. For a check of the validity of the most important alignments, see EPU Preconditions Guide (Start > Programs > FEI Company > EPU > Preconditions Guide).

Launching EPU

Launch EPU from the Windows® Start menu:
Start > All Programs > FEI Company > Epu > EPU application.

After starting the application, a splash screen displays.

Figure 2 EPU Splash Screen
While the application is loading, the system checks to confirm that all subsystems are satisfactory. If the check fails, e.g., because the vacuum system is not ready or because the stage is not homed, a dialog displays with a message indicating the problem.

**Acquisition and Optics Settings**

After EPU software startup, the EPU ribbon bar displays.

![EPU Ribbon Bar](image)

Before setting up a new run, complete the tasks in the Preparation tab. The first one deals with the adjustment of the Optics and Acquisition settings. For each of the possible magnification levels used during an automatic data collection session, a preset exists. Set the illumination conditions of the microscope and the conditions under which the CCD is used to give images for each preset. Parameters regarding the CCD are:

- Camera name, binning, readout-area, pre-exposure time (only for FEI cameras), pre-exposure pause (only for FEI cameras), and exposure time.

Some cameras require additional parameters:

- **Ceta camera**: Frames Summed and Noise reduction (used to adjust the dynamic range or to decrease readout noise at low dose). *Figure 4* shows those of the Ceta camera (used to adjust the dynamic range or to decrease readout noise at low dose).

![Camera Specific Settings May Extend the General Camera Settings](image)
Direct Detection Cameras: For non-LM presets, the mode of the camera can be chosen to be either Linear or Counted. LM presets (e.g., Atlas) always work in Linear mode. Additionally, for the Data Acquisition preset, an extra mode is available: Counted/Super Resolution; as well as the option to define Dose Fractions. The total number of frames depends on the exposure time and the camera internal frame rate. For cameras that only support equidistant fractionation, the number of dose fractionation can be chosen and the fractions should be exactly equidistant (the number of fractions should be an exact divider of the total number of frames). For cameras that support varidistant fractionation (e.g., Falcon 3EC), the option to enable Fractions is provided instead. The fractionation scheme is then defined on a separate view. See “Falcon Intermediate Images” on page 50.

For detailed information, refer to the help given in the microscope's user interface or camera manuals.

Adjust the column optics using the parameters:

- Magnification, spot size, illumination area (on Titan) or illumination value (C2% of maximum power on Tecnai or Talos), defocus (which is the intended defocus that will be temporarily added when an exposure is taken), TEM imaging mode (Microprobe or Nanoprobe).
- Use the Set and Get buttons to send the parameters to the instrument or to retrieve them from the microscope.
- Enter the intended defocus for image acquisition in the designated text box. Parameters are also set when acquiring an image via Preview.

With Import or Export, you can store or retrieve the settings for all presets to/from disk.

The various presets are described here in order of magnification, as they appear in the dropdown list in Figure 3. In practice, it is helpful to start with the Data Acquisition preset because adjusting the dose and making a parallel beam will determine the choice of C2 aperture, for example.
In principle, EPU allows the use of a different camera for each preset if there are several CCDs available for the chosen microscopy mode (in EFTEM mode, you can only use the GIF CCD). For practical purposes, however, you should restrict your choice to using the same CCD for foil hole centering, auto focus, drift measurement, and data acquisition to relieve the stress on the vacuum and hardware.

**Description of Presets**

**Atlas Presets**

An atlas will be recorded to give an overview of the specimen and to find grid squares suitable for automatic data recording. Such a grid square should fulfill the following conditions:

- Thin specimen area, electron translucent
- No broken carbon film
- Ice-filled holes

FEI recommends using the microscope’s user interface and the flu-screen/flu-cam/TIA to select a low magnification that allows imaging the maximum unobstructed field of view on the CCD. Check the counts in an image using TIA with approximately the CCD settings that will be used for atlas acquisition. To copy the microscope optical settings over into the EPU ribbon bar, click the Get button and modify the CCD settings as desired.

A binning of 2 is recommended in the atlas acquisition to speed up the process. As guidance: an atlas of 6x6 1K images takes 10–12 minutes. Typically, a magnification (LM 60–80 X) is used so a 5x5 (7x7) tiled atlas will be recorded. Depending on instrument configuration, camera, and chosen magnification, the exact number of tiles recorded may vary. Tiles can be subdivided into smaller tiles (so called sub–tiles) using image shift for positioning. The eventual number of tiles and sub–tiles is reported in the status window during atlas acquisition.

**NOTE**

Falcon is not selectable for LM imaging. The Grid Square and Atlas setups are only definable at LM magnifications.
Grid Square Preset

The purpose of the grid square setup is to be able to find or define suitable target areas for data acquisition. The selection of target areas (manually or via an automatic ice thickness filter, see “Ice Thickness Filter Adjustment” on page 88) is done using this setup. Choose a magnification to image as much of a grid square as possible (a typical magnification is LM 400–600 X, illuminated area 150–200 µm, but numbers depend on the type of grid and the microscope configuration). A parallel beam is advantageous, as a convergent beam may lead to an effective change of magnification when out of focus.

Hole/Eucentric Height Preset

This preset is used for:

- Performing automatic adjustment of eucentric height during automated run
- Acquiring low dose images of target areas for inspection
- Quantifoil support grids:
  - Image, find and center holes (the positions of which were defined at the grid square level)
  - Define a template of acquisition positions and a focus position. This template will be applied to every hole during data acquisition.
  - Use a magnification here with at least one hole completely visible in the image. However, the algorithm for finding holes is more reliable if other holes are also visible (at least half visible).

Choose a beam such that no harm is done (dose smaller than 0.1 e⁻ / Å²/sec). Typically, the magnification will be in the low SA range, beam diameter about 10–15 µm. A defocus of about -10–20 µm is recommended to enhance contrast. A parallel beam is advantageous, as a convergent beam may lead to an effective change of magnification when out of focus.

Data Acquisition Preset

Particular care must be taken to set the illumination conditions for the actual data acquisition. Adjust the required resolution and dose on the sample by selecting magnification, C2-aperture, beam diameter, spot size, TEM mode (Microprobe/Nanoprobe). The illumination should be parallel.

The illuminated area should be selected to fulfill specific needs. For example:

- A small illuminated area prevents double-beam exposure when there are closely spaced target areas.
A larger illuminated area is good when there are widely spaced targets and it is important for the beam to also hit some carbon.

**Figure 6  Choices of Camera Models for Data Acquisition Setup**

Information about the dose is given in the EPU image info panel, and the dose is indicated on the status panel of the microscope’s user interface. Dose is correct if, for a given illumination, the magnification is set such that the beam covers the full fluorescent screen. Information about the illuminating beam (size and whether it is parallel or condensed) can be read from the Beam Settings Control cluster or the status display of the microscope’s UI.

On Tecnai or Talos the beam diameter is not given, but can be obtained from measuring the beam size at 10000 X on the large fluorescent screen. At 10000 X the conversion is: 1 cm on the flu-screen equals 1 µm at the specimen plane. However, the parallelism of illumination can only be approached for particular settings on Tecnai or Talos (FEI Application Note, de Haas, 2011).

<table>
<thead>
<tr>
<th>NOTE</th>
<th>Ensure that the intensity zoom is off when going down to 10000 X mag.</th>
</tr>
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<tr>
<th>NOTE</th>
<th>Examine the FFT of the image to evaluate the image quality. See “Image Display” on page 106.</th>
</tr>
</thead>
</table>
**Autofocus Preset**

To avoid problems due to slight misalignments, set the illumination conditions identically to the acquisition conditions (spot size, TEM imaging mode, and intensity or beam diameter). It can be advantageous to set the beam diameter a little bit wider because the Autofocus routine will tilt the beam,¹ which will lead to a small shift of the illuminated area when not already close to focus.

---

¹ The Autofocus procedure is similar to the one pioneered by Koster, van den Bos, and van der Mast (1987). The Autofocus measures the image shift that is induced when the beam is tilted. This shift can be related to a defocus by calibration. Focus is defined by zero beam tilt induced image shift.
The CCD parameters can be adjusted differently. For example, it is not necessary to use the full readout area if the range of focus variations to be covered is limited (the range of beam tilt-induced shifts that can be measured will be halved, but that will still be sufficient on a flat sample). Thus, instead of increasing the beam diameter, simply reduce the CCD area, with the advantage that readout speed is increased for many CCDs.

| NOTE | To speed up the shift measurements, the images are internally reduced to 2k pixels width. Therefore, choosing binning 1 on a 4k x 4k CCD would not increase accuracy. |

**Drift Measurement Preset**

To have EPU stop and wait until drift is within a threshold set by the user, a setup is defined that allows EPU to record images for drift measurement at a meaningful magnification, illumination size, and CCD setting.

This preset is used for:

- Drift measurements during data acquisition (applicable only to Quantifoil type specimens)
- Drift measurements that are started as standalone functions (*Auto Functions* tab). This is useful to determine the typical settling time that the application must wait after moving the stage to the next target for data acquisition.

Select a CCD setting that will result in a fast readout in order to avoid overhead in the drift measurement. Using only half the CCD frame helps in this case. In addition, binning will increase the readout speed, but FEI recommends staying at binning 1 because the accuracy is needed.

If the drift threshold is set very low, it may be necessary to increase the magnification such that it takes less time until a small drift is detectable as a shift in the image.

Also see “*Standalone Drift Measurement*” on page 91.
4 Calibrate Image Shifts

Overview

The calibration determines corrective image shifts between the defined optical settings (i.e., the presets) used in EPU. Usually, a good lens series alignment should ensure that switching magnification will not shift the image of the feature. However, EPU needs an especially precise alignment and certain factors induce deviations from a perfect alignment:

- The alignment is done on a camera or flu screen that can have a different center than the camera used for EPU.
- For the transition between HM and LM modes, a shift is easily induced by readjustment of the LM rotation center.
- When aligning the microscope, the normalization of the lenses may have been handled differently (especially on Tecnai and Talos systems)
- An inaccuracy in rotation center alignment will induce small shifts when EPU presets use defocus.

Resetting Calibration

If a calibration is available, whenever an optical state is set via EPU (either with the Set button or an image acquisition), the corrective image shift is applied.

| NOTE | Defocus is applied only when actually acquiring images |

The image shift calibration needs to be redone:

- Whenever optical presets that are used in EPU are changed (magnification and defocus)
- Whenever another CCD is used (magnification center may shift)
- If another microscope alignment or FEG-register is loaded
- When a feature does not stay centered when switching the EPU presets and acquiring images

The image shift calibration can be invalidated by clicking **Reset Calibration**. Corrections only use the image shift deflection coils and will not be visible in the Image Settings Control cluster of the microscope UI, which uses a combined image and beam shift.
The applied image shift is shown in the System Status display on the microscope UI (User Image Shift).

| NOTE | When you have stopped working with EPU, starting any direct alignment procedure will immediately remove any remaining user image shift. |

**Performing Calibration**

**Search and Center Feature**

Search for a feature that will be recognizable both in LM mode and at high magnification. Center this feature at Data Acquisition magnification by performing the following procedure:

1. Select **Gridsquare** and click **Preview**.
2. Right-click on the feature in the image and choose **Move stage here**.

![Figure 8 Stage Control by Mouse](image)

3. Select the “Hole” preset and click **Preview**. The feature is usually visible. If not, use the fluscreen/flucam to search and center it and click **Preview**.
4. Right-click on the feature in the image and choose **Move stage here**.
5. Select the **Data Acquisition** preset and click **Preview**. The feature should be visible (provided the lens series alignment is acceptable).
Calibrate

1. Select the Calibrate Image Shifts task.

   *Figure 9  Starting the Shift Calibration*

2. Click Start Calibration. An image taken with the Data Acquisition preset will be shown on the left.

3. Move the red circle to mark a position on the recognizable feature.

   *Figure 10  Centering the Feature*

4. As a test, click Re-acquire.
5. If the feature was centered as described above, it should be nicely centered now (within the accuracy of the stage movement). Otherwise, repeat the procedure again for the backlash correction to be consistent.

6. Click **Proceed**. An image taken with the Hole Acquisition preset will be shown on the right display.

7. On the right display, move the red circle to the feature (zoom in, if necessary).

8. Click **Proceed**. A new Hole settings image will be shown in the left display, and the feature should be centered now (image shift is used).

9. An image taken with the Grid Square acquisition preset will be shown on the right display.

**Figure 11 Center Grid Square Acquisition Relative to Hole Acquisition**

10. Continue until all presets have been processed.
11. After the last **Proceed**, the status window displays a message reporting successful calibration.

*Figure 12  Status Panel Reports Successful Calibration*
5 Acquire an Atlas

Overview

An Atlas is an overview of a sample composed by acquiring low-magnification images at different stage positions (tiled acquisition). The atlas is used for navigation purposes and determining which grid squares to include during an EPU run. This chapter describes how to set up an Atlas.

Setting up an Atlas

Before setting up an Atlas session to record a map of the loaded sample, set it to eucentric height (preferably in the center of the grid) and focus.

