thermoscientific

EPU

User Manual

PN 1025707
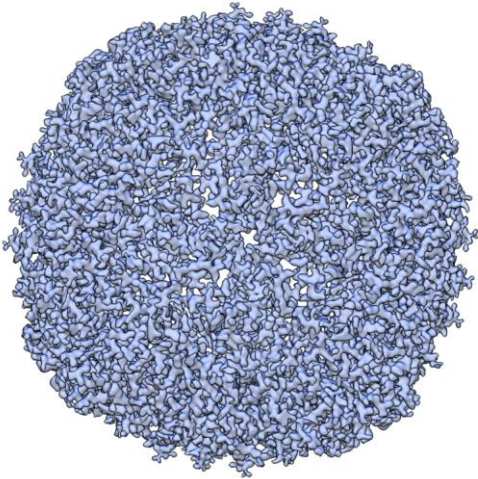
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Thermo Fisher
SCIENTIFIC

Contents

1   Introduction

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.

3D SPA reconstruction of Apoferritin at a resolution of 2.14 Å.
Data acquired on a Krios G3i with Falcon 3EC camera in counting mode.
Reference information on EMDataBank (EMD4213,
http://emsearch.rutgers.edu/atlas/4213_downloads.html)

For the high resolution reconstruction of a particle, large amounts of well-aligned images need to be summed and averaged. Biological material is highly sensitive to electron radiation. To prevent damage to the specimen, an extremely low dose of electrons must be applied, which may result in a low signal-to-noise ratio in the recorded images. It would be a demanding and time consuming task for a microscopist to acquire the images that the reconstruction software needs to complete its task with a satisfactory result. Fortunately, automation software is available to drastically reduce the time and effort, and at the same time improve the reliability of harvesting such large quantities of high quality data.

EPU is a Thermo Fisher Scientific software product for automated high quality data acquisition in a Single Particle Analysis (SPA) workflow. SPA is an approach to 3D image creation, during which a large number of vitrified, low-contrast complexes are imaged under low electron dose conditions. After conformational classification and particle averaging, this results in a high-resolution 3D representation.

The acronym EPU is from the Latin "E Pluribus Unum" — *Out of Many, One*. This name reflects the actual pathway: a single 3D particle structure is extracted from numerous 2D images with multiple particles per image. EPU takes care of the most time-consuming step in the SPA workflow: the acquisition of useful images. The actual reconstruction is performed by various open source software applications.

EPU facilitates the following steps in the data acquisition procedure:

- Screening of multiple specimens to select the specimens and specimen areas with the highest potential for high quality data acquisition.
- Automated or customized selection of the optimal acquisition areas on the specimen.
- High throughput, high resolution data collection.

1.1 Target Audience for this Software User Manual

This manual is aimed at users of Thermo Scientific and FEI Transmission Electron Microscopes with at least basic microscopy skills.

It is expected that:

- The user is proficient in operating the microscope.
- The user is proficient in preparing the microscope for the acquisition of high quality data on all cameras and detectors.
- The user is proficient in:
  - Evaluating image and data quality.
  - Identifying the cause of any imperfections in an acquired images.
  - Making adjustments to correct for the imperfections.
    (This excludes the use of tools and functionalities that are only available to Thermo Fisher Scientific engineers).

For online training materials to help you improve your Cryo-EM skills, visit the EM-learning.com website (https://em-learning.com/).

For questions, remarks and support regarding the EPU software, please contact the EPU support desk at EPU@thermofisher.com (mailto:EPU@thermofisher.com).

1.2 System and Software Compatibility

The EPU software is available for the following Thermo Scientific and FEI TEM systems, provided that they are running up-to-date system software.

Microscope Family	Microscope Types
Titan	Titan, Themis, Krios, Halo
Talos	F200, L120, Arctica, Glacios
Tecnai	*Not supported*

For detailed system and software version compatibility information, refer to the EPU Release Notes.

Not all features and functions in this user manual are available on all systems and all supported microscope software versions.

2   Getting Started

2.1   Prepare for an EPU Session

Before starting an EPU session, make sure the following prerequisites are met and preparations are completed.

1. Verify that the **Magnification Calibrations** are completed for the LM and SA ranges.

2. Verify that an **Alignments File** and a **FEG Register** are loaded that match with the High Tension and Extractor settings of the system.

3. Verify that the **camera** is cooled and at a stable temperature.

4. In the **TEM User Interface** > **Direct Alignments** control panel, the following alignments must be checked and if necessary adjusted:

    *   Eucentric height

    *   Focus

    *   Rotation center for LM magnifications

    *   Rotation center for SA magnifications

For an extensive preconditions check, refer to section Detailed Preconditions on page 188 in this user manual.
Detailed instructions for the individual alignments and calibrations are available in the online help for the TEM User Interface.

5. Close all software applications on the Microscope PC that are not essential to working with EPU. This is necessary to maximize the available memory and CPU resources.

6. In applications that are required for acquisition, close all files and images that are not relevant to EPU.

7. In the **TEM User Interface** > **Low Dose** control panel, make sure **Low Dose** is **off**.
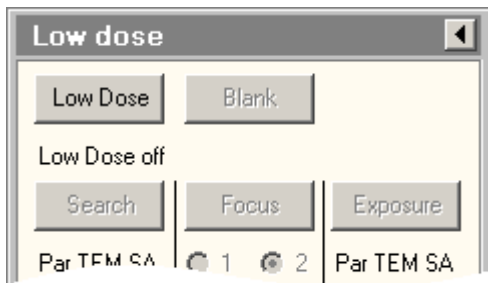
2.2   Start the EPU Software

1. Verify that the preconditions are met and preparations are completed for a successful EPU session.

2. Start **EPU**
   Shortcuts to the EPU software can be found on the desktop and in the Windows Start menu.

   - A splash screen appears while EPU runs the startup checks.
     If one or more checks fail, the splash screen displays the related messages.
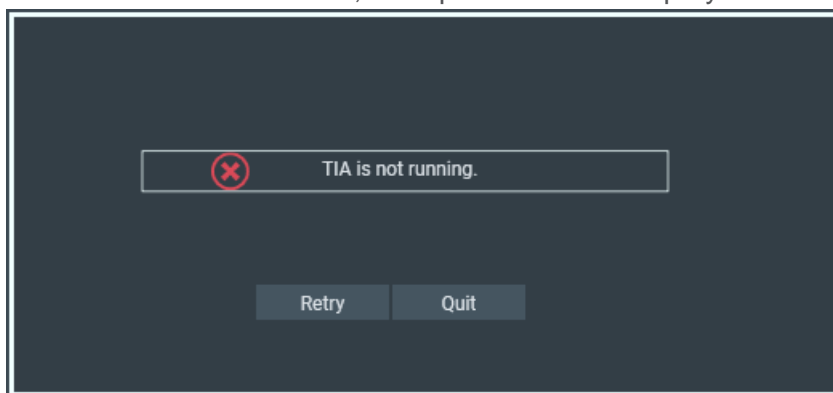
   - Solve the reported issue(s).

   - Select **Retry**

2.3   Log in on Thermo Scientific Athena

The Thermo Scientific Athena software is part of the Data Management Portal (DMP) functionalities.

To connect to Athena, follow the instructions below:

1. In the lower-right corner of the EPU user interface, select **Athena Login**
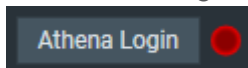
The red dot indicates that there is no active connection to Athena yet.

The *Thermo Scientific Athena* login screen appears.
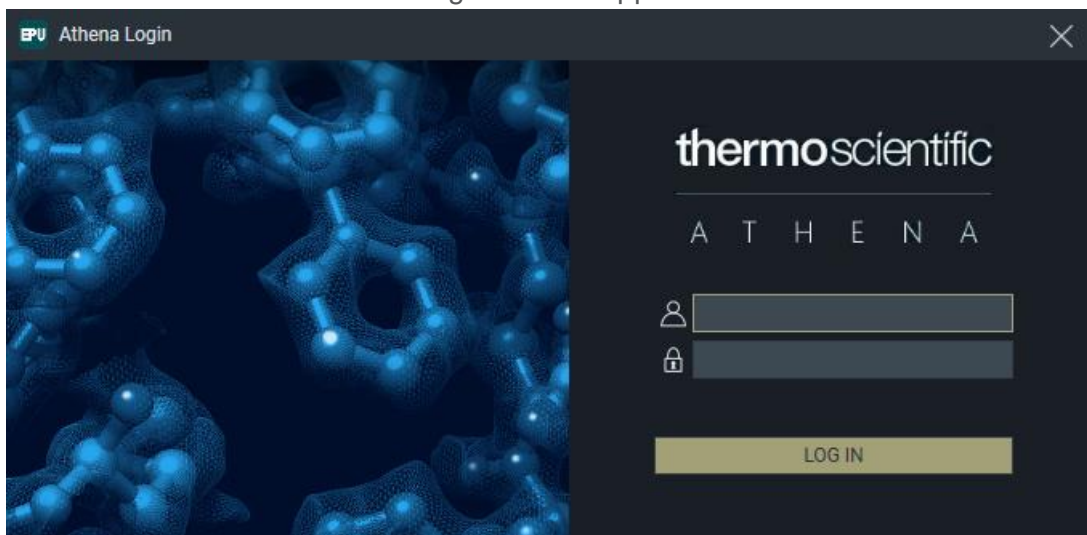
2. Enter the **username** and **password** for Athena.
    If the login is successful, the Athena connection indicator turns green and the username is displayed.
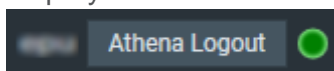
3   The User Interface

3.1   The User Interface Panels

The EPU user interface guides the user through all actions that are needed to prepare and execute an Automated Acquisition run.

Tab Selection
The tabs are typically worked through from left to right. Each tab provides a set of tasks.

Task Selection
Tasks are typically executed from top to bottom. The set of available tasks depends on the selected tab.

Task Execution
The content of the Task Execution pane depends on the active task. It can display an input dialog, one or multiple acquired images, or progress information for an ongoing function.

Ribbon Bar
The Ribbon Bar offers a set of controls that are necessary or helpful for completing the active task.

Side Panels
The Side Panels pane contains a set of collapsible panels. The set of available side panels depends on the active task and/or the selected image in the Task Execution pane.

3.2   The Messages Side Panel

The Messages panel shows a list of *Error* and *Notification* messages in chronological order.
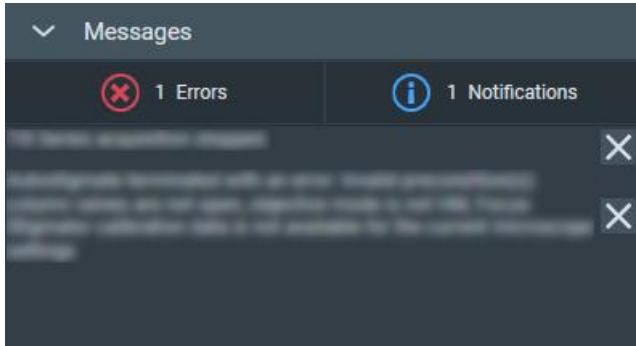
By default, all Errors and Notifications are displayed.
Select **Notifications** to hide the Notification messages.
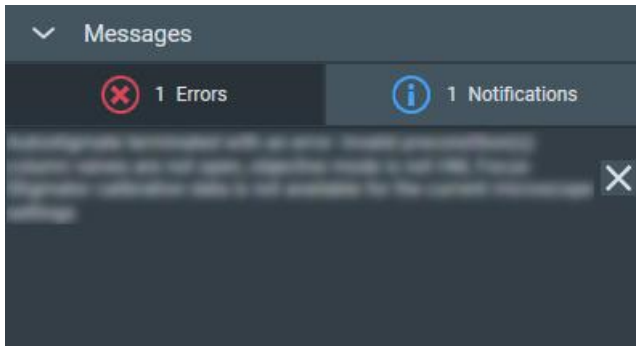
The same applies to the Error messages.

To clear messages that are no longer relevant:

- Select the **cross** at the right-side of each individual message.

- Right-click on **any message** and select **Delete All Messages**

3.3   The Status Side Panel

The Status panel displays various types of messages in chronological order, such as:

- Errors and Notifications.
- Progress messages for ongoing automated procedures.
- Intermediate and final results of automated procedures.
- Instructions and recommendations to the user.

3.4   The Histogram Side Panel

In case two or more images are present in the Task Execution panel, the Histogram applies only the selected image. The selected image is recognizable by the sand-colored image title and frame.

3.4.1   The Histogram Panel for All Images, Except Live Diffraction Images

The Histogram side panel shows a spectrum of the selected image in the Image Display.
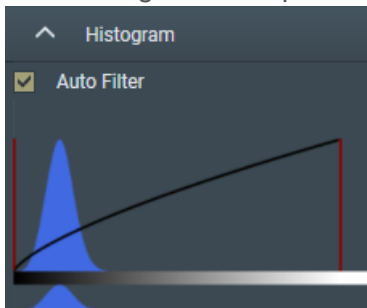
The Histogram side panel offers the following functionalities:

Auto Filter:

- Ticked:
  EPU automatically calculates the optimal contrast and brightness settings and applies these values when a new image is acquired or selected.

- Cleared:
  Manual adjustments of the contrast, brightness or gamma values are also applied to the next acquisition.

When ticked, it is possible to adjust contrast, brightness and gamma for the current image. To reset contrast, brightness and gamma to their default values, clear *Auto Filter* and tick it again.

Black Level:
Drag the **red line at the left-side** of the main histogram to adjust the black level. Pixels with an intensity below the black level value are displayed with zero intensity (black).

White Level:
Drag the **red line at the right-side** of the main histogram to adjust the white level. Pixels with an intensity above the white level value are displayed with maximum intensity (white).

Gamma:
Drag the **diagonal black line** up or down to adjust the Gamma curve.
If the Black Level and/or White Level are adjusted, then the Gamma curve is scaled proportionally in the range between the Black Level and White Level values.

Zoom:
In the lower histogram, drag a **range** to zoom in on a section of the spectrum.
Click outside the zoom range in the lower histogram to reset the zoom level.
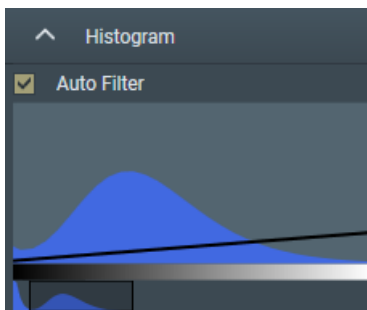
3.5 The Image Information Side Panel

The Image Information panel displays a small basic set of the image meta data.

∧ Image Information	
Applied Defocus	0
Dose	517.68 e/nm²
Exposure Time	1.00 s
Image Size	1855,1919
Pixel Size	142.63 pm
Maximum	65535
Mean	7434.76
Minimum	0

3.6 The Image Display

The availability of functionalities for the Image Display depends on the active task, the applied Acquisition and Optics Preset and the selected image.

Zoom Slider

Drag the slider with the mouse to change to the zoom level.

Color Enhancement

Applies a color mapping to the intensity values in the image. This makes it easier to recognize intensity gradients and areas with similar intensity.

Show / Hide the Inset Window

By default, the Inset window displays the FFT of the image.

FFT Filter

The FFT Filter optimizes the contrast and brightness of the FFT, so the Thon Rings are shown clearer. The FFT Filter does not affect the image for which the FFT is displayed.

Note	**This filter is not adjustable and has not been optimized for performance and low resource usage.** **Activate the FFT Filter only when necessary. Deactivate the FFT Filter when there is no direct need to display the FFT for an image.**

Swap Inset and Main images

Swap the images in the Inset and the Main windows. If the Inset window is hidden, then the Main window will toggle between the FFT and the normal camera image.

Zoom to Fit

Adjust the zoom level, so the entire image fits in the image display frame.
Zoom to fit is also available in the right-click context menu of the image display.

Zoom 1:1

Adjusts the zoom level to 100%, so the image is displayed in actual size.
Zoom 1:1 is also available in the right-click context menu of the image display.

Show / Hide Panning Inset Window

Show or hide the panning inset window.

3.6.1 Zoom In and Zoom Out

1. Place the **mouse cursor** at the **region of interest** in the Image Display.

2. Scroll up or down with the **mouse wheel**.
    The image will zoom in or out around the cursor location.

3.6.2 Navigate and Pan in a Zoomed Image

To navigate and pan in an image, either drag the image with the mouse, or use the panning inset.

1. Select the **panning inset**

2. Drag the **dark gray square** across the **panning inset**
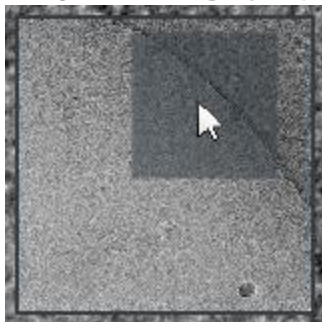
3.6.3 The Red Crosshair
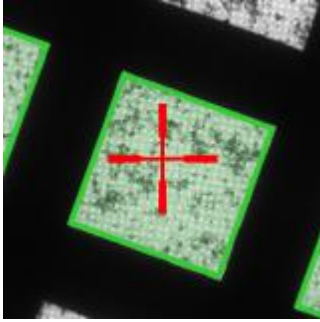
The red crosshair is commonly referred to as the *stage position*. Although the red crosshair moves when the stage moves, it does not mark the stage position itself. The red crosshair marks the *center of the field of view* if a new image were acquired, provided that there is no Image/Beam Shift.

3.6.4 Export an Image to File

All images that are displayed in the Task Execution panel can be exported to file.

1. Right-click in the image to open the context menu.
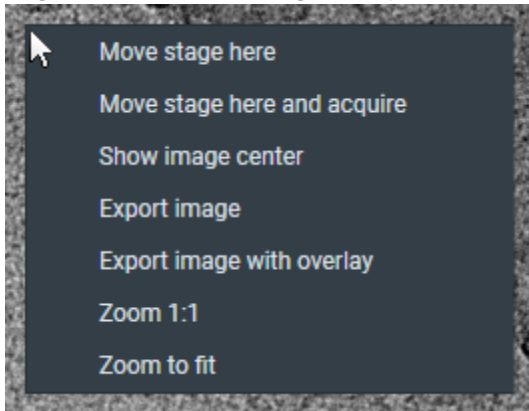
The options in the context menu depend on the active task and image type.

2. Select either:

- **Export image**
  Create a file of the image. The image is saved with the original resolution.

- **Export image with overlay**
  Create a file of the image with scales, markers and other visual aids. The image is saved in the displayed resolution. This may be less detailed than the original image.

4   The Preparation Tab

The Preparations tab provides a set of tasks and functionalities to set up the microscope and the EPU application for successful automated acquisition.

4.1   The Acquisition and Optics Settings Task

For each step in the preparation and automated acquisition process, a Preset must be prepared that fulfills a specific set of requirements. Each Preset consists of:

- Optics Settings.
- Camera Settings.
- Advanced camera and/or exposure settings
  (not for all camera types).

4.1.1   Description of the Camera Settings

4.1.1.1   Camera Settings for All Camera Types
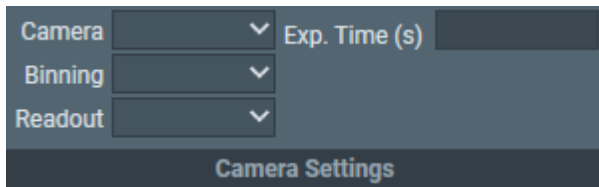
For the Thermo Scientific Falcon 3EC and Falcon 4 camera, the Exp. Time (s) is available in the Exposure Settings.

Camera
Select the camera that is used for the selected Preset.

Binning
Select the sensor-to-image pixel grouping mode.

The options are:

- **1**: each pixel in the acquired image corresponds to a single sensor pixel.

- **2**: the signal of 4 sensor pixels (2x2) is integrated into a single image pixel.

- **4**: the signal of 16 sensor pixels (4x4) is integrated into a single image pixel.

A higher **Binning** value:

- Does not affect the field of view.

- Decreases the image resolution.

- Increases the image acquisition speed for CCD cameras.
  For CMOS cameras the image acquisition speed is practically independent of the Binning value.
  CMOS cameras are:

  - All FEI and Thermo Scientific Falcon and Ceta cameras.

  - Gatan K2, K3 and OneView cameras.

- Increases the signal strength of the image pixels.

Readout
Select the area section of the camera sensor that is used for image acquisition.

The options are:

- **Full**: on a 2048x2048 sensor, all the signal of all pixels is used.

- **Half**: on a 2048x2048 sensor, the signal of a 1024x1024 area around the center is used.

- **Quarter**: on a 2048x2048 sensor, the signal of a 512x512 area around the center is used.

A smaller *Readout* value:

- Decreases the field of view.

- Does not affect the image resolution.

- Increases the image acquisition speed for CCD cameras.
  For CMOS cameras the image acquisition speed is practically independent of the Readout area.
  CMOS cameras are:

  - All FEI and Thermo Scientific Falcon and Ceta cameras.

  - Gatan K2, K3 and OneView cameras.

- Does not affect the signal strength of the image pixels.

Exp. time (s)
For the Thermo Scientific Falcon 3EC and Falcon 4 camera, the Exp. Time (s) is available in the Exposure Settings.

Specify the time during which the camera sensor is exposed to the electron beam.

A longer Exposure Time value:

- Does not affect the field of view.

- Does not affect the image resolution.

- Decreases the image acquisition speed.
  Depending on the camera type and other settings, the *frame rate* is not necessarily affected.

- Increases the signal strength of the image pixels.

EPU validates the specified value. If necessary, EPU adjusts the specified value to the nearest valid value.

4.1.1.2 Exposure Settings for the Thermo Scientific Falcon 3EC Camera and Falcon 4 Camera

Mode

Select the integration mode.
When not in the LM magnifications range, the following options are available:

- **Linear**: the normal integrating mode.

- **Counted**: Electron Counting mode.
  The Counted mode gives better performance of the detector, but requires low dose rates. The Counted option delivers sub-pixel accuracy without increasing the image size. It describes the detected electrons by a normalized pattern that is positioned with sub pixel accuracy.

Fractions

Select whether or not to save Dose Fraction images.
Direct detectors read a lot of 'elemental' frames that can be integrated into Dose Fractions. These fractions can be saved as separate images, next to the integrated image. The total number of frames depends on the exposure time and the camera's internal frame rate. The following options are available:

- **No**:
  Do not save dose fractions.

- **Manual**:
  Specify the number of Dose Fractions in **Fractions (Nr)**.

- **Auto**:
  The Dose Rate is used to calculate the number of Dose Fractions. To ensure that the Dose Fraction images can be aligned properly, every Dose Fraction has at least 1 e/px.

- **Maximum**:
  The maximum number of Dose Fractions is equal to the number of frames.

- **EER**: Electron Event Registration mode.
  Falcon 4 only
  In EER mode, the camera records the coordinates of the individual electrons that hit the Sensor Package. The following data is stored on the Storage Server:

  - An `*.eer` file with thecoordinates and a Gain Reference image.

  - An integrated image of the recorded specimen area.

  In EER mode, the acquired data volume is much smaller than in Counted mode:. The camera does not acquire Fractions, and the `*.eer` file is much smaller than an image file with same data content.
  To process `*.eer` files, specialist software is required.

Align

Select whether or not the camera frames are aligned before summing.
When **Align** option **Yes** is selected:

- The camera calculates the image shift between consecutive frames.

- The camera applies a matching correction to each frame before it is summed into an integrated image or a Dose Fraction image.

The result of the **Align** function depends on the selected **Fractions** mode:

- When **Fractions** is **No**, the frames are aligned before summing into an integrated image.

- When **Fractions** is **Manual** or **Auto**, the frames are aligned before summing into a Dose Fraction image. After that, the Dose Fractions are aligned before summing into the integrated image. An XML file with the applied shifts is saved next to the Dose Fraction images.

- When **Fractions** is **Maximum**, the Dose Fractions are aligned before summing into the integrated image, and an XML file with the applied shifts is saved next to the Dose Fraction images.

Note	**The Align function does not work when the exposure time is too short.**

Exp. Time (s) and Dose

The values of the Exposure Time and Dose parameters are coupled via the measured Dose Rate. When the Exposure Time value is changed, the measured Dose Rate is used to automatically calculate the corresponding Dose value, and vice versa.

As long as the Dose Rate is not known yet, the automatic Dose or Exposure Time calculation cannot be performed. As soon as the Dose Rate is measured, the *leading* parameter is used to calculate the other. In the example screen image below, *Dose* is the leading parameter.
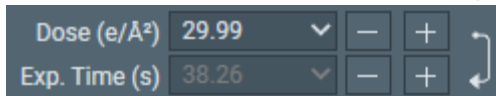
- The arrow points from Dose to Exposure Time.
- Exposure Time is grayed out.

To make *Exposure Time* leading:

- Select the **arrow** to reverse it.
- Select the **Exp. Time (s)** input field.
- Select **[+]** or **[–]** to adjust the Exposure Time value.
  The **[+]** or **[–]** buttons multiply or divide the current value by 2.

Fractions (Nr)

- When **Fractions** is **Manual**: the number of Dose Fractions must be specified manually.
- When **Fractions** is **Auto**: the number of Dose Fractions is calculated automatically.

The specified number of Dose Fractions is only applied when acquiring images with the Data Acquisition Preset.

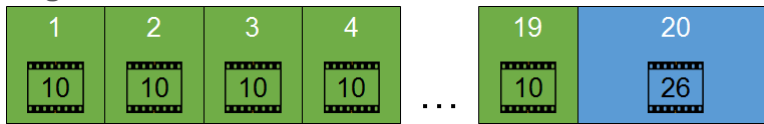The number of frames in each Dose Fraction image is determined as follows:

- Initially, the frames are distributed equally over the Dose Fractions.

  - When **Fractions** is **Auto**, the average dose in each Dose Fraction must be at least 1 e/px. This requires that the the Dose Rate is known.

  - When **Align** is **Yes**, the number of frames per Dose Fraction in the initial distribution must be a multitude of 6.

- Remaining frames are added to the last Dose Fraction.

The combination of these rules may lead to unexpected fractionation schemes. Run a test acquisition to check the result.

For best results, the Dose Fractions should be as equidistant as possible. If necessary, adjust the Dose or Exposure Time, or specify a different number of Dose Fractions to reach a more balanced fractionation scheme.

In the example below, 216 frames are distributed in 20 Dose Fractions. This example does *not* meet the optimal equidistant goal:

- **Align** is **No**

  - `216 / 20 = 10.8`
    Each Dose Fraction contains 10 frames.

  - The remainder is `216 - (20×10) = 16` frames.
    These are added to the last Dose Fraction, so that the total number of frames in the last Dose Fraction is 26 frames.

- **Align** is **Yes**
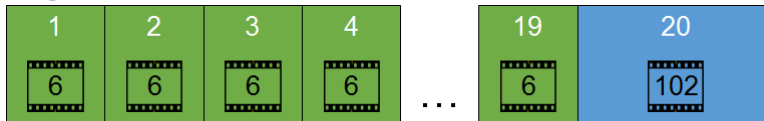
  - `216 / 20 = 10.8`
    Each Dose Fraction contains 6 frames.

  - The remainder is `216 - (20×6) = 96` frames.
    These are added to the last Dose Fraction, so that the total number of frames in the last Dose Fraction is 102 frames.

4.1.1.3 Dose Rate for Thermo Scientific Falcon 3EC and Falcon 4 Cameras

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Measure

Select **Measure** to determine the Dose Rate that the camera sensor receives. The color bar indicates if the measured dose rate is suitable for high quality data acquisition.

- Blue: the Dose Rate is too low. Images may not be usable for 3D reconstruction.
- Green: the Dose Rate is suitable for high quality data acquisition.
- Red: the Dose Rate is too high. The detector is over-exposed.

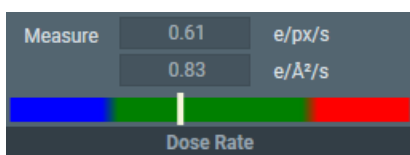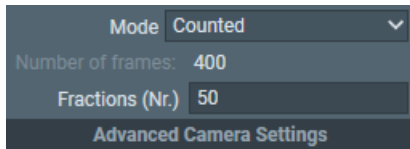The measured Dose Rate is only valid for the current Optics Settings. When the Optics Settings are changed, the Dose Rate must be measured again.

4.1.1.4 Advanced Camera Settings for the Gatan K2 and K3 Camera

Mode

Select the integration mode.
When not in the LM magnifications range, the following options are available:

- **Linear**: the normal integrating mode.
  The Linear mode is only used by the Gatan K2 camera.
  The Gatan K3 camera always uses Counted mode or Counted/Super Resolution mode.

- **Counted**: use electron counting.
  The Counted mode gives better performance of the detector, but requires low dose rates.

- **Counted/Super Resolution**:
  This mode is only available in the Data Acquisition Preset, and when **Fractions (Nr)** is set to a value higher than 1.
  In **Counted/Super Resolution** mode, the position of an electron is assigned to a pixel to achieve sub-pixel resolution. This information is saved only in the Dose Fraction images. Because of this additional information, the Dose Fraction images will have double the width and height in pixels.

Number of frames

This is not a user setting, it is a calculated value. Number of frames depends on:

- The internal frame rate of the camera
- The total **Exposure time**.

Fractions (Nr)

Specify the number of Dose Fraction images.
The specified value is only applied when acquiring images with the Data Acquisition Preset.
The value of **Fraction (Nr)** must be an exact divider of the **Number of frames** value.
When acquiring a preview image, the Dose Fraction images are saved in the `Preparation Preview` sub-folder of the camera root folder.

4.1.1.5 Advanced Camera Settings for the Thermo Scientific Ceta Camera
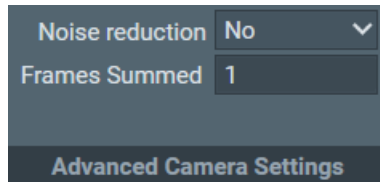
The online help function in the TEM User Interface contains additional information about the settings for the Ceta camera, with and without Ceta Speed Enhancement (Ceta-2).

Noise reduction

Select **Yes** to decrease the readout noise at low dose. Enabling Noise reduction decreases the maximum frame rate by up to 50%.
Noise reduction is only available when **Frames Summed** is **1**.

Frames Summed

Specify the number of frames that is summed during acquisition. Specifying a higher number of frames increases the dynamic range in the acquired image.

4.1.2 Description of the Optics Settings

Optics Settings for microscopes with a Condenser Lens System with *three* lenses:
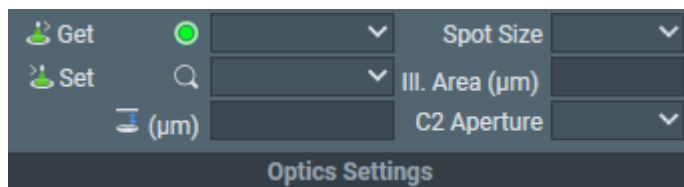
Optics Settings for microscopes with a Condenser Lens System with *two* lenses:
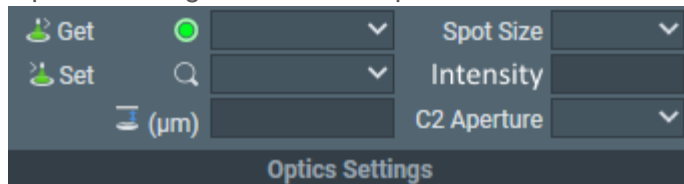
Get
Imports the Optics Settings parameter values from the microscope.

Set

Applies the Optics Settings parameter values to the microscope.

Probe Mode

The definition and values of this parameter are identical to the same-name parameter in the TEM User Interface.

The microscope system software remembers the last used defocus value for Microprobe and for Nanoprobe mode. When the returning to a probe mode, the last used defocus value for that probe mode is automatically restored.

To prevent that the result of the Autofocus function is lost, the following Presets and Autofunctions must use the same Probe Mode as the Data Acquisition Preset:

- Presets:
  - Autofocus
  - Drift Measurement
- Autofunctions:
  - Eucentric Height
  - Autofocus

Magnification

The definition and values of this parameter is identical to the same-name parameter in the TEM User Interface.

Defocus

The definition and values is identical to the same-name parameter in the TEM User Interface.

In most Presets it is useful to apply a *small* defocus for enhanced contrast. Too much defocus may invalidate the alignments and calibrations.

The Defocus value in the Data Acquisition Preset is only applied to the acquisition of the Preview image. During the Automated Acquisition run, EPU uses the values from the Defocus list.

Spot Size

The definition and values of this parameter is identical to the same-name parameter in the TEM User Interface.

Illuminated Area

This parameter is only available on microscopes that have a Condenser Lens System with three lenses. The definition and value of this parameter is identical to the same-name parameter in the microscope software.

As a general rule, aim for a beam diameter that is just large enough to fully cover the camera field of view, without suffering from distortions or aberrations along the edges. If the beam diameter is larger than the camera field of view, then parts of the specimen that are not imaged at that time are needlessly exposed to the electron beam. This may destroy valuable specimen area, which is then lost for high quality data acquisition.

Intensity

This parameter is only available on microscopes that have a Condenser Lens System with two lenses. The value of the Intensity parameter is an indication for the beam diameter. It is usually set with the Intensity knob on the handpanels.

As a general rule, aim for a beam diameter that is just large enough to fully cover the camera field of view, without suffering from distortions or aberrations along the edges. If the beam diameter is larger than the camera field of view, then parts of the specimen that are not imaged at that time are needlessly exposed to the electron beam. This may destroy valuable specimen area, which is then lost for high quality data acquisition.

The beam diameter can be estimated by measuring the beam size on the large fluorescent screen. At 10000 X magnification, 1 cm on the FluScreen equates to 1 μm at the specimen plane. However, the parallelism of illumination can only be approached for particular settings (*FEI Application Note, de Haas, 2011*).

Keep the following restrictions in mind:

- If the Probe, Magnification, Defocus or Spotsize value changes, then the Intensity value may require adjustment as well.

- For magnifications of 10.000 X and lower,
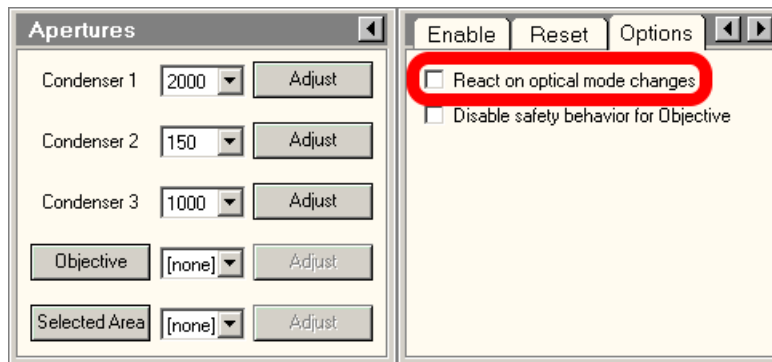 make sure that *TEM User Interface > Beam Settings > Int. Zoom* is off.
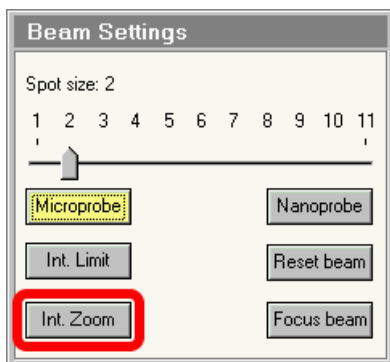
C2 Aperture

The definition and values of this parameter is identical to the same-name parameter in the TEM User Interface.

The C2 aperture selection is only available if the C2 Aperture Mechanism is enabled at the time EPU is started. If the C2 Aperture mechanism is enabled or disabled after EPU has started, then the status is *not* updated to EPU.

The C2 aperture selection is only available for the Data Acquisition Preset and the Atlas Preset.

- The selected aperture for the Data Acquisition Preset is automatically applied to all other Presets, except the Atlas Preset.
- The default value for the Atlas is the largest C2 aperture. If desired, select a different aperture.

Note	Do not use the *React on optical mode changes* function to change the apertures automatically.

Among others, the **React on optical mode changes** function automatically applies the C2 aperture that was selected the previous time that the optical mode was used. This may conflict with the *C2 Aperture* value in the *Acquisition and Optics Presets*.

The **React on optical mode changes** function may also retract the Objective aperture mechanism. If Phase Plates are used in the experiment, then retracting the Objective aperture mechanism means that the Phase Plates are moved away from the beam.

4.1.2.1 Guidelines for the Optics Settings when Using Phase Plates

A Volta Phase Plate is generated by exposing the Phase Plate to the beam. This means there are limitations to the Optics Settings parameters of the Acquisition and Optics Presets when working with a Phase Plate:

- The beam must be parallel, so that the unscattered beam focuses into a small spot on the Phase Plate.
- The focus position on the phase plate has to stay within tight limits, which in turn puts tight limits on the stability of the beam direction. For reference, the stability limits for imaging with a Phase Plate are far stricter than those for coma free imaging.

In practice, the optics system of a microscope is not ideal. Changing the beam diameter may induce a slight tilting of the beam, which may cause the beam to partially leave the Volta Phase Plate. To prevent this effect:

- Always use a parallel beam to illuminate the Phase Plate.

- For a selection of Presets, the Optics Settings must be partially or fully identical.
  If a Phase Plate related restriction is applicable to a Preset, then this is described in the chapters for that Preset.

It is possible to use a convergent beam when working on the microscope, as long as it does not hurt the existing Phase Plate and does not activate a new one.

4.1.3 The Recommended Order to Define the Acquisition and Optics Presets

The recommended order to define the Presets is not the same as their order in the Presets list:

1. Data Acquisition
2. Hole/EucentricHeight
3. Autofocus
4. Drift Measurement
5. Thon Ring
6. Zero Loss (only for EFTEM mode)
7. GridSquare
8. Atlas

In an Automated Acquisition run, the data acquisition step is the value-creating action. To get the best quality images for 3D reconstruction, the Data Acquisition Preset must be optimized without sacrifices to the other Presets.

Although the optics system is highly reproducible, it is always better to avoid changes that are not strictly necessary. To achieve maximum stability, use the Data Acquisition Preset as the basis for all other Presets that are used during the Automated Acquisition run.

4.1.4 Define the Data Acquisition Preset

The Data Acquisition Preset is used to acquire the images that will be used for 3D reconstruction of the particles in the specimen. The parameter values of the Data Acquisition Preset depend only on the requirements for successful 3D reconstruction.

| Parameters	Typical Settings and Recommendations

Optics Settings	• TEM Imaging Mode: Nanoprobe.
	Nanoprobe is well suited for a narrow parallel beam at high magnifications.
	• Parallel beam.
	• Intensity Zoom is Off.
	• Magnification must match the required resolution.
	• Illuminated Area:
	    • A small Illuminated Area prevents double exposure when the Acquisition Areas are close together.
	    • A larger Illuminated Area is preferred when the Acquisition Areas are are widely spaced and it is important for the beam to also hit some carbon.
	• Spot Size must match the Illuminated Area to achieve the required Dose Rate.
	• Defocus:
	The Defocus value is only used during preparation for the Automated Acquisition run. The data acquisitions during the run use the Defocus values that are specified for the Acquisition Areas.
	
	When using Phase Plates, the Optics Settings must be defined with on-plane conditions.
	For instructions, refer to:
	
	• Guidelines for the Data Acquisition Preset when Using Phase Plates with a Condenser Lens System with Three Lenses on page 34
	• Guidelines for the Data Acquisition Preset when Using Phase Plates with a Condenser Lens System with Two Lenses on page 37.

Camera Settings	• Binning: 1 • Readout: Full
Apertures	• C2: start with the 50 μm aperture. • Objective: When using Phase Plates, Make sure the dropdown list for the Objective aperture mechanism contains at least five Phase Plates: PhP1 - PhP5. If present, it can be safely assumed that the Phase Plate positions are defined accurately.

Use the procedure below to set the Data Acquisition Preset.

1. In the **Preparation** tab, select the **Acquisition and Optics Settings** task.

2. In the **Preset Selection** section of the Ribbon Bar, set **Presets** to Data Acquisition.

3. Start a *live image view*
   For Falcon and Ceta cameras, use **Velox**
   For other cameras, use the **TEM User Interface** > **CCD/TV Camera** control panel and **TIA**, or use **Gatan Digital Micrograph**

   a. Select the **Camera** that is used for this Preset.

   b. Select **Binning: 1**

   c. Select **Readout area**: **Full**

   d. Start the *live image view.*

   e. Select **Binning**: **1**

   f. Select **Readout area**: **Full**

4. Move the specimen to an area that can be sacrificed for experimenting with the optics settings.

5. Select **Search** to start continuous acquisition.

6. Use the handpanels to attain an image that meets the requirements.

   • Use the FluScreen and/or the FFT in TIA, Velox or Digital Micrograph to assess the image quality.

   • The Defocus value is only used during preparation for the Automated Acquisition run. The data acquisitions during the run use the Defocus values that are specified for the Acquisition Areas. For a detailed description and instructions, refer to How to Use an Acquisition and Defocus Scheme.

7. Verify that the *Dose Rate* measurement is valid and stable.
   For cameras that can measure the Dose Rate, monitor the Dose Rate in the acquisition software:

   • For Falcon cameras, use EPU or Velox.

   • For Gatan cameras, use Digital Micrograph.

   For cameras that do not report the Dose Rate, follow the instructions below:

   a. Adjust **Intensity** and/or **Spot Size**, so that:

   • The beam illuminates the entire FluScreen.

   • The Screen Current is at 0.2 nA or higher.

   If the FluScreen is not fully illuminated with sufficient intensity, then the *Dose Rate* value in the TEM User Interface is not accurate.

    b. If not visible yet, add **Dose rate [e-/Å2s]** to the **TEM User Interface status panel**

- Right-click in the **status panel** where you wish to display the *Dose rate* value.

- Select **Dose rate** > **Dose rate [e-/Å2s]**
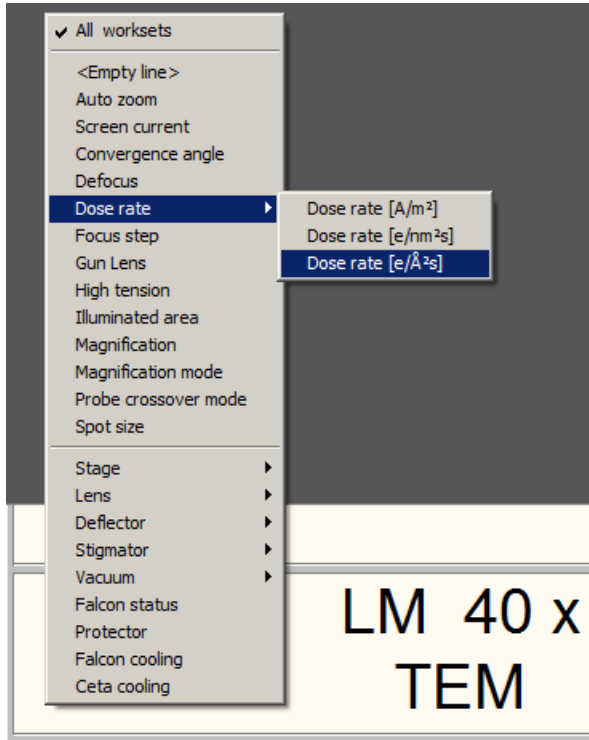
    c. Note the current **Dose Rate** value.

    d. With the **Magnification** knob, increase the magnification by two index steps.

    e. Verify that the **Dose Rate** value is unchanged.

    f. Decrease the magnification by two index steps, back to the initial value.

    g. Verify that the **Dose Rate** value is unchanged.

8. Verify that the beam is parallel:

   a. Verify that **Beam Settings** control panel > **Illumination** is **Parallel**.
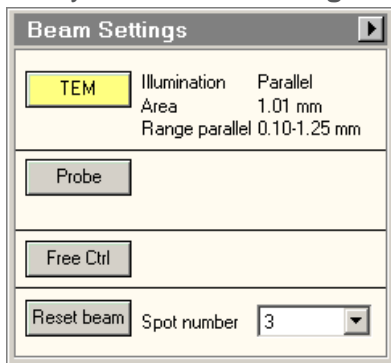
    b. If the microscope has a Condenser Lens System with *two* lenses,
then also verify that the **specimen** and the **Objective aperture** are both focused.
If no Objective aperture is inserted, then:

- Select a small Objective aperture.

- Verify that the aperture is focused.

- Return the Objective mechanism to its initial position.

9. Select **Optics Settings** > **Get** to import the current optical settings from the microscope.

10. In **Camera Settings**:

    a. Select the **Camera**

    b. Select **Binning** and **Readout**

    c. If the camera is *not* a Thermo Scientific Falcon 3EC or Falcon 4,
then specify the **Exp. Time (s)**.
For Thermo Scientific Falcon 3EC and Falcon 4 cameras, this parameter is specified in
Exposure Settings, after the Dose Rate has been measured.

11. If a Thermo Scientific Falcon 3EC or Falcon 4 camera is used,
then select **Dose Rate** > **Measure**
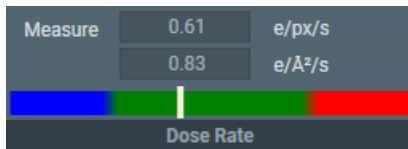
If the measured Dose Rate is *not* in the green zone:

    a. Adjust the illumination.
Either:

- Use the handpanels to adjust the **Intensity** and/or **Spot Size**, then select **Optics Settings** > **Get**

- In the **Optics Settings**, adjust the **Illuminated Area** or **Intensity**, and/or **Spot Size** then select **Set**

    b. Select **Measure** again to update the Dose Rate value.
If no Dose Rate value is known yet and *Measure* is skipped, then the Preview acquisition includes a Dose Rate measurement.

12. Depending on the selected Camera, also specify the additional camera-specific parameters:

- Thermo Scientific Falcon 3EC / Falcon 4: **Exposure Settings** with the **Exp. Time (s)** and **Dose** parameters.

- Thermo Scientific Ceta: **Advanced Camera Settings**

- Gatan K2 / K3: **Advanced Camera Settings**

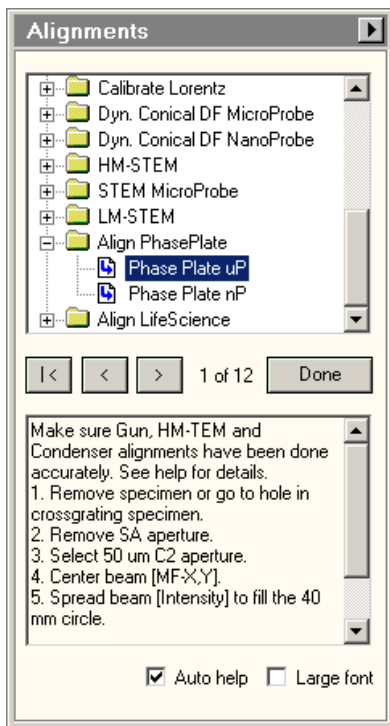13. In the **Acquisition** section, select **Preview**.
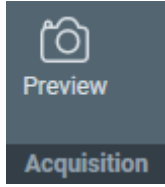
4.1.4.1 Guidelines for the Data Acquisition Preset when Using Phase Plates with a Condenser Lens System with Three Lenses

Perform the Phase Plate Microprobe (uP) Alignment

On Titan systems, perform the Phase Plate Microprobe (uP) alignment procedure:

1. Select the **TEM User Interface** > **Alignments** control panel

2. Select **Auto help** to display detailed instructions for accurate execution of the alignments below.

3. Select the **Align PhasePlate** > **Phase Plate uP** alignment.
   In this alignment procedure:

   a. Very accurately align the **diffraction lens focus** at the **highest camera length**.
      This alignment ensures that the phase plate is exactly in focus.

   b. Very accurately align the **beam shift pivot points**.
      This alignment ensures a stable beam position relative to the phase plate, when an image-beam shift is applied.

After the Phase Plate Microprobe (uP) alignment procedure is completed, continue with the Phase Plate Nanoprobe (nP) alignment procedure.

Perform the Phase Plate Nanoprobe (nP) Alignment

Perform the Phase Plate Nanoprobe (nP) alignment procedure.

Note	On Titan systems, Phase Plate Nanoprobe (nP) alignment procedure can only be done after the Phase Plate Microprobe (uP) alignment procedure is completed.

1. Select the **TEM User Interface** > **Alignments** control panel

2. Select **Auto help** to display detailed instructions for accurate execution of the alignments below.

3. Select the **Align PhasePlate** > **Phase Plate nP** alignment.
   In this alignment procedure:

   a. Very accurately align the **diffraction lens focus** at the **highest camera length**.
      This alignment ensures that the phase plate is exactly in focus.

   b. Very accurately align the **beam shift pivot points**.
      This alignment ensures a stable beam position relative to the phase plate, when an image-beam shift is applied.
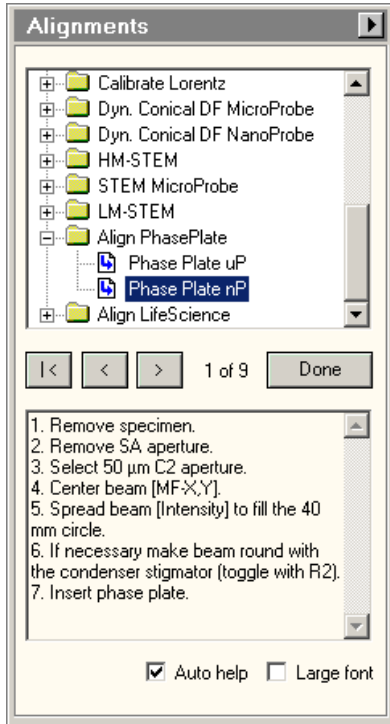
### Phase Plate On-Plane Illumination on Titan

When the beam is focused onto the Phase Plate, the illumination is called on-plane. In all other cases, the illumination is called off-plane.
For the desired illumination conditions, the on-plane conditions also need to be verified.
On-plane illumination of the phase plate implies parallel illumination of the specimen.

Follow the steps below to attain on-plane illumination:

1. If the *Data Acquisition Preset* is already completed, then:

    a. Select **EPU** > **Preparation** >
       Acquisition and Optics Settings > **Presets** > **Preset**: **Data Acquisition**

    b. Select **Optics Settings** > **Set**

2. If the *Data Acquisition Preset* is not defined yet,
   then use the TEM User Interface and/or the Handpanels to:

    a. Select the **TEM imaging mode**

    b. Select the desired **Magnification**, **Spot Size** and **Intensity** for high quality data acquisition.

3. In the **TEM User Interface** > **Beam Settings** control panel, verify that **Illumination** is **Parallel**
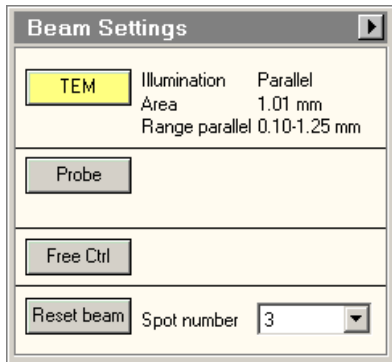
4. Select **Handpanels** > **Diffraction**

5. With the **Handpanels** > **Magnification** knob, set the highest camera length.

6. Select **Handpanels** > **Eucentric Focus**

7. In the **TEM User Interface** > **Phase plate** control panel:
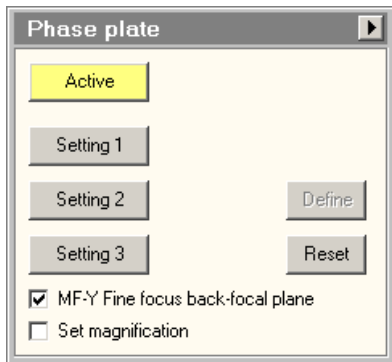
    a. Select **Active**

    b. Tick **MF-Y Fine focus back-focal plane**

8. With the **Handpanels** > **Multifunction Y** knob, focus the beam to the smallest possible spot on the FluScreen.

9. Select **Handpanels** > **Diffraction** again to return to imaging mode.

Note	During the Automated Acquisition run, make sure that the *Phase plate* control panel is in *Active* status.

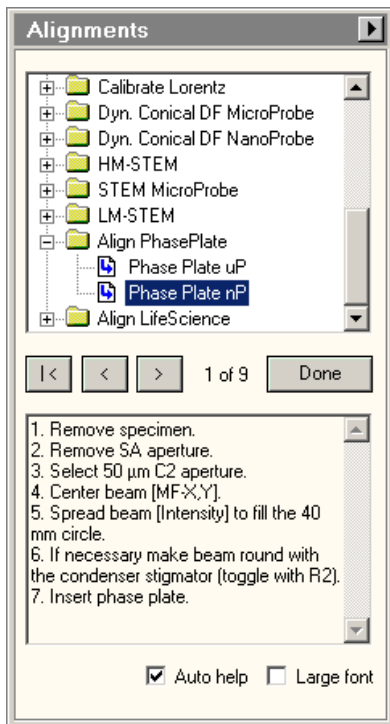| **Note**	If the spot size and/or illuminated area are changed, the on-plane condition needs to be verified again.

4.1.4.2 Guidelines for the Data Acquisition Preset when Using Phase Plates with a Condenser Lens System with Two Lenses

Perform the Phase Plate Nanoprobe (nP) Alignment

Perform the Phase Plate Nanoprobe (nP) alignment procedure.

Note	On Titan systems, Phase Plate Nanoprobe (nP) alignment procedure can only be done after the Phase Plate Microprobe (uP) alignment procedure is completed.

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1. Select the **TEM User Interface** > **Alignments** control panel

2. Select **Auto help** to display detailed instructions for accurate execution of the alignments below.

3. Select the **Align PhasePlate** > **Phase Plate nP** alignment.
    In this alignment procedure:

    a. Very accurately align the **diffraction lens focus** at the **highest camera length**.
        This alignment ensures that the phase plate is exactly in focus.

    b. Very accurately align the **beam shift pivot points**.
        This alignment ensures a stable beam position relative to the phase plate, when an image-beam shift is applied.

Phase Plate On-Plane Illumination on Talos

On Talos systems, the on-plane condition can be set without using the **Phase plate** control panel. Follow the steps below to attain on-plane illumination:

1. If the *Data Acquisition Preset* is already completed, then:

    a. Select **EPU** > **Preparation** >
        Acquisition and Optics Settings > **Presets** > **Preset**: **Data Acquisition**

    b. Select **Optics Settings** > **Set**

2. If the *Data Acquisition Preset* is not defined yet,
    then use the TEM User Interface and/or the Handpanels to:

    a. Select the **TEM imaging mode**

    b. Select the desired **Magnification**, **Spot Size** and **Intensity**

3. In the **TEM User Interface** > **Beam Settings** control pane> **Tune** tab, verify that the beam is set to **Nanoprobe**.

4. Insert the **FluScreen**

5. Verify that the beam is parallel:

    a. Select **Handpanels** > **Diffraction**

    b. With the **Handpanels** > **Magnification** knob, set an intermediate camera length.

    c. On the **FluScreen,** verify that the beam is forming a probe.

6. With the **Handpanels** > **Magnification** knob, set the highest camera length.

7. Select **Handpanels** > **Eucentric Focus**

8. With the **Handpanels** > **Intensity** knob, narrow the beam to a probe.

9. Select **Handpanels** > **Diffraction** again to return to imaging mode.

Note   If the Spot Size and/or Intensity are changed, then the on-plane condition must be verified again.

4.1.5   Define the Hole/EucentricHeight Preset

The Hole/EucentricHeight Preset is used to perform the Auto-eucentric Height function during the automated run.

On Lacey Carbon specimens, Hole/EucentricHeight Preset is also used to:

*   Acquire low dose images of Acquisition Areas for inspection.

On Quantifoil specimens, the Hole/EucentricHeight Preset is also used to:

*   Find and center individual Foil Holes that have been selected at the Grid Square level.
*   Acquire low dose images of Foil Holes for inspection.
*   Specify the Template that defines the locations for Autofocus, Drift Measurement and one or more Data Acquisition Areas.

The Hole/EucentricHeight Preset must meet the following requirements:

*   For Quantifoil samples: the field of view must contain at least one complete Foil Hole. The algorithm for marking and centering a hole is more reliable if the neighboring holes are also partly visible.
*   The field of view must contain an area of carbon foil for successful execution of the Auto-eucentric function.
*   The contrast and brightness must be good enough to clearly identify the edge of the carbon foil around a hole.
*   The Dose rate must be small enough to prevent damage to the Data Acquisition Area(s) in the Foil Hole.

Parameters	Typical Settings and Recommendations
Optics Settings	- TEM Imaging Mode: the same as the Data Acquisition Preset. - Illuminated Area: typically 10–15 µm. On Quantifoil samples: twice as large as the Foil Hole interspacing. - Magnification: low SA range, so the Illuminated Area matches the field of view. - Defocus: 10-20 µm. Defocus helps to enhance contrast, so it is easier to detect the edge of the carbon foil around a hole. - Parallel beam. - Dose must be smaller than 0.1 [e- / Å2sec]. When using Phase Plates, the Hole/EucentricHeight Preset must be defined with an off-plane illumination, so that an activated Phase Plate is not harmed.
Camera Settings	- Binning: 1 - Readout: Full
Apertures	Same as the Data Acquisition Preset.

Use the procedure below to set the Hole/EucentricHeight Preset.

1. Apply the Data Acquisition Preset values to the microscope:

   a. Select the **Preparation** > **Acquisition and Optics Settings** task.

   b. Select **Preset Selection** > **Presets**: **Data Acquisition**

   c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets**: **Hole/EucentricHeight**.

3. Start a *live image view*
   For Falcon and Ceta cameras, use **Velox**
   For other cameras, use the **TEM User Interface** > **CCD/TV Camera** control panel and **TIA**, or use **Gatan Digital Micrograph**

   a. Select the **Camera** that is used for this Preset.

   b. Select **Binning: 1**

   c. Select **Readout area**: **Full**

   d. Start the *live image view.*

   e. Select **Binning: 1**

4. Select **Search** to start continuous acquisition.

5. Move the specimen to an area that can be sacrificed for experimenting with the optics settings.

6. Use the handpanels to create an image that meets the field of view requirements.
   This will typically be the case at a **Magnification** in the lower SA range, and an **Illuminated Area** of 10–15 μm.

7. Adjust the illumination parameters to meet the contrast and brightness requirements.

8. Verify that the **Dose rate** matches the requirements.

Note	Make sure that the beam covers the entire FluScreen, and that the Screen Current is at least 0.2 nA. If the FluScreen is not fully illuminated with sufficient intensity, the Dose rate value in the Microscope User InterfaceTEM User Interface is not accurate.

9. If the Dose Rate is higher than 0.1 [e- / Å2sec]:

   a. Insert the **FluScreen**

   b. Select a higher **Spot Size** number.

   c. Use the **Intensity** knob to adjust the beam diameter.
      Make sure that the entire FluScreen is illuminated and that the Screen Current does not drop below 0.2 nA.

   d. Retract the **FluScreen** again.

10. Verify that the beam is parallel:

   a. Verify that **Beam Settings** control panel > **Illumination** is **Parallel**.
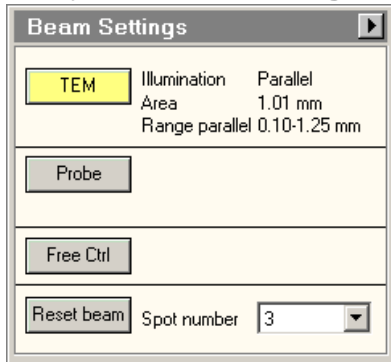
   b. If the microscope has a Condenser Lens System with *two* lenses,
   then also verify that the **specimen** and the **Objective aperture** are both focused.
   If no Objective aperture is inserted, then:

   - Select a small Objective aperture.

   - Verify that the aperture is focused.

   - Return the Objective mechanism to its initial position.

11. Acquire an image with the following camera settings:

   - **Bias/Gain** correction: **Bias/Gain**

   - The same **Integration time**, **Binning** and **Readout area** as used for the live image view.

12. Verify that the acquired image meets the requirements above.
   If necessary:

   a. Use the Handpanels to adjust **Magnification**, **Intensity** and/or **Spot Size**

   b. Acquire a **new image** and verify it against the requirements above.