NOTE
Ideally, pressing Eucentric Focus on the hand panel should bring the specimen to focus. Otherwise, using the Wobbler in a high LM magnification is practical.

Figure 13 Session Setup for EPU Atlas
After clicking **Apply**, select **Atlas Acquisition** in the left panel and click **Acquire**. The Atlas acquisition begins, and the status window displays the number of images to be acquired.

Click **Stop** at any time during the acquisition, e.g., if the settings are incorrect. This will take a few seconds to stop the acquisition entirely. Then alter the relevant settings in the preparation UI and restart the acquisition.

The atlas acquisition in EPU 1.3 starts from within the vicinity of the center of field of field of view and progresses in a clockwise spiral fashion. Such an acquisition scheme is chosen for the following reasons:

1. Minimizing the time in which useful central region is acquired.
2. Minimizing the stage moves required during each atlas acquisition.

Provision to automatically stop the atlas after a specified number of tiles is provided in the top ribbon panel (see *Figure 14 on page 29*).

To move to a specific location, right click on the region of interest and select **Move stage here** in the context menu.

*Figure 14  Finished Atlas*
To import a previously acquired atlas, click **Load Sample** and navigate to the folder where the atlas is stored and select *atlas.dm* file as shown below.

*Figure 15  Re-loading Previously Acquired Atlas*
6  Calibrate $I_0$

Overview

EPU can automatically select target areas with the correct ice thickness. The specimen under examination consists of a frozen solution that contains the objects/molecules of interest. It forms a layer of ice on the carbon foil and especially across the holes on the carbon film. For data acquisition, target areas are selected such that the thickness of the ice is ideal (too thick does not produce good images, and too thin does not contain molecules).

The purpose of this calibration is to be able to rely on the ice thickness image filters that are set in the Location Selection task (see page 46), even after days of data collection. The thresholds set in the filters refer to the CCD counts in the image. However, if over time the intensity of the gun changes, a normalization of the counts is required.

Drive the stage to an area with no specimen and measure the intensity of the illumination in a hole ($I_0$), which intensity is then used for normalization of the counts in the image. Measuring the counts of the CCD image of a hole provides a measure of the intensity of the illumination.

NOTE

$I_0$ calibration is optional. If not done, the ice filter min/max parameters simply work on gray scales of the image, rather than as fractions of $I_0$. This is fine as long as the beam intensity does not fluctuate too much.
Setup

1. Find a gap on the sample, e.g., a broken grid square. This is referred to as the $I_0$ region.

   **HINT**
   The easiest way to find such location is to first acquire the atlas, look for a gap there, and use **Move Stage Here** to go to that location. Use a gap/hole of at least several micrometers. A foil hole without any ice is too small.

2. From the Preparation tab, click **Calibrate $I_0$** in the left pane.

   ![Figure 16 $I_0$ Preparation Tab](image16)

3. Select the **GridSquare** preset from the $I_0$ calibration ribbon.

   ![Figure 17 $I_0$ Calibration Ribbon](image17)

4. Click **Preview** to acquire an image at the current location.

5. If the $I_0$ region is not positioned in the center, use the mouse to the center it, right click, and select **Move Stage Here**. Then acquire a new image.

   **NOTE**
   For the sake of reproducibility, do not center the $I_0$ region using the joystick because the joystick moves the stage without any backlash correction.
6. The $I_0$ calibration is measured by averaging the signal inside the circle. To change the size of the centered circle, double-click on a position in the image. Increasing the size of the circle contributes to a stable average, but make sure the circle is well inside the gap/hole to allow for some positioning tolerance.

**HINT**

Allow for a margin of at least 250 nm at both sides of the $I_0$ region.

7. Click **Get stage position and diameter**. This stores the stage position and circle diameter in EPU.

8. Click either **Calibrate current** or **Calibrate all** to measure the $I_0$ value for the grid square and (in the latter case) foil hole optical presets.

9. The images are re-acquired and $I_0$ values are measured and logged to the status window.

**$I_0$ Calibration Notes**

- Remove and disable $I_0$ calibrations by clicking **Remove $I_0$ measurements**.
- For both magnifications (hole and grid square), the same stage position and circle diameters is used.
- During automatic data acquisition, $I_0$ is recalibrated at regular intervals. Also during automatic acquisition, $I_0$ is recalibrated prior to starting with a new grid square if the former $I_0$ measurement is older than 2 hours.
- $I_0$ calibration images acquired during the automatic run are saved in the EPU session directory.
- During EPU session setup, calibration can be rerun by a single button click (see **“Focus Calibration” on page 34**).
- Currently, $I_0$ for foil hole magnification is only measured for reference. It will be used in the future for target area analysis.
7 Calibrate Focus and Eucentric Correction

Overview

A properly working automatic focus is essential for running EPU. In addition, eucentric height must be adjusted during automatic data acquisition via a focus measurement (the stage is brought to eucentric focus by using its Z-drive).

For a complete description of auto functions, see “Auto Functions” on page 90.

Focus Calibration

The focus calibration is required to make focus measurements work accurately and converge quickly.

Preparation

1. Search an area of the specimen with only carbon that is thin (< 20 nm). Avoid thick features.
2. Bring the specimen to eucentric height.
3. Select a magnification. Calibration usually works well on a magnification and imaging mode, as typically used for EPU data acquisition.
5. Focus the specimen using the weakest contrast. When the specimen is nicely focused, the contrast is minimal.

Calibration

1. Select the Auto Functions tab in the EPU ribbon bar.
2. Select the acquisition preset to use (preferably Autofocus).
3. Click Focus Calibration.
4. Click OK on the Manual Focus dialog that displays.
The calibration now proceeds. Images with tilted beam and cross correlation images are displayed. The encircled bright spot in the cross-correlation image (Figure 18) corresponds to the shift between the first two images.

Figure 18  Focusing Calibration

Note: Ensure that these shifts are not too small (> 10% of the image width) so the measurements can be accurate. If necessary, increase the magnification of the preset used.

Upon completion, click Resume to accept and store calibration.

Figure 19  Confirming Calibration
The routine calibrates both focus and stigmators. To analyze its success, examine the last cross correlation display (zoom in a little). It shows a set of four green lines that represent the shifts that were measured during the focus calibration.

*Figure 20  Cross-Correlation Image Display*

If the preparation was carefully done and the shifts were measured successfully, the green lines should be all perpendicular and of approximately same length, as to form a symmetric cross. The bright spot with the red circle corresponds to the last measured shift during the stigmator calibration. In this case, the maximum in the cross correlation is clearly visible, indicating that the shifts could be measured without problem.
If the lines are not perpendicular, the astigmatism has not been corrected completely. The image in Figure 21 shows an example where some astigmatism remains.

**Figure 21  Astigmatism Remaining in Cross-Correlation Image**

Although the auto function works well in this case, it may be worthwhile to run Auto Focus once and repeat the calibration procedure. Set Desired Defocus to 0 and Auto Stigmate to Yes.

| NOTE | When using mixed TEM imaging modes during data acquisition (i.e., Microprobe for foil hole acquisition/eucentric height to get a wide parallel beam at low magnification and Nanoprobe at data acquisition to get a narrow parallel beam at high magnification), the focus calibration must be run for both these TEM modes. Temporarily alter the setting used for the focus calibration do to so. |

See also “Auto Focus Parameters” on page 90.
Eucentric Correction Calibration

The eucentric focus calibration is optional. It is meant to capture the difference between the microscope’s eucentric focus preset and the actual focus when the specimen is brought to eucentric height with help of the alpha tilt wobbler. This will be of high importance once the tilted acquisition is incorporated into EPU.

NOTE

Instead of calibrating the eucentric focus, you can also adjust the lens series alignment.

1. Carefully bring the specimen to eucentric height using the stage wobbler.
2. Focus the specimen.
3. Select the foil hole/eucentric height preset.
4. Click Eucentric Correction Calibration to start the calibration procedure.
8 Data Acquisition

Overview

Successful data acquisition depends on proper EPU setup and correctly following the steps for square and location selection described in this chapter.

General Directions

Successful data acquisition also depends on observing the following general directions.

Disc Space

Make sure that sufficient disc space is available before starting the run. Amounts in excess of 100 GB (much more if fractions are acquired) per day are no exception. If sufficient disc space is not available, EPU pauses and asks for another storage location.

Sample Quality

A well-prepared sample with good ice thickness and particle distribution is necessary to acquire high quality data. The success rate of EPU data acquisition itself also depends on the sample quality: crystalline ice or contamination with ice particles can lead to failure in finding and centering foil holes.

Alignments and Calibration

If you are experiencing problems, refer to “Troubleshooting” on page 76. In addition, “Preconditions” on page 7 can help in checking some important microscope alignments.

Make sure the calibrations were done at a central stage position, at eucentric height, and in focus (preferably with a parallel beam).
Optical Settings

In most cases, it is useful to acquire images with some defocus applied for contrast. However, adding too much defocus can cause problems in that alignments and calibrations may no longer be valid.

Liquid Nitrogen

To avoid failure of an unattended run, make sure that the liquid nitrogen supply is sufficient to finish the run. EPU frequently checks if a refill of the dewars is required (if applicable) and will request a refill if needed. However, if the main supply runs out, EPU cannot detect this and will continue while the sample temperature increases.
**EPU Setup**

Switch to the EPU tab. The order of the tasks in the EPU navigation pane indicates the workflow for setting up a data acquisition.

*Figure 22  EPU Session Startup*

A session can contain several data acquisition runs, which may use different parameters.
Define a Session

1. Under Session Settings (Figure 22), specify **Automatic** or **Manual** if you want to set up a fully automatic run or select suitable target locations manually. It is possible to switch between automatic and manual runs in one session.

2. Under Output, decide where the data is to be stored and in what format (this can also be changed between runs).

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<td><strong>For Direct Detection Cameras:</strong> Intermediate images are not saved in this folder. The camera has a fixed location where intermediate images can be saved. EPU saves the images at this location under the same folder structure to facilitate easy merging. If you want to have all the data in one place, you can select the fixed location of the camera as your output folder.</td>
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3. Under Specimen, enter some properties of the specimen support film to be used in the session. EPU supports two types: lacey carbon and Quantifoil, the latter coming in multiple variations that differ in hole size and spacing.

4. Under Email settings, enter the email addresses separated by commas, semicolons or spaces. Click the check box to enable email notifications to these recipients at the end of a run. Use the ‘Test’ button to confirm if the required email settings have been configured. These settings can be updated even after a run is started.

<table>
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<td>The email service uses the FEI Email Component that is installed along with the microscope server. The email queue and sender services have to be configured correctly for the email notifications to be sent correctly from EPU. Refer to the FEI Email Component manual for instructions on how to configure the email services.</td>
</tr>
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</table>
**Square Selection**

The EPU session automatically loads the last atlas. The atlas can be exchanged (a new one acquired or an old one loaded), as long as no data has been acquired. Once data runs have taken place, the EPU session continues with this atlas (even if a new one has been acquired) to maintain data consistency.

The Square Selection view displays the atlas overview image, similar to what is displayed in the Atlas tab. However, all detected grid squares are selected for acquisition by default and therefore outlined in green (*Figure 23*). Atlas tiles are outlined in yellow.

The general idea is that only those squares should be selected that contain vitreous ice of the right thickness and without contamination. The selection can be manipulated with the **Select all**, **Unselect all**, **Invert** buttons, and the context menu. The context menu permits you to “open” a tile in its original resolution or to zoom in on it (*Figure 24*).

**NOTE**

Squares can be selected by **Ctrl** + mouse click and deselected by **Shift** + mouse click.
Right-click on a tile to display context menu.

Figure 24  Opened Enlarged Tile and Context Menu
Select **Move stage to grid square** in the context menu to navigate to the selected grid square, as shown below.

*Figure 25  Drive Stage Using Atlas and Context Menu*

---

**Select Target Area**

The selection of suitable target areas differs for Quantifoil and lacey carbon samples.

- **Lacey carbon**: In automatic acquisition mode, these areas are defined by the ice thickness filters and data is acquired systematically by using a generated pattern of stage positions for data acquisition.

- **Quantifoil**: First select suitable foil holes (see below, *Hole Selection (Quantifoil Only)*) and then define the target areas for acquisition (*“Template Definition (Quantifoil Only)” on page 59*).
Hole Selection (Quantifoil Only)

1. Navigate to the grid square of your choice in the Square Selection task.
2. Click **Hole selection** in the navigation pane on the left.
3. Click **Acquire** to obtain an image.
4. When the image displays, click **Measure hole size** in the Measurements ribbon bar.

   This overlays the image with two yellow circles connected by a straight line. The initial size of the circles corresponds to the foil hole size and spacing as defined in the session.
5. The yellow circles must be resized and moved to match the hole size and spacing exactly, as shown below. Zoom into the image to position the measure tool more accurately.