13. In **EPU**:

   a. Select **Optics Settings** > **Get** to import the current optics values from the microscope.

   b. In **Camera Settings**, select the same **Camera**, **Binning**, **Readout** and **Exp. time (s)** values as used for the previously acquired image.

   c. Select **Acquisition** > **Preview**
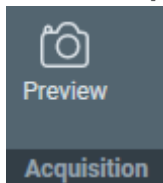
14. Verify that the **image quality** is identical to the previously acquired image.

4.1.5.1 Guidelines for the Hole/EucentricHeight Preset when Using Phase Plates

When using Phase Plates, the Hole/EucentricHeight Preset must be defined with an *off-plane* illumination, so the currently activated Phase Plate is not harmed.

On Titan systems:
Use NanoProbe and verify that **TEM User Interface** > **Beam Settings** control panel > **Illumination** is **Parallel**.
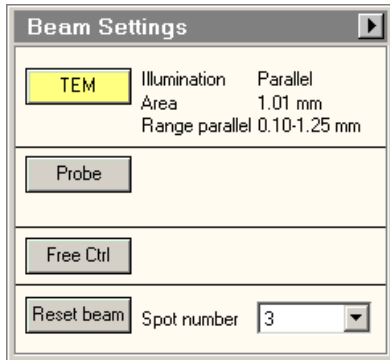
Above a certain illuminated area (~8 µm for a 50 µm C2 aperture) the beam changes from parallel to spreading.

On Talos systems, verification of the off-plane condition is done in Diffraction mode.

To verify the off-plane condition:

1. Switch to **MicroProbe**

2. Set sufficient **Defocus** to enhance contrast.

3. Select **Handpanels** > **Diffraction**

4. With the **Handpanels** > **Magnification** knob, set the highest camera length.

5. Verify that the diffraction spot size is at least as large as the 40 mm circle on the FluScreen or FlucamViewer.

4.1.6 Define the Autofocus Preset

The Autofocus Preset is used for:

- Calibration of the Autofocus function.
- Execution of the Autofocus step during the automated run.

The Autofocus function determines the amount of image shift while beam is tilted. After calibration, the amount of image shift is a measure for the amount of defocus.

The Autofocus Preset must meet the following requirements:

- The Optics Settings for the Autofocus Preset must be as close to the Optics Settings of the Data Acquisition as possible.
- The combination of beam diameter and field of view must be such, that the entire field of view is illuminated at all times during execution of the Autofocus function.

Recommendations for the beam diameter and the field of view:

Sample flatness and stability	Optics Settings > Illuminated Area or Intensity	Camera Settings > Readout
Better than average	Same as the Data Acquisition Preset	Half
Average	Same as the Data Acquisition Preset	Full
Worse than average	Slightly larger than the Data Acquisition Preset	Full

Parameters	Typical Settings and Recommendations
Optics Settings	- Illuminated Area / Intensity: see recommendations above. - Probe Mode: if the AFIS functionality is present and calibrated, then use the same Probe Mode as for the Data Acquisition Preset.

Camera Settings	- See recommendations above
	- Binning: 1 or 2
	  - CCD cameras:
	    When the Readout setting is Full on a 4k X 4k camera, select Binning 2. To speed up shift measurement calculations, the image size is reduced internally to 2k X 2k pixels. Binning 1 does not increase the accuracy of the Autofocus function, but the image acquisition may take more time.
	  - CMOS cameras:
	    Compared to Binning 1, selecting Binning 2 does not noticeably speed up image acquisition. There is also no loss of accuracy, as long as Binning 1 or 2 is used.

Apertures	Same as the Data Acquisition Preset.

Use the procedure below to define the Autofocus Preset.

1. Apply the Data Acquisition Preset values to the microscope:

    a. Select the **Preparation** > **Acquisition and Optics Settings** task.

    b. Select **Preset Selection** > **Presets**: **Data Acquisition**

    c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets: Autofocus**

3. In **Camera Settings**, select the same **Camera**, **Binning** and **Readout** as used in the Data Acquisition Preset.

4. Depending on the selected camera, select the same camera-specific values as used in the Data Acquisition Preset:

    - Thermo Scientific Falcon 3EC / Falcon 4:
      Duplicate the parameter values in the **Exposure Settings** section.

    - Thermo Scientific Ceta:
      Duplicate the parameter values in the **Advanced Camera Settings** section.

    - Gatan K2 / K3:
      Duplicate the parameter values in the **Advanced Camera Settings** section.

    Settings that are not used in the Autofocus Preset can be duplicated without negative consequences. When a value is not applicable to the Autofocus Preset, it will be ignored.

5. Select **Acquisition** > **Preview**

The Autofocus Preset cannot be finalized without executing the Autofocus calibration and/or Autofocus function. Depending on the flatness and stability of the sample, the Camera Settings and/or the Optics Settings parameters may need adjustment. If adjustments are necessary, this will be done during Autofocus calibration and/or during Stand-alone execution of the Autofocus function.

4.1.6.1 Guidelines for the Autofocus Preset when Using Phase Plates

When using Phase Plates, changes in the Condenser Lens System must be avoided. This makes it even more important to use the same Optics Settings parameter values as the Data Acquisition Preset.

Since the Autofocus function acquires images with a tilted beam, it will create satellite spots on the phase plate film. These spots are sufficiently far removed from the actual Volta area in the optical center to have a negative effect on the quality of the data acquisitions.

If Phase Plates are used in combination with a Direct Detection camera in electron counting mode, the beam intensity can be very low and exposure time for data acquisition can be very long. Nevertheless, Autofocus will work with an integration time that is significantly shorter than the time that is specified in the Data Acquisition preset. For the Autofocus function a very small dose is sufficient to reliably perform cross-correlation based shift measurements. The equivalent of a couple of dose fractions should be sufficient.

4.1.7 Define the Drift Measurement Preset

The Drift Measurement Preset is used for:

- Stand-alone execution of the Drift Measurement function in the Auto Functions tab.

- Execution of the Drift Measurement function during the automated run.
  The automated run postpones data acquisition until the drift speed has dropped below a configurable threshold.

The Drift Measurement function tracks the position of recognizable features and patterns in consecutive images, and compares the detected position shift with a specified threshold value.

The Drift Measurement Preset must meet the following requirements:

- The Optics Settings for the Drift Measurement Preset must be as close to the Optics Settings of the Data Acquisition as possible.

- The exposure time must be small enough to minimize blur in the images that are used for the Drift Measurement.
  In a blurry image, it is hard to accurately identify recognizable features and patterns.

- The time interval between consecutive images must be short enough to allow for fast measurements.

- If the Drift Threshold is set very low, the resolution of the acquired images must be high enough to enable detection of very small shifts.

- For Quantifoil samples: the beam diameter must be small enough, so that the Drift Measurement Area can be placed close to the Data Acquisition Area(s) without risking multiple exposure of the Data Acquisition Area(s).

Parameters	Typical Settings and Recommendations
Optics Settings	If Phase Plates are used, then the Optics Settings for the Drift Measurement Preset must be identical to the Data Acquisition Preset, including the on-plane illumination conditions. If Phase Plates are not used, then the below recommendations are applicable. • Probe Mode: if the AFIS functionality is present and calibrated, then use the same Probe Mode as for the Data Acquisition Preset. • TEM Imaging Mode: same as the Data Acquisition Preset. • Magnification: If the drift threshold is very low, it may be necessary to select a higher magnification than is used for the Data Acquisition preset. • Illuminated Area / Intensity for Quantifoil samples: Decrease the beam diameter if possible, but make sure the entire field of view is illuminated.
Camera Settings	• Binning: 1 Although a higher Binning value could increase the readout speed, keep Binning at 1 for maximum accuracy. • Readout: Half. This increases the readout speed.
Apertures	Same as the Data Acquisition Preset.

Use the procedure below to define the Drift Measurement Preset.

1. Apply the Data Acquisition Preset values to the microscope:

   a. Select the **Preparation** > **Acquisition and Optics Settings** task.

   b. Select **Preset Selection** > **Presets**: **Data Acquisition**

   c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets**: **Drift Measurement**

3. In **Camera Settings**:

   a. Select the same **Camera** as used in the Data Acquisition Preset.

   b. Select **Binning**: **1**

   c. Select **Readout**: **Half**

4. Depending on the selected camera, select the same camera-specific values as used in the Data Acquisition Preset:

- Thermo Scientific Falcon 3EC / Falcon 4:
  Duplicate the parameter values in the **Exposure Settings** section.

- Thermo Scientific Ceta:
  Duplicate the parameter values in the **Advanced Camera Settings** section.

- Gatan K2 / K3:
  Duplicate the parameter values in the **Advanced Camera Settings** section.

Settings that are not used in the Drift Measurement Preset can be duplicated without negative consequences. When a value is not applicable to the Drift Measurement Preset, it will be ignored.

5. Select **Acquisition** > **Preview**

The Drift Measurement Preset cannot be finalized without executing the Drift Measurement function. Depending on the specified Drift Threshold and the measurement results, the Optics Settings and/or Camera Settings parameters may need adjustment. If adjustments are necessary, this will be done during Drift Measurement calibration and/or during Stand-alone execution of the Drift Measurement function.

4.1.8 Define the Thon Ring Preset

The Thon Ring Preset is used for:

- Stand-alone execution of the Autocoma function.
- Stand-alone execution of the Autostigmate function.

The Autocoma and Autostigmate functions are intended for optimization of the system alignments before starting an automated run. Both functions are not executed during the automated run, which means that also the Thon Ring Preset is not applied during the automated run.

The Thon Ring Preset must meet the following requirements:

- Thon Rings are clearly visible in the FFT of the acquired images.

The recommendations below are specified relative to the Data Acquisition Preset.

Parameters	Typical Settings and Recommendations

Optics Settings	- When using Phase Plates, the Optics Settings for the Thon Ring Preset must be defined equal to the Data Acquisition Preset to prevent accidental overexposure of an activated Phase Plate.
	- Spot Size:
	  - When using the survey camera: select Spot Size 3 or higher.
	  - When using the high sensitivity camera: check the dose rate to select a suitable Spot Size.
	  - Do not use Spot Size 1 or 2.
	- Illuminated Area / Intensity:
	If desired, slightly decrease the beam diameter to increase the signal strength. When the beam is too narrow, less Thon Rings will be visible.
	- Defocus: typically between -1 μm and -3 μm.

Camera Settings	Binning: 2

Use the procedure below to set the Thon Ring Preset.

1. Apply the Data Acquisition Preset values to the microscope:

    a. Select the **Preparation** > **Acquisition and Optics Settings** task.

    b. Select **Preset Selection** > **Presets**: **Data Acquisition**

    c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets**: **Thon Ring**

3. Select **Optics Settings** > **Get**

4. Prepare a *live FFT view*.
   For Falcon and Ceta cameras, use **Velox**
   For other cameras, use the **TEM User Interface** > **CCD/TV Camera** control panel and **TIA**, or use **Gatan Digital Micrograph**

    a. Select the same **Camera** as used in the Thon Ring Preset.

    b. Use the following camera settings:

- **Binning**: **2**
- **Readout area**: **Full**

    c. Start the *live image acquisition*

    d. Move the **specimen** to an area with **thin, uninterrupted, amorphous carbon foil** that is *not* close to a grid bar.

    e. Display the **FFT** of the live image.

5. Assess the quality of the **Thon Rings**
If necessary adjust the optics parameters to improve the sharpness of the Thon Rings.

6. Verify that the beam is parallel:

    a. Verify that **Beam Settings** control panel > **Illumination** is **Parallel**.

    b. If the microscope has a Condenser Lens System with *two* lenses,
then also verify that the **specimen** and the **Objective aperture** are both focused.
If no Objective aperture is inserted, then:

- Select a small Objective aperture.
- Verify that the aperture is focused.
- Return the Objective mechanism to its initial position.

7. Acquire an image with the following camera settings:

- **Bias/Gain** correction: **Bias/Gain**
- The same **Integration time**, **Binning** and **Readout area** as used for the live image view.

8. Verify that the acquired image meets the requirements above.
If necessary:

    a. Use the Handpanels to adjust **Magnification**, **Intensity** and/or **Spot Size**

    b. Acquire a **new image** and verify it against the requirements above.

9. In **EPU**:

    a. Select **Optics Settings** > **Get** to import the current optics values from the microscope.

    b. In **Camera Settings**, select the same **Camera**, **Binning**, **Readout** and **Exp. time (s)** values as used for the previously acquired image.

    c. Select **Acquisition** > **Preview**

10. Verify that the **image quality** is identical to the previously acquired image.

11. In the **Image Display**, select **Show/Hide Inset** and/or **Swap Inset and Main** to display the FFT for the acquired image.

12. Check the quality of the Thon Rings.
Select **Filter FFT** to improve the contrast and brightness of the FFT if the Thon Rings .

13. If necessary, adjust the Optics Settings.
Either:

- Use the handpanels to adjust the **Intensity** and/or **Spot Size**,
then select **Optics Settings** > **Get**

- In the **Optics Settings**, adjust the **Illuminated Area** or **Intensity**, and/or **Spot Size** then select **Set**

4.1.9   Define the Zero Loss Preset

The Zero Loss Preset is only available when the microscope is in EFTEM mode at the time EPU is started. The Zero Loss Preset is used by the Auto Zero-Loss auto function. During an Automated Acquisition run, EPU executes the Auto Zero-Loss function at specified interval to maintan a proper alignment of the energy slit in the filter with the Zero Loss Peak.

If Phase Plates are used, then the Optics Settings for the Zero Loss Preset must be identical to the Data Acquisition Preset to prevent accidental overexposure of an activated Phase Plate.

If no Phase Plates are used, then it still recommended to use the same Optics and Camera Settings as the Data Acquisition Preset.

Parameters	Typical Settings and Recommendations
Optics Settings	Same as the Data Acquisition Preset.
Camera Settings	Same as the Data Acquisition Preset.
Apertures	Same as the Data Acquisition Preset.

Use the procedure below to define the Zero Loss Preset.

1. Apply the Data Acquisition Preset values to the microscope:

   a. Select the **Preparation** > **Acquisition and Optics Settings** task.

   b. Select **Preset Selection** > **Presets**: **Data Acquisition**

   c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets: Autofocus**

3. In **Camera Settings**, select the same **Camera**, **Binning** and **Readout** as used in the Data Acquisition Preset.

4. Depending on the selected camera, select the same camera-specific values as used in the Data Acquisition Preset:

   • Thermo Scientific Falcon 3EC / Falcon 4:
   Duplicate the parameter values in the **Exposure Settings** section.

   • Thermo Scientific Ceta:
   Duplicate the parameter values in the **Advanced Camera Settings** section.

   • Gatan K2 / K3:
   Duplicate the parameter values in the **Advanced Camera Settings** section.

   Settings that are not used in the Zero Loss Preset can be duplicated without negative consequences. When a value is not applicable to the Zero Loss Preset, it will be ignored.

5. Select **Acquisition** > **Preview**

4.1.10 Define the GridSquare Preset

The GridSquare Preset is used to find and select Target Areas that are suitable for data acquisition.

The GridSquare Preset must meet the following requirements:

- The field of view must contain an entire, centered Grid Square.

- The field of view must contain a part of each neighboring Grid Square.
  This is needed to make sure that a Grid Square image still contains an entire Grid Square in case the positioning of the specimen has drifted over time. As a rule of thumb, the field of view of the Grid Square image must be approximately twice as large as a single Grid Square.

- The contrast and brightness must be good enough to assess the ice thickness.

- For Quantifoil samples, the contrast and brightness must be good enough to detect Foil Holes.

Parameters	Typical Settings and Recommendations
Optics Settings	- TEM Imaging Mode: same as the Data Acquisition Preset. - Magnification: LM 400X - LM 600X. - Illuminated Area: approximately 150-200 μm. - Parallel beam. When using Phase Plates, high doses on the Phase Plate must be prevented, so avoid focusing the beam to a small spot.
Camera Settings	- Binning: 1 - Readout: Full
Apertures	Same as the Data Acquisition Preset.

Use the procedure below to set the GridSquare Preset.

1. Apply the Data Acquisition Preset values to the microscope:

   a. Select the **Preparation** > **Acquisition and Optics Settings** task.

   b. Select **Preset Selection** > **Presets**: **Data Acquisition**

   c. Select **Optics Settings** > **Set**

2. Select **Preset Selection** > **Presets**: **GridSquare**

3. Start a *live image view*
   For Falcon and Ceta cameras, use **Velox**
   For other cameras, use the **TEM User Interface** > **CCD/TV Camera** control panel and **TIA**, or use **Gatan Digital Micrograph**

   a. Select the **Camera** that is used for this Preset.

   b. Select **Binning: 1**

   c. Select **Readout area**: **Full**

   d. Start the *live image view.*

   e. Select **Binning** 1.

   f. Select **Readout area** Full.

4. Select **Search** to start continuous acquisition.

5. Use the **handpanels** to adjust the **Magnification** and/or **Intensity** to meet the requirements for contrast and brightness, and for the field of view.
   This will typically be the case at a Magnification of LM 400X - LM 600X with an Illuminated Area of 150-200 μm.

6. Verify that the beam is parallel:

   a. Verify that **Beam Settings** control panel > **Illumination** is **Parallel**.

   b. If the microscope has a Condenser Lens System with *two* lenses,
      then also verify that the **specimen** and the **Objective aperture** are both focused.
      If no Objective aperture is inserted, then:

      - Select a small Objective aperture.

      - Verify that the aperture is focused.

      - Return the Objective mechanism to its initial position.

7. Acquire an image with the following camera settings:

   - **Bias/Gain** correction: **Bias/Gain**

   - The same **Integration time**, **Binning** and **Readout area** as used for the live image view.

8. Verify that the acquired image meets the requirements above.
   If necessary:

   a. Use the Handpanels to adjust **Magnification**, **Intensity** and/or **Spot Size**

   b. Acquire a **new image** and verify it against the requirements above.

9. In **EPU**:

   a. Select **Optics Settings** > **Get** to import the current optics values from the microscope.

   b. In **Camera Settings**, select the same **Camera**, **Binning**, **Readout** and **Exp. time (s)** values as used for the previously acquired image.

   c. Select **Acquisition** > **Preview**

   ![Preview / Acquisition button]

10. Verify that the **image quality** is identical to the previously acquired image.

4.1.11 Define the Atlas Preset

An automated run starts by acquiring an Atlas. The Atlas is an overview of the specimen. The Atlas is used to detect and select GridSquares for automatic data acquisition. It is assembled by stitching a series of images (so-called *Tiles*) together.

For these purposes, the Atlas Preset must meet the following requirements:

- The field of view must cover the largest possible specimen area. The field of view must contain an area of at least 3x3 entire Grid Squares.
  The Grid Square detection algorithm becomes more reliable when more Grid Squares are captured in the field of view.

- The field of view must be fully illuminated. This also means that the image does not contain any cut-offs.
  To fulfill this requirement it may be necessary to not only set the Atlas Preset, but also to perform the Atlas Optical Alignment calibration.

- The grid bars of the carbon foil are clearly recognizable at the edges of each image.

- The contrast and brightness must be good enough to assess the ice thickness.

- The contrast and brightness must be good enough to detect broken carbon foil.

The picture below shows a section of an Atlas. Small offsets can be visible at the edges of the stitched images.

Parameters	Typical Settings and Recommendations
Optics Settings	• TEM Imaging Mode: Microprobe Microprobe is better suited for a wide parallel beam at lower magnifications. • Magnification: LM 60X - LM 200X. • Parallel beam. With defocus, a convergent beam may lead to an effective change of magnification. When using Phase Plates, avoid focusing the beam to a small spot, so high doses on the Phase Plate are prevented.
Camera Settings	• Binning: 2 • Readout: Full
Apertures	If possible, use the same apertures as the Data Acquisition Preset. If it is not possible to meet the illumination requirements with that aperture, then select a larger aperture. Note that EPU can not change the selected apertures. Use the *TEM User Interface > Apertures* control panel to select the required apertures before starting the Atlas acquisition or the automated acquisition run.

Use the procedure below to define the Atlas Preset.

1. Select the **Preparation** > **Acquisition and Optics Settings** task.

2. Select **Preset Selection** > **Presets**: **Atlas**

3. In the **TEM User Interface** > **Apertures** control panel:

    • Set **Condenser 2** to the largest aperture.

    • Set **Objective** to the retracted position.

4. Start a *live image view*
   For Falcon and Ceta cameras, use **Velox**
   For other cameras, use the **TEM User Interface** > **CCD/TV Camera** control panel and **TIA**, or use **Gatan Digital Micrograph**

    a. Select the **Camera** that is used for this Preset.

    b. Select **Binning: 1**

    c. Select **Readout area**: **Full**

    d. Start the *live image view.*

    e. Select **Binning: 2**.
       With older camera types, image acquisition with Binning 1 may take more time. The acquisition time of the latest generation CMOS cameras does not depend on the Binning value. Although Binning 1 would give a higher accuracy, Binning 2 is sufficient for the purpose of the Atlas.

    f. Select **Readout area: Full**.

5. Select **Search** to start continuous acquisition.

6. Select **Magnification**: **LM 60X**,
    or a magnification close to this value.

7. With the handpanels adjust the **Intensity** and/or **Spot Size** and/or **Magnification**, so the requirements above are fulfilled.

Note	Do *not* apply Beam Shift to eliminate asymmetric cut-offs. Beam Shift values are *not* stored in the Preset.

8. If the system has a **Cryobox** and the image still has cut-offs at a magnification of LM 200X or higher, then the center of the Cryobox aperture may have an offset relative to the system's optical axis. To compensate for this offset, continue as follows:

    a. Complete the **Atlas Preset** procedure as if there were no cut-offs.
        The purpose of this procedure is no longer to fulfill all the requirements for the Atlas Preset. Instead the goal is now to prepare an Atlas Preset that can be used as a starting point for the Atlas Optical Alignment calibration. This means that the contrast and brightness requirements still apply, but that any remaining cut-offs do not have to be removed by adjusting only the Intensity, Spot Size and Magnification.

    b. Perform the **Atlas Optical Alignment**
        The Atlas Optics Alignment determines the offset of the Cryobox aperture relative to the system's optical axis. It then calculates the amount of beam-shift that is needed to let the beam pass through the center of the Cryobox aperture.
        Refer to chapter Perform the Atlas Optics Alignment on page 60.

    c. Return to this **Atlas Preset** procedure at step 9 to adjust the **Intensity** and/or **Spot Size** and/or **Magnification**.
        It may be necessary to loop through steps 9 and 10 more than once to achieve an optimized Atlas Preset.

9. Acquire an image with the following camera settings:

    - **Bias/Gain** correction: **Bias/Gain**

    - The same **Integration time**, **Binning** and **Readout area** as used for the live image view.

10. Verify that the acquired image meets the requirements above.
    If necessary:

    a. Use the Handpanels to adjust **Magnification**, **Intensity** and/or **Spot Size**

    b. Acquire a **new image** and verify it against the requirements above.

11. In **EPU**:

    a. Select **Optics Settings** > **Get** to import the current optics values from the microscope.

    b. In **Camera Settings**, select the same **Camera**, **Binning**, **Readout** and **Exp. time (s)** values as used for the previously acquired image.

    c. Select **Acquisition** > **Preview**
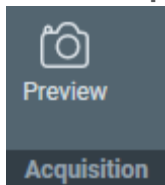
12. Verify that the **image quality** is identical to the previously acquired image.

4.1.12 Import and Export the Acquisition and Optics Presets

With the Import and Export functions it is possible to archive the values for all current Presets, and to load them again at a later time. This way, setting up a new Automated Acquisition run for a regularly used specimen type can be done much easier and much faster.

Presets
Import
Export
Preset Selection
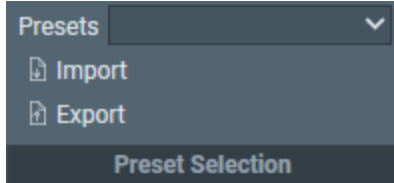
Export

Writes the current parameter values for all Presets to a file (`*.sxml`) and save it to an appointed drive and folder. The Presets file contains:

- The Optics Settings for all Presets.

- The Camera Settings for all Presets.
  Depending on the selected camera the Advanced Camera Settings and/or Exposure Settings are also included.

Import

Overwrite all current parameter values for all Presets with the values from the selected Presets file.

- There is no *Undo* function. It may be wise to export the current Presets to a file before importing a different Presets file.

- Presets files that are created with previous software versions are supported with limitations. When EPU can not import or convert a legacy Presets file it will display an error message.

Note	A Presets file contains the parameter values for *all* Presets. It is not possible to export or import the settings for a single Preset.

4.2 The Atlas Optics Alignment Task

On systems with a Cryobox, a cut-off may become visible in low magnification images. This cut-off appears when the center of the Cryobox aperture has an offset relative to the system's optical axis.

The Atlas Optics Alignment determines the offset of the Cryobox aperture relative to the system's optical axis. It then calculates the amount of Image/Beam Shift that is needed to compensate for the offset, so that the beam passes through the center of the Cryobox aperture. The Image/Beam Shift value that results from the Atlas Optical Alignment calibration is only applied when the Atlas Preset is used to acquire images. It does not affect other Presets.

After the Atlas Optics Alignments is completed, the Atlas Preset can typically use a lower magnification. This means less images are needed to cover the entire specimen, which saves time.

4.2.1 Perform the Atlas Optics Alignment

To perform the Atlas Optics Alignment procedure, follow the steps below:

1. In the **TEM User Interface** > **Apertures** control panel:
   - Set **Condenser 2** to the largest aperture.
   - Set **Objective** to the retracted position.

2. Check if the Atlas Preset is prepared to start the Atlas Optical Alignment calibration:

   a. In the **Preparation** tab > **Acquisition and Optics Settings** task > **Preset Selection** section of the Ribbon Bar, set **Presets** to **Atlas**.
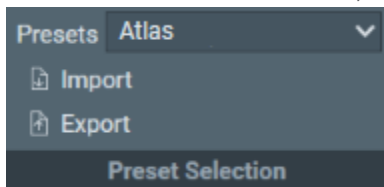
   b. In the **Acquisition** section, select **Preview**.

---
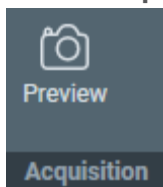
    c. If the acquired image does not meet the requirements for the Atlas Preset, follow the instructions in Define the Atlas Preset on page .
The requirement that no cut-offs must be visible does not apply yet.

3. Select the **Atlas Optics Alignment** task.
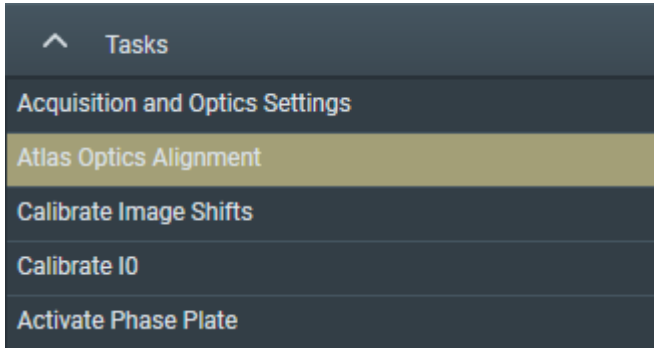
4. In the **Atlas Optics Alignment** section of the Ribbon Bar, select **Acquire**.
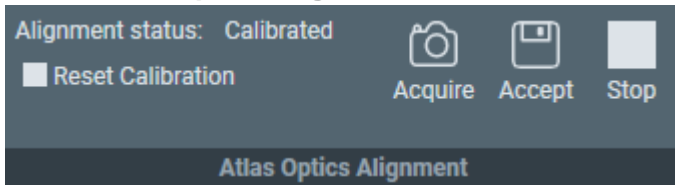
If necessary, select **Stop** to abort the automated procedure.

5. In the acquired image, check for cut-offs.
A Cryobox aperture cut-off is very similar to a C2 aperture or Objective aperture cut-off. It appears as a dark area with a round edge.
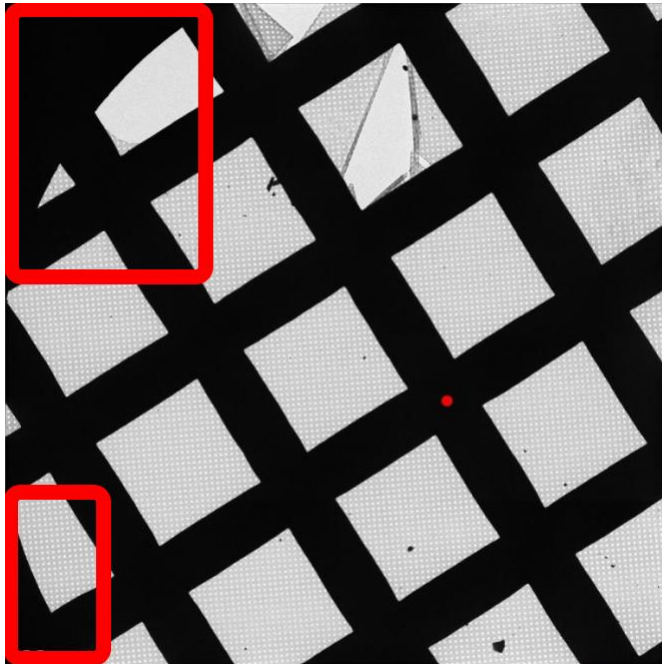
The red dot represents the position of the system's optical axis relative to the center of the Cryobox aperture.

6. If one or more cut-offs are present:

    a. Estimate where the physical center of the Cryobox aperture is.

    b. In the image, double-click on the estimated location.
       The red dot moves to the indicated location.

    c. Select **Acquire** again.

    d. Check again for cut-offs. If necessary, repeat steps a, b and c.

7. When the image is free from cut-offs, select **Accept**.

8. Return to chapter Define the Atlas Preset on page 55 at step 9 to complete the Atlas Preset.

Note	**The Atlas Optics Alignment calibration is based on the Optics Settings of the Atlas Preset. If the Optics Settings of the Atlas Preset are changed, the calculated beam shift value may not be accurate anymore, and the calibration must be renewed.**

To renew the Atlas Optical Alignment calibration:

9. Select **Reset Calibration**.

10. Perform steps 4 - 7 again.

4.3 The Calibrate Image Shifts Task

Even on a well-aligned system, a centered feature may shift away from the image center when a new image is acquired at a different magnification. A good lens series alignment minimizes the amount of shift, but a small amount of shift may still be present due to the following factors:

- The alignment is done on the FluScreen or on a different camera than the camera that is used for the image acquisition. Different cameras on the same system can have small offsets relative to each other.