*Figure 27   Measuring Hole Size*

6. When the size, spacing, and orientation of the measure tool are correct, click **Find Holes**. After a few seconds, a grid of holes will be overlaid.
**Automatic Selection**

For automatic location selection, define grey values using the histogram control in the filter ice quality pane ("Ice Thickness Filter Adjustment" on page 88). An upper and a lower threshold value can be set. The filters will be applied immediately and the change in the selection of holes can be seen in the image display (below).

_Figure 28 Foil Holes Selected by Ice Thickness Filters_

At run time, the selected filters will be automatically applied to every grid square. The filters can be normalized with respect to the image intensity in a hole by use of the $I_0$-calibration, so that the hole selection does not depend on gun current.
**Manual Selection**

If you have switched the session to **Manual Selection**, you can still use the mouse and ice quality histogram to select/deselect foil holes that are suited for data acquisition initially. In addition, there are selection buttons in the menu that can be used, for example, to deselect all holes.

Use the grid square image for navigation and inspect the grid or foil holes for particle content, ice thickness, charging, etc., by using the microscope’s UI and TIA at imaging conditions of your choice. Using the context menu, select and add/remove individual foil holes.

*Figure 29  Context Menu for Manual Hole Selection*

**NOTE**

- The manual selection procedure must be performed for every grid square that has been selected for the run.
- Before acquiring a grid square image, ensure that eucentric height has been adjusted properly, as the target positions will be stored as X, Y, Z coordinates (and a big change in Z will always have impact on the X,Y coordinates).
- Manual selection of target areas can be a way to separate LM and HM work completely, since the automatic run will never need to switch to LM.
- To quickly add target areas, hold down Ctrl when clicking; to quickly remove target areas, hold down Shift when clicking.
- If you have generated a pattern of target areas (lacey carbon) or done the ‘find holes’ holes procedure (quantifoil), then manual selection by clicking will tend to stick to the previously found hole positions for precision. Otherwise, they can be set freely.
Falcon Intermediate Images

On systems equipped with an FEI Falcon™ camera, a special acquisition mode is available allowing the EPU to program and read out the intermediate images that—on normal acquisition—are integrated into one image. Since these intermediate images are buffered before the camera is read-out, this functionality can be used for high-speed recording of consecutive, low-dose exposures. This functionality is also referred to as movie-mode or dose fractionation and can help to study beam induced effects like charging, sample damage, etc.

Saving all individual frames can be unpractical in terms of throughput and storage space utilization. In many cases, it is not really needed as there can be too little information at each frame. In that case, a number of slots can be defined, each of which can store a dose fraction, which is an integration of a number of frames. The number of dose fractions available for storage depends on the specific camera model (unlimited for Falcon III, limited for earlier models). This concept is depicted schematically in the drawing below.

Figure 30   Falcon Intermediate Images Concept

When setting up the acquisition, specify per dose fraction which range of frames to integrate; these integrated images are eventually read out and saved to disk. The number of frames per dose fraction may vary and can be as small as a single frame.
Enabling Dose Fractions

The dose fractions functionality is available for final data acquisition with the Falcon camera. If enabled, additional controls are unlocked.

**Falcon I & II**

1. Select the **Preparation** tab and click the **Acquisition and Optics** task in the navigation panel, ensuring the BM–Falcon camera is selected for the **Data Acquisition** preset.

![Figure 31 Data Acquisition Preset, Falcon I & II](image-url)
2. Navigate to the EPU tab page and click the Session Setup task. Note a new check box is available called Store Falcon Intermediate Images. Click this check box to enable the functionality and click the Apply button to apply the new settings.

Figure 32 Session Setup Task
The **Falcon Frames** task appears after enabling the intermediate images.

**Figure 33  Falcon Frames**

---

**Falcon III**

1. Select the **Preparation** tab and click the **Acquisition and Optics Settings** task. Select the **Data Acquisition** preset and make sure that the BM-Falcon camera is selected. Set **Fractions** to **Yes**. The **Direct Detector Dose Fractions** task will appear in the Task list when Fractions are enabled.

**Figure 34  Data Acquisition Preset, Falcon III**
Setting Up the Dose Fractions

Navigate to the **Falcon Frames** task to program the dose fractions (see previous image).

**Falcon I & II**

1. The ribbon shows the total exposure time of the acquisition and the number of frames to be acquired. The total exposure time is identical to the exposure time specified on the **Preparation** tab for the **Data Acquisition** preset.

2. To allocate the frames to the available dose fractions, a data grid is provided. Each row corresponds to a dose fraction; the columns specify the beginning and ending frame numbers.

   ![Figure 35: Dose Fraction Grid, Falcon I & II](image)

The total number of frames taken in the indicated exposure time are calculated. You can specify a lower number of frames to be distributed over the fractions. The distribution of frames over the fractions can be set by Single Frames; Equal Dosage, and in case there are more frames then fractions, the left over frames can be accumulated in the last fraction.

**NOTE**

- Frame 0 cannot be used.
- The frame numbers in each consecutive dose fraction must be in ascending order and the ranges may not overlap.
- A dose fraction can consist of a single frame, which is specified using the same frame numbers for **Begin** and **End**.
- To inspect the number of frames and exposure time of a dose fraction, select the corresponding row and hover over it with the mouse cursor. A tool tip appears with the information.
3. To test the intermediate frame acquisition, click the **Preview** button. This acquires the intermediate images plus the integrated image. If some of the specified frame numbers are invalid, it is reported in the status window.

*Figure 36  Preview Button*

4. To inspect the acquired intermediate image, use the image viewer on the same page. Use the **Previous** and **Next** buttons to browse through the intermediate images. The last image of the sequence is the integrated image.
5. To finish the process, specify how to store the intermediate images during EPU acquisition using **Storage Options** (either as separate files or as a single stack (.mrc only)).

![Storage Options](image)

The files are stored in the regular EPU output folder with the other images. An image stack does not contain the integrated image as this is already part of the normal EPU output data.

**Throughput Considerations**

The transfer of the intermediate images (from camera to temporary buffer to final storage location) is executed in parallel to the automated acquisition. However, at various points during the automated acquisition, EPU may need to wait for these background processes to catch up. The waiting time will depend on the amount of data being transferred and how fast the data can be transferred. For example, a network storage location is typically slower than a directly attached storage device. The amount of data relates directly to the number of intermediate images per exposure and the number of exposures per location. If throughput is key, take these points into account when setting up the automated run.

**Falcon III**

1. Select the **Preparation** tab and click the **Direct Detector Dose Fractions** task.

2. The ribbon shows the total exposure time of the acquisition and the number of frames to be acquired. The total exposure time is identical to the exposure time specified on the **Preparation** tab for the **Data Acquisition** preset.

3. Specify the number of dose fractions in the ribbon. The number of fractions should be equal to or smaller than the number of frames.
4. Use the table to allocate the frames to the available dose fractions. Each row corresponds to a dose fraction. The columns specify the beginning and ending frame numbers.

**Figure 38  Dose Fraction Grid, Falcon III**

The total number of frames taken in the indicated exposure time are calculated. You can specify a lower number of frames to be distributed over the fractions. The distribution of frames over the fractions can be set by Single Frames; Equal Dosage, and in case there are more frames then fractions, the left over frames can be accumulated in the last fraction.

**NOTE**

- The first and last frame(s) are affected by opening/closing the shutter and are not uniformly illuminated.
- The frame numbers in each consecutive dose fraction must be in ascending order and the ranges may not overlap.
- A dose fraction can consist of a single frame, which is specified using the same frame numbers for Begin and End.
- Hover over a dose fraction to see a tooltip with information about the dose fraction.
5. Use the **Validate** button to validate the dose fractions specification. Errors will be colored red and listed in the status window. Hover over an error to get a suggestion about how to fix it.

6. Click the **Preview** button to test image acquisition with dose fractions. Both the fraction images and the integrated image are acquired. The Validate option is always executed first when you click **Preview**.

7. Use the image viewer to inspect the dose fractions and the integrated image. The zero index is the integrated image.

8. Right-click in the image display to get a context menu in which the dose fractions and the integrated image can be saved.
9. No other applications can use the Falcon III camera when the dose fractions are viewed in the image viewer. Switch tabs, select another task or click the Finish button to release the Falcon III camera.

10. Go to the EPU tab and select the Session task.

11. The dose fractions will be saved on the storage server that is provided together with the camera. Refer to the Note on page 42.

**Template Definition (Quantifoil Only)**

The Template Definition task allows you to customize particle data acquisition. You create a template defining at which locations, relative to a foil hole, acquisitions will be made and auto focus will be performed. The acquisition areas can be graphically placed by clicking in the image. To set up a template:

1. Click **Template Definition**. The ribbon appears.

   ![Template Definition Ribbon](image)

2. Click **Acquire** to retrieve an image at the current position.

3. Click **Find Hole Center** to recognize the foil hole in the image that is closest to the center. A yellow circle will be placed on the position in the image where the foil hole was found.
4. Click **Find and Center Hole**. This will initiate a stage move to center the foil hole. An image will be automatically acquired and displayed. Use **Find Hole Center** again to find the foil hole in the new image.

5. **Adjust Maximum Image Shift**: Image shift can induce coma and astigmatism. Limit the amount of image shift applied, particularly on image-corrected systems. If all template areas cannot be reached solely by image shift below this limit, then stage movements will be involved.

6. **Adjust Delay after Image Shift**: This is a relaxation time to avoid that the image is drifting after use of the magnetic deflectors.

7. **Adjust Delay after Stage Shift**: There will be specimen drift for some time after a stage move. High resolution data should only be taken after an appropriate settling time. Alternatively, drift can be measured and data acquisition paused until a specified drift limit is reached (see “Drift Measurement” on page 66).
8. Position a **focus area** and multiple **acquisition areas** by first selecting the item to be positioned from the menu and then left-clicking in the image. The areas can still be dragged/moved afterwards.

9. Select the focus area and adjust Autofocus Area Settings.

   *Figure 41  Focus Area Parameters*

   - **Recurrence:** You can choose to perform an auto focus at every foil hole, never, or “after distance,” i.e., only when the acquisition has moved on the specimen by more than a specified distance.

   - **Distance:** The distance threshold use for the “after distance” option of Autofocus recurrence.

   - **Focus using:** The specimen can be focused by using the objective lens current or by adjusting the stage Z.

   - **Auto Stigmate:** In principle, auto focus can also correct astigmatism. However, under realistic conditions (low dose, bubbling ice, etc.) this option may not deliver results that are accurate enough.
10. Click Auto to place a pattern of acquisition areas inside the foil hole.
   - When you select a previously placed area, all the generated areas will have the same parameters (exposure time, focus pairs, etc.). Figure 42 shows an example of an acquisition template.

![Figure 42 Example of Acquisition Template](image)

- On Titan microscopes, both the CCD area and the beam are indicated. On Tecnai and Talos microscopes, the beam diameter cannot be calibrated and the beam area display will simply match the CCD area. Select the correct spacing by checking the beam size on the fluscreen.

11. You can select each of the acquisition areas and:
   - Change integration time if you want to deviate from the values chosen in the general setup.
   - Use Add/Remove to configure multiple exposures, such as focus or dose pairs.
   - Setup defocus variations per exposure, as explained in Defocus Variation.

**NOTE**

The changing of integration time per acquisition area is disabled if the Falcon camera is used with sub-frame recording enabled.
Defocus Variation

The EPU software supports a variety of scenarios to specify the applied defocus per recorded image. In basic use cases, a fixed defocus value is assigned to each acquisition area. If multiple exposures have been defined per acquisition area, the value can be set for each exposure individually.

For more advanced scenarios, configure the defocus settings to alternate between consecutive foil holes. This is accomplished via lists of defocus values that, on data acquisition, are cycled when moving from foil hole to foil hole.

NOTE

The defocus list is not a shared entity, but each exposure on each acquisition area has its own list of values.

To illustrate the concept, a schematic drawing is shown below where each acquisition area has a list of two defocus values.

The sequence of applied defocus values set up with two acquisition areas, each with a list of two defocus values, is shown below:

Figure 43  Sequence of Recordings and Defocus Setting During Template Handling at Data Acquisition

- For the first foil hole (A), each acquisition area is recorded with the first defocus value in the list, e.g., –4 µm for area I and –3 µm for area II
- For the second foil hole (B), all areas are recorded with the second defocus value in the list, e.g., –2 µm for area I and –1 µm for area II
- For the third foil hole (C), the used defocus restarts at the beginning of the list (–4 µm and –3 µm respectively)
**Same Defocus Values per Acquisition Area**

A common use case is to have the same defocus values per acquisition area. To facilitate this use case, the defocus list of the currently selected area is copied when creating additional acquisition areas. Hence, if the same list of values is needed for each acquisition area, first set up one acquisition area completely before creating the other areas. However, when editing the values later on keep in mind that each area (and exposure) has its own list.

**Different Defocus Values Per Acquisition Area**

As the defocus lists are specified per acquisition area (and per exposure), more advanced use cases are supported. The image below demonstrates a scenario, where each acquisition area has a different number of defocus values in the list.

- For the first foil hole (A), each acquisition area is recorded with the first defocus value in the list (e.g., -1 µm for area I, -4 µm for area II and -3 µm for area III)
- For the second foil hole (B), acquisition area I is acquired again with its only value in the list (e.g., -1 µm), area II and III with the second value in the list (-2 µm and 0 µm respectively)
- For the third foil hole (C), area I is again acquired with its only value in the list (-1 µm), area II with the third value in the list (-1 µm) and area III will start again at the first value in its list (-3 µm), etc.

**NOTE**

Currently, if a foil hole is skipped (for example because it could not be centered, auto focus failed, etc.), then the corresponding focus value will also be skipped.
Recording Focus Pairs

A common use case is the recording of focus pairs. Typically, two exposures are set per acquisition area, each with a different defocus value. The first exposure may cycle from foil hole to foil hole, the second is assigned a high value to assist particle picking. In the image below illustrates a scenario with only one acquisition area.

**Figure 45  Sequence of Applied Defocus Values Set with Two Exposures and Varying Defocus Per Exposure**

- For the first foil hole (A), two exposures are recorded each with its own defocus value (–3 µm and –6 µm respectively)
- For the second foil hole (B), the first exposure uses the second defocus value in the list (–2 µm), the second exposure uses the fixed high value (–6 µm)
- For the third foil hole (C), the first exposure cycles to third value in the list (–1 µm), while the second exposure again uses the fixed value (–6 µm)
- For the next foil hole the cycle restarts

Managing the Defocus Lists

- Add a new value to the focus list by adding a value to the second defocus edit box and clicking on the Add defocus value icon.

**Figure 46  Adding Defocus Value**
Use **Remove focus value** to remove the value taken over from the general setup if it does not suit your needs.

**Figure 47   Removing Defocus Value**

---

**Drift Measurement**

After moving the specimen, a certain amount of drift occurs. Its origin is partly mechanical and partly due to a disturbance in thermal equilibrium. The drift will reduce with time.

A drift measurement area can be placed on the template for the purpose of measuring the drift and pausing EPU data acquisition until it is low enough to guarantee high quality data.

Adjust the following parameters (click on the drift area marker first):

**Drift Threshold:** EPU will only continue after this threshold is reached or after 10 minutes. (If the measurement times out, EPU will proceed to the next foil hole.)

**Recurrence:** There are two options:

- Measure drift after each stage movement, i.e., after navigating to each foil hole
- Measuring drift once per grid square (a longer drift settling time is to be expected after movement between grid squares). For movements from one foil hole to the other (in this grid square), the stage settling time is applied.
Since detecting low drift under experimental conditions (specimen may change under illumination, read-out time of the CCD limits the measurement intervals), selecting the first option may reduce throughput. In that case, use the stage settling time parameter instead, after having determined an adequate time constant with the standalone drift auto function. See also “Drift Measurement Preset” on page 22 and “Standalone Drift Measurement” on page 91.

If the selection is to limit the image shift and navigate to data acquisition areas by stage, then the second option is the only available option.

**NOTE**

For the lacey carbon specimen, the settling time is the only parameter to stabilize the specimen drift. It will not be possible to place a drift measure position in the template for lacey carbon, as there is no template for it.
Template Execution (Quantifoil Only)

Acquire one template to test that EPU is setup correctly. Select the Template Execution task and click Preview. This runs the template, including finding and centering the hole on the current stage position. If any irregularities are observed during the template execution, solve them before continuing to the next step.

**NOTE**

Make sure you are at least close to eucentric height (within a few microns); otherwise, automatic focusing may fail.

![Template Execution Ribbon](image)
**Area Selection (Lacey Carbon Only)**

In the lacey carbon approach, the Quantifoil **Location Selection** task is replaced by **Area Selection**. Because the carbon film does not contain predefined holes, you create a pattern of equally spaced target areas (you can choose the distance).

Click **View Pattern** to plot circles (symbolizing the beam diameter) and rectangles (indicating the field of view of the CCD/plate camera) covering the entire image.

![Area Selection View](image)

---

**NOTE**

On Tecnai and Talos microscopes, the beam diameter is not yet calibrated and therefore is just drawn to fit the CCD area. Check the fluscreen to see what the beam area actually is.

Starting from this pattern, the selection of the suitable areas is identical to the one described for Quantifoil (page 46). For ice filtering using the ice quality histogram, only the area in the field of view of the CCD is considered by the filters.

In the Area Selection view, you can also adjust the parameters for auto focus and add multiple exposures (e.g., focus pairs). This is very similar to what is described above for the Quantifoil template definition (page 59).

Adjust the stage shift delay to your needs, ensuring that it is long enough so that data acquisition does not suffer from drift after a stage move.
During data acquisition, EPU will run auto focus on the first area, which will thus be sacrificed. The stage then moves to the next area and the acquisition(s) done as specified. If required, auto focus will now be run on the already exposed area, the stage will be moved to the next area, high resolution data will be acquired, etc.

**Automatic and Manual Selection**

The difference between automatic and selection of target areas is the same as for Quantifoil samples. See “Hole Selection (Quantifoil Only)” on page 46 for details.

**Automated Acquisition**

Click **Start Run** on the Automated Acquisition menu. An automated acquisition can run for several days in a row, if required.

When you are certain that the data acquisition runs satisfactorily, click **Close Col. Valves**. EPU will close the column valves when data acquisition is completed.

**NOTE**

Click **Pause** or **Stop** at any time to interrupt data acquisition. After **Stop**, you can readjust most parameters (e.g., change the beam diameter or the exposure times). When you then restart the acquisition, the data run will be continued from where it was abandoned. Even if EPU has been shut down, data acquisition can still be continued from when it was interrupted.

When EPU skips or finishes data acquisition on a grid square, this square is marked internally as “closed” to maintain the integrity of the data. However, it is possible to make these grid squares again accessible for data acquisition without creating a completely new session. In the Location Selection view, right-click on the grid square to display the context menu and select the **Open** option.
**Data Acquisition**

**Automated Acquisition**

In the Automatic Target Area selection (see *Figure 22 on page 41*), for every selected grid square the following occurs:

1. If two hours have passed, \( I_0 \) is measured to normalize the ice filters.
2. The stage moves to the center of the grid square.
3. The eucentric height is adjusted (at low SA magnification). If the eucentric height fails, the grid square is skipped.
4. An image of the grid square is taken.
5. Target areas on the grid square are determined and selected by the ice thickness filters.
6. The automated data acquisition will then take data on all selected target areas as defined in *“Template Definition (Quantifoil Only)” on page 59* and *“Area Selection (Lacey Carbon Only)” on page 69*. If the centering of a foil hole fails (Quantifoil only) or the auto focus is not successful, the target area is skipped.

**Manual Selection**

In Manual Target Area selection, the sequence of events is much simpler because the selection process has already been performed. There is no switching back to LM magnifications, and there is no adjustment of eucentric height (the target locations should be stored with the grid square at eucentric height).
9 Viewing Results

Overview

You can view the EPU results by switching to the Results Viewer tab or via Windows® Picture Viewer.

Using the Results Viewer

Use the Load Session button in the top ribbon to load EPU results. In the dialogue box, browse the directory where the EPU session was stored and open the EpuSession.dm file.

Figure 52 Opening EPU Sessions
After a session has been loaded, the corresponding atlas and the first tile with data is displayed in the left panel of the UI and the first grid square is displayed in the central image display (see figure 9-2).

**Previous** and **Next** control buttons can be used to navigate between grid squares (or foil holes or data images, whichever is shown in the central display, see below).

**Figure 53  Results Viewer**
For viewing foil hole images, right-click on the current grid square image and select **Show foil holes** in the context menu.

*Figure 54  Navigation to Foil Hole Images*

Similarly, navigation from a foil hole image to its parent grid square or to the corresponding data images can be achieved by selecting **Show grid square** and **Show data images**.

*Figure 55  Navigation to Data Images and Toggling High*

High resolution images can be viewed by clicking **Show high resolution** in the context menu of a low resolution image (see image above). If already in high resolution mode, then the reversion to low resolution mode is made by clicking **Show low resolution**.

*Figure 56  Reverting Back to Low Resolution Images*
Using Windows to View Images

Every image acquisition within EPU results in a number of stored files:

- The high quality mrc or tif file
- An xml file with metadata
- A jpeg file with compressed image data
- Additional mrc or tif image(s) with the dose fractions if Falcon’s Intermediate Images functionality is used

In Windows Explorer®, navigate to the directory in which the images are saved.

![EPU Data Directory](image)

Browse through all the jpeg images and select the image to be inspected.

- **Windows 7**: Right-click on the selected image and select Preview. This opens the Windows Photo Viewer. Use its navigation buttons to view next or previous images.
- **Windows XP**: Right-click on the selected image and select **Open With > Windows Picture and Fax Viewer** from the context dialog. *Previous* and *Next* buttons load images.
10 Troubleshooting

Overview

This chapter lists problems that may be encountered when setting up an automated acquisition or during the acquisition itself. Solutions are provided.

Known Issues

The following table contains known issues, possible causes, and solutions.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atlas:</strong></td>
<td></td>
</tr>
</tbody>
</table>
| Atlas does not look as it should: | 1. **Cause:** The specimen may not be in focus. Large deviations in defocus will cause the images to rotate and, since illumination is not parallel in this step, the apparent magnification will also change.  
**Solution:** Refocus.  
2. **Cause:** If the specimen is focused, the calibrations are probably not valid anymore and must be redone. (Has there been a big change in alignments or was another alignment file loaded?)  
**Solution:** Recalibrate.  
3. **Cause:** Distortion can be inherent to images at very low magnification.  
**Solution:** Acquire an atlas at increased magnification.  
4. **Cause:** The view is partly blocked (e.g., by an aperture) at the selected magnification.  
**Solution:** Acquire an atlas at increased magnification; confirm with a preview (or TIA) that the field-of-view of a single tile is unobstructed.  
5. **Cause:** The system's beam and image shift are not well-aligned, causing loss of illumination.  
**Solution:** Re-align the beam and image shifts for LM mode using the Microscope UI (Alignments Control, Calibrate LM–Image/Beam calibration). |
| “Move to” command does not center feature or grid square. | See 1, 2, 3 above.  
**Test:** Check this at the magnification at which the atlas is acquired (use the CCD, not the flu screen, to be sure).  
**Solution:** Same as above. |
Table 1  Troubleshooting (2 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| “Move to” command (in Atlas) does not center grid square | **Description:** Atlas looks OK, but navigation to the grid square is inaccurate. Grid square is not centered on CCD when acquiring an image in the Location/Area Selection view.  
**Possible Causes:**  
1. Some image shift is applied (check in the Image Settings control panel of the microscope UI).  
   **Solution:** Reset it.  
2. If you see the same problem at Atlas magnification (see above), specimen was not focused when taking the atlas (see above) or calibrations are not valid anymore.  
   **Solution:** Focus and/or redo the calibrations.  
3. Rotation center is off (≥ any change in focus can result in image shifts). Usually, to enhance contrast you will have applied a defocus of a few mm during image acquisition.  
   **Solution:** Adjust rotation center.  
4. Nonparallel beam or excessive defocus is used. (This results in small centering errors.)  
   **Solution:** Work with a parallel beam and do not use excessive defocus. There may always be some small discrepancy because positions are calculated and not all stage characteristics may be taken into account.  
5. Imperfect LM lens series alignment and EPU image shift calibration. When changing magnifications, a feature should stay centered (especially true when switching between atlas and grid square magnifications).  
   **Test:**  
   - Select the **Preparation** tab.  
   - Select the **Acquisition and Optics Settings** task.  
   - Use the **Acquire** buttons for easy switching with correct lens normalizations and image shift corrections.  
   **Solution:** Repeat EPU image shift calibration or when shifts are large, adjust the LM lens series alignment. |
### Troubleshooting (3 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| “Move Stage to” command does not center foil hole or feature. | Check:  
- If you see this problem only in the Template Definition view (at higher magnification), see ““Move Stage to …” command in Location Selection / Area Selection view does not center foil hole or feature in Template Definition view.” on page 79.  
- If you see this while staying at the magnification used for imaging the grid square, proceed here.  
  1. **Cause**: Too much defocus can change image rotation. A non-parallel beam will lead to additional change in magnification.  
     **Solution**: Make sure to work with no or just a few mm defocus. (Focus specimen correctly by using the wobbler and specify the wanted defocus in the optics settings of EPU.) If possible, use a parallel beam.  
  2. **Cause**: Mechanical play in stage.  
     **Solution**: Make sure that before taking the grid square image, the grid was centered with a Move Stage... command in the atlas (compensates for any backlash problems).  
  3. **Cause**: Calibrations are no longer valid (microscope alignments have changed?).  
     **Solution**: Redo calibrations.  
**Note**: Since the feature positions are calculated and not all characteristics of the stage are known exactly, a feature will not be centered 100% accurately. A micron deviation is not uncommon. |
| Measure foil holes: the original size of yellow glasses does not match at all. | 1. **Cause**: Correct Quantifoil type was not selected at start of session or the size of the holes deviates from specification.  
     **Solution**: Set the size correctly in this view.  
  2. **Cause**: Size of the holes deviates from specification.  
     **Solution**: Set the size correctly in this view. |
## Known Issues

### Template Definition:

**Problem:**

“Move Stage to ...” command in Location Selection/Area Selection view does not center foil hole or feature in Template Definition view.