- Readjustment of the LM rotation center can cause a shift when switching between LM and HM modes.

- The use of defocus during the Automated Acquisition run can cause a small shift due to inaccuracies in the rotation center alignment.

- During lens series alignment, the normalization of the lenses may have been handled differently than during Automated Acquisition.

For optimum performance of the Automated Acquisition run, any remaining shifts need to be compensated. The Image Shifts Calibration measures the shift between the various the Presets, and calculates the required corrective image shifts. This corrective image shift is applied when a different Preset is set to the microscope.

All shifts are relative to the Data Acquisition Preset, so reconstruction is always based on zero-shift images.

The applied image shift is displayed in the *TEM User Interface > System Status* control panel as the *User image shift* value.

The *TEM User Interface > Image Settings* control panel > *Image shift* value is a combination of image shift and beam shift. User image shift only uses the image deflector coils, so it is not shown in the Image Settings control panel.

4.3.1 When to Reset and Renew the Image Shift Calibration

The Image Shifts Calibration needs to be renewed:

- When the Optics Settings of a Preset are updated (magnification and defocus).

- When a different camera is selected in the Camera Settings of a Preset. The magnification center may shift.

- When the Alignment file that was used to define the Presets is updated, or when a different Alignment file is loaded.

- When the FEG Register that was used to define the Presets is updated, or when a different FEG Register is loaded.

- When a feature does not stay centered after selecting a different Preset.

To clear the Image Shifts Calibration, select **Reset Calibration**.
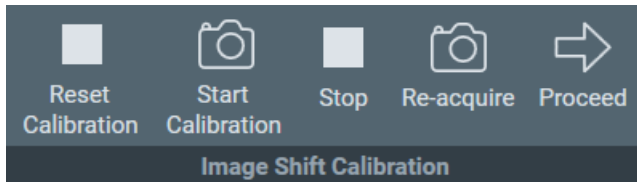
4.3.2 Prepare for Image Shift Calibration

The Image Shift Calibration acquires images with each Preset. In each image, the exact location of an easily recognizable feature must be pinpointed. The shifts between the pinpointed locations are used to compensate for image center offsets at various magnifications.

To prepare for the Image Shift Calibration, follow the procedure below:

1. Select **Preparation** tab > **Acquisition and Optics** task.

2. Select **Preset Selection** > **Presets**: **Data Acquisition**
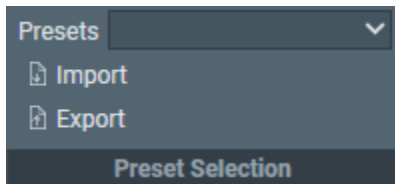
3. Select **Optics Settings** > **Set**

4. Insert the **FluScreen**

5. Move the specimen to find an **easily recognizable feature**, and center it in the image on the FluScreen.

| Note | **Select an asymmetric feature that is easily recognizable at LM magnifications.** |

If you are not sure about the recognizability of the feature details at low magnifications, switch to LM magnifications for a quick check.

6. Select **Preset Selection** > **Presets**: **GridSquare**

7. Select **Acquisition** > **Preview**
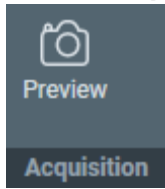
8. In the acquired image, right-click on the feature and select **Move stage here**
   If necessary, zoom in to make it easier to pinpoint the feature.
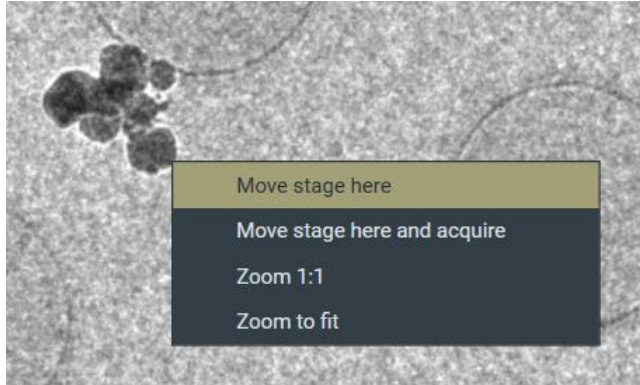
9. Select **Preset Selection** > **Presets**: **Hole/EucentricHeight**

10. Select **Acquisition** > **Preview**

11. In the acquired image, check if the selected feature is easily recognizable.
    It doesn't have to be centered, but on a well-aligned system it should be well within the field of view.
    When not in view:

    a. Insert the **FluScreen**

    b. Move the specimen to find and center the recognizable feature.

    c. Select **Preview** again.

12. In the acquired image, right-click on the feature and select **Move stage here**

13. Select **Preset Selection** > **Presets**: **Data Acquisition**

14. Select **Acquisition** > **Preview**

15. In the acquired image, check if the selected feature is visible near the image center.

4.3.3 Perform the Image Shift Calibration

1. If the Prepare for Image Shift Calibration on page has not been performed yet, do so first.

2. Insert the **FluScreen**

3. In the **Preparation** tab > **Acquisition and Optics** task:

   a. Select **Preset Selection** > **Presets**: **Data Acquisition**
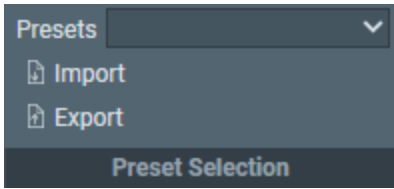
   b. Select **Optics Settings** > **Set**

4. If necessary, re-adjust the position of the **easily recognizable feature** so that it is centered on the FluScreen.

5. Select **Preparation** tab > **Calibrate Image Shifts** task.
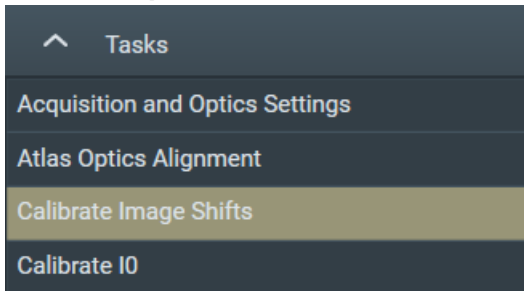
6. Select **Image Shift Calibration** > **Start Calibration**
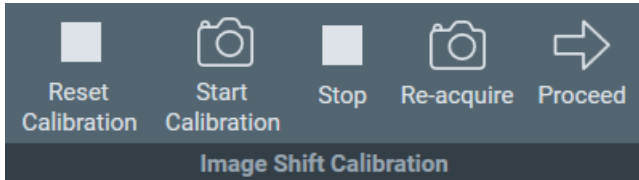
   The calibration procedure acquires the first image and displays it on the left side of the Task Execution panel.
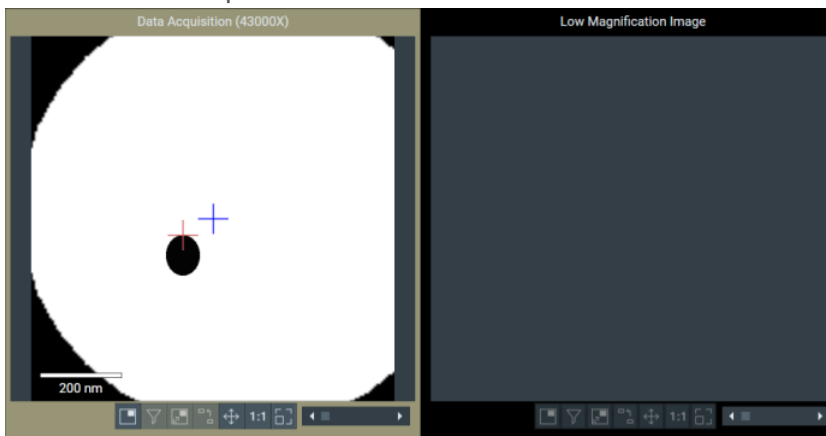
7. Center the recognizable feature in the center of the image.

    a. In the **left-side image**, double-click on the **recognizable feature**.
        The red crosshair moves to the selected position. The red crosshair can be relocated as often as necessary.
        If desired, zoom in for better accuracy.

    b. Select **Re-acquire**
        EPU uses a *backlash corrected stage move* to center the marked feature and then acquires a new image.

    c. If the feature is not properly centered, select **Re-acquire** again.
        On a well-aligned and calibrated system this should solve the offset.
        Note that the CompuStage is not infinitely accurate. A very small offset may still exist at the highest magnification. This is acceptable.

8. After the feature is properly centered, the shifts between the Presets can be determined:

    a. Select **Proceed**
        EPU acquires a new image using the next Preset, and displays it on the right-side of the Task Execution panel.

    b. In the new image on the right-side, double-click on the **distinctive location** of the **recognizable feature**.
        The red crosshair moves to the selected position.

        *   The red crosshair can be relocated as often as necessary.

        *   Zoom in for better accuracy.

    c. Repeat step *a* and step *b* until the image shifts between all Presets have been calibrated.
        For every shift:

        *   The image on the left-side serves as a reference.

        *   Use the new image on the right-side to mark the distinctive location on the recognizable feature.

    d. After the last shift is calibrated, the Status panel reports the successful completion of the calibration.

4.4 The Calibrate I0 Task

A Cryo-EM specimen consists of a solution that contains the particles of interest, which is flash-frozen on a carbon foil. It forms a layer of vitreous ice that spans across the holes on the carbon foil. The thickness and quality of the ice layer is a critical parameter for efficient, high quality data acquisition:

- Ice that is too transparent, is typically too thin.
  Images from such ice layer do not contain sufficient quantities of particles.

- Ice that is not transparent enough is typically too thick, or is not vitreous.
  Images from such ice layers typically have insufficient contrast.

An Ice Quality Filter is used to automatically select areas that are suitable for high quality data acquisition. This filter uses the illumination intensity to assess the thickness and quality of the ice layer.

When the beam passes through ice, the illumination intensity decreases. The ratio between the pre-specimen illumination intensity (*source intensity*) and the post-specimen illumination intensity (*recorded intensity*) is a reliable indicator for the thickness and quality of the ice layer.

In an acquired image, the recorded intensity for each pixel is measured in *counts*, and is visualized as a gray scale value. The Ice Quality Filter applies an upper and a lower boundary to the average gray scale value of each candidate area. Areas that are inside the suitable range are automatically selected for data acquisition.

The source intensity can fluctuate a little over time, which transfers directly to the average gray scale values. As a consequence, using the absolute gray scale value as the selection criterion may cause suitable areas to be exclude, and unsuitable areas to be selected.

The I_0 calibration enables the ice thickness filter to compensate for source intensity fluctuations. It provides a reference value (I_0), based on the counts in an image that is acquired while the beam passes through an area of the specimen where no carbon foil and no ice are present. Because there is no material between the source and the camera sensor, the I_0 reference value represents the source intensity.

If an I_0 reference value is available, then the upper and lower boundaries of the Ice Quality Filter are defined relative to that I_0 reference value. When the source intensity changes, the suitable range of gray scale values changes proportionally with it.

- During the preparation of an Automated Acquisition run, the I_0 reference value can be refreshed manually.

- During an Automated Acquisition run, the I_0 reference value is refreshed automatically:

  - At regular intervals

  - Before starting with a new Grid Square, if the most recent I_0 measurement is older than 2 hours.

  The I_0 calibration images are saved in the EPU session directory.

The I_0 calibration is an optional task. If no I_0 reference value is available, the gray scale values of the Ice Quality Filter boundaries are fixed. This is fine, as long as the source intensity does not fluctuate too much.

4.4.1 Perform the I0 Calibration for the GridSquare Preset

Note   It is more efficient to perform the I_0 calibration *after* the Atlas is acquired.

In an Atlas, areas that are suitable for the I_0 measurement can be found quite easy. It is also easier to move the specimen to a suitable area.

To prepare and perform the I_0 Calibration, follow the procedure below.

1. If an Atlas has been acquired already:

    a. Select **Atlas** tab > **Square Selection** task.

    b. Right-click on a **Grid Square** with a large gap and select **Move stage to grid square**.
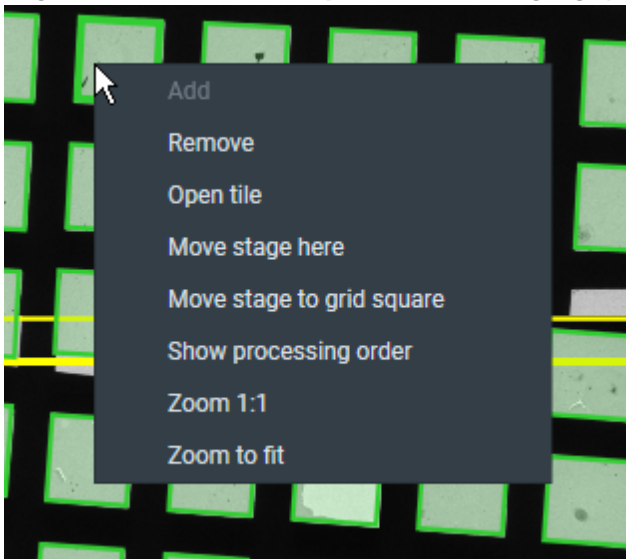
2. If *no* Atlas has been acquired yet:

    a. Insert the FluScreen. .

    b. Set **Magnification** to a low LM magnification.

    c. Move the specimen to a Grid Square with a gap of at least several micrometers wide, and center the gap on the FluScreen.

    d. In the **Preparation** tab > **Acquisition and Optics Settings** tab > **Preset Selection** section, select **Preset**: **GridSquare**.

e.   In the **Optics Settings** section, select **Set**.

f.   If necessary, move the specimen to re-center the Grid Square.

3.   Select **Preparation** tab > **Calibrate I0** task.

∧ Tasks
Acquisition and Optics Settings
Calibrate Image Shifts
Calibrate I0
Activate Phase Plate
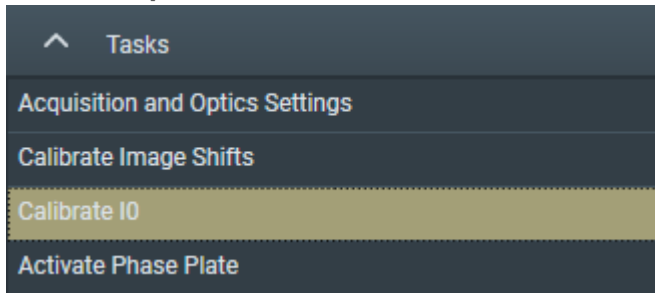
4.   In the **Acquisition Settings Selection** section of the Ribbon Bar, select **Acquisition Settings**: **GridSquare**.

Acquisition Settings GridSquare ∨	📷
Status: calibrated 2018-07-03, 15:37	Preview
Acquisition Settings Selection	
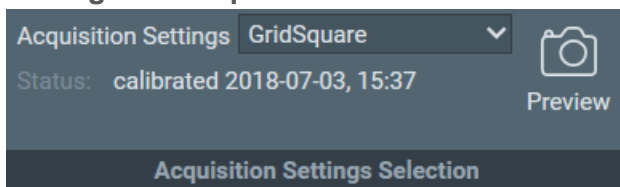
5.   Select **Preview** to acquire an image at the current location.

6.   Center the region that will be used for reference intensity (I_0) measurement:

a.   In the image, right-click in the center of a suitable area and select **Move stage here**.

b.   Select **Preview** again to acquire a new image.

Note	**Do not center the I_0 region by moving the specimen with the joystick.**

The **Move stage here** function uses a backlash correction routine to ensure stage position reproducibility. During the Automated Acquisition run the same backlash correction routine is used to revisit the selected location for I_0 calibration updates.

7.   Set the diameter of the I_0 region:

a.   Double-click on the **center of the I0 region**.
A circle appears.

b.   Increase the **diameter** of the circle, but make sure it stays at least 250 nm away from the surrounding carbon foil.

The I_0 reference value is determined by averaging the signal inside the circular boundary. A larger diameter increases the reliability of the measurement.

8.   Select **Get Stage Position and Diameter**.

Remove I0 Measurements	Get Stage Position and Diameter	Calibrate Current	Calibrate All	Stop
		Calibration		

---
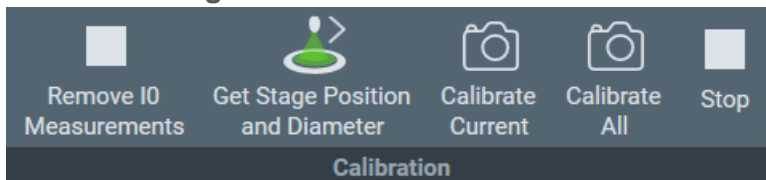
9. Select **Calibrate Current** to measure I_0 value for the 'GridSquare' preset, or click 'Calibrate All' to measure I_0 for both the 'GridSquare' and 'Hole' presets. New images are acquired and I_0 values are measured. The results are stored and displayed in the status window.

4.4.2 Perform the I0 Calibration for the Hole/EucentricHeight Preset

The procedure to calibrate I_0 for the Hole/EucentricHeight Preset is the same as for the GridSquare Preset. For the Hole/EucentricHeight Preset, the same stage position and area diameter are used for calibration and during the Automated Acquisition run.

Currently, I_0 for the Hole/EucentricHeight Preset is only measured for reference. In the future it may be used for Target Area analysis.

4.5 The Activate Phase Plate Task

The use of a Phase Plate substantially enhances the low-to-medium frequencies when imaging weak phase objects. This frequency enhancement results in a higher image contrast. A higher contrast makes identifying and aligning features of interest easier and more reliable.

With an activated Phase Plate, image contrast is maintained under optical focus, so it should be possible to acquire data that does not need any Contrast Transfer Function (CTF) correction.

Resolution is severely limited by focus accuracy. To avoid specimen damage the focus cannot be adjusted on the region of interest. Therefore, the preferred strategy is to acquire data with a certain amount of defocus and to apply a CTF correction.
See for example, "Using the Volta phase plate with defocus for cryo-EM single particle analysis" (Danev et al, eLife 2017;6:e23006) (https://elifesciences.org/articles/23006).

The duration of an Automated Acquisition run generally exceeds the lifespan of a Phase Plate area. During an Automated Acquisition run procedure, EPU selects and activates a new Phase Plate at regular intervals. A new Phase Plate must be activated to make sure that:

*   The next data acquisition starts with a decent phase shift, so that the acquired image has sufficient contrast for fast, reliable and accurate feature identification.

*   The change in phase shift during the first acquisition stays within limits. At the beginning of the Phase Plate development, phase shift changes rapidly. Activating the new Phase Plate before acquiring an image stabilizes the phase shift.

To successfully activate new Phase Plates during an Automated Acquisition run, the following parameters must be specified:

Activation time

For a successful EPU experiment, the Phase Plate should establish a phase shift range of $0.5\pi \pm 0.3\pi$. To achieve a phase shift of 0.5π, the Phase Plate typically needs to be irradiated with an electron dose of 50 nC – 200 nC. The required activation time can be calculated from the electron dose number:

```
t = dose / (beam current)
```

For example: with a 1 nA beam current, an activation time of 120 seconds is needed to reach 120nC.

Accelerate and **Accelerate factor**

- The **Accelerate** function temporarily selects the largest C2 aperture to increase the beam current. After the phase plate activation is completed, the initial C2 aperture is selected again.

- The **Accelerate factor** is the area ratio between the initial C2 aperture and the largest C2 aperture.

The Accelerate function *only* changes the selected C2 aperture. The Optics settings are not affected.

4.5.1   Determine the Phase Plate Activation Time

Note	The Phase Plate activation time is best determined with the *Sherpa > AutoCTF* function**.**

For regular users it is possible to check the activation time in the **Preparations** tab > **Activate Phase Plate** task. To do so, follow these steps:

1. Correct for astigmatism if necessary.
   Follow the instructions in Run the Autostigmate Auto Function on page 85.

2. In the **TEM User Interface** > **Apertures** control panel, select an **Objective** mechanism > **Phase Plate** position.

3. Retract the **FluScreen**.
   The beam will be blanked now.

4. Select the **Preparation** tab > **Activate Phase Plate** task.
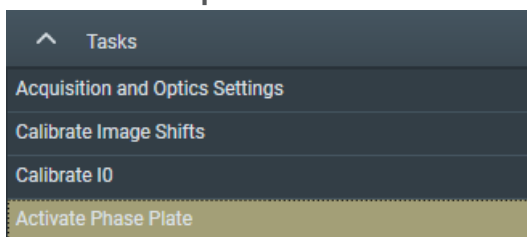
5. Select **Next Position** to move the Objective aperture mechanism to a fresh area on the phase plate.
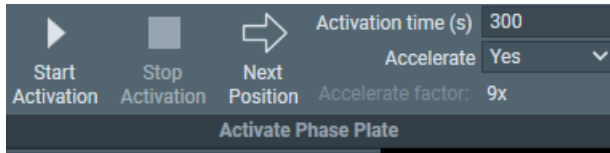
6. Wait for the drift of the aperture mechanism to settle.
   For a small move, this takes about 30 seconds.

Note	When making a large move with the aperture mechanism, such as changing to a different Phase Plate, or re-inserting after the mechanism has been retracted, it may take up to 5 minutes for drift to settle. Please refer to the Phase Plate documentation for the latest specifications.

7. Specify the **Activation time (s)** value.

8. If desired, select **Accelerate**: **Yes**

9. Select **Start Activation**.
   An image is acquired and displayed in the top left section of the Task Execution panel.

10. Wait for the Activation time to expire.
    EPU acquires a new image and displays it in the top right section of the Task Execution panel.
    The FFT of both images are displayed side-by-side in the bottom section of the Task Execution panel, so they are easy to compare. If the Thon rings are shifted inward by half a period, then Phase Plate activation is completed successfully.
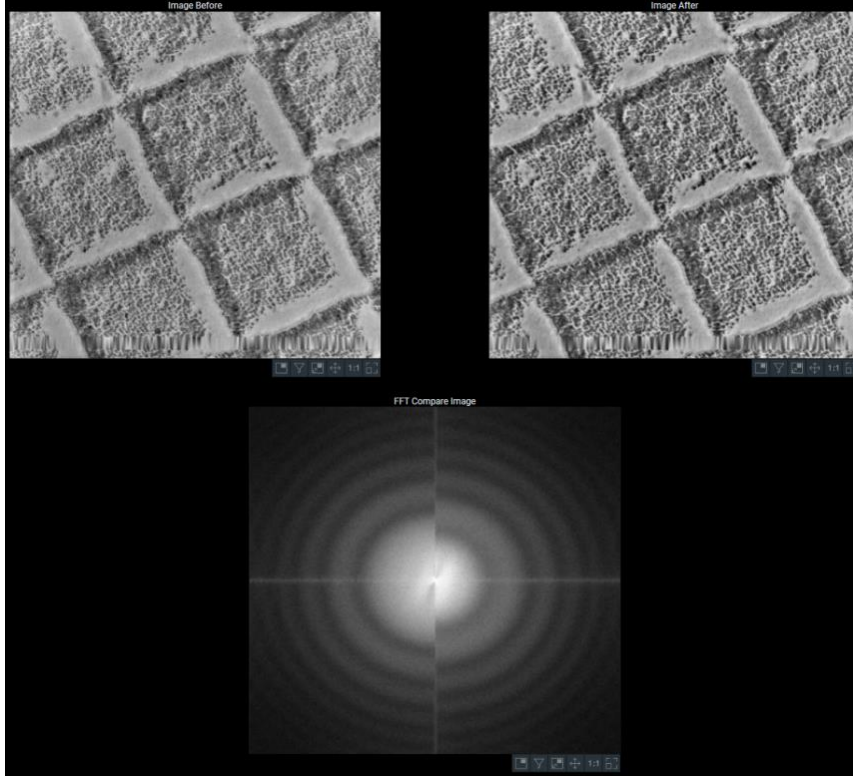
Left half: FFT of an image at the beginning of the activation.
Right half: FFT of an image at end of the activation.

5 The Auto Functions Tab

The Auto Functions tab provides tasks:

- To calibrate Auto Functions.

- To execute Auto Functions as stand-alone tasks, outside the context of setting up an Automated Acquisition run.

For each calibration and stand-alone execution of an Auto Function uses an Acquisition and Optics Preset. Except for the *Auto-eucentric by stage tilt* task, the Defocus value that is specified in the selected Preset is not applied during calibration or stand-alone execution of the Auto Function.

5.1 The Calibration Tasks

5.1.1 The Autofocus Calibration Task

5.1.1.1 Prepare for Autofocus Calibration

The Autofocus calibration calibrates the Autofocus function, and measures and corrects astigmatism.

Note	**The Autofocus calibration result is stored in the Windows Registry. It can only be completed successfully by Thermo Fisher Scientific engineers, or after logging in with the S***upervisor* **account.**

For the best result, the Autofocus calibration requires an area on the specimen:

- Where no ice is present.

- Where only carbon foil with a decent amount of gold particles is visible.

If the currently loaded specimen does not meet all the above requirements, then please exchange the specimen. For example, use the *Combined Test Specimen (Agar S142)* that is delivered with the microscope. This is a holey carbon foil specimen with gold particles and graphitized carbon.

To prepare for the Autofocus calibration, use the Handpanels and the TEM User Interface to perform the procedure below:

1. Select **Right Handpanel** > **Eucentric Focus**

2. Select the **Handpanel User Button** that is assigned to **Reset Defocus**
   This is usually **R2**

3. Move the specimen to an area that meets the requirements listed above.

4. Manually set the specimen to eucentric height.

5. If necessary, use the **TEM User Interface** and the **Handpanels** to accurately correct for astigmatism.

6. Accurately focus the specimen.

5.1.1.2 Perform the Autofocus Calibration for the Autofocus Preset

To perform the Autofocus calibration, follow the procedure below:

1. If necessary, select **Auto Functions** > **Calibrations** to expand the Calibrations section.
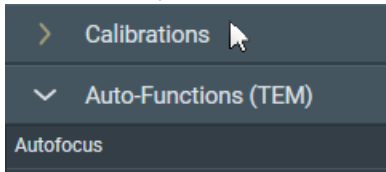
2. Select the **Auto Functions** > **Calibrations: Autofocus** task.

3. Select **Preset Selection** > **Preset**: **Autofocus**
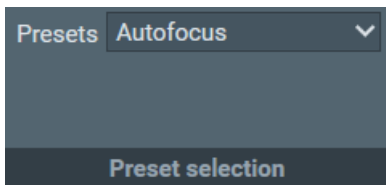
The Autofocus calibration ignores the Defocus value of the selected Preset.

4. Select **Execution** > **Start**
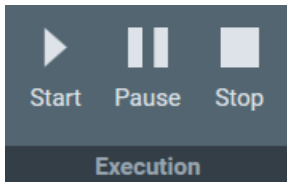
5. Follow the instructions on screen.
EPU may request to manually adjust focus and correct astigmatism.
After completing a manual adjustment, select **Resume**
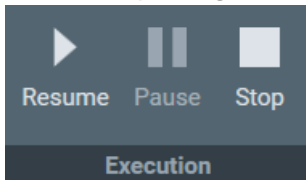
The Autofocus calibration procedure acquires images with negative beam tilt and positive beam tilt, and calculates their cross-correlation. The encircled bright spot in the Cross-Correlation image corresponds to the shift between the beam tilt images.
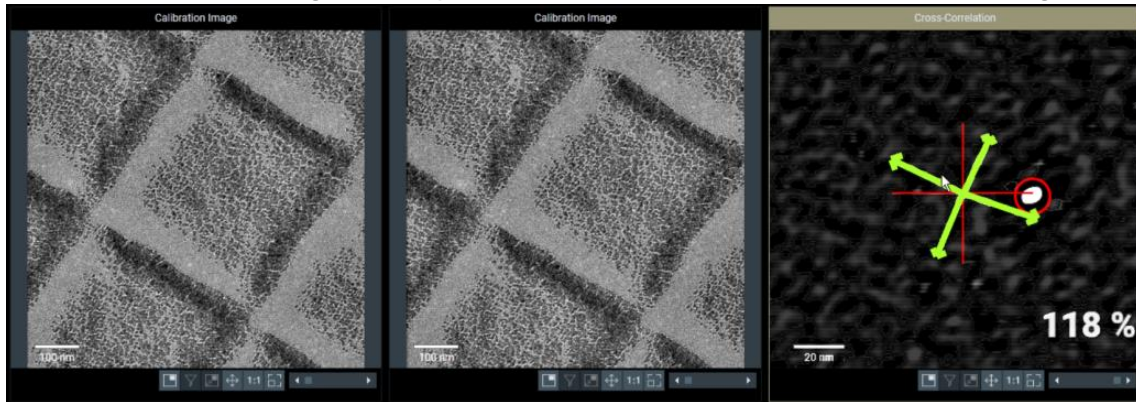
6. In the Cross-Correlation image, estimate the length of the shift.

7. Compare the length of the cross-correlation shift to the width of the beam tilt images. For accurate calibration results, the shift must be at least 10% of the beam tilt image width.
   If the shift in the Cross-Correlation image is less than 10% of the image width:

   a. Select **Stop** to abort the calibration procedure.

   b. Estimate the length of the shift in the Cross-Correlation image.

   c. Select **Preparation** > **Acquisition and Optics Settings** > **Preset Selection** > **Presets**: **Autofocus**

   d. Increase the **Optics Settings** > **Magnification**

   e. Select **Acquisition** > **Preview**

   f. Make sure the width of the acquired image is less than 10 times the length of the cross-correlation shift.
   If not, increase **Magnification**, select **Preview** and check again.

   g. Return to the **Auto Functions** > **Calibrations: Autofocus** task and select **Execution** > **Start** again.

8. At the end of the procedure, select **Resume** to accept and store the calibration results.

9. Inspect the last Cross-Correlation image. If necessary, zoom in for a more accurate look.

   a. Verify that the **green arrows** form a **symmetric cross**, with the arms at 90° angles and of approximately the same length. This indicates that there is no significant astigmatism.
   If the arms are not at 90°, or are of different lengths, then correct the astigmatism and perform the Autofocus calibration again.

   b. Verify that the **red circle** marks a **clear and bright spot**. This indicates that the shift between the beam tilt images is measured without problems.