**Possible Cause(s) and Solution(s):**

1. **Check:** If you see this problem also at the magnification at which the grid square is imaged, see information under “Grid Square (Location Selection/Area Selection View):” on page 77. Otherwise~
   - **Cause:** Some image shift is applied (check this in Image Settings control panel of the microscope UI).
   - **Solution:** Reset it.

2. **Cause:** Bad alignment of LM lens series against HM lens series and bad EPU image shift calibrations.
   - **Test:** When switching between Location Selection view and Template Definition view magnifications, a feature should stay centered on the CCD.
   - Select the Preparation tab in EPU.
   - Select the Acquisition and Optics Settings task.
   - Use the Acquire buttons for easy switching with correct lens normalizations and image shift corrections.
   - **Solution:** Repeat EPU image shift calibration or when the shifts are large, adjust LM lens series alignment.

---

**Problem:**

“Move Stage here...” command in Template Definition view does not center feature.

**Possible Cause(s) and Solution(s):**

1. **Cause:** Mechanical play in stage.
   - **Solution:** Try at least twice to make sure that a backlash correction was performed.

2. **Cause:** Working far from eucentric focus will render the magnification calibrations invalid. Large defocus will slightly rotate the image and (with a non-parallel beam) magnification changes.
   - **Solution:** Make sure the sample is at eucentric height, focused, rotation center is well corrected, and the defocus chosen in the Optics settings of EPU should not exceed 20 µm.

3. **Cause:** Calibrations are invalid (maybe the microscope alignment changed?).
   - **Solution:** Redo calibrations.

4. **Cause:** Stage may have a hardware problem and does not move correctly over these smaller distances.
   - **Solution:** Call Service.
## Table 1  Troubleshooting (5 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow circle does not match foil hole size</td>
<td>1. <strong>Cause</strong>: Measure Foil Holes/Find Foil Holes in the Location Selection view was not performed.</td>
</tr>
<tr>
<td></td>
<td><strong>Solution</strong>: Return to Location Selection and do it.</td>
</tr>
<tr>
<td></td>
<td>2. <strong>Cause</strong>: Specimen is not at eucentric height for the current sample position and not focused.</td>
</tr>
<tr>
<td></td>
<td><strong>Solution</strong>: Bring specimen to eucentric height and focus it.</td>
</tr>
<tr>
<td></td>
<td>3. <strong>Cause</strong>: Too large defocus is used and beam is not parallel. (The apparent magnification of</td>
</tr>
<tr>
<td></td>
<td>the image may change under these circumstances.)</td>
</tr>
<tr>
<td></td>
<td><strong>Solution</strong>: Select a defocus selected in the Template Optics settings of EPU of less than 20 µm.</td>
</tr>
<tr>
<td></td>
<td>If possible, choose a parallel beam.</td>
</tr>
<tr>
<td></td>
<td>4. <strong>Cause</strong>: The Measure Foil Hole step was inaccurate.</td>
</tr>
<tr>
<td></td>
<td><strong>Solution</strong>: Repeat this step (Location Selection view) with a slight change in the size of the</td>
</tr>
<tr>
<td></td>
<td>yellow glasses.</td>
</tr>
<tr>
<td></td>
<td>5. <strong>Cause</strong>: Calibrations are invalid (maybe the microscope alignment changed?).</td>
</tr>
<tr>
<td></td>
<td><strong>Solution</strong>: Redo calibrations.</td>
</tr>
</tbody>
</table>
Table 1  Troubleshooting (6 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| “Find Foil Hole” does not succeed. | 1. **Check**: Does the yellow circle match the hole size? If not, see above, *Yellow circle does not match foil hole size.*
2. **Cause**: Measure Foil Holes/Find Foil Holes in the Location Selection view not done. (The “find foil hole” algorithm uses information about hole size and spacing.)
   **Solution**: Switch to the Location Selection view and perform Measure Foil Holes and **Find Foil Holes**.
3. **Cause**: The circle size is wrong. (The algorithm of finding holes may be quite sensitive to the exact hole size.)
   **Solution**: Repeat the **Measure Foil Holes** step (Location Selection view). Change the size of the yellow glasses slightly.
4. **Cause**: Magnification is too high.
   **Solution**: Use a magnification in which neighboring foil holes are at least partly visible. This will increase the stability of the algorithm since the spacing and orientation of the foil hole pattern can be exploited.
5. **Cause**: Magnification is too low. (The current algorithm does not work with too many foil holes visible.)
   **Solution**: Increase the magnification such that only the foil hole and its nearest neighbors are visible on the CCD image.
6. **Cause**: The current specimen location contains too many features (crystalline ice, etc.) that cause the algorithm to fail.
   **Solution**: Find a cleaner sample area.
7. **Cause**: Contrast is too low.
   **Solution**: Add or increase the defocus in the optics settings. (Make sure to start from focused specimen, specimen at approximately eucentric height).
8. **Cause**: Image quality too bad (noise). The human eye is very good at integrating over features, so noise may easily be underestimated at this magnification.
   **Solution**: Take image with higher dose.
9. **Cause**: Foil holes appear dark when the Allow Dark Foil Holes option is not selected.
   **Solution**: If the foil hole appears darker than the foil itself, select the option **Allow Dark Foil Holes** (in ribbon of Location Selection view).
<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| Foil hole centering fails (or hole is not correctly centered) | Tracing this problem is a combination of tracing problems in:  
1. “Find Foil Hole” (see “Find Foil Hole” does not succeed.” on page 81. Can be tested separately).  
2. “Move stage here” (see “Move Stage here...” command in Template Definition view does not center feature.” on page 79. Can be tested separately).  
In addition, the following could be the case:  
**Cause:** The foil hole was not fully visible initially and the fitted circle degenerated to an ellipse. In this case the centering will not be perfect.  
**Solution:** Reduce the magnification. |
| Pattern acquisition fails.                   | 1. **Cause:** Foil hole cannot be recentered. (Pattern acquisition requires the foil hole to be centered within 300 nm.)  
**Test:** Click **Find and Center Foil Hole** button.  
**Solution:** See above, *Foil hole centering fails (or hole is not correctly centered).*  
2. **Cause:** Auto Focus fails.  
**Solution:** See below, “Pattern acquisition: Auto Focus fails.” on page 83. |
Table 1  Troubleshooting (8 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| Pattern acquisition: Auto Focus fails. | 1. **Cause:** Specimen is too far off from eucentric height and focus. Usually during an automatic run of EPU, after every move to a new grid square, the eucentric height is adjusted. During the setup phase, you moved to a grid square, but may not have adjusted eucentric height.  
**Solution:** Adjust eucentric height.  
2. **Cause:** Auto focus does not converge because the beam is visible in the images. There may be a couple of reasons for this.  
**Test:** Acquire a single image with the auto focus presets (from the Preparation tab). If the beam edge is visible on this image, then~  
**Cause:** The beam shift was not aligned.  
**Solution:** Use the Direct Alignments panel to align the beam shift at the auto focus or data acquisition magnification.  
**Note:** If the beam moves when you change Intensity of illuminated area, then you have to align apertures or condenser system as well.  
If the beam edge is not visible in the test, then it will become visible only when the beam is tilted or shifted.  
3. **Cause:** Beam tilt pivot point misaligned.  
**Solution:** Use Direct Alignments panel to align the beam tilt pivot point.  
**Tip:** Adjust eucentric height and focus first. There is a little problem in that the pivot point is well aligned only at a certain focus setting.  
You may have to allow for some inaccuracy by choosing a slightly larger beam diameter in the focus optics setting since in the automatic data acquisition you will not always start close to focus.  
4. **Cause:** The image/beam shift calibration is not done.  
**Test:** When you add some image shift in the Image Settings control cluster, do you then see the beam edge moving into the field of view?  
**Solution:** Perform the Calibrate HM-TEM > Image/Beam calibration (use the Alignments control panel of the microscope UI).  
5. **Cause:** Auto focus does not converge, although the beam edge is not visible; invalid focus calibration  
**Solution:** Redo focus calibration.  
(Test with the standalone Auto Focus routine.) |
Table 1  Troubleshooting (9 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| **Pattern acquisition:** beam edge visible in data acquisition | 1. **Test:** Acquire a single image with the Data Acquisition presets (Preparation tab). If the beam edge is visible on this image, this means~  
  **Cause:** Beam shift was not aligned correctly in the first place.  
  **Solution:** Use the Direct Alignments panel to align the beam shift data acquisition magnification.  
  **Note:** If the beam moves when you change intensity of illuminated area, align apertures or condenser system as well.  
  If the beam edge is not visible, then it will only become visible when the beam is shifted during the pattern acquisition.  
  2. **Test:** When you add some image shift in the Image Settings control cluster, do you then see the beam edge moving into the field of view? If so~  
  **Cause:** Image/beam shift calibration is not done.  
  **Solution:** Perform HM-TEM > Image/Beam calibration (Alignments control panel of the microscope UI). |
| **Pattern acquisition:** acquisition areas are not placed correctly. | 1. **Cause:** If you use Auto Focus by Stage Z Adjustment, maybe the first time Z had to be adjusted a lot, which leads to displacements in X and Y.  
  **Solution:** Try again. This time the Z adjustment should be small.  
  2. **Cause:** If you use Auto Focus by Change of Objective Lens Current, focusing may shift the feature (hole) when the rotation center is not well aligned.  
  **Solution:** Use the Direct Alignments panel to align the rotation center.  
  3. **Cause:** EPU image shift calibration no longer valid or not done.  
  **Test:** Use the Preparation tab to acquire images with the Hole/Eucentric Height preset and Data Acquisition preset. Does a given feature stay centered? If yes, then the lens series alignment is not well done and the deviations are not captured by the EPU image shift calibration.  
  **Solution:** Redo the EPU image shift calibrations. If the shift is very large, redo the microscope HM image shift alignment first.  
  4. **Cause:** Image beam shift calibration not valid. (If you use image/beam shift only to place the acquisition areas, placement should generally be quite accurate.)  
  **Solution:** Perform HM-TEM > Image Beam calibration (Alignments control panel of microscope UI).  
  5. **Cause:** Magnification calibration not valid.  
  **Test:** Test the validity of the calibrations by using the Move Stage Here... feature. However, in these stage movements, some inaccuracy may have to be accepted due to the mechanics of the stage.  
  **Solution:** Redo the magnification calibrations. |
Table 1  Troubleshooting (10 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated Acquisition:</td>
<td></td>
</tr>
</tbody>
</table>
| Grid squares are all skipped. | 1. **Cause:** The eucentric height procedure is failing.  
**Test:** The performance of the auto-eucentric height procedure can be tested standalone (use the Auto Functions tab in EPU). During automated data acquisition, the procedure is performed using the Hole/Eucentric Height optical preset (a different defocus may be applied). The procedure used is the one that is based on a focus measurement and, therefore, a valid focus calibration is needed.  
**Solution:** Increasing the number of counts per exposure may help if the images are very noisy. The focus calibration may have to be redone.  
2. **Cause:** No holes are found. A grid square image is acquired, and auto eucentric height succeeds, but then no foil holes were found. Measure Foil Holes procedure was not set up correctly.  
**Solution:** Redo the “Measure foil holes” procedure that should have been done during the setup phase (Location Selection task).  
3. **Cause:** No foil holes are selected. A grid square image is acquired, holes are found, but none is selected by the ice thickness filters.  
**Solution:** Redefine the filters (go to the Location/Area Selection). If the intensity changes due to an instability of the gun, use the $I_0$ calibration in EPU in addition. It will periodically measure the intensity in a hole and correct the ice filters accordingly. |
## Troubleshooting