5.1.1.3 Perform the Autofocus Calibration for Other Presets

The Autofocus function is not only used in the Automated Acquisition run. It can also be executed as a stand-alone Auto Function with any Preset.

The result of the Autofocus calibration is coupled to the Probe Mode in which it is executed. If both Probe Modes are used in the Presets, then the Autofocus calibration must be performed with a Preset that uses Nanoprobe mode and a Preset that uses Microprobe mode. The procedure is exactly the same, except for the selected Preset.

5.1.1.4 How to Improve the Autofocus Calibration Result

If the green arrows in the final Cross-correlation image are not at 90 degree angles relative to each other, the astigmatism has not been corrected completely.
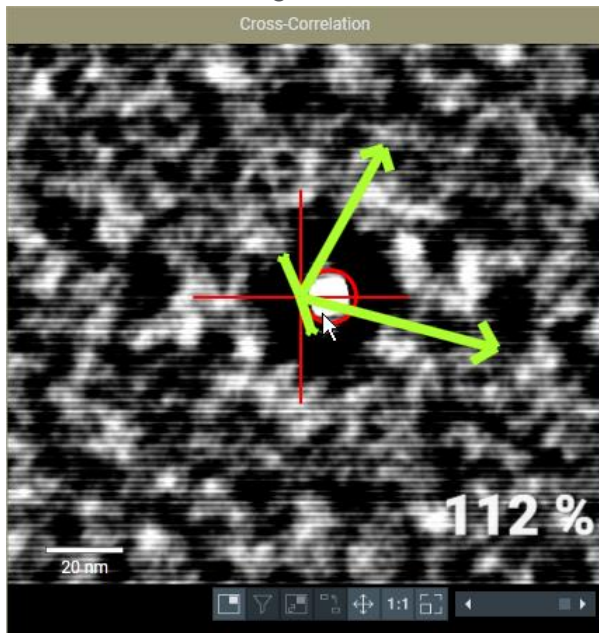
Although the Autofocus function may work well enough for automated data acquisition, it may be worthwhile to improve the stigmation and the accuracy of the calibration.

1. Select the **Auto Functions** > **Auto Functions (TEM): Autofocus** task.

^ Calibrations
Autofocus
Eucentric correction

^ Auto-Functions (TEM)
Autofocus
Auto-eucentric by beam tilt
Auto-eucentric by stage tilt
Autostigmate
Autocoma
Drift stabilization

2. In **Auto Function Settings**:

Desired Defocus (µm)	-2.000	Auto Stigmate	Yes
Iterate to (µm)	-5.000	Use Three Image Method	No
Focus Method	Objective lens		
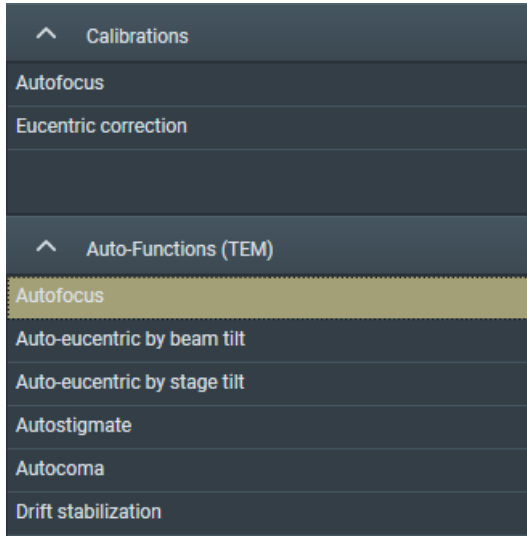
Auto Function Settings
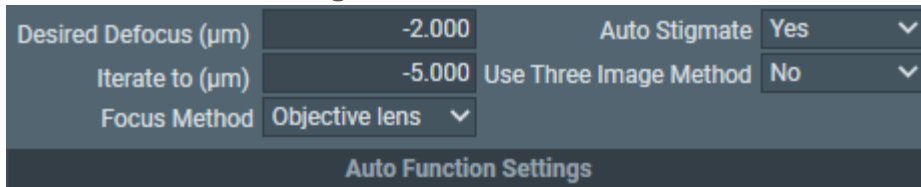
   a. Specify **Desired Defocus**: **0.0**

   b. Select **Auto Stigmate**: **Yes**.

3. Select **Execution** > **Start** to run the Autofocus function.

▶ ❚❚ ■
Start Pause Stop

Execution
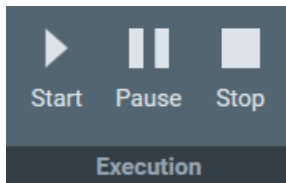
4. Repeat the calibration procedure.

5.1.2 The Eucentric Correction Calibration Task

There are several methods to bring a specimen to eucentric height. Two common methods are:

- Via stage tilt, using the CompuStage Alpha tilt axis wobbler.
  This is the most accurate method. It brings the specimen to the *mechanically defined* eucentric height.
  The stage tilt method is best suited for low to medium magnifications.

- Via beam tilt, using the beam tilt wobbler.
  This method is better suited for high magnifications.

At higher magnifications, it can be difficult or even impossible to use the stage tilt method in an automated routine, especially when the starting position is too far off eucentric height. Two reasons are:

- The initial shift between images that are acquired at opposite Alpha tilt positions would be so large that there is little or no overlap, so no meaningful cross-correlation can be calculated. Without a meaningful cross-correlation, any adjustments to the CompuStage Z-position would be guesswork.

- Due to mechanical tolerances, the CompuStage is not infinitely accurate. At low magnifications, the field of view of the camera is large enough to absorb these mechanical tolerances, especially after running a backlash correction routine. As the magnification increases, the field of view of the camera approaches the same order of magnitude as the tolerances of the CompuStage. This means it is no longer certain that the region of interest on the specimen is returned to the field of view after using the Alpha tilt wobbler.

The beam tilt method is the preferred method at higher magnifications, because it can use very small tilt angles to limit initial image shift and does not suffer from mechanical tolerances.

To get the same result from both methods:

- The beam tilt pivot points must be accurately aligned at eucentric focus height.
- Eucentric focus height and the mechanical eucentric height must be exactly the same.

On an accurately aligned system, the stage tilt method and the beam tilt method result in the same Z-position. In reality, the result of the beam tilt method can have a small offset relative to the mechanically defined eucentric height. The purpose of the Eucentric correction calibration is to measure this offset, so that the *Auto-eucentric by beam tilt* Auto Function can compensate for it.

The Eucentric correction calibration is optional. If there is a noticeable offset between the results of the stage tilt method and the beam tilt method, then it is also possible to renew the microscope's lens series alignments.

5.1.2.1 Prepare for the Eucentric Correction Calibration

1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Auto-eucentric by stage tilt**

2. Select **Preset Selection** : **Presets**: **Hole/EucentricHeight**

3. Select **Execution** > **Start**
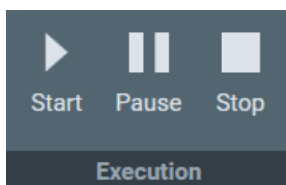
4. Wait until the *Auto-eucentric by stage tilt* function is completed.
   Check the **Status** panel to monitor progress and intermediate results.

5. Use the **Handpanels** > **Focus** knob to accurately focus the specimen.

5.1.2.2 Perform the Eucentric Correction Calibration

1. If necessary, select **Auto Functions** > **Calibrations** to expand the Calibrations section.

2. Select **Eucentric correction**
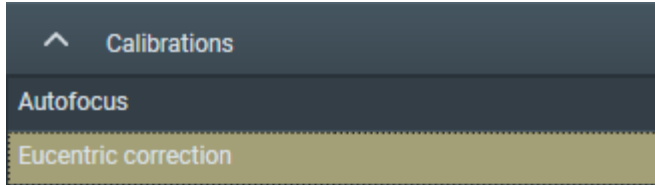
3. Select **Preset Selection** > **Presets**: **Hole/EucentricHeight**.

4. Select **Execution** > **Start**
   No manual actions are required during the calibration procedure.

5. Wait until the calibration procedure is completed.
   Check the **Status** panel to monitor progress and intermediate results.

5.2 The Auto Functions (TEM) Stand-Alone Execution Tasks

The functions in the **Auto Functions (TEM)** group of tasks can be executed outside the workflow context. To set up a properly performing Automated Acquisition run it is not absolutely necessary to tune and execute them here, but it may help to fine-tune an already good set of system alignments.

5.2.1 Run the Autofocus Auto Function

The Autofocus function works in two steps.

First, the Autofocus function establishes focus. The Autofocus algorithm iterates to an *initial* defocus value, typically –5 μm. By aiming for a small defocus:

- The images that are used to measure and adjust the current defocus show a good amount of contrast.

- The Cross-Correlation image shows a significant peak that is not at zero-shift.

The initial defocus value is specified in **Auto Functions** tab > **Auto-Functions (TEM)**: **Autofocus** task > **Auto Function Settings** section > **Iterate to**
In a stand-alone execution, the Autofocus function can also correct for astigmatism and/or for drift. During an Automated Acquisition run these corrections are skipped.

After the algorithm established the initial defocus, the Autofocus function applies the *final defocus* value. Which final defocus value is used depends on where in the EPU workflow the Autofocus function is executed:

- In the **Auto Functions** tab > **Auto-Functions (TEM)**: **Autofocus** task, EPU uses the **Auto Function Settings** > **Desired Defocus** value.

- To acquire an image from an Acquisition Area during the Automated Acquisition run, EPU uses the defocus value from the Defocus list. The Defocus list is specified in the *EPU > Area Selection* task for Lacey Carbon specimens, or in the *EPU > Template Definition* task for Quantifoil specimens.

- At any other time, EPU uses the defocus value from the active Preset.

To change defocus from the initial value to the final value, the Autofocus function either:

- Adjusts the Objective lens current, similar to using the *Handpanels > Focus* knob.

- Adjusts the stage Z-position, similar to the *Auto-eucentric by beam tilt* autofunction.

To run the Autofocus function, follow the procedure below:

1. Select the **Auto Functions** > **Auto Functions (TEM): Autofocus** task.
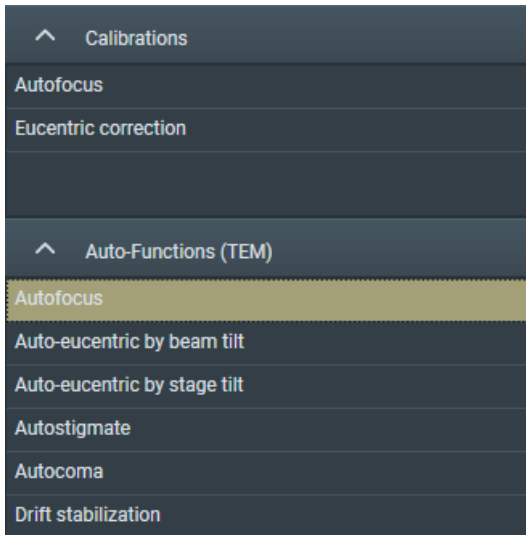
2. Select the **Preset Selection** > **Preset** that will be used for the stand-alone execution of the Autofocus function.
   The Auto Functions can be executed with any Preset.

3. In the **Auto Function Settings** section:
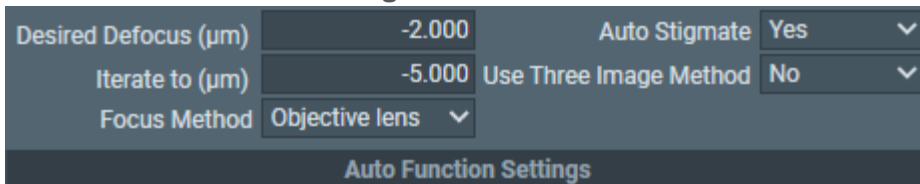
   a. Specify the final defocus value in **Desired Defocus**

   b. Specify the initial defocus value in **Iterate to**

    c.   Select the **Focus Method** that will be used to change from the initial *Iterate to* value to the final *Desired Defocus* value**.**

    d.   If desired, select **Auto Stigmate**: **Yes** to correct for astigmatism during the Autofocus execution.
        Including astigmatism during Autofocus works well on a stable area of the specimen such as a piece of carbon film.
        The Auto Stigmate action is *not* executed during an Automated Acquisition run. On CryoEM specimens, the ice quality changes between consecutive exposures, which may cause stigmation to fail or to give inaccurate results.

    e.   If desired, select **Use Three Image Method**: **Yes** to include drift correction. EPU will acquire an extra image to measure drift.

4.   Select **Execution** section> **Start**
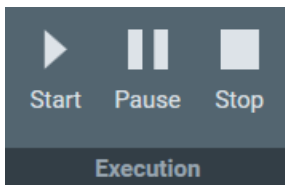
5.2.2 Run the Auto-eucentric by beam tilt Auto Function

The *Auto-eucentric by beam tilt* function is used to set the specimen to eucentric height. Before using this function, the *Eucentric correction* calibration must be completed. Refer to The Eucentric Correction Calibration Task on page 79 for background information and instructions how to perform the Eucentric correction calibration.

This method is more suited for higher magnifications. At low magnifications the method can be used also, but the accuracy would not be very good.

To run the *Auto-eucentric by beam tilt* Auto Function, follow the procedure below:

1.   Select the **Auto Functions** > **Auto-eucentric by beam tilt** task.

---
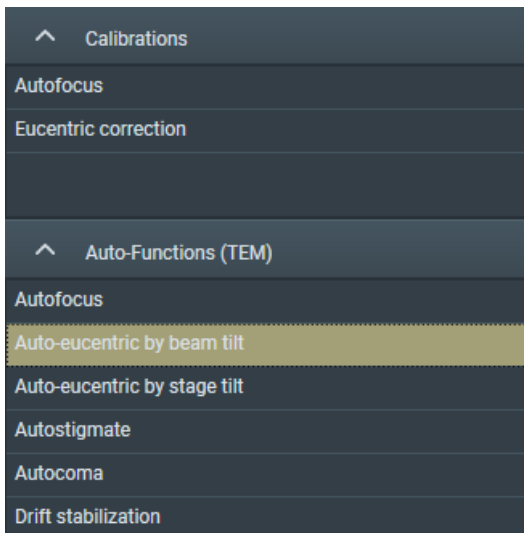
2. Select the **Preset Selection** > **Presets** with which the Auto Function will be executed.

3. In the **Auto Function Settings** section:

Desired Defocus (µm)	0.000
Iterate to (µm)	-5.000
Use Three Image Method	No
Auto Function Settings	
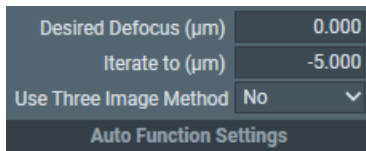
For a description of the parameters, refer to Run the Autofocus Auto Function on page 81.

a. Specify the **Desired Defocus**

b. Specify the **Iterate to** defocus value

c. If desired, select **Use Three Image Method**: **Yes** to include drift correction. EPU will acquire an extra image to measure drift.

4. Select **Execution** > **Start**

▶	⏸	⏹
Start	Pause	Stop
Execution		
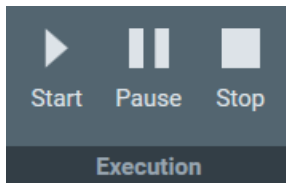
5.2.3 Run the Auto-eucentric by stage tilt Auto Function

The **Auto-eucentric by stage tilt** function is used to set the specimen to eucentric height. This method is more suited for lower and mid range magnifications.

Note	**The** Auto-eucentric by stage tilt **function is not used during Automated Acquisition.**

To run the **Auto-eucentric by stage tilt** Auto Function, follow the procedure below:

1. Select **Auto Functions** > **Auto-eucentric by stage tilt**

∧ Calibrations
Autofocus
Eucentric correction
∧ Auto-Functions (TEM)
Autofocus
Auto-eucentric by beam tilt
Auto-eucentric by stage tilt
Autostigmate
Autocoma
Drift stabilization
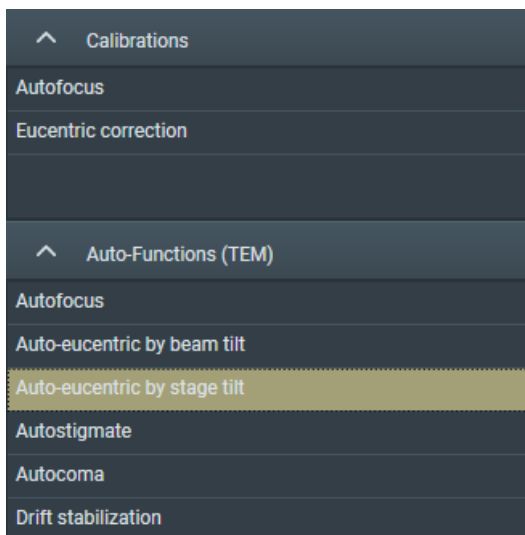
2. Select the **Preset Selection** > **Presets** that will be used to execute the Auto Function. The Auto Functions can be executed with any Preset.

3. Specify the **Auto Function Settings** > **Final stage tilt**
This is the maximum tilt angle at which the CompuStage Alpha tilt axis wobbles. The default value is 15 degrees. Larger and smaller values can be entered, depending on the start conditions.
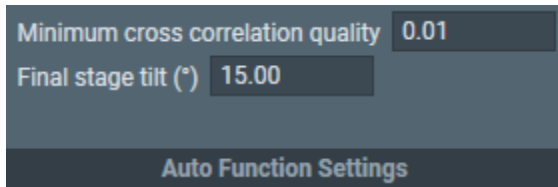
It is strongly recommended to leave the **Minimum cross correlation quality** at the default value (0.01). This is the threshold value at which the *Auto-eucentric by stage tilt* function declares a successful result.

4. Select **Execution** > **Start**
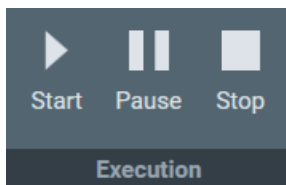
5.2.4 Run the Autostigmate Auto Function

The Autostigmate function uses Thon ring-based aberration corrections. This method corrects astigmatism with higher accuracy than the Auto Stigmate option in the Autofocus function.

To run the **Autostigmate** Auto Function, follow the procedure below:

1. Verify that the *Autofocus* calibration is completed.
If not, refer to The Autofocus Calibration Task on page 75 for instructions.

2. Move the specimen to an area of continuous amorphous carbon foil that is *not* close to a grid bar.

3. In the **Auto Functions** tab, select **Autostigmate**
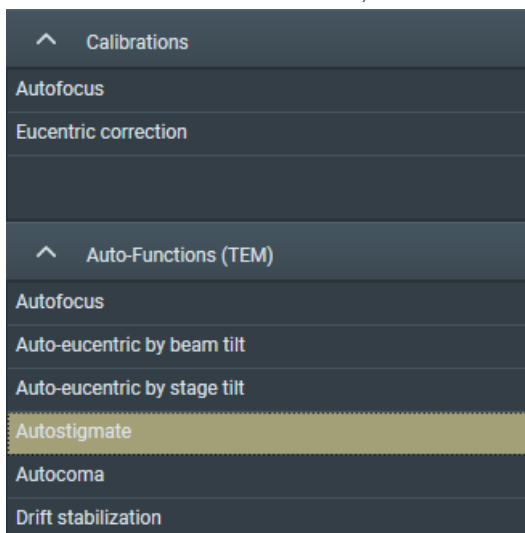
4. Select **Preset Selection** > **Presets: Thon Ring**.

5. Select **Execution** >**Start**.
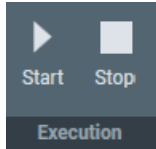
EPU acquires an image and then attempts to fit Thon Rings.

6. Wait for the Autostigmate function to complete.
The Autostigmate function does not have a Pause option.

The Autostigmate function is successful when Thon Rings can be properly fitted. If fitting fails, then a white cross is drawn across the Thon Rings image.
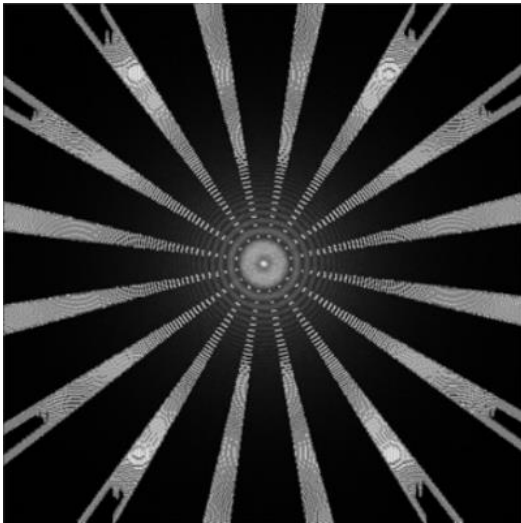
Stigmation is successful
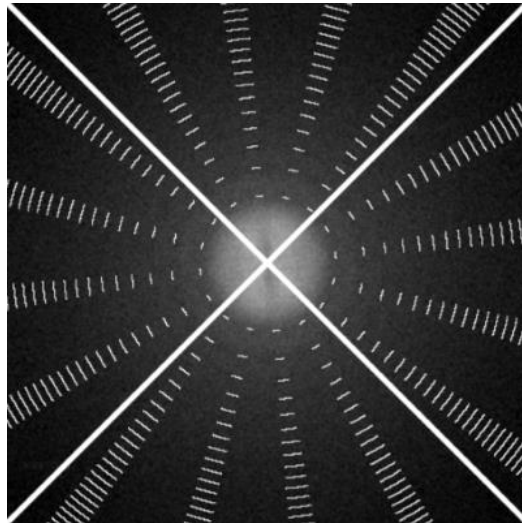
Stigmation has failed.

5.2.5 Run the Autocoma Auto Function

Coma-free alignment is important since coma decreases the achievable reconstruction resolution. If coma is not corrected, a beam shift or beam tilt will result in different objective astigmatism values across an image.

Note	For an optimum result, run the Autostigmate function before *and* after running the Autocoma function.

To run the **Autocoma** Auto Function, follow the procedure below:

1. Run the **Autostigmate** Auto Function as described in chapter Run the Autostigmate Auto Function on page 85.

2. Make sure that the specimen is still positioned at an area of amorphous carbon foil.

3. Verify that the **Objective aperture** is accurately centered.
   If necessary, manually center the **Objective aperture**

4. Select the **Auto Functions** > **Auto Functions (TEM): Autocoma** task
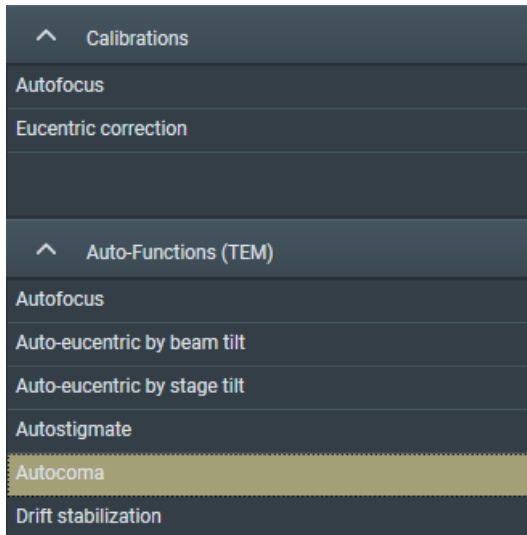
5. Select **Preset Selection** > **Presets: Thon Ring**
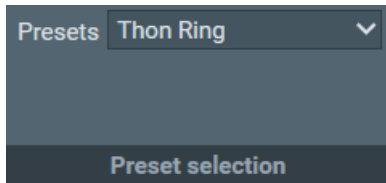
6. Select **Execution** > **Start**
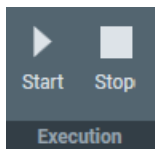
   EPU acquires an image and then attempts to fit Thon Rings.

7. Wait for the Autocoma function to complete.
   The Autocoma function does not have a Pause option.

8. Verify that the **Objective aperture** is still accurately centered.
   During execution of the Autocoma, the Objective aperture may be automatically retracted and inserted again.
   If necessary, manually center the **Objective aperture**

9. Run the **Autostigmate** Auto Function again, as described in chapter Run the Autostigmate Auto Function on page 85.

5.2.6 Run the Drift Stabilization Auto Function

After a stage move, a certain amount of mechanical and thermal drift occurs.

- Friction in the stage mechanics needs to settle and relax.
- A small amount of heat is generated by the motors, which needs to dissipate and disappear.

To acquire images with the highest possible quality, data acquisition must be postponed until drift has decreased to an acceptable level. The Drift stabilization task provides a good estimation for the *Delay after Stage Shift* value that EPU uses during the Automated Acquisition run.

- Quantifoil: *Delay after Stage Shift* is typically used to postpone the next action after a long move, for example to a new Grid Square.
- Lacey Carbon: *Stage Shift Delay* is typically used to postpone high quality data acquisition after the specimen is moved the next Acquisition Area.

The Drift Measurement function acquires a series of images, and then measures the image shift between consecutive images to calculate the drift speed.

- If the drift speed decreases below a specified threshold value before it times out, then the Drift stabilization function reports OK.
- If the drift speed times out before it reaches the specified threshold value, then the Drift stabilization function reports a failure.

To run the Drift stabilization Auto Function, follow the procedure below:

1. Select the **Auto Functions** > **Drift stabilization** task.
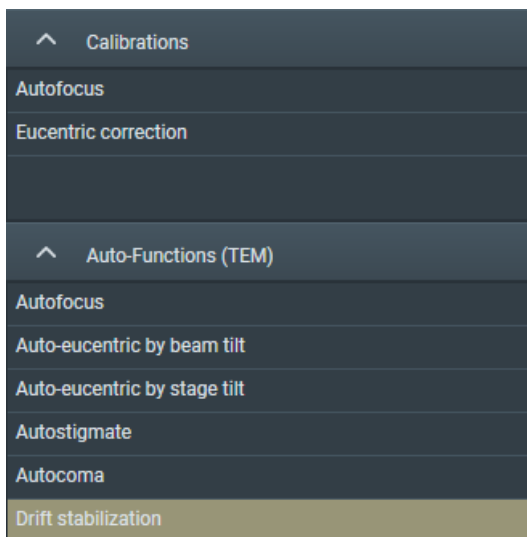
2. Select **Preset Selection** > **Presets: Drift Measurement**

3. In the **Auto Function Settings** section:

Max. Remaining Drift (nm/s)	0.067
Time Out (s)	300.0
Auto Function Settings	
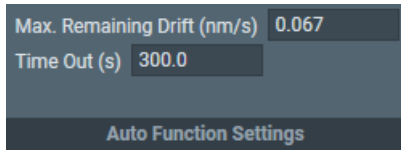
a. Specify **Max. Remaining Drift (nm/s)**
This is the threshold value at which the Drift stabilization function reports a successful result.

b. Specify **Time Out (s)**
This is the time after which the Drift stabilization function reports a failure.
During the Automated Acquisition run, the Time Out is fixed at 600 seconds.

4. Prepare the CompuStage for a move at a similar speed and across a similar distance as during the Automated Acquisition run:

a. With the **Handpanel** > **Magnification** knob, set the magnification to the same value as the GridSquare Preset.
The maximum speed of the CompuStage depends on the current magnification. The magnification that is specified in the Drift Measurement Preset will be not set to the microscope until the Drift stabilization Auto Function is started.

b. Move the specimen to an area of continuous amorphous carbon foil that is not close to a grid bar.

c. Select **Add**.
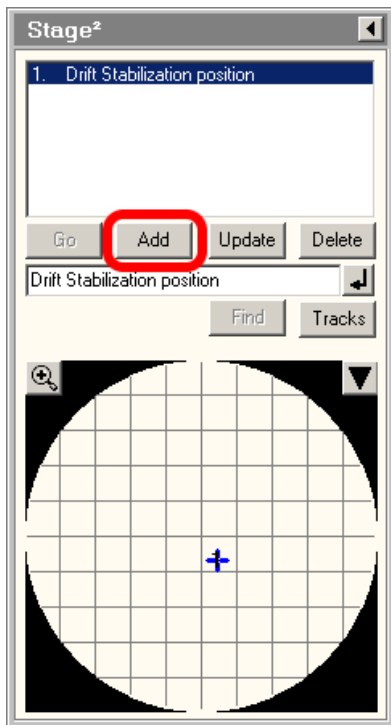
Optionally specify a name.

If it is necessary or desirable to repeat the Drift Stabilization then the suitable specimen area is easily retrieved.

d. In the **TEM User Interface** > **Stage2** control panel > **Set** tab, specify an **X** position and a **Y** position at an appropriate distance from the current position.
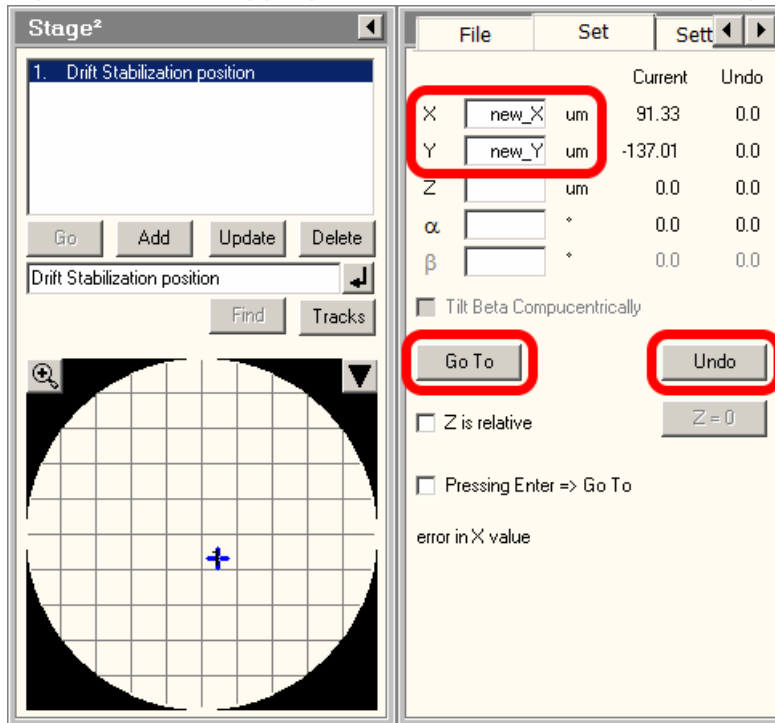
*   To mimic a large move, add 160 μm to both X and Y.
    Specimens with a Grid Square width of 80 μm are commonly used. Adding two Grid Square lengths assures there is a decent margin in the measured stabilization time.

*   To mimic a short move, add 10 μm to both X and Y.
    On Lacey Carbon, an Acquisition Area spacing of 5 μm is commonly used, but this depends on the beam diameter in the Data Acquisition Preset.

e. Select **Go To** and wait for the CompuStage to complete the move.

5. Select **Stage2** control panel > **Undo** to move back to the previous position.

6. Wait for the move to complete, then immediately select **Execution** > **Start**
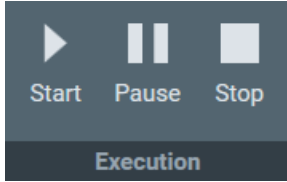
The Drift stabilization Auto Function will run until either the Time Out is exceeded or the specified Max. Remaining Drift is reached. The drift measurement results are plotted in a graph. The Max. Remaining Drift is indicated by the red line.
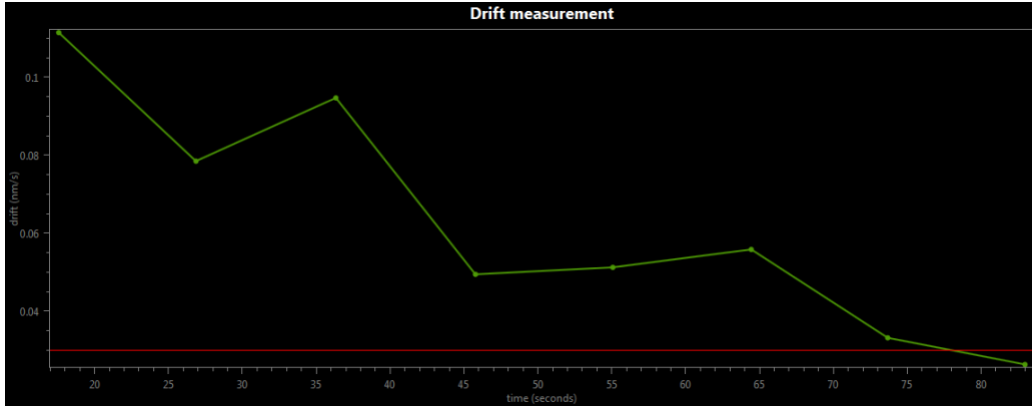
5.2.7 Run the Auto Zero-Loss Function

The Auto Zero Loss function maintains the alignment of the energy slit in the filter with the Zero Loss Peak. This requires that the filter alignment must be accurate and recent.

Note	The Auto Zero-Loss function is only available when the system is in EFTEM mode when EPU is started.

Follow the steps below to run the Auto Zero-Loss function:

1. Select the **Auto Functions** > **Auto Zero-Loss** task.
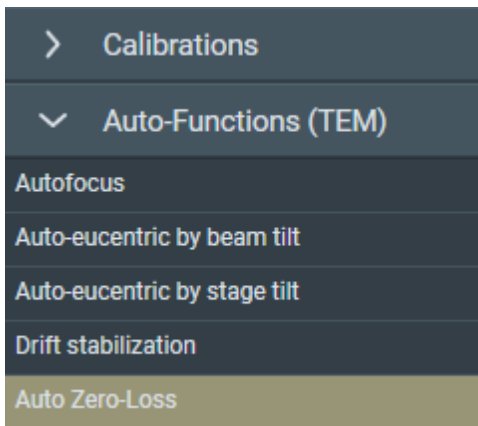
2. Select **Preset Selection** > **Presets: Zero Loss**
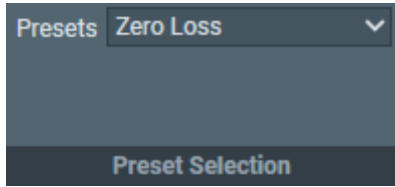
3. Select **Start**
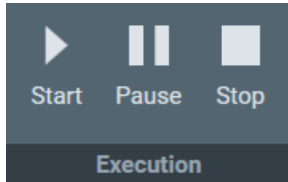
4. Wait until the Auto Zero Loss function is completed successfully,
   or:

   - Select **Pause** to inspect the progress and intermediate result,
     then select **Resume** to let the Auto Zero Loss function continue.

   - Select **Stop** to abort the Auto Zero Loss function and revert the alignment value.

6 The Atlas Tab

An *Atlas* is an overview of a specimen. In EPU, the Atlas is used to:

- Navigate from Grid Square to Grid Square during the preparation and execution of an Automated Acquisition run.

- Estimate the potential to yield high quality data during the Automated Acquisition run. The success of an Automated Acquisition run depends among others on:

  - Good ice quality and thickness, with low to no contamination.

  - Good particle distribution.

An Atlas is created by acquiring a set of large area images, and stitching them together into a map of the specimen. The magnification that is typically used for Atlas acquisition allows for an assessment of the ice quality and thickness. It does not allow for a direct assessment of the presence and distribution of particles, although the thickness of the ice layer is usually a good indicator for particle density.

6.1 The Setup Session Task

To setup a new Atlas session, follow the procedure below:

1. Select the **Atlas** tab > **Session Setup** task.
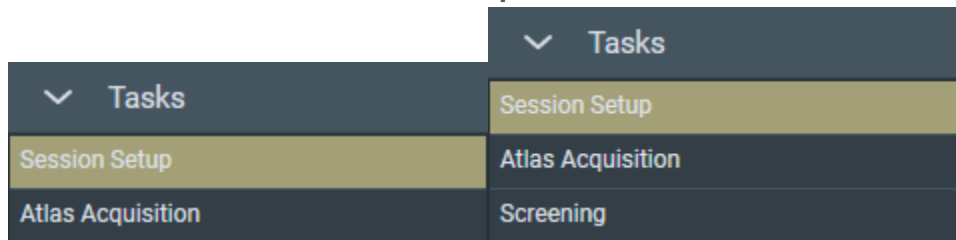
2. In the Ribbon Bar, select **New Session**.
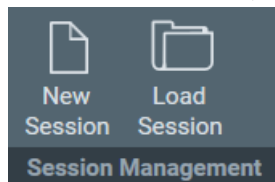
3. Enter a **Name** for the session.

4. If desired, enter additional information in **Description**.

5. Select the **Image format** that will be used to store the acquired images.

   - **MRC**: Electron microscopy image format. The MRC format includes an extensive set of metadata about the microscope and the microscope settings.
     Refer to The MRC2014 Image Format on page 216.

   - **TIFF**: Raster image format: TIFF file format (https://en.wikipedia.org/wiki/TIFF).

6. Specify the **Output folder**
   In the specified folder, EPU creates a sub-folder with the session's *Name*.

Note	Do *not* rename or move the Output folder.

   In this folder, EPU stores the following files:
   - The Atlas session file `ScreeningSession.dm`
   - The images and metadata for the entire Atlas and for all Tiles.

   For a screening session, the session file and folder contains Atlases and Tiles for all screened specimens.

7. If desired, tick **Set as default storage folder**.
   If *Set as default storage folder* is ticked, then the specified folder is used as the default *Output folder* for succeeding Atlas sessions.

8. Select **Apply** to create the new Atlas Session with the specified settings.

6.2   The Screening Task

If the microscope is equipped with an AutoLoader, then the Screening task is available. The Screening task is an optional step in the EPU flow. If the specimens have not been pre-selected before they are loaded into the AutoLoader, then it is recommended to perform a Screening Session before starting the Atlas Acquisition task. The result of a Screening Session helps to select and prioritize specimens that have the highest potential for large quantities of high quality data.

In a Screening Session, EPU automatically acquires Atlases for the specimens in multiple Slot Positions. In each acquired Atlas, EPU evaluates the size, brightness and contrast of the Grid Squares. These visual properties loosely correlate to the physical properties that determine the suitability of a Grid Square for high quality data acquisition, such as:

- Ice thickness (average brightness of a Grid Square).
- Contaminations (local dark areas in the Grid Square).
- Cracks (sharply defined local bright areas in the Grid Square).
- Empty holes (local bright areas that coincide with foil holes).

EPU groups the Grid Squares in categories with similar visual properties. This categorization makes it easier to estimate the potential of the specimen for high quality data acquisition, and to create the selection of Grid Squares that will be processed during the Automated Acquisition run.

6.2.1   Reset the Defocus Value

Follow the steps below to reset the Defocus value:

1. On the **Handpanels**, select **Eucentric Focus**

2. In the **TEM User Interface**, select **Stage2** control panel > **Control** > **Holder** to return all axes to their 0-positions.

3. Search for a recognizable feature near the center of the specimen, and center it on the FluScreen.

4. Bring the specimen at or close to eucentric height.

5. In **EPU**:

   a. Select the **Auto Functions** > **Auto-eucentric height by beam tilt** task.

   b. Select **Preset Selection** > **Presets**: **Hole/EucentricHeight**

   c. Select **Execution** > **Start**

   d. Wait for the **Auto-eucentric height by beam tilt** function to finish.

6. Select the **Handpanel User Button** that is assigned to **Reset Defocus**
   This is usually **R2**

6.2.2 Acquire Atlases for Multiple Specimens

To acquire Atlases for multiple specimens in a Screening session, follow the procedure below:

1. Select the **Atlas** > **Screening** task.
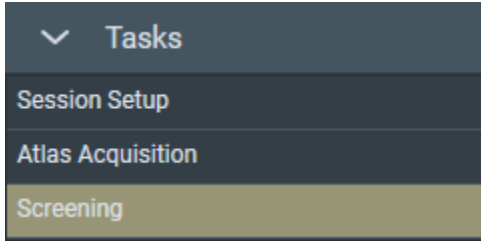
   The Task Selection panel displays all Slot Positions in the AutoLoader.

   - Empty slot:
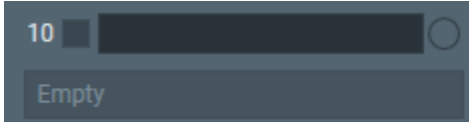
   - Occupied slot:
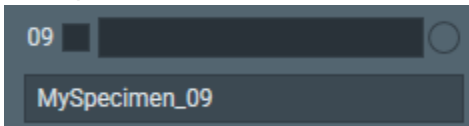
2. For each occupied slot:

   a. If desired, tick the **Slot Position** to schedule it for Atlas acquisition.
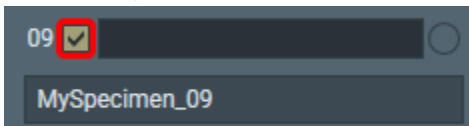
      Empty Slot Positions can not be selected.

   b. If desired, edit the default **description**
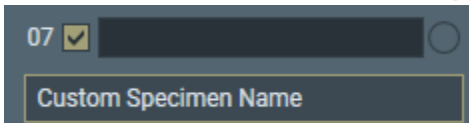
      Only on systems with Titan 3.X / Talos 2.X software:
      At the creation of a new Atlas session, EPU copies the slot descriptions that are present the *TEM User Interface > AutoLoader* control panel to the slots in the Screening task. After the Atlas session is created, EPU and the TEM User Interface do not synchronize their slot descriptions.

3. If desired, specify the **Number of tiles**
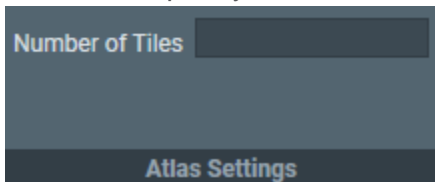
   The specified *Number of tiles* applies to all Slot Positions. It is not possible to specify a different value per Slot Position.
   It is also not possible to specify a *Start Position* like in the Atlas Acquisition task. In a Screening Session, each Atlas Acquisition starts at the *Close to center* position.

---

4. Select **Acquisition** > **Start** to begin the screening procedure.
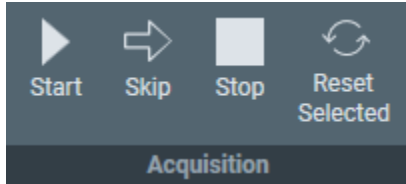
If the Objective Aperture Mechanism is enabled and inserted, then EPU retracts the aperture before acquisition starts. After acquisition is completed, the Objective aperture is inserted again.
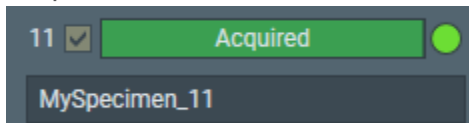
For each included Slot Position, EPU loads the specimen and acquires an Atlas. The progress bar displays the progress or the result of the Atlas acquisition.
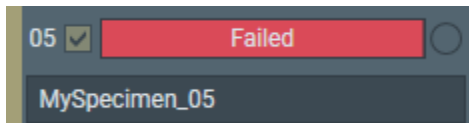
- Acquiring
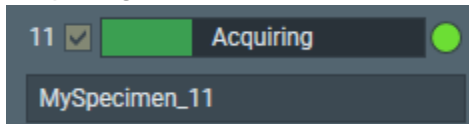
The **green LED** shows that the specimen from this Slot Position is currently loaded on the CompuStage.

- Acquired

- Failed

- Aborted
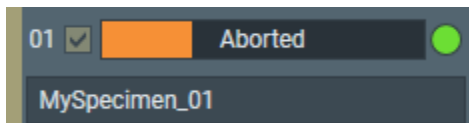
While the screening procedure is running, it is possible to:

- Schedule or unschedule Slot Positions for which Atlas acquisition has not started yet.

- Select any Slot Position to view the Atlas.
  To select a Slot Position, left-click anywhere in the Slot Position, except for the checkbox, the description or the specimen type.
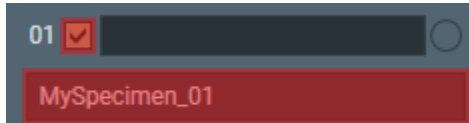
The left edge of the Slot Position becomes sand-colored and the Atlas for the specimen appears in the Image Display.
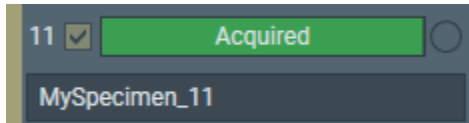
Viewing the Atlas for a specimen does not affect an ongoing Atlas Acquisition for a different specimen.

5. Wait until all selected Slot Positions are processed.
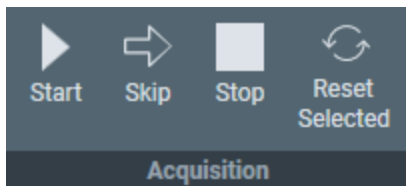
6.2.3 Reset the Slot Position Status

After a Slot Position is processed by the Screening task, it is possible to reset the status back to the initial value. This means that the following information is erased or returned to its initial value:

- Slot Position status

- Atlas

- Selected Grid Square categories
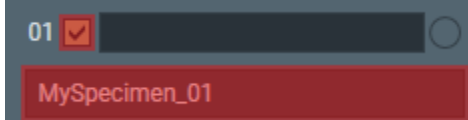
To reset the status of a Slot Position:

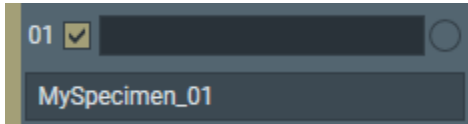1. Select the **Slot Position**

2. Select **Reset Selected**

The status, Atlas, name and selection Grid Square categories are erased, or are returned to their initial values.

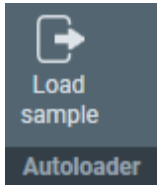### 6.2.4 Load a Specimen on the Stage

1. Select the **Atlas** > **Screening** task to display all Slot Positions in the AutoLoader.

2. Select the **Slot Position** at which the desired specimen is located.
   To select a Slot Position, left-click anywhere, except for the checkbox or the description or the specimen type.
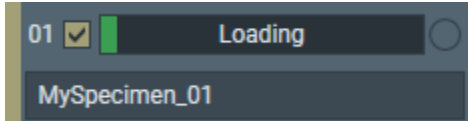

   The edge of the Slot Position becomes sand-colored and the Atlas for the specimen appears in the Image Display.
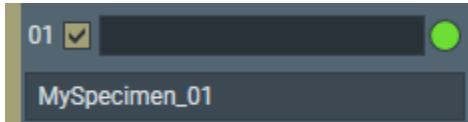

3. Select **Load sample**

   The status of the selected Slot Position changes to *Loading*

4. Wait until the loading procedure is completed and the **LED** is **green**

6.2.5 Pre-select the Grid Squares for the Automated Acquisition Run

Th eScreening functionality assess the quality of the Grid Squares, and assigns Grid Squares with similar characteristics to th esame category. The Atlas for each Slot Position has a set of checkboxes with the number of Grid Squares in each category.
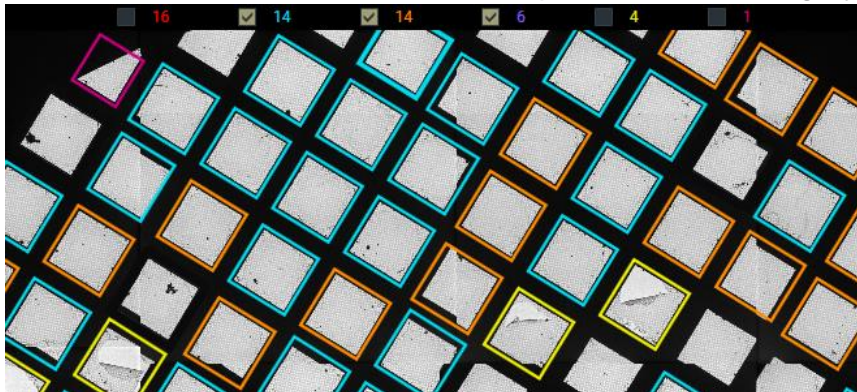
If present, the *Broken Grid Squares* category is always *red*. The other Grid Square categories have randomly assigned colors. These colors do not indicate the suitability of the Grid Square category for high quality data acquisition. The colors are help to mark the Grid Squares in each category.

EPU assumes that less than 50% of the Grid Squares is broken. If more than 50% of the Grid Squares would be marked as broken, than the *Broken* category is omitted and all Grid Squares are assigned to the non-broken categories.

In the Screening task, it is possible to make a pre-selection of suitable Grid Squares for the Automated Acquisition run. If the Atlas from the Screening Session is used for the Automated Acquisition run, then the *EPU > Square Selection* task loads the Grid Squares in the selected Grid Square categories as the initial selection.

To create a pre-selection of Grid Squares for the *EPU > Square Selection* task:

1. Select a **Slot Position** with status Acquired
    To select a Slot Position, left-click anywhere, except for the checkbox, the description or the specimen type.
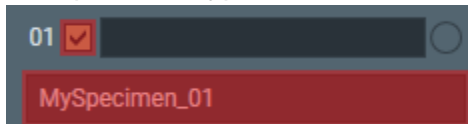
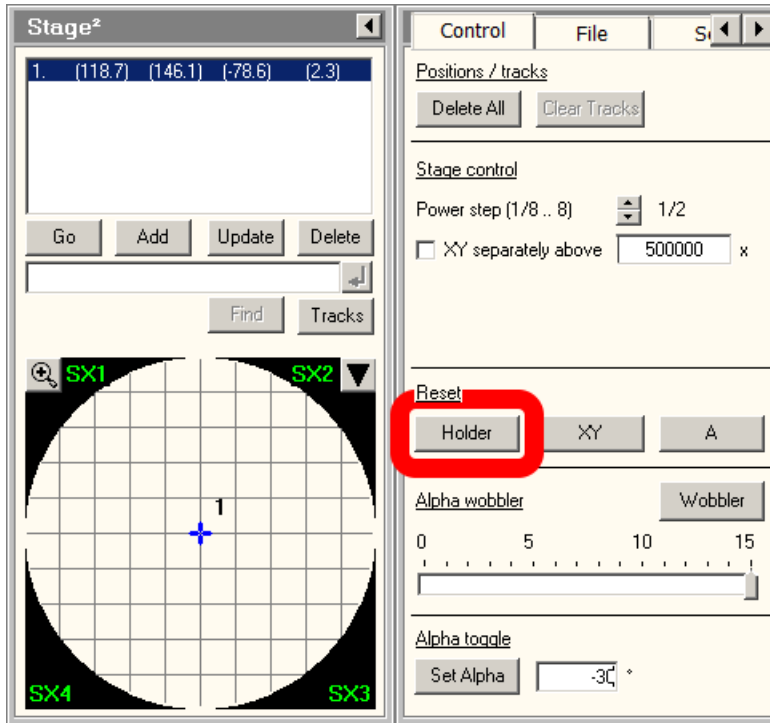   The Atlas for the specimen appears in the Image Display.

2. In the Image Display, above the Atlas:

    *   Tick the **Grid Square categories** that appear suitable for high quality data acquisition.

    *   Clear the **Grid Square categories** that do not appear suitable for high quality data acquisition.

3. Repeat steps 1 and 2 for all **Slot Positions** with status **Acquired**

6.3 The Atlas Acquisition Task

6.3.1 Reset the Defocus Value

Follow the steps below to reset the Defocus value:

1. On the **Handpanels**, select **Eucentric Focus**

2. In the **TEM User Interface**, select **Stage2** control panel > **Control** > **Holder** to return all axes to their 0-positions.


3. Search for a recognizable feature near the center of the specimen, and center it on the FluScreen.

4. Bring the specimen at or close to eucentric height.

5. In **EPU**:

    a. Select the **Auto Functions** > **Auto-eucentric height by beam tilt** task.

    b. Select **Preset Selection** > **Presets**: **Hole/EucentricHeight**

    c. Select **Execution** > **Start**

    d. Wait for the **Auto-eucentric height by beam tilt** function to finish.

6. Select the **Handpanel User Button** that is assigned to **Reset Defocus**
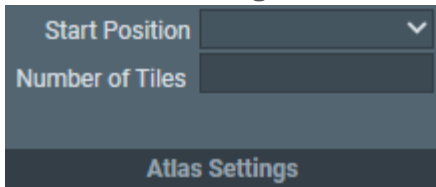   This is usually **R2**

6.3.2   Acquire a New Atlas

To acquire a new Atlas for a single specimen, follow the procedure below:

1. Select the **Atlas** > **Atlas Acquisition** task.
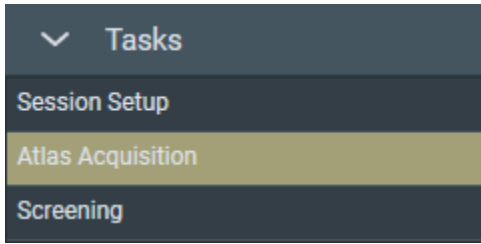
2. In the **Atlas settings** section:

    a. Select the **Start position**:

    - **Close to center**
      Atlas acquisition starts close to the center of the specimen.

    - **Close to current**
      Atlas acquisition starts close to the current stage position.

    b. If desired, specify the **Number of tiles** to restrict the area that will be covered in the new Atlas.

3. Select **Acquisition** > **Start** to begin Atlas acquisition.
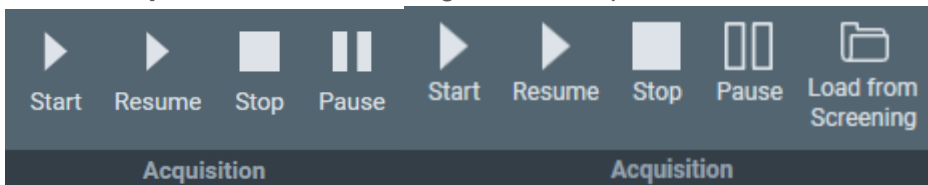
    - If the C2 aperture mechanism is enabled, then EPU first selects the largest C2 aperture. After the acquisition is completed EPU returns the aperture mechanism to its initial position.

    - If the Objective aperture mechanism is enabled and inserted, then EPU first selects the retracted position. After the acquisition is completed EPU returns the aperture mechanism to its initial position.

4. If the image quality is not good enough, or if stitching the tiles does not result in a good Atlas:

    a. Select **Stop**
    This can be done at any time during the Atlas acquisition and may take a few seconds.

    b. Select the **Preparation** > **Acquisition and Optics Settings** task,
   then select **Preset Selection** > **Presets**: **Atlas**

  c. Adjust the **Optics Settings** and or **Camera Settings** parameters.

Note	If the Optics Settings parameters of the Atlas Preset are changed, and the system has a Cryobox, then the Atlas Optics Alignment must be performed again.

  d. Return to the **Atlas** tab.

  e. Select **Acquisition** > **Start**
    A new Atlas acquisition starts. All tiles that were acquired before adjusting the Atlas Preset are discarded.

The Atlas acquisition starts from the selected Start position and progresses in a counter-clockwise spiral fashion. This method minimizes the time in which a useful region is acquired, and minimizes stage moves between consecutive acquisitions.

After each acquisition, the Atlas is updated with a new tile.
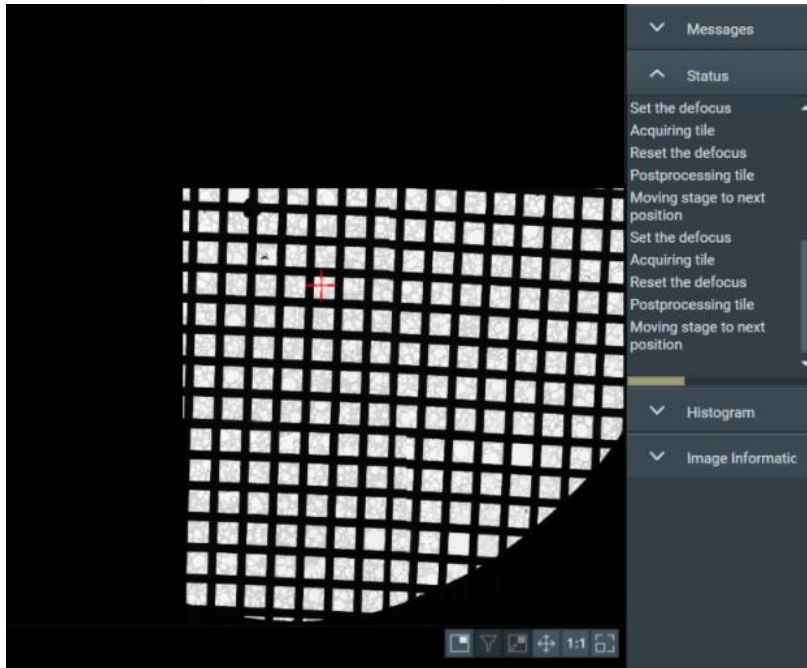
The Status panel provides information about the ongoing actions and progress. Depending on system configuration and the settings in the Atlas Preset, the exact number of tiles in a full Atlas may vary.

6.3.3   Use an Existing Atlas from the Screening Task

If the system has an AutoLoader, then the Screening task is available and Atlases may have been acquired for the specimens in multiple Slot Positions.
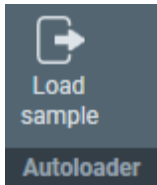
1. Select the **Atlas** > **Screening** task to display all Slot Positions in the AutoLoader.

2. Select the **Slot Position** at which the desired specimen is located.
    To select a Slot Position, left-click anywhere, except for the checkbox or the description or the specimen type.
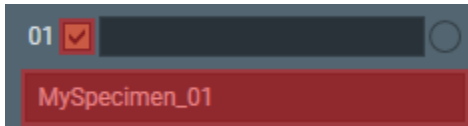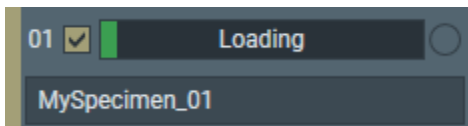

    The edge of the Slot Position becomes sand-colored and the Atlas for the specimen appears in the Image Display.
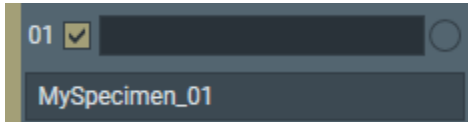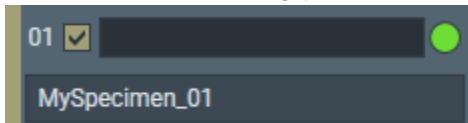

3. Select **Load sample**

    The status of the selected Slot Position changes to *Loading*

4. Wait until the loading procedure is completed and the **LED** is **green**


5. Inspect the **Atlas**

6. If desired, create or adjust the Grid Squares selection that appear suitable for high quality data acquisition.
   Above the Atlas:

   - Tick the **Grid Square categories** that appear suitable for high quality data acquisition.

   - Clear the **Grid Square categories** that do not appear suitable for high quality data acquisition.

   If present, the *Broken Grid Squares* category is always *red*. The other Grid Square categories have randomly assigned colors.
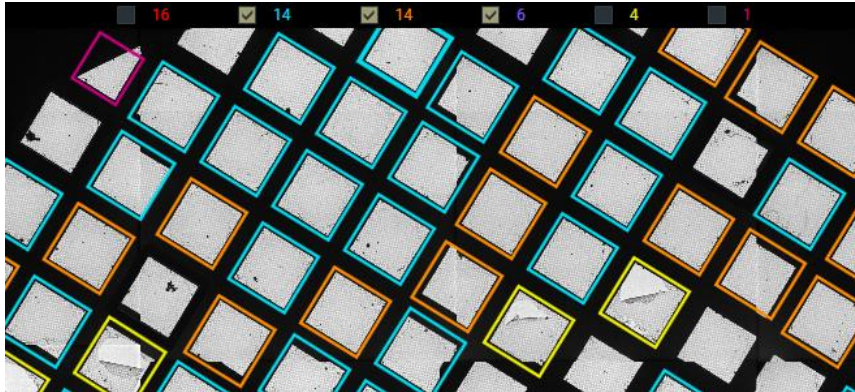
7. Select **Atlas** > **Atlas Acquisition** > **Load from Screening**
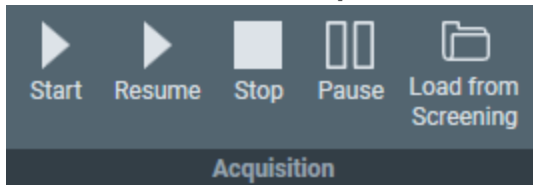
   EPU loads the Atlas for the Slot Position from which the specimen is loaded, and sets it as the active Atlas for the Automated Acquisition run.
   EPU also loads the selected Grid Square categories as the default selection for the Square Selection task.