### Known Issues

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
</table>
| **Too many foil holes; target areas are skipped.** | 1. **Cause:** They are not found in the grid square image in the first place because step(s) were omitted in the setup phase. **Solution:** Repeat setup steps.  
2. **Cause:** They do not pass the ice filtering. Either the illumination conditions or acquisition settings have changed after the filters have been selected or the filters were tuned on a grid square with too thin/thick ice. **Solution:** Go to the Location/Area Selection view and adjust the ice filter settings.  
3. **Cause:** Foil holes were selected in the grid square image, but could not be found or centered at the foil hole magnification. A foil hole is skipped if it could not be centered within certain accuracy. **Solution:** For troubleshooting, see “Find Foil Hole” does not succeed.” on page 81 and “Foil hole centering fails (or hole is not correctly centered)” on page 82.  
4. **Cause:** Auto focus fails. A foil hole is skipped if the focus could not be determined. **Solution:** For trouble shooting, see “Pattern acquisition: Auto Focus fails.” on page 83. During the automatic data acquisition, auto focus may also fail if the lens series focus presets are (very) ill-defined. The routine is based on the measurement of beam tilt-induced image shifts that are proportional to the amount of defocus (it works like the wobbler on the hand panel). For valid measurements, the images must overlap by at least 60%, which means that the range that the auto focus can cover is limited, especially when run at high magnification. Initially, eucentric height and focus are adjusted at the magnification used for centering the foil hole (before an image of the grid square is acquired), but later the auto focus routine is run at a much higher magnification typical for data acquisition. If the magnification switch results in a large focus change, auto focus may fail. In this case, correct the objective lens presets of your lens series (part of the lens series alignment). |

| Acquisition areas are not placed correctly. | Solutions:  
1. See “Pattern acquisition: acquisition areas are not placed correctly.” on page 84.  
2. When you have chosen Z adjustment as the method of auto focus, Z adjustments will always induce some X and Y movements of the specimen. Big adjustments can be a consequence of a non-flat or tilted specimen (little can be done about that). |
Table 1  Troubleshooting (12 of 12)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause(s) and Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam is shifted in Autofocus routine.</td>
<td><img src="image" alt="Diagram of beam shifted in Autofocus routine" /></td>
</tr>
</tbody>
</table>

For high magnifications (> ~75 kX) and small beam sizes as shown in the image above, it can happen during the Autofocus routine that the beam seems slightly shifted, resulting in a not completely illuminated detector. This is usually due to (1) beam tilt pivot points not set correctly and/or (2) a too large value in Autofunctions > Autofocus > Iterate to. To resolve:

- Make sure you are at eucentric height (run Autofunctions > Eucentric Height or set manually).
- Click Eucentric focus on the hand panel; a well aligned TEM will now be in focus. If not:
  - Carefully focus manually or use Autofunctions > Autofocus at a lower magnification.
- Click Reset defocus (defocus reading in UI is now 0).
- Perform Direct Alignments > beam tilt pivot points.
- Go to the desired optical settings for Autofocus.
- Check the number in Iterate to in Autofunctions > Autofocus.
- Manually change defocus to this number and check the movement of the beam.
- If it is too large (as show in the image above), try the following (in the order shown below):
  - Find the largest defocus value for which the beam stays on the detector and reduce Iterate to to the number found, ~or~
  - Reduce the camera area to Half or Quarter, ~or as a last resort~
  - Increase the beam diameter.
Appendix A

Overview

This appendix provides additional material on the following topics:
- Adjusting ice filter thickness, see below
- Automatic functions, see page 90
- Standalone drift measurement, see page 91

Ice Thickness Filter Adjustment

For automatic selection of suitable target areas, a range of gray values must be specified in the Filter Ice Quality histogram. If a target area mainly contains pixels that fall into the range of the selected gray scale, it is accepted as having a suitable ice thickness. In cases in which an I₀-calibration is used, the gray values correspond to fractions of I₀ and any change in illumination intensity will be accounted for automatically.

To adjust the filter:

1. If not already done, acquire a grid square image.
2. For a Quantifoil sample:
   - Click Measure Hole Size, if not already done earlier.
   - Click Find foil holes.
3. For a lacey carbon sample, click View Pattern.
4. In the Filter Ice Quality histogram, move the red selection markers with the mouse to select the approximate range of gray values. The selection of target areas in the image display will be updated (if there are many target areas, this may take some time).
For a fine adjustment of the ice filters, zoom into the histogram by selecting a range in the lower band of the histogram display. Double-click on the lower band to undo the zooming.

*Figure 58  Histogram of Ice Filter Adjustments (Zoomed)*

The target area used for filtering is the main fraction (inner 90%) of either the area of a detected hole (Quantifoil) or the area that would be seen on the acquired image (lacey carbon).
Auto Functions

The Auto Functions tab in EPU provides access to automatic function calibrations. Auto functions may also be executed as standalone tasks. The relevant ribbon area is shown in red below.

![Auto Functions](image)

The three auto functions are:

- **Auto Focus**: Adjusts the focus.
- **Auto Eucentric**: Based on a focus measurement and adjusts the stage Z such that the specimen is at eucentric focus. (If the eucentric correction calibration is available or negligible, the result should roughly be the same as with Auto Eucentric Stage Tilt.)
- **Auto Eucentric Stage Tilt**: Adjusts the eucentric height by tilting the stage between +15 and -15 degrees and minimizing specimen shift by adjusting the stage Z.

Access the Preset dropdown menu to select the imaging preset to be used when executing the auto functions.

- The **Autofocus** preset is typically used for focus measurement based techniques, which tilt the beam.
- The **Hole/Eucentric Height** preset is used for the method that tilts the stage.
- The extra defocus specified in these settings is not necessarily applied during execution of the auto functions.

Auto Focus Parameters

The following Auto Focus parameters can be adjusted.

- **Desired**: After execution of Auto Focus the specimen should be imaged with a desired focus as specified here in micrometers.

<table>
<thead>
<tr>
<th>NOTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>This does not include the extra defocus specified in the imaging settings. In the context of an automatic EPU data acquisition, the desired defocus will be set to zero.</td>
</tr>
</tbody>
</table>
■ **Iterate to:** This is the defocus that must be adjusted first during execution of the auto focus routine. It ensures that the expected shift in the cross-correlation is always clearly different from zero and that there will be a guaranteed contrast in the images in the last iteration of the measuring procedure. The “desired” defocus is then adjusted by simply changing the objective lens current.

■ **Method:** Auto focus can use the objective lens current to adjust the focus or it can use the Z-drive of the stage. In the latter case, the procedure is similar to Auto Eucentric.

■ **Auto Stigmate:** No means that only the focus will be adjusted; Yes means that astigmatism will also be corrected. This works on a well prepared stable area of the specimen such as a piece of carbon film. It is not recommended during automatic data acquisition when the ice is changing between exposures.

### Standalone Drift Measurement

Standalone drift measurement can be used to determine a characteristic settling time for the stage.

To set up a drift measurement, select **Drift stabilization** in the navigation panel and access the Presets dropdown menu to select the imaging preset **Drift Measurement**. Adjust the drift threshold (Max. Remaining Drift) and drift Time Out. Start the measurement by clicking the **Start** button in the ribbon. The measurement will run until either the specified drift is reached or the timeout is exceeded.

The result of the measurement is a graph that shows the drift over time.

*Figure 60  Drift Measurement Progress*
Appendix B

MRC Image Format in EPU

EPU uses the MRC2014 data format. The official format specification can be found at: http://www.ccpem.ac.uk/mrc_format/mrc_format.php.

MRC files have a main header and an extended header. The main header contains generic image information like: the image dimensions and the pixel format (see the MRC2014 format specification). The extended header contains information about the microscope state at acquisition time (e.g., magnification, accelerating voltage, stage position, beam shift, and additional image acquisition information such as binning and exposure). The manufacturer specific format of the extended header is Table 2.

The image views use the top left corner as zero position. The bottom right corner contains the last pixel. The image data in the MRC2014 files is stored in the same order; the first pixel value (read from the beginning till the end of the file) is from the top left corner and the last pixel value is from the bottom right corner. The pixels are written line by line, starting with the top line (see the image below). Be aware that third-party applications like IMOD and Fiji/ImageJ, can display MRC images in different way. A common discrepancy for example, is the orientation among the central horizontal plane by choosing a different location for the zero-position (top-left or bottom-left corner).
Appendix B  ■  Format FEI Specific Metadata in Extended Header

Format FEI Specific Metadata in Extended Header

The size of the extended header is specified by the “NSYMBT” field in the main header. The extended header of MRC files generated by EPU contains FEI-specific information about the microscope state. The table below shows which data can be found in the extended header.

Table 2  Format Extended Header (1 of 7)

<table>
<thead>
<tr>
<th>Name</th>
<th>Offset (dec)</th>
<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metadata size</td>
<td>0</td>
<td>0x0000</td>
<td>Int32</td>
<td>NA</td>
<td>Metadata size in bytes</td>
</tr>
<tr>
<td>Metadata version</td>
<td>4</td>
<td>0x0004</td>
<td>Int32</td>
<td>NA</td>
<td>Version ID of the metadata format 0 = Initial version which is described here</td>
</tr>
<tr>
<td>Bitmask 1</td>
<td>8</td>
<td>0x0008</td>
<td>UInt32</td>
<td>NA</td>
<td>Individual bits indicate which metadata fields are set</td>
</tr>
<tr>
<td>Timestamp</td>
<td>12</td>
<td>0x000C</td>
<td>Float64</td>
<td>Bitmask 1 – #0</td>
<td>Time when the image was taken</td>
</tr>
<tr>
<td>Microscope type</td>
<td>20</td>
<td>0x0014</td>
<td>16 chars</td>
<td>Bitmask 1 – #1</td>
<td>Identifier for microscope type (Krios, Talos, Titan, Metrios, etc.)</td>
</tr>
<tr>
<td>D-Number</td>
<td>36</td>
<td>0x0024</td>
<td>16 chars</td>
<td>Bitmask 1 – #2</td>
<td>Microscope identifier</td>
</tr>
<tr>
<td>Application</td>
<td>52</td>
<td>0x0034</td>
<td>16 chars</td>
<td>Bitmask 1 – #3</td>
<td>Application name (e.g. “FEI Tomography”)</td>
</tr>
<tr>
<td>Application version</td>
<td>68</td>
<td>0x0044</td>
<td>16 chars</td>
<td>Bitmask 1 – #4</td>
<td></td>
</tr>
<tr>
<td>Gun</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT</td>
<td>84</td>
<td>0x0054</td>
<td>Float64</td>
<td>Bitmask 1 – #5</td>
<td>High tension in Volts</td>
</tr>
<tr>
<td>Dose</td>
<td>92</td>
<td>0x005C</td>
<td>Float64</td>
<td>Bitmask 1 – #6</td>
<td>Dose in E/m²</td>
</tr>
</tbody>
</table>

Bool = Boolean of 1 byte (0 = false, other value = true)
Int32 = Signed integer of 4 bytes
UInt32 = Unsigned integer of 4 bytes
Float64 = Floating point number of 8 bytes
### Format Extended Header (2 of 7)

<table>
<thead>
<tr>
<th>Name</th>
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<th>Offset (hex)</th>
<th>Format</th>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha tilt</td>
<td>100</td>
<td>0x0064</td>
<td>Float64</td>
<td>Bitmask 1 – #7</td>
<td>Holder Alpha tilt along axis in degrees</td>
</tr>
<tr>
<td>Beta tilt</td>
<td>108</td>
<td>0x006C</td>
<td>Float64</td>
<td>Bitmask 1 – #8</td>
<td>Holder Beta tilt along axis in degrees</td>
</tr>
<tr>
<td>X-Stage</td>
<td>116</td>
<td>0x0074</td>
<td>Float64</td>
<td>Bitmask 1 – #9</td>
<td>Stage X position in meters</td>
</tr>
<tr>
<td>Y-Stage</td>
<td>124</td>
<td>0x007C</td>
<td>Float64</td>
<td>Bitmask 1 – #10</td>
<td>Stage Y position in meters</td>
</tr>
<tr>
<td>Z-Stage</td>
<td>132</td>
<td>0x0084</td>
<td>Float64</td>
<td>Bitmask 1 – #11</td>
<td>Stage Z position in meters</td>
</tr>
<tr>
<td>Tilt axis angle</td>
<td>140</td>
<td>0x008C</td>
<td>Float64</td>
<td>Bitmask 1 – #12</td>
<td>Angle of tilt axis in image in degrees</td>
</tr>
<tr>
<td>Dual axis rotation</td>
<td>148</td>
<td>0x0094</td>
<td>Float64</td>
<td>Bitmask 1 – #13</td>
<td>Measured rotation angle after b flip in degrees (Tomography only)</td>
</tr>
<tr>
<td>Pixel size X</td>
<td>156</td>
<td>0x009C</td>
<td>Float64</td>
<td>Bitmask 1 – #14</td>
<td>Pixel size X in meters</td>
</tr>
<tr>
<td>Pixel size Y</td>
<td>164</td>
<td>0x00A4</td>
<td>Float64</td>
<td>Bitmask 1 – #15</td>
<td>Pixel size Y in meters</td>
</tr>
<tr>
<td>Optics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defocus</td>
<td>220</td>
<td>0x00DC</td>
<td>Float64</td>
<td>Bitmask 1 – #22</td>
<td>Defocus in meters</td>
</tr>
<tr>
<td>STEM Defocus</td>
<td>228</td>
<td>0x00E4</td>
<td>Float64</td>
<td>Bitmask 1 – #23</td>
<td>STEM defocus in meters</td>
</tr>
<tr>
<td>Applied defocus</td>
<td>236</td>
<td>0x00EC</td>
<td>Float64</td>
<td>Bitmask 1 – #24</td>
<td>Relative defocus applied by application in meters</td>
</tr>
<tr>
<td>Instrument mode</td>
<td>244</td>
<td>0x00F4</td>
<td>Int32</td>
<td>Bitmask 1 – #25</td>
<td>0 = TEM, 1 = STEM</td>
</tr>
<tr>
<td>Projection mode</td>
<td>248</td>
<td>0x00F8</td>
<td>Int32</td>
<td>Bitmask 1 – #26</td>
<td>0 = Imaging, 1 = Diffraction</td>
</tr>
<tr>
<td>Objective lens mode</td>
<td>252</td>
<td>0x00FC</td>
<td>16 chars</td>
<td>Bitmask 1 – #27</td>
<td>LM, HM, Lorentz</td>
</tr>
<tr>
<td>High magnification mode</td>
<td>268</td>
<td>0x010C</td>
<td>16 chars</td>
<td>Bitmask 1 – #28</td>
<td>Mi, SA, Mh</td>
</tr>
<tr>
<td>Probe mode</td>
<td>284</td>
<td>0x011C</td>
<td>Int32</td>
<td>Bitmask 1 – #29</td>
<td>0 = micro, 1 = nano</td>
</tr>
</tbody>
</table>