6.4 Load an Existing Atlas Session

Note   **If the specimen has already been (partly) processed in an Automated Acquisition run, then do not load an existing Atlas session for a new EPU session with that specimen.**

The Atlas file does not contain data about which tiles and Grid Squares have been processed already in a preceding Automated Acquisition run with the current specimen. Processed areas may be too damaged to yield new high quality data. Revisiting these areas is therefore not a productive use of system time.

To load an existing Atlas Session, follow the procedure below:

1. Select the **Atlas** > **Session Setup** task.
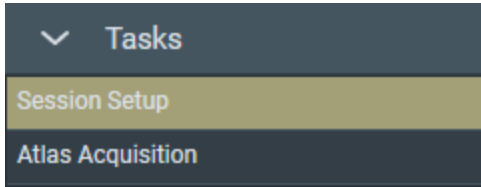
2. Select **Session Management** > **Load Session**.
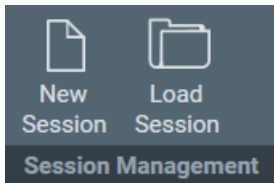
3. Navigate to the Output folder of the desired Atlas Session and select the `ScreeningSession.dm` file.

Note	**Do not load an `Atlas.dm` file.**

If the Screening functionality is not available, then the `ScreeningSession.dm` file contains a single Atlas.

7 The EPU Tab

The EPU tab provides a set of tasks to setup and run an Automated Acquisition Session for a single specimen. Before starting a new session in the EPU tab:

- The tasks in the Preparations tab and the Auto Functions tab must be completed.
- An Atlas of the specimen must be available.

The set of tasks in the EPU tab depends on the specimen carrier: *Quantifoil* or *Lacey Carbon*. The actions that need to be completed in each task depend also on the session type: *Automated* or *Manual* target selection.

Task	Specimen Carrier	Session Type	
		Automated	**Manual**
Session Setup	All	The Session Setup task is generic for both Specimen Carrier types and for both Session Types.	
Square Selection	All	The Square Selection task is generic for both Specimen Carrier types and for both Session Types.	
Area Selection	Only for Lacey Carbon	Once, valid for all selected Grid Squares: • Define and generate an Acquisition Area pattern. • Set the filters.	*Each individual* selected Grid Square: • Define an generate an Acquisition Area pattern. • Set the filters for initial selection. • Customize the selection manually.
Hole Selection	Only for Quantifoil	Once, valid for all selected Grid Squares: • Specify the Foil Hole diameter and spacing. • Find the Foil Holes. • Set the filters.	*Each individual* selected Grid Square: • Specify the Foil Hole diameter and spacing. • Find the Foil Holes. • Set the filters for initial selection. • Customize the selection manually.
Template Definition	Only for Quantifoil	How to define a Foil Hole Template does not depend on the Session Type.	
Template Execution	Only for Quantifoil	How to validate the Foil Hole Template does not depend on the Session Type.	
Automated Acquisition	All	The Automated Acquisition task is generic for both Specimen Carrier types and for both Session Types.	

7.1 The Session Setup Task

7.1.1 Description of the Session Setup Options

7.1.1.1 The EPU Session Preferences File

To prepare and setup a new Automated Acquisition run, EPU offers an option to re-use a considerable subset of the parameter values and calibration results from a preceding session. These values and results can be stored in an EPU Session Preferences file. The use of an EPU Session Preferences file has the following consequences for the EPU workflow:

- The order in which the tabs and tasks must be completed is changed.

- For many tasks in the regular EPU workflow, execution of the task can now be replaced by only a verification of the loaded values.

The recommended workflow sequence for a *New from preferences* session is as follows:

1. Create an EPU Session with the **New from preferences** function.

2. Verify the **Acquisition and Optics Presets**.
   If one or more presets are adjusted, then the related calibrations and other parameters need to be adjusted as well!

3. Acquire an **Atlas**.

4. Continue with the tasks in the **EPU** tab.
   For some tasks, the parameter values may be loaded from the EPU Session Preferences file.

The EPU Session Preferences file uses the `*.epuSessionPreferences` file extension. This file can contain the following values:

Tab	Task	Included Values
Preparation	Acquisition and Optics Settings	All Presets, same as an Preparations > Preset Selection > Export.
	Atlas Optics Alignment	All values
	Calibrate Image Shifts	All values
	Calibrate I0	All values
	Activate Phase Plate	None
Auto Functions	Calibrations: Autofocus	None
	Calibrations: Eucentric correction	None

Tab	Task	Included Values
	Auto-Functions (TEM): Autofocus	None
	Auto-Functions (TEM): Auto-eucentric by beam tilt	None
	Auto-Functions (TEM): Auto-eucentric by stage tilt	None
	Auto-Functions (TEM): Autostigmate	None
	Auto-Functions (TEM): Autocoma	None
Atlas	Session Setup	None
	Atlas Acquisition	None
	Screening	None
EPU	Session Setup	All values, except: • Session name and Description • Athena settings • Storage folder • Email settings
	Square Selection	None
	Area Selection	All values and filter selections.
	Hole Selection	All values and filter selections.
	Template Definition	All values
	Template Execution	None
	Automated Acquisition	Phase Plate selections and values .

7.1.1.2 Acquisition Mode for Quantifoil Specimens

EPU has two methods to locate and center a position on the specimen:

- Optical centering with Image/Beam Shift:
  The specimen remains stationary while the beam shifts to illuminate the Feature of Interest and project it in the center of the camera field of view.
  Although this is very fast, Image/Beam Shift may induce coma under sub-optimal conditions. The amount of coma is typically not significant for small offsets, but for larger offsets this may reduce the image quality.

- Mechanical centering with Stage Shift:
  The beam remains stationary while the specimen moves, so that the Feature of Interest is located in the center of the camera field of view.
  Although the aberrations that are associated with Image/Beam Shift are absent, mechanical centering is considerably slower than optical centering.

If certain conditions are met, then EPU can use optical centering also for relatively large offsets, and thus replace most Stage Shifts by Image/Beam Shifts.
These conditions are:

- Aberration Free Image Shift (AFIS) is present, and is calibrated by a Thermo Fisher Scientific engineer.

- A wide usable Image/Beam Shift range is available:

  - The system does *not* have an Image Corrector.
    An Image Corrector restricts the usable Image Shift range.

  - Phase Plates are *not* used.
    The use of Phase Plates is not compatible with the beam tilt angles that are needed for coma correction.

For Lacey Carbon specimens, EPU automatically determines when to use Stage Shift and when to use Image/Beam Shift to optimize accuracy and throughput.

For Quantifoil specimens, *EPU > Session Setup* offers a manual selection between two *Acquisition Mode* options:

- Accurate Hole Centering

- Faster Acquisition

The Acquisition Modes use different methods to center and visit the Foil Holes for data acquisition. The execution of the Foil Hole Template is the same for both Acquisition Modes.

Accurate Hole Centering

In Accurate Hole Centering mode, EPU processes each individual Foil Hole independently from its surrounding Foil Holes.

EPU first locates the individual Foil Hole, and then accurately centers it in the camera field of view. This procedure requires magnification changes and lens normalizations, and uses small Stage Shifts to mechanically adjust the position of the specimen.

The Accurate Hole Centering procedure does not use Image/Beam Shift to center a Foil Hole. If the Template Areas are within reach of the Maximum Image Shift, then Image/Beam Shift may still be used to center the Drift Measurement, Autofocus and Acquisition Areas during execution of the Foil Hole Template

Faster Acquisition

In Faster Acquisition mode, EPU processes the Foil Holes in groups.

The Faster Acquisition procedure uses Stage Shifts to center multiple Foil Holes at once as a group. This reduces the number of Stage Shifts, magnification changes and lens normalizations.

- If the conditions in Acquisition Mode for Quantifoil Specimens on page 111 are *not* met, then EPU uses Stage Shifts to visit the individual Foil Holes in the centered group.

- If the conditions in Acquisition Mode for Quantifoil Specimens on page 111 are fulfilled, then EPU uses Image/Beam Shifts to visit the individual Foil Holes within the centered group. This reduces the number of Stage Shifts even further.

If Image/Beam is used to visit the individual Foil Holes, then the stage remains stationary. The metadata of all acquired images in the centered group then contains the same stage coordinates.

To separate and group the images per Foil Hole or per Acquisition Area, the file names of the acquired images must be processed.

The format of the file names is:
`FoilHole_[Hole ID]_Data_[Acquisition Area ID]_[date]_[time].mrc`

For example: in `FoilHole_31545690_Data_31547881_31547882_20190601_0819.mrc`

- `[Hole ID]` is `31545690`

- `[Acquisition Area ID]` is `31547881_31547882`

- `[date]` is `20190601` in `yyyymmdd` format

- `[time]` is `0819` in 24-hour `hhmm` format

If Faster Acquisition mode is selected, then the Automated Acquisition run starts with an automatic calibration to maximize centering accuracy. This calibration takes 2–3 minutes. In a longer automated run, this extra time is easily overcome by the much faster Foil Hole processing method. For short runs that acquire images from only a few Foil Holes, the Accurate Hole Centering is often faster.

7.1.1.3 Pixel Format for Gatan Dose Fraction Images

The pixel format of the images that are acquired with the Gatan K2 camera depends on the **Advanced Camera Settings** > **Mode** in the Acquisition and Optics Preset and the **Session Setup** > **Dose fraction output format**. The pixel format has a direct impact on the image file sizes.

MRC Image Pixel Format for a Gatan K2 Camera

Advanced Camera Settings > Mode	Dose fraction output format		
	Gain Normalized	Non-Gain Normalized	Non-Gain **N**ormalized Packed
Linear	32-bit float	16-bit unsigned integer + dark reference image(s) + gain reference image(s)	*not supported*
Counted	32-bit float	16-bit unsigned integer + gain reference image(s)	8-bit unsigned integer + gain reference image(s)
Counted / Super Resolution	32-bit float	8-bit unsigned integer + gain reference image(s)	4-bit unsigned integer + gain reference image(s)

MRC Image Pixel Format for a Gatan K3 Camera

Advanced Camera Settings > Mode	Dose fraction output format		
	MRC Gain Normalized	MRC Non-Gain **N**ormalized	MRC Non-Gain **N**ormalized Packed
Counted	8-bit unsigned integer	8-bit unsigned integer + gain reference image per Grid Square	4-bit unsigned integer + gain reference image per Grid Ssquare
Counted / Super Resolution	8-bit unsigned integer	8-bit unsigned integer + gain reference image per Grid Square	4-bit unsigned integer + gain reference image per Grid Square

TIFF Image Pixel Format for a Gatan K3 Camera

Advanced Camera Settings > Mode	Dose fraction output format	
	MRC Gain Normalized	MRC Non-Gain **N**ormalized

Advanced Camera Settings > Mode	Dose fraction output format	
	MRC Gain Normalized	MRC Non-Gain **N**ormalized
Counted	8-bit unsigned integer	8-bit unsigned integer
Counted / Super Resolution	8-bit unsigned integer	8-bit unsigned integer

All Gatan K3 TIFF images use lossless LZW file compression.

7.1.2 Create a New Session without an EPU Session Preferences File

To setup a new Automated Acquisition session without using a Session Preferences file, follow the procedure below:

1. Select the **EPU** tab > **Session Setup** task.
    If no session is active yet, all other tasks are disabled.

2. In **Session Management**, select **New Session**.

The **Session** form appears in the Task Execution panel.

3. In the **General session settings** section:

   a. Enter a **Session name**

   b. If desired, enter a **Description** for the new session.

   c. Select the **Type**
   The Type setting defines how suitable areas are selected for Data Acquisition.

      • **Automated**: suitable areas are automatically detected, assessed and selected by filters and algorithms. For most sessions, the Automated session type results in the most efficient acquisition of high quality data.

      • **Manual**: suitable areas must be hand-picked by the user. Use this mode when the number of areas from which high quality data must (or can) be acquired is very limited.
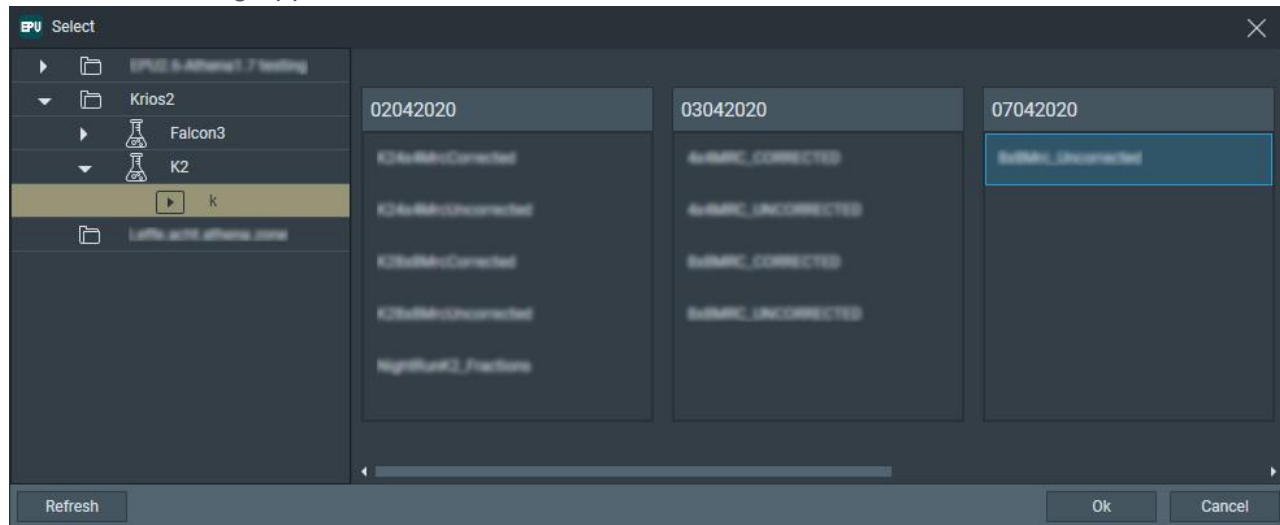
      It is not possible to use a mix of automated and manual area selection in a single Automated Acquisition run. It is possible though to follow-up an automated session by a manual session or vice versa, as long as the specimen remains loaded and the Atlas is not replaced or renewed.

   d. Select the **Acquisition Mode**
   For a description of the available options, refer to Acquisition Mode for Quantifoil Specimens on page 111.

   e. Tick or clear **Use Phase Plates**

4. If available, in the **Athena Settings** section:

   a. At **Selected Dataset**, select **Select**
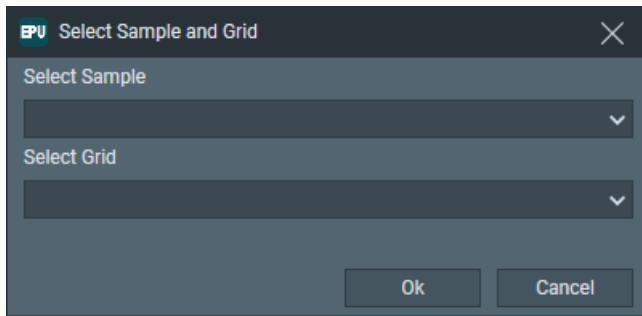   The *Select* dialog appears.

   b. Browse to the desired **Dataset** and select **OK**
   Selected Workflow displays the path to the *Selected Dataset*

   c. If desired, at **Selected Grid**, select **Select**
   The *Select Sample and Grid* dialog appears.

For a successful Automated Acquisition run, it is not required to select a Sample and Grid. A Dataset is sufficient.

d. Select the desired **Sample** and **Grid** and select **OK**

e. If available and desired, tick **Enable Quality Monitor** to use the EPU Quality Monitor (EQM).

5. In the **Output** section:

a. Select the **Image Format**.

- **MRC**: Electron microscopy image format.
  The MRC format includes an extensive set of meta-data about the image, the microscope settings, and the used detector.
  For a description of the Thermo Scientific meta-data, refer to The MRC2014 Image Format on page 216.
  For a description of the generic meta-data, refer to MRC/CCP4 file format for images and volumes.

- **TIFF**: Generic raster image format.
  A description of the format can be found on the web, e.g. at TIFF file format (https://en.wikipedia.org/wiki/TIFF).

Most 3D reconstruction software supports the MRC2014 format. If your reconstruction software does not support the MRC format, select the TIFF format.

Note	When using the Dose Fractions functionality that is available for Direct Detection cameras, the TIFF image format cannot be used.

b. For systems with a Gatan K2 camera only: select the **Dose fractions output format**. Refer to the section below for a description of the options.

c. Specify or select a **Storage folder**.
All session data and acquired images are stored in the specified folder, except for Dose Fraction images. Dose Fraction images are saved on the Storage Server (Falcon 3EC and Falcon 4) or on the Gatan PC (Gatan K2 and K3).

Note	Do *not* rename or move the EPU Session Storage folder.

EPU loads the active EPU session at startup. If the folder path is changed, EPU cannot find the session data. It is not possible to manually load an EPU session from a different location.

    d. If desired, tick or clear **Default folder**.
When ticked, the specified *Storage folder* is the default location for succeeding sessions.

6. In the **Specimen** section:

    a. Select the **Specimen carrier**: **Lacey Carbon** or **Quantifoil**.

    b. If **Quantifoil** is selected,
specify the **Quantifoil type**:

- A variety of predefined hole diameter and spacing dimensions is available.

- If none of the predefined options match your specimen, select **Custom**.

The hole diameter and spacing dimensions can be adjusted later to accurately match the actual sample and imaging conditions.

7. In the **Email** section:

    a. Specify the **Recipients**
Addresses must be separated by commas, semi-colons or spaces.

    b. Tick or clear the **Send email...** checkbox.

    c. If desired, select **Test** to confirm if the entered email addresses are valid and if the email services are configured correctly.
The notification emails are sent via the email service components that are installed on the Microscope PC and on the Support PC. If the test email is not delivered to at least one of the specified addresses, contact Thermo Fisher Scientific.

An email is sent:

- When the run is completed.

- If the run terminates automatically, for example if the column valves are closed due to vacuum issues, or if there is no more available disk space on the Storage Server or the Gatan PC.
If the run is stopped manually in the EPU user interface, no emails will be sent.

The email settings can be updated after the Automated Acquisition run has started.

8. Select **Apply**

7.1.3 Create a New Session from an EPU Session Preferences File

To setup a new Automated Acquisition session based on a EPU Session Preferences file, follow the procedure below.

1. Select the **EPU** tab > **Session Setup** task.
   If no session is active yet, all other tasks are disabled.

2. In **Session Management**, select **New from preferences**.
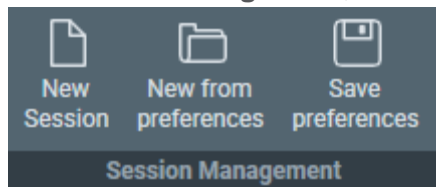
3. Browse to the desired `*.epuSessionPreferences` file and select **Open**.

The **Session** form appears in the Task Execution panel.

All available values in the selected `*.epuSessionPreferences` file are loaded, except:

- The General session settings > Session name and Description
- The Athena Settings
- The Output settings > Storage folder
- The Email Settings section

The Athena Settings section is only visible when the Athena client software is installed on the Microscope PC. If the Athena Settings are disabled, then first log in on Thermo Scientific Athena.

For instructions, refer to Log in on Thermo Scientific Athena on page 8. For a successul Automated Acquisition run it is not required to select an Athena Dataset.

4. In the **General session settings** section:

   a. Enter a **Session name**

    b. If desired, enter a **Description** for the new session.

5. If available, in the **Athena Settings** section:

    a. At **Selected Dataset**, select **Select**
       The *Select* dialog appears.

    b. Browse to the desired **Dataset** and select **OK**
       Selected Workflow displays the path to the *Selected Dataset*

    c. If desired, at **Selected Grid**, select **Select**
       The *Select Sample and Grid* dialog appears.
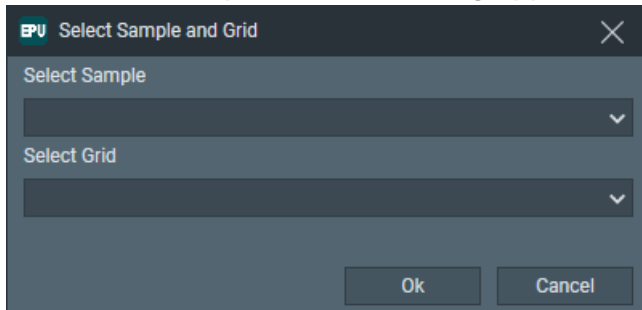
For a successful Automated Acquisition run, it is not required to select a Sample and Grid. A Dataset is sufficient.

    d. Select the desired **Sample** and **Grid** and select **OK**

    e. If available and desired, tick **Enable Quality Monitor** to use the EPU Quality Monitor (EQM).

6. In the **Output** section:

   a. Specify or select a **Storage folder**.
   All session data and acquired images are stored in the specified folder, except for Dose Fraction images. Dose Fraction images are saved on the Storage Server (Falcon 3EC and Falcon 4) or on the Gatan PC (Gatan K2 and K3).

Note	Do *not* rename or move the EPU Session Storage folder.

   EPU loads the active EPU session at startup. If the folder path is changed, EPU cannot find the session data. It is not possible to manually load an EPU session from a different location.

   b. If desired, tick or clear **Default folder**.
   When ticked, the specified *Storage folder* is the default location for succeeding sessions.

7. In the **Email** section:

   a. Specify the **Recipients**
   Addresses must be separated by commas, semi-colons or spaces.

   b. Tick or clear the **Send email...** checkbox.

   c. If desired, select **Test** to confirm if the entered email addresses are valid and if the email services are configured correctly.
   The notification emails are sent via the email service components that are installed on the Microscope PC and on the Support PC. If the test email is not delivered to at least one of the specified addresses, contact Thermo Fisher Scientific.

   An email is sent:

   - When the run is completed.

   - If the run terminates automatically, for example if the column valves are closed due to vacuum issues, or if there is no more available disk space on the Storage Server or the Gatan PC.
   If the run is stopped manually in the EPU user interface, no emails will be sent.

   The email settings can be updated after the Automated Acquisition run has started.

8. Select **Apply**

It is possible to change the loaded values in the Session Setup task as needed.
Refer to Create a New Session without an EPU Session Preferences File on page 115 for a description of the parameters and their values.

It is also possible to adjust any of the other values that are loaded in other tabs and tasks.
Refer to The EPU Session Preferences File on page 109 for:

- An overview of the supported values and results.

- The changes in the EPU Workflow when using an EPU Session Preferences file.

7.1.4 Limitations to Loading or Acquiring an Atlas after Session Setup

After completing the Session Setup task, EPU loads the most recently acquired Atlas as the basis for the Automated Acquisition run. As long as no data has been acquired yet, it is possible to acquire a new Atlas or to load a different, existing Atlas. Be aware though that the validity of an Atlas stops when the specimen is unloaded.

Note	If the specimen has already been (partly) processed in an Automated Acquisition run, then do not load an existing Atlas session for a new EPU session with that specimen.

The Atlas file does not contain data about which tiles and Grid Squares have been processed already in a preceding Automated Acquisition run with the current specimen. Processed areas may be too damaged to yield new high quality data. Revisiting these areas is therefore not a productive use of system time.

Once data acquisitions have taken place, the Automated Acquisition session relies on the underlying Atlas to keep track of the processing progress and status and to maintain data consistency.

7.2 The Square Selection Task

The purpose of the Square Selection task is to select only those Grid Squares that meet the requirements for high quality data acquisition:

- The specimen area is thin enough to be electron translucent.
- The specimen area contains no broken carbon foil.
- For Cryo-EM samples:
  - The holes in the carbon foil are filled with vitreous ice.
  - The Grid Square contains no contamination.

The Square Selection task is the same for Lacey Carbon and for Quantifoil type specimens.

7.2.1 Select Grid Squares for High Quality Data Acquisition

Grid Square selection is a manual task. To select the Grid Squares that meet the requirements for high quality data acquisition, follow the procedure below.

1. Select the **EPU** tab > **Square Selection** task.
   The most recent Atlas appears in the Task Execution panel.
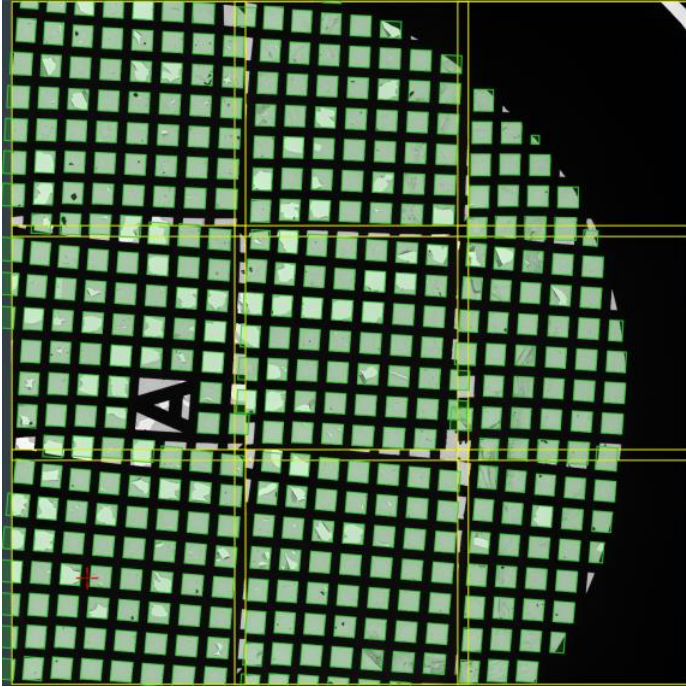
In the Atlas image:

* Atlas Tiles have a yellow outline.
  The Atlas Tiles typically overlap.

* Selected Grid Squares have a green outline and shading.

   * If the Atlas is loaded from the Screening Session, then the default selection includes only the Grid Squares of the selected categories.
     For an explanation of the Grid Square categories, refer to Pre-select the Grid Squares for the Automated Acquisition Run on page .

   * If a new Atlas is acquired, the default selection includes all detected Grid Squares.

* When hovering over a Grid Square, a tooltip displays additional information.
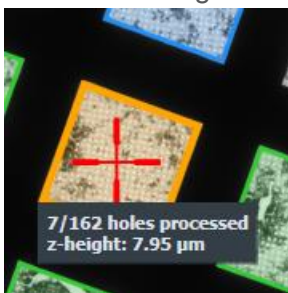
2. If desired, select **Unselect All** to start with an empty selection.
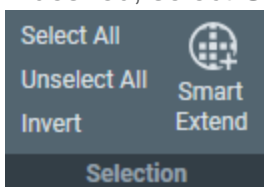
3. Customize the selection of Grid Squares from which data must be acquired.
   Blue and orange Grid Squares have been (partly) processed in an Automated Acquisition run. They cannot be included or removed anymore.

   - Use your own judgment to manually add or remove individual Grid Squares:

     - Use the context menu:
       Right-click on a Grid Square, then select **Add** or **Remove** from the context menu.
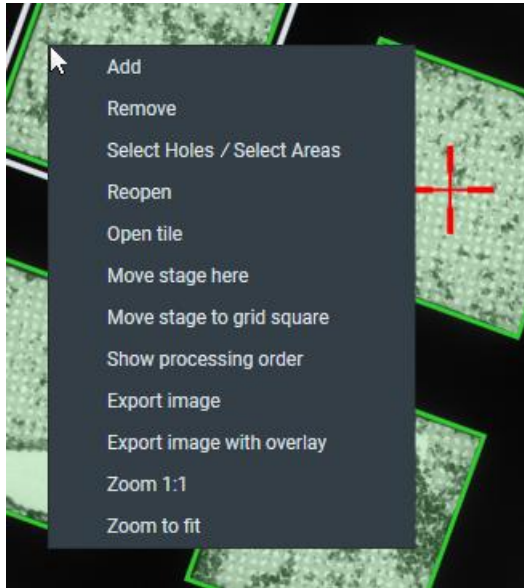
     - Use the keyboard and mouse:
       To add, hold the **Control** key and select one or more **gray Grid Squares**.
       To remove, hold the **Shift** key and select one or more **green Grid Squares**.

   - If the Atlas is loaded from a Screening Session, then *Grid Square Suggestion* is active. Grid Square Suggestion is a machine learning algorithm that identifies Grid Squares that are not included in the selection yet, but that have similar properties as the existing selection. These best-match Grid Squares are marked with a dotted green-and-white border.
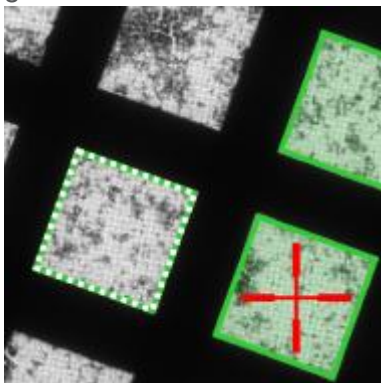
     To add a suggested Grid Square to the selection, either:

     - Add it manually as described above.

     - Select **Smart Extend** to add the best matching Grid Square from the suggested set.

The Grid Square Suggestion and Smart Extend functions are only available if the following conditions are all met:

- The Atlas is loaded from a Screening Session.

- The existing selection includes at least one Grid Square.
  For a good result, start with a selection of at least three Grid Squares. A larger baseline will result in a better match.

- There is at least one detected Grid Square available that is not broken, and that is not included in the selection.

7.2.2 Zoom in on an Individual Atlas Tile Image

In the Atlas overview image it can be difficult to assess the quality of a single Grid Square. For a higher resolution image of an Atlas Tile:

Right-click on an **Atlas Tile**.
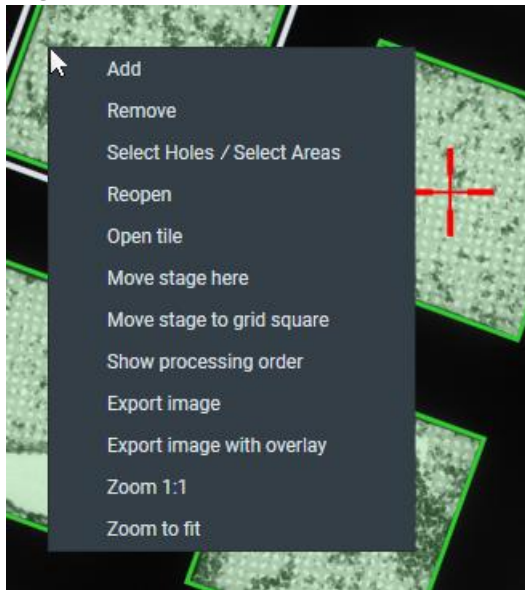
1. Select **Open tile**.

2. Add individual Grid Squares to the selection, and/or remove individual Grid Squares from the selection.

3. Right-click in the **Atlas Tile** image.

4. Select **Close tile** to return to the Atlas overview image again.

7.2.3 Change the Grid Square Processing Order

The order in which the selected GridSquare are processed in the Automated Acquisition run can be edited. To view and change the processing order, follow the instructions below:

1. In the **Processing Order** section of the Ribbon Bar, select **Show**.
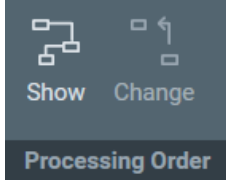
The processing order appears in the Atlas overview image.
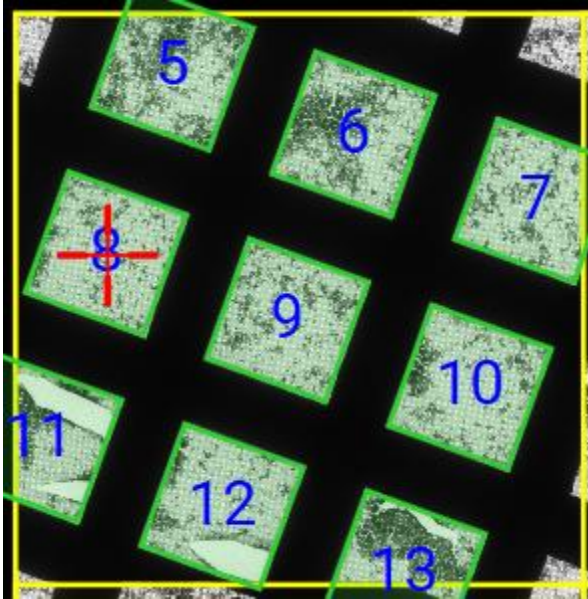
The processing order is determined on Atlas level, not per individual Atlas Tile. When an individual Atlas Tile is opened then the image display will show the sequence numbers for the Grid Squares in that specific Tile.

2. Select **Processing Order** > **Change**.

3. In the image display, select the **Grid Square** that must be processed first.
    The selected Grid Square is moved to the number 1 position in the processing order. The sequence number of the other Grid Squares in the Atlas is automatically updated. This is also true when an individual Atlas Tile is opened while selecting the new number 1 Grid Square.

    *   Example A:

        *   In the Atlas overview image, Grid Square 25 is selected as the new number 1.

        *   Grid Squares 1 - 24 are moved to positions 2 - 25.

    *   Example B:

        *   An Atlas Tile is opened in which Grid Squares 130 - 138 are located.

        *   Grid Square 134 is selected as the new new number 1 in the overall processing order.

        *   On Atlas level, Grid Squares 1 - 133 move one position to positions 2 - 134.

4. Select the Grid Square that must be processed next. This Grid Square is moved to the number 2 position in the overall processing order.

5. Continue selecting Grid Squares in the desired sequence.
    Not all Grid Squares in the entire Atlas have to be assigned a new position in the processing order. After the Grid Squares you want to process first have been moved to the first positions in the processing order, then stop selecting Grid Squares.

6. Select **Change** again to leave the processing order change mode.

7.3   The Area Selection Task for Lacey Carbon Specimens
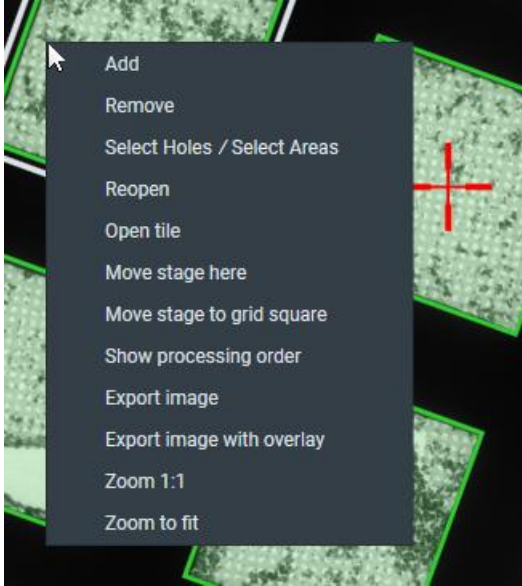
7.3.1   Define the Acquisition Area Pattern and Filter Settings for an Automated Selection Session

If the *Session Setup > Type* is *Automated*, then EPU uses the built-in filters and algorithms to select suitable Acquisition Areas.

To configure the filter settings, follow the instructions below. This procedure must be executed for only one of the selected Grid Squares. During the Automated Acquisition run, EPU uses the same filter settings for all selected Grid Squares.
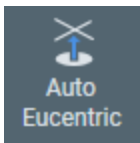
1. Acquire a representative Grid Square image:

    a. Select the **EPU** tab > **Square Selection** task.

    b. In the **Image Display**, identify a Grid Square that appears representative for the condition of the specimen.

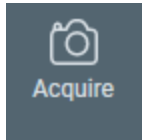c. Right-click on the identified Grid Square and select **Select Areas**

EPU switches to the *Area Selection* task for the selected Grid Square.

d. Select **Auto Eucentric**

- If the specimen is at a different location, then EPU first moves the specimen to the center of the selected Grid Square.

- The Auto Eucentric function includes the acquisition of a new Grid Square image.

- While the Auto Eucentric function runs, the *Auto Eucentric* button changes to *Stop Auto Eucentric*. If selected, the Auto Eucentric function is aborted and the specimen returns to its previous Z position.

- If the specimen is already at eucentric height, or if there is a different reason to skip the Auto Eucentric Height function, then select **Acquire** to only acquire a new Grid Square image.

If an Atlas is loaded from the Screening task, then there is a chance that the orientation of the specimen does not match well enough with the orientation of the loaded Atlas. If the orientation of the grid, or the content of the imaged area does not match with the Atlas, then refer to Correct a Mismatch in the Orientations Between a Re-used Atlas and a Reloaded Specimen on page 185.
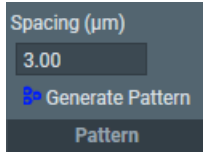
2. If desired, modify the **Defocus List**
   The Defocus List is applied to all Acquisition Areas on the specimen.
   For instructions, refer to Modify the Defocus List for a Lacey Carbon Specimen on page .

3. Generate a pattern of Acquisition Areas:

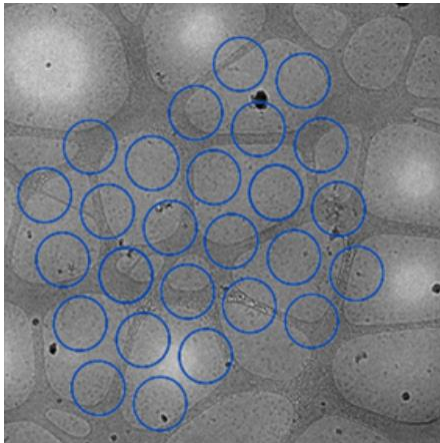   a. Specify the **Spacing** value.
      This is the Center-to-Center distance of adjacent Acquisition Areas.

      • On microscopes with a Three Lens Condenser system, the size of the Acquisition Area is defined by the Illuminated Area which is specified in the Data Acquisition Preset.

      • On microscopes with a Two Lens Condenser system, the Acquisition Area is defined as the smallest circle that fully covers the camera field of view at the Data Acquisition Preset magnification. The beam is typically slightly wider than the camera field of view.

   b. Select **Generate Pattern**.
      EPU draws hexagonal pattern of Acquisition Areas across the entire Grid Square image.

      Depending on the microscope type and configuration, the pattern contains circles and rectangles. Rectangles mark the field of view of the camera. Circles mark the beam diameter.

      • On microscopes with a Three Lens Condenser system, the size of the Acquisition Area is defined by the Illuminated Area which is specified in the Data Acquisition Preset.

      • On microscopes with a Two Lens Condenser system, the Acquisition Area is defined as the smallest circle that fully covers the camera field of view at the Data Acquisition Preset magnification.

   c. If the Acquisition Areas are too close to each other or too far apart, then adjust **Spacing** and select **Generate Pattern** again.

4. Set or adjust the Ice Quality Filter:

   a. Select **Preset Ice Filter** to reset the Ice Quality Filter boundaries.

   b. In the **Filter Ice Quality** histogram:

- Drag the **red lower boundary marker** to exclude Acquisition Areas that are too dark.
- Drag the **red upper boundary marker** to exclude Acquisition Areas that are too bright.

After every change, the Ice Quality filter updates the selection of suitable Acquisition Areas immediately. Depending on the number of areas in the Grid Square image, this may take some time.

5. Select **Remove Areas Close To Grid Bar** to exclude Acquisition Areas near the edge of the Grid Square.
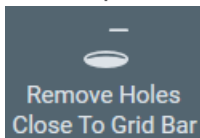
If the result of the *Remove Holes Close to Grid Bar* filter is not satisfactory, then select the filter button again to de-activate it. This will automatically generate a new pattern and apply the Ice Quality filter.

7.3.2 Handpick the Acquisition Areas for a Manual Selection Session

If *Session Setup > Type* is *Manual*, then each selected Grid Square must be visited to handpick all target Acquisition Areas. For each selected Acquisition Area, the X, Y and Z position of the area center is stored. During the Automated Acquisition run, EPU does not re-evaluate the suitability of the selected Acquisition Areas.

After the suitable Grid Squares are selected in the *Square Selection* task, the procedure to handpick the target Acquisition Areas is as follows:

1. In the **Square Selection** task:
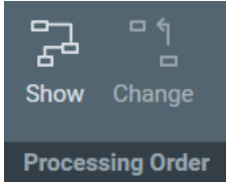
    a. Select **Processing Order** > **Show**

    b. If desired, change the Grid Square processing order.
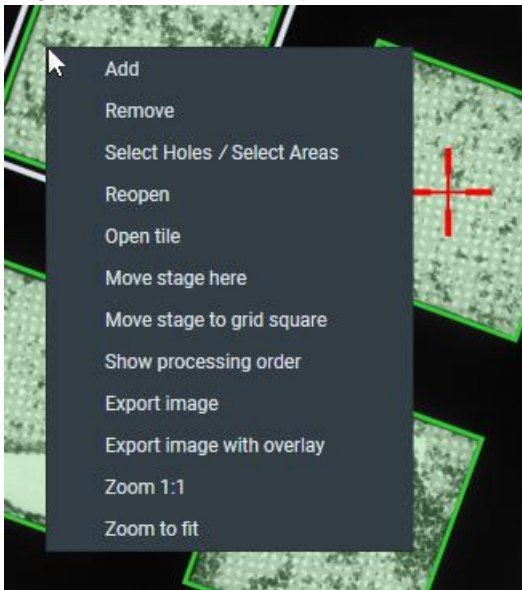   For instructions, refer to Change the Grid Square Processing Order on page .

    c. Right-click on **Grid Square 1** and select **Select Areas**

    EPU switches to the *Area Selection* task for the selected Grid Square. The lower-left corner of the Area Selection Image Display displays the processing order number of the Grid Square and Z-position of the selected Grid Square.

2. In the **Area Selection** task:

    a. Select **Auto Eucentric**

- If the specimen is at a different location, then EPU first moves the specimen to the center of the selected Grid Square.

- The Auto Eucentric function includes the acquisition of a new Grid Square image.

- While the Auto Eucentric function runs, the *Auto Eucentric* button changes to *Stop Auto Eucentric*. If selected, the Auto Eucentric function is aborted and the specimen returns to its previous Z position.

- If the specimen is already at eucentric height, or if there is a different reason to skip the Auto Eucentric Height function, then select **Acquire** to only acquire a new Grid Square image.
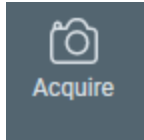

If an Atlas is loaded from the Screening task, then there is a chance that the orientation of the specimen does not match well enough with the orientation of the loaded Atlas. If the orientation of the grid, or the content of the imaged area does not match with the Atlas, then refer to Correct a Mismatch in the Orientations Between a Re-used Atlas and a Reloaded Specimen on page 185.

b. If desired, generate the Acquisition Area pattern.
For instructions, refer to Create the Initial Acquisition Area Selection for a Manual Selection Session on page 134.

c. Customize the selection of target Foil Holes.
For instructions, refer to Customize the Selection of Acquisition Areas on page 137.

d. Select **Navigate** > **Next Square** to move to the next Grid Square.
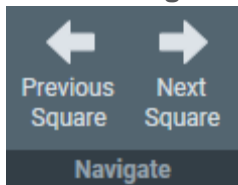
In the Square Selection image display, the white border moves to the next Grid Square. The specimen does not move until *Auto Eucentric* or *Acquire* is selected.

e. Repeat steps *a – e* above until all Grid Squares are prepared.

3. If desired, inspect or adjust the Acquisition Area selection for a prepared Grid Square:

a. Navigate to the desired Grid Square.
Either:

- Select **Navigate** > **Previous Square** until the desired Grid Square is reached.
In the Square Selection image display, the white border moves to the previous Grid Square. The specimen does not move until *Auto Eucentric* or *Acquire* is selected.

- Select the **Square Selection** task
> right-click on the desired **Grid Square**
> select **Select Areas**

EPU displays the Grid Square image for the selected Grid Square.

Note	Unless the current selection of Acquisition Areas must be cleared:
	• Do *not* select *Acquire*
	• Do *not* select *Generate*
	• If *Remove Holes Close to Grid Bar* is active,
	then do *not* select *Remove Holes Close to Grid Bar*

b. If a new Grid Square image is acquired, then generate a new initial Acquisition Area selection.
For instructions, refer to Create the Initial Acquisition Area Selection for a Manual Selection Session on page 134.

c. Customize the selection of Acquisition Areas.
For instructions, refer to Customize the Selection of Acquisition Areas on page 137.

7.3.2.1 Create the Initial Acquisition Area Selection for a Manual Selection Session

In a Manual Selection Session, the actions below must be repeated for each selected Grid Square.

1. If desired, modify the **Defocus List**
The Defocus List is applied to all Acquisition Areas on the specimen.
For instructions, refer to Modify the Defocus List for a Lacey Carbon Specimen on page 141.

2. Generate a pattern of Acquisition Areas:

a. Specify the **Spacing** value.
This is the Center-to-Center distance of adjacent Acquisition Areas.

- On microscopes with a Three Lens Condenser system, the size of the Acquisition Area is defined by the Illuminated Area which is specified in the Data Acquisition Preset.

- On microscopes with a Two Lens Condenser system, the Acquisition Area is defined as the smallest circle that fully covers the camera field of view at the Data Acquisition Preset magnification. The beam is typically slightly wider than the camera field of view.

b. Select **Generate Pattern**.
EPU draws hexagonal pattern of Acquisition Areas across the entire Grid Square image.

Depending on the microscope type and configuration, the pattern contains circles and rectangles. Rectangles mark the field of view of the camera. Circles mark the beam diameter.

- On microscopes with a Three Lens Condenser system, the size of the Acquisition Area is defined by the Illuminated Area which is specified in the Data Acquisition Preset.

- On microscopes with a Two Lens Condenser system, the Acquisition Area is defined as the smallest circle that fully covers the camera field of view at the Data Acquisition Preset magnification.

c. If the Acquisition Areas are too close to each other or too far apart, then adjust **Spacing** and select **Generate Pattern** again.

3. Set or adjust the Ice Quality Filter:

    a. Select **Preset Ice Filter** to reset the Ice Quality Filter boundaries.

    b. In the **Filter Ice Quality** histogram:

    •   Drag the **red lower boundary marker** to exclude Acquisition Areas that are too dark.

    •   Drag the **red upper boundary marker** to exclude Acquisition Areas that are too bright.

    After every change, the Ice Quality filter updates the selection of suitable Acquisition Areas immediately. Depending on the number of areas in the Grid Square image, this may take some time.

4. Select **Remove Areas Close To Grid Bar** to exclude Acquisition Areas near the edge of the Grid Square.
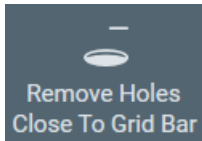
    If the result of the *Remove Holes Close to Grid Bar* filter is not satisfactory, then select the filter button again to de-activate it. This will automatically generate a new pattern and apply the Ice Quality filter.

Note	When the Generate Pattern function is performed, all Foil Holes that match with the Ice Quality Filter are automatically included in the target Foil Hole selection. Any previous customization of the target Foil Hole selection is cleared.

If desired, customize the initial selection of target Acquisition Areas as described in Customize the Selection of Acquisition Areas on page

7.3.2.2 Customize the Selection of Acquisition Areas

Before starting the manual selection, check if the manual selection tools are available in the Ribbon Bar.

If not, set the **EPU** tab > **Session Setup** task > **General session setting** > **Type** to **Manual**.

Note	The following steps must be repeated for *each* individual selected GridSquare.

1. Perform all the steps that are described in Define the Acquisition Area Pattern and Filter Settings for an Automated Selection Session on page 128.

Note	Make sure the specimen is accurately set to eucentric height.

The positions of the selected areas are stored as (X,Y,Z) coordinates. If the specimen is not at eucentric height during manual selection, the X and Y coordinates of the selected areas may have an offset. This may cause failure of the Autofocus and/or Eucentric Correction functions during the Automated Acquisition run, which may negatively impact the amount and quality of the acquired data.

2. Create a selection of Acquisition Areas.
    Either:

    - Select the suitable areas in the generated pattern.

    - Unselect the bad areas in the generated pattern.

    - Select the bad areas in the generated pattern, then invert the selection.

    - Create a selection of suitable areas without using a generated pattern.

    Instructions for each of these selection procedures are described below.

If the Ice Quality Filter boundaries are changed after a manual selection has been made, then the selected set is not updated. Selected areas that fall outside the new boundaries will not be removed from the selection. Likewise, areas that are not part of the selection will not be added to selection if their average gray scale value now falls inside the new boundaries.

Select the Suitable Areas in the Generated Pattern

When the number of bad areas is larger than the number of suitable areas:

1. Select **Unselect All** to clear the selected set.

2. Add the suitable areas to the selection:

   - To add a single area to the selected set, right-click on an area, then select **Add** from the context menu.

   - To add one or more separate areas to the selected set, hold the **Control** key and select the individual suitable areas.

   - To add multiple adjacent areas, select **Selection Brush**, hold the **Control** key and swipe across the region of suitable areas.

Unselect the Bad Areas in the Generated Pattern

When most areas in the generated pattern are suitable for high quality data acquisition, it can be more efficient to remove the ones that are *not* suitable:

1. Select **Select All** to add all areas to the selected set.

2. Remove the bad areas from the selection:

   - To remove a single area from the selected set, right-click on an area and select **Remove** from the context menu.

   - To remove one or more separate areas from the selected set, hold the **Shift** key and select the individual bad areas.

   - To remove multiple adjacent areas, select **Selection Brush** and swipe across the region of bad areas.

Select the Bad Areas in the Generated Pattern, then Invert the Selection

When the bad areas in the generated pattern are easier to identify than the suitable areas:

1. Select **Unselect All** to clear the selected set.

2. Select the bad areas as if they were added to the selection:

   - To add a single area to the selected set, right-click on an area and select **Add** from the context menu.

   - To add one or more separate areas to the selected set, hold the **Control** key and select the individual areas.

   - To add multiple adjacent areas, select **Selection Brush** , hold the **Control** key and swipe across the region of bad areas.

3. Select **Invert**.

Add and Remove Acquisition Areas with the Selection Brush

The instruction in Add and Remove Foil Holes with the Selection Brush on page 156 are applicable for a Quantifoil specimen. These instructions are also applicable for the generated pattern of Acquisition Areas on a Lacey Carbon specimen.

**Create a Selection of Acquisition Areas Without Using a Generated Pattern**

When the generated pattern does not hit all the suitable areas in the Grid Square, it is possible to freely create Acquisition Areas at any location.

1. Select **Acquire** to start with a fresh Grid Square image.
   Do *not* select View Pattern.

2. Create Acquisition Areas.
   Either:

   - Right-click in the center of a suitable area, then select **Add** from the context menu.

   - Hold the **Control** key, then select the center of one or more suitable areas.

7.3.2.3 Inspect an Acquisition Area to Assess Suitability

To decide whether or not to include an Acquisition Area in the selection, a closer look may be needed to assess particle content, ice thickness, charging, etcetera.

1. In the Grid Square image:

   a. Right-click on the Acquisition Area you wish to inspect.

   b. Select **Move stage to location** or **Move stage to here**.
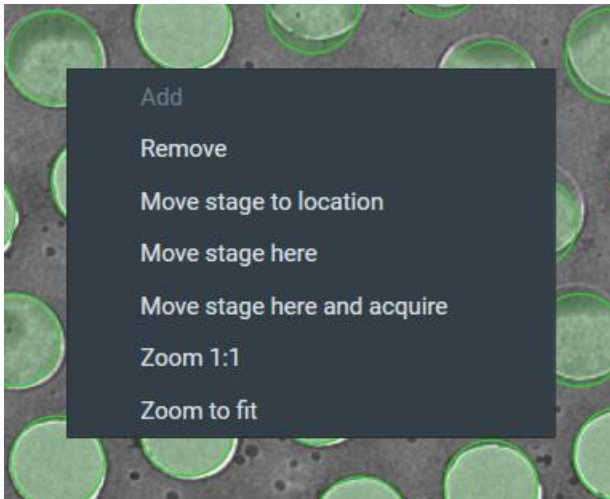
   - **Move stage to location**: centers the Acquisition Area in which the right mouse button was clicked.
     (*The image above is taken with a Quantifoil specimen, but the functionality is the same.*)

   - **Move stage here**: centers the exact position at which the right mouse button was clicked.

---

2. Select **TEM User Interface** > **CCD/TV Camera** control panel > **Acquire** to acquire a single image.
    To avoid unnecessary exposure:

    - Do not select Search.

    - Do not use the FluScreen to view the specimen.

3. If necessary, change magnification and/or illumination, and acquire a new image.

Note	Every exposure causes damage to the specimen. To maintain the highest quality, try to keep the accumulated dosage to a minimum.

7.3.3 Set the Autofocus and Drift Stabilization Values

On a Lacey Carbon specimen there is no repetitive pattern of identically shaped Foil Holes. Because of this, the Autofocus and Drift Measurement functions cannot be performed on predefined dedicated areas.

- Autofocus:
  After data has been acquired from an Acquisition Area, that Acquisition Area becomes the Autofocus Area for the next Acquisition Area.

- Drift Measurement:
  On Lacey Carbon specimens, the Drift Measurement function is not performed. Instead, a timed delay is used to wait for drift to decrease after a stage move. Use the **Auto Function** tab > **Drift stabilization** task to determine a realistic value for a short move. Refer to Run the Drift Stabilization Auto Function on page 88 for instructions.

Set the Autofocus and Drift Stabilization values as follows:

1. In the **Autofocus Area Settings** section of the Ribbon Bar:

    a. Select **Recurrence**:

        - **Always**: Autofocus is performed at every Foil Hole.

        - **Never**: Do not run the autofocus function at all.

        - **After distance**: only perform the autofocus function when the acquisition has moved on the specimen by more than a specified distance.

    b. If **Recurrence** is set to **After Distance**, specify **Distance**.

   c. Select **Focus using**:

- **Objective Lens**: EPU adjusts the focal plane to the Z-position of the specimen.

- **Stage Z axis**: EPU adjusts the Z-position of the specimen to the focal plane.

2. In the **Data Acquisition Settings** section of the Ribbon Bar,
specify the **Delay after Image Shift** and **Delay after Stage Shift**:

|	
Delay after Image Shift (s)	0.50
Delay after Stage Shift (s)	5.00
Data Acquisition Settings	
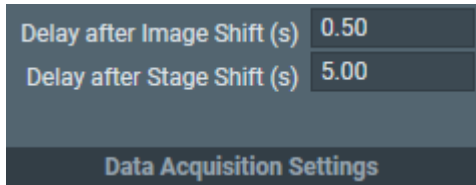
7.3.4 Modify the Defocus List for a Lacey Carbon Specimen

EPU acquires one image per Acquisition Area. By default, this image is acquired with zero defocus. The defocus value that is specified in the Data Acquisition Preset is not used during the Automated Acquisition run.

To acquire images with a non-zero defocus, specify one or more defocus values in the Defocus List. During the Automated Acquisition run, EPU cycles through the Defocus List to acquire images with different defocus values. On Lacey Carbon specimens, EPU cycles through a single Defocus List that is applicable for all Acquisition Areas.

Follow the instructions below to specify or modify the Defocus List:

1. Select the **Defocus list**

2. Use the **arrow keys** or the **mouse** to move the cursor to the desired location in the list.

3. Specify the desired **Defocus value(s)**

Defocus list (µm)
0.0, -1.0, -3.0
Data Acquisition Area
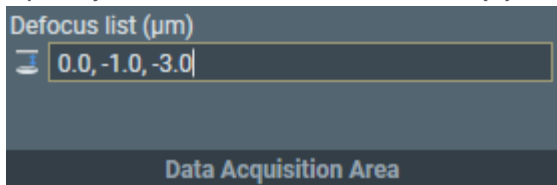
Use a **comma** or **space** to separate multiple values

4. Select **Enter** or click outside the list to store the updated Defocus list.
EPU validates the list. If invalid values are present, then EPU corrects or removes the invalid values.

It is not possible to modify the Defocus list while an Automated Acquisition run is ongoing or paused. To modify the Defocus list, the run must first be stopped.

7.4   The Hole Selection Task for Quantifoil Specimens

7.4.1   Define the Foil Hole Selection Filters for an Automated Selection Session

If the *Session Setup > Type* is *Automated*, then EPU uses the built-in filters and algorithms to select suitable Foil Holes. At the start of each Grid Square, EPU evaluates the position and condition of the Foil Holes, so that suitable Foil Holes are identified and selected just before data acquisition begins.

To configure the Hole Selection parameters and filter settings, follow the instructions below. This procedure must be executed for only one of the selected Grid Squares. During the Automated Acquisition run, EPU uses the same Hole Selection parameters and filter settings for all selected Grid Squares.

1. Acquire a representative Grid Square image:

    a. Select the **EPU** tab > **Square Selection** task.

    b. In the **Image Display**, identify a Grid Square that appears representative for the condition of the specimen.

   c. Right-click on the identified Grid Square and select **Select Holes**
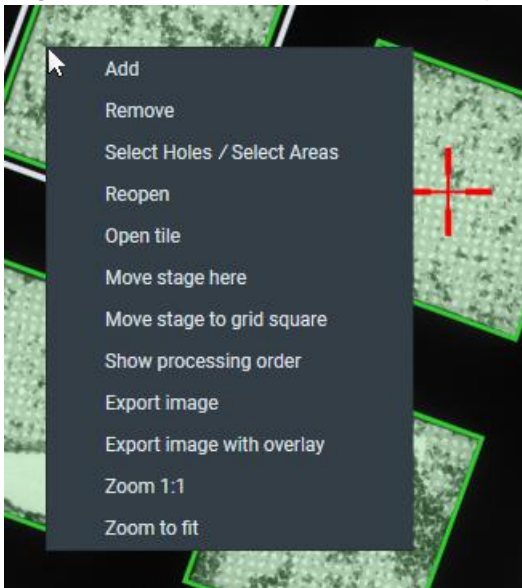
   EPU switches to the *Holes Selection* task for the selected Grid Square.
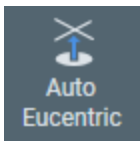
    d. Select **Auto Eucentric**

- If the specimen is at a different location, then EPU first moves the specimen to the center of the selected Grid Square.

- The Auto Eucentric function includes the acquisition of a new Grid Square image.

- While the Auto Eucentric function runs, the *Auto Eucentric* button changes to *Stop Auto Eucentric*. If selected, the Auto Eucentric function is aborted and the specimen returns to its previous Z position.

- If the specimen is already at eucentric height, or if there is a different reason to skip the Auto Eucentric Height function, then select **Acquire** to only acquire a Grid Square image.
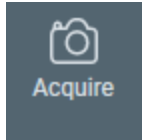

If an Atlas is loaded from the Screening task, then there is a chance that the orientation of the specimen does not match well enough with the orientation of the loaded Atlas. If the orientation of the grid, or the content of the imaged area does not match with the Atlas, then refer to Correct a Mismatch in the Orientations Between a Re-used Atlas and a Reloaded Specimen on page 185.
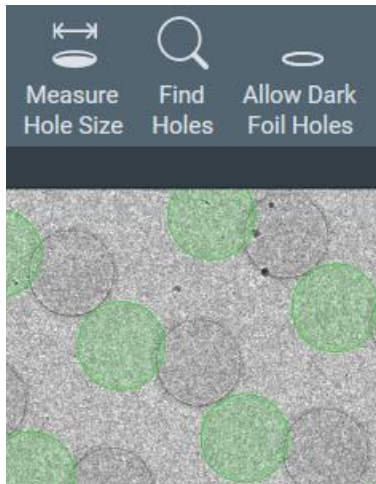
2. Specify the Foil Hole diameter and center-to-center interspacing:

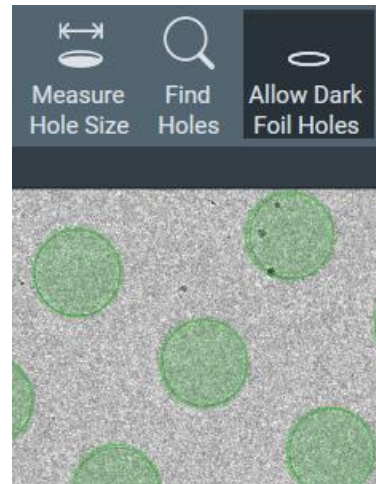   a. Select **Measure Hole Size**.

   A measurement tool