**Bool = Boolean of 1 byte (0 = false, other value = true)**  
**Int32 = Signed integer of 4 bytes**  
**UInt32 = Unsigned integer of 4 bytes**  
**Float64 = Floating point number of 8 bytes**
## Table 2  Format Extended Header (3 of 7)

<table>
<thead>
<tr>
<th>Name</th>
<th>Offset (dec)</th>
<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFTEM On</td>
<td>288</td>
<td>0x0120</td>
<td>Bool</td>
<td>Bitmask 1 – #30</td>
<td>Is true when the magnifications are adapted to the energy filter</td>
</tr>
<tr>
<td>Magnification</td>
<td>289</td>
<td>0x0121</td>
<td>Float64</td>
<td>Bitmask 1 – #31</td>
<td>Nominal magnification</td>
</tr>
<tr>
<td>Bitmask 2</td>
<td>297</td>
<td>0x0129</td>
<td>UInt32</td>
<td>NA</td>
<td>Individual bits indicate which metadata fields are set</td>
</tr>
<tr>
<td>Camera length</td>
<td>301</td>
<td>0x012D</td>
<td>Float64</td>
<td>Bitmask 2 – #0</td>
<td>Nominal camera length</td>
</tr>
<tr>
<td>Spot index</td>
<td>309</td>
<td>0x0135</td>
<td>Int32</td>
<td>Bitmask 2 – #1</td>
<td>TEM: beam diameter in meters STEM: not used Undefined on 2 lens condenser systems</td>
</tr>
<tr>
<td>Illuminated area</td>
<td>313</td>
<td>0x0139</td>
<td>Float64</td>
<td>Bitmask 2 – #2</td>
<td>TEM: beam diameter in meters STEM: not used Undefined on 2 lens condenser systems</td>
</tr>
<tr>
<td>Intensity</td>
<td>321</td>
<td>0x0141</td>
<td>Float64</td>
<td>Bitmask 2 – #3</td>
<td>Uncalibrated measure of beam diameter on 2 lens condenser systems</td>
</tr>
<tr>
<td>Convergence angle</td>
<td>329</td>
<td>0x0149</td>
<td>Float64</td>
<td>Bitmask 2 – #4</td>
<td>Undefined on 2 lens condenser systems</td>
</tr>
<tr>
<td>Illumination mode</td>
<td>337</td>
<td>0x0151</td>
<td>16 chars</td>
<td>Bitmask 2 – #5</td>
<td>None, Parallel, Probe, Free Undefined on 2 lens condenser systems</td>
</tr>
<tr>
<td>Wide convergence angle range</td>
<td>353</td>
<td>0x0161</td>
<td>Bool</td>
<td>Bitmask 2 – #6</td>
<td>Undefined on 2 lens condenser systems</td>
</tr>
<tr>
<td>EFTEM Imaging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slit inserted</td>
<td>354</td>
<td>0x0162</td>
<td>Bool</td>
<td>Bitmask 2 – #7</td>
<td></td>
</tr>
<tr>
<td>Slit width</td>
<td>355</td>
<td>0x0163</td>
<td>Float64</td>
<td>Bitmask 2 – #8</td>
<td>Slit width in eV</td>
</tr>
<tr>
<td>Acceleration voltage offset</td>
<td>363</td>
<td>0x016B</td>
<td>Float64</td>
<td>Bitmask 2 – #9</td>
<td>in V</td>
</tr>
<tr>
<td>Drift tube voltage</td>
<td>371</td>
<td>0x0173</td>
<td>Float64</td>
<td>Bitmask 2 – #10</td>
<td>in V</td>
</tr>
</tbody>
</table>

**Bool** = Boolean of 1 byte (0 = false, other value = true)  
**Int32** = Signed integer of 4 bytes  
**UInt32** = Unsigned integer of 4 bytes  
**Float64** = Floating point number of 8 bytes
### Table 2  Format Extended Header (4 of 7)

<table>
<thead>
<tr>
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<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy shift</td>
<td>379</td>
<td>0x017B</td>
<td>Float64</td>
<td>Bitmask 2 – #11</td>
<td>in eV</td>
</tr>
</tbody>
</table>
| Image shift X         | 387          | 0x0183       | Float64  | Bitmask 2 – #12 | TEM: pure image shift in logical units  
STEM: image-beamshift in logical units, used as corrective shift |
| Image shift Y         | 395          | 0x018B       | Float64  | Bitmask 2 – #13 |                                                                            |
| Beam shift X          | 403          | 0x0193       | Float64  | Bitmask 2 – #14 | TEM: image & beam shift are combined (in logical units), so the beam shift also shifts the image  
STEM: DC beamshift in logical units |
| Beam shift Y          | 411          | 0x019B       | Float64  | Bitmask 2 – #15 |                                                                            |
| Integration time      | 419          | 0x01A3       | Float64  | Bitmask 2 – #16 | Camera or dose fraction exposure time                                      |
| Binning Width         | 427          | 0x01AB       | Int32    | Bitmask 2 – #17 |                                                                            |
| Binning Height        | 431          | 0x01AF       | Int32    | Bitmask 2 – #18 |                                                                            |
| Camera                |              |              |          |                 |                                                                            |
| Camera name           | 435          | 0x01B3       | 16 chars | Bitmask 2 – #19 | TEM: Name of the camera  
STEM imaging: <empty>                                                        |
| Readout area left     | 451          | 0x01C3       | Int32    | Bitmask 2 – #20 |                                                                            |
| Readout area top      | 455          | 0x01C7       | Int32    | Bitmask 2 – #21 |                                                                            |
| Readout area right    | 459          | 0x01CB       | Int32    | Bitmask 2 – #22 |                                                                            |
| Readout area bottom   | 463          | 0x01CF       | Int32    | Bitmask 2 – #23 |                                                                            |
| Ceta noise reduction  | 467          | 0x01D3       | Bool     | Bitmask 2 – #24 |                                                                            |

**Bool** = Boolean of 1 byte (0 = false, other value = true)  
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**UInt32** = Unsigned integer of 4 bytes  
**Float64** = Floating point number of 8 bytes
### Table 2  Format Extended Header (5 of 7)

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<th>Name</th>
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<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceta frames summed</td>
<td>468</td>
<td>0x01D4</td>
<td>Int32</td>
<td>Bitmask 2 – #25</td>
<td>Number of frames summed for dynamic range</td>
</tr>
<tr>
<td>Direct detector electron counting</td>
<td>472</td>
<td>0x01D8</td>
<td>Bool</td>
<td>Bitmask 2 – #26</td>
<td></td>
</tr>
<tr>
<td>Direct detector align frames</td>
<td>473</td>
<td>0x01D9</td>
<td>Bool</td>
<td>Bitmask 2 – #27</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 0</td>
<td>474</td>
<td>0x01DA</td>
<td>Int32</td>
<td>Bitmask 2 – #28</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 1</td>
<td>478</td>
<td>0x01DE</td>
<td>Int32</td>
<td>Bitmask 2 – #29</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 2</td>
<td>482</td>
<td>0x01E2</td>
<td>Int32</td>
<td>Bitmask 2 – #30</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 3</td>
<td>486</td>
<td>0x01E6</td>
<td>Int32</td>
<td>Bitmask 2 – #31</td>
<td></td>
</tr>
<tr>
<td>Bitmask 3</td>
<td>490</td>
<td>0x01EA</td>
<td>UInt32</td>
<td>NA</td>
<td>Individual bits indicate which metadata fields are set</td>
</tr>
<tr>
<td>Camera param reserved 4</td>
<td>494</td>
<td>0x01EE</td>
<td>Int32</td>
<td>Bitmask 3 – #0</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 5</td>
<td>498</td>
<td>0x01F2</td>
<td>Int32</td>
<td>Bitmask 3 – #1</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 6</td>
<td>502</td>
<td>0x01F6</td>
<td>Int32</td>
<td>Bitmask 3 – #2</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 7</td>
<td>506</td>
<td>0x01FA</td>
<td>Int32</td>
<td>Bitmask 3 – #3</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 8</td>
<td>510</td>
<td>0x01FE</td>
<td>Int32</td>
<td>Bitmask 3 – #4</td>
<td></td>
</tr>
<tr>
<td>Camera param reserved 9</td>
<td>514</td>
<td>0x0202</td>
<td>Int32</td>
<td>Bitmask 3 – #5</td>
<td></td>
</tr>
<tr>
<td>Phase Plate</td>
<td>518</td>
<td>0x0206</td>
<td>Bool</td>
<td>Bitmask 3 – #6</td>
<td>Indicates whether phase plate was used for data acquisition</td>
</tr>
</tbody>
</table>

**Definitions:**
- **Bool** = Boolean of 1 byte (0 = false, other value = true)
- **Int32** = Signed integer of 4 bytes
- **UInt32** = Unsigned integer of 4 bytes
- **Float64** = Floating point number of 8 bytes
### Table 2  Format Extended Header (6 of 7)

<table>
<thead>
<tr>
<th>Name</th>
<th>Offset (dec)</th>
<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEM Detector name</td>
<td>519</td>
<td>0x0207</td>
<td>16 chars</td>
<td>Bitmask 3 – #7</td>
<td></td>
</tr>
<tr>
<td>Gain</td>
<td>535</td>
<td>0x0217</td>
<td>Float64</td>
<td>Bitmask 3 – #8</td>
<td></td>
</tr>
<tr>
<td>Offset</td>
<td>543</td>
<td>0x021F</td>
<td>Float64</td>
<td>Bitmask 3 – #9</td>
<td></td>
</tr>
<tr>
<td>STEM param reserved 0</td>
<td>551</td>
<td>0x0227</td>
<td>Int32</td>
<td>Bitmask 3 – #10</td>
<td></td>
</tr>
<tr>
<td>STEM param reserved 1</td>
<td>555</td>
<td>0x022B</td>
<td>Int32</td>
<td>Bitmask 3 – #11</td>
<td></td>
</tr>
<tr>
<td>STEM param reserved 2</td>
<td>559</td>
<td>0x022F</td>
<td>Int32</td>
<td>Bitmask 3 – #12</td>
<td></td>
</tr>
<tr>
<td>STEM param reserved 3</td>
<td>563</td>
<td>0x0233</td>
<td>Int32</td>
<td>Bitmask 3 – #13</td>
<td></td>
</tr>
<tr>
<td>STEM param reserved 4</td>
<td>567</td>
<td>0x0237</td>
<td>Int32</td>
<td>Bitmask 3 – #14</td>
<td></td>
</tr>
<tr>
<td>Scan settings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dwell time</td>
<td>571</td>
<td>0x023B</td>
<td>Float64</td>
<td>Bitmask 3 – #15</td>
<td>Dwell time per pixel in seconds</td>
</tr>
<tr>
<td>Frame time</td>
<td>579</td>
<td>0x0243</td>
<td>Float64</td>
<td>Bitmask 3 – #16</td>
<td>Frame time in seconds (currently it will not be used)</td>
</tr>
<tr>
<td>Scan size left</td>
<td>587</td>
<td>0x024B</td>
<td>Int32</td>
<td>Bitmask 3 – #17</td>
<td></td>
</tr>
<tr>
<td>Scan size top</td>
<td>591</td>
<td>0x024F</td>
<td>Int32</td>
<td>Bitmask 3 – #18</td>
<td></td>
</tr>
<tr>
<td>Scan size right</td>
<td>595</td>
<td>0x0253</td>
<td>Int32</td>
<td>Bitmask 3 – #19</td>
<td></td>
</tr>
<tr>
<td>Scan size bottom</td>
<td>599</td>
<td>0x0257</td>
<td>Int32</td>
<td>Bitmask 3 – #20</td>
<td></td>
</tr>
<tr>
<td>Full scan FOV X</td>
<td>603</td>
<td>0x025B</td>
<td>Float64</td>
<td>Bitmask 3 – #21</td>
<td>Field of view in meters</td>
</tr>
<tr>
<td>Full scan FOV Y</td>
<td>611</td>
<td>0x0263</td>
<td>Float64</td>
<td>Bitmask 3 – #22</td>
<td></td>
</tr>
</tbody>
</table>

Bool = Boolean of 1 byte (0 = false, other value = true)
Int32 = Signed integer of 4 bytes
UInt32 = Unsigned integer of 4 bytes
Float64 = Floating point number of 8 bytes
## Table 2  Format Extended Header (7 of 7)

<table>
<thead>
<tr>
<th>Name</th>
<th>Offset (dec)</th>
<th>Offset (hex)</th>
<th>Format</th>
<th>Is present flag</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDX Elemental maps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Element</td>
<td>619</td>
<td>0x026B</td>
<td>16 chars</td>
<td>Bitmask 3 – #23</td>
<td></td>
</tr>
<tr>
<td>Energy interval lower</td>
<td>635</td>
<td>0x027B</td>
<td>Float64</td>
<td>Bitmask 3 – #24</td>
<td></td>
</tr>
<tr>
<td>Energy interval higher</td>
<td>643</td>
<td>0x0283</td>
<td>Float64</td>
<td>Bitmask 3 – #25</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>651</td>
<td>0x028B</td>
<td>Int32</td>
<td>Bitmask 3 – #26</td>
<td></td>
</tr>
<tr>
<td>Dose fractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Is dose fraction</td>
<td>655</td>
<td>0x028F</td>
<td>Bool</td>
<td>Bitmask 3 – #27</td>
<td></td>
</tr>
<tr>
<td>Fraction number</td>
<td>656</td>
<td>0x0290</td>
<td>Int32</td>
<td>Bitmask 3 – #28</td>
<td></td>
</tr>
<tr>
<td>Start frame</td>
<td>660</td>
<td>0x0294</td>
<td>Int32</td>
<td>Bitmask 3 – #29</td>
<td></td>
</tr>
<tr>
<td>End frame</td>
<td>664</td>
<td>0x0298</td>
<td>Int32</td>
<td>Bitmask 3 – #30</td>
<td></td>
</tr>
<tr>
<td>Reconstruction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input stack filename</td>
<td>668</td>
<td>0x029C</td>
<td>80 chars</td>
<td>Bitmask 3 – #31</td>
<td></td>
</tr>
<tr>
<td>Bitmask 4</td>
<td>748</td>
<td>0x02EC</td>
<td>UInt32</td>
<td>NA</td>
<td>Individual bits indicate which metadata fields are set</td>
</tr>
<tr>
<td>Alpha tilt min</td>
<td>752</td>
<td>0x02F0</td>
<td>Float64</td>
<td>Bitmask 4 – #0</td>
<td></td>
</tr>
<tr>
<td>Alpha tilt max</td>
<td>760</td>
<td>0x02F8</td>
<td>Float64</td>
<td>Bitmask 4 – #1</td>
<td></td>
</tr>
</tbody>
</table>

*Bool = Boolean of 1 byte (0 = false, other value = true)*  
*Int32 = Signed integer of 4 bytes*  
*UInt32 = Unsigned integer of 4 bytes*  
*Float64 = Floating point number of 8 bytes*
Appendix C

Description of .xml Files Generated by EPU

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Custom Data</td>
<td>Dose</td>
<td>Average number of electrons per meter square</td>
</tr>
<tr>
<td></td>
<td>Applied defocus</td>
<td>Defocus (in meters) applied through EPU during acquisition</td>
</tr>
<tr>
<td>Intensity Scale</td>
<td>-</td>
<td>Not used</td>
</tr>
</tbody>
</table>
| Reference Transformation| Reference transformation matrix | • a11  
• a12  
• a13  
• a21  
• a22  
• a23 Matrix used internally in EPU that represents the calibration context of the microscope |
| Unit                   | • Prefix power        | Used internally in EPU                                                      |
|                        | • Symbol              |                                                                             |
| Spatial Scale          | Offset                | • X  
• Y  • Currently not updated by EPU                                          |
|                        | Pixel size            | • X  
• Y  • Pixel size along the X direction of image (includes camera binning), in meters |

NOTE: These files are obsolete; all the information contained on the xml files can be now found on the FEI-specific metadata in the mrc extended header. We advise that you use the latest, as the xml files will be removed in the next release of EPU.

Each .xml file generated by EPU contains descriptive metadata of the state of the microscope during that specific acquisition. The size of each file is approximately 6 KB, which may vary by a few bytes depending on the choice of, e.g., acquisition parameters. The table below describes the contents of a standard EPU .xml file.
### Table 3  Description of .xml Files (2 of 6)

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope Data</td>
<td>Acquisition</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acquisition date and time</td>
<td>Acquisition date in yyyy-mm-dd format and time in 24 hours format</td>
</tr>
<tr>
<td></td>
<td>Camera</td>
<td>• Binning adopted during acquisition</td>
</tr>
<tr>
<td></td>
<td>Camera location</td>
<td>• Default=Wide angle, currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td>Dark gain correction</td>
<td>• Default=None, currently not implemented in EPU</td>
</tr>
<tr>
<td></td>
<td>Exposure time</td>
<td>• Exposure time in seconds</td>
</tr>
<tr>
<td></td>
<td>Inserted</td>
<td>• Default=None, currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td>Name</td>
<td>• Camera used during acquisition</td>
</tr>
<tr>
<td></td>
<td>Pre-exposure pause time</td>
<td>• Time in seconds. <strong>Note:</strong> Pre-exposure is applicable only for FEI cameras and only if a pre-exposure is specified in EPU. For other cameras, this field will have a default value of Zero. Also, pre-exposure pause time and pre-exposure time are valid only if this field is true. (For details of pre-exposure and shutter behavior, refer TEM help.)</td>
</tr>
<tr>
<td></td>
<td>Pre-exposure supported</td>
<td>• Pre-exposure in EPU is only supported on FEI cameras; this field is therefore false for non-FEI cameras. Also, pre-exposure pause time and pre-exposure time are valid only if this field is true. (For details of pre-exposure and shutter behavior, refer TEM help.)</td>
</tr>
<tr>
<td></td>
<td>Pre-exposure time</td>
<td>• Time in seconds. <strong>Note:</strong> Pre-exposure is applicable only for FEI cameras, if specified in EPU. For other cameras, this field will have a default value of Zero.</td>
</tr>
<tr>
<td></td>
<td>Readout area</td>
<td>• Readout area (width and height) adopted during acquisition, in pixel; remains specific to camera used during acquisition. X, Y represent the origin point of the readout area.</td>
</tr>
<tr>
<td></td>
<td>Shutter</td>
<td>• Shutters used during acquisition, pre-specimen, post-specimen, or both</td>
</tr>
</tbody>
</table>
### Table 3  Description of .xml Files (3 of 6)

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscope Data (cont.):</strong></td>
<td>Plate camera</td>
<td>Exposure time In seconds</td>
</tr>
<tr>
<td>Scan settings</td>
<td>• Reduced area</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Resolution</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Scan area</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Scan rotation</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td>Scanning detector</td>
<td>• Gain</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Inserted</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Name</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td>• Offset</td>
<td>• Not applicable to EPU</td>
</tr>
<tr>
<td><strong>Gun</strong></td>
<td>Accelerating voltage</td>
<td>Accelerating voltage in volts as indicated in microscope UI</td>
</tr>
<tr>
<td>Extraction voltage</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>Filament</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>Gun lens</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>Source type</td>
<td></td>
<td>Default=Thermionic; currently not updated by EPU</td>
</tr>
<tr>
<td>Wehnelt bias</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td><strong>Instrument</strong></td>
<td>Acquisition software</td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>Acquisition software version</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>Instrument ID</td>
<td></td>
<td>Hardware dongle no</td>
</tr>
<tr>
<td>Instrument model</td>
<td></td>
<td>Currently, this field consists of computer name on which EPU/</td>
</tr>
<tr>
<td></td>
<td></td>
<td>microscope software is installed. This might change in future versions of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPU.</td>
</tr>
<tr>
<td><strong>Optics</strong></td>
<td>Beam diameter</td>
<td>Beam diameter in meters (only for Titan family of microscopes)</td>
</tr>
<tr>
<td>Beam shift</td>
<td>• xCoordinate</td>
<td>• Image beam shift in TEM optical units along x-coordinate</td>
</tr>
<tr>
<td>(image beam shift)</td>
<td>• yCoordinate</td>
<td>• Image beam shift in TEM optical units along y-coordinate</td>
</tr>
</tbody>
</table>
### Table 3  Description of .xml Files (4 of 6)

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscope Data (cont.):</strong></td>
<td><strong>Beam tilt</strong></td>
<td>• xCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• yCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Camera length</strong></td>
<td>Default=0; currently not applicable to EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Column operating mode</strong></td>
<td>Always TEM mode for EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Column operating sub-mode</strong></td>
<td>Default=Bright field; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Condenser stigmator</strong></td>
<td>• xCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• yCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Defocus</strong></td>
<td>Default as indicated from microscope UI</td>
</tr>
<tr>
<td></td>
<td><strong>Diffraction focus</strong></td>
<td>Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Diffraction shift</strong></td>
<td>• xCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• yCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Diffraction stigmator</strong></td>
<td>• xCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• yCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Focus</strong></td>
<td>Focus value of microscope, in meters</td>
</tr>
<tr>
<td></td>
<td><strong>Gun stigmator</strong></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Illumination mode</strong></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Illumination probe sub-mode</strong></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td><strong>Image shift</strong></td>
<td>• xCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• yCoordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Image shift in TEM optical units along X coordinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Image shift in TEM optical units along Y coordinate</td>
</tr>
</tbody>
</table>
### Table 3  Description of .xml Files (5 of 6)

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscope Data</strong> (cont.)</td>
<td>Intensity</td>
<td>Intensity settings (related to C2 lens) that determine the beam diameter in Tecnai family of microscopes. For Titan family, the beam diameter is stored instead.</td>
</tr>
<tr>
<td>Objective lens mode</td>
<td></td>
<td>Low magnification (LM) or high magnification (HM)</td>
</tr>
<tr>
<td>Objective stigmator</td>
<td>xCoordinate</td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td>yCoordinate</td>
<td>• Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td>Probe mode</td>
<td></td>
<td>Probe mode, micro or nano, adopted during acquisition</td>
</tr>
<tr>
<td>Projector mode</td>
<td></td>
<td>Default=Diffraction; currently not updated by EPU</td>
</tr>
<tr>
<td>Spot index</td>
<td></td>
<td>Spot size used for image acquisition</td>
</tr>
<tr>
<td>STEM defocus</td>
<td></td>
<td>Default=0; not applicable to EPU</td>
</tr>
<tr>
<td>STEM field of view</td>
<td></td>
<td>Not applicable to EPU</td>
</tr>
<tr>
<td>STEM magnification</td>
<td></td>
<td>Not applicable to EPU</td>
</tr>
<tr>
<td>TEM magnification</td>
<td></td>
<td>TEM nominal magnification, as indicated in microscope UI</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
<td>Description</td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td>ID</td>
<td></td>
<td>Currently not updated by EPU</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td>Holder</td>
<td>Double or single tilt holder; currently not updated by EPU</td>
</tr>
<tr>
<td>Position</td>
<td>A</td>
<td>• Alpha tilt angle in radians</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>• Beta tilt angle in radians</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>• Position of sample stage along X coordinate, in meters</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>• Position of sample stage along Y coordinate, in meters</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>• Position of sample stage along Z coordinate, in meters</td>
</tr>
<tr>
<td>Sample loader</td>
<td></td>
<td>Default=None; currently not updated by EPU</td>
</tr>
</tbody>
</table>
### Table 3  Description of .xml Files (6 of 6)

<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuum</td>
<td>Projection chamber pressure</td>
<td>Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td>Sample pressure</td>
<td>Default=0; currently not updated by EPU</td>
</tr>
<tr>
<td></td>
<td>Vacuum mode</td>
<td>Default=Ready; currently not updated by EPU</td>
</tr>
</tbody>
</table>
Appendix D

Image Display

The image display panel has the following controls:

<table>
<thead>
<tr>
<th>Controls</th>
<th>Icon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enable/disable picture-in-picture mode. By default, the FFT is displayed in the inset window.</td>
<td><img src="image1.png" alt="Icon" /></td>
</tr>
<tr>
<td>Swap the images in the main and inset windows.</td>
<td><img src="image2.png" alt="Icon" /></td>
</tr>
<tr>
<td>Fit to window.</td>
<td><img src="image3.png" alt="Icon" /></td>
</tr>
<tr>
<td>Zoom to actual size.</td>
<td><img src="image4.png" alt="Icon" /></td>
</tr>
</tbody>
</table>

Zoom and Pan

- Zoom in to and out of the main image using the mouse wheel, the ‘+’ and ‘-’ keys or the zoom controls in the display.
- Pan across the image by moving the yellow rectangle in the inset at the bottom. When the image is zoomed in, this inset window is automatically displayed. It can be hidden by disabling the button.

Adjust the image brightness and contrast by dragging the red and black lines in the image histogram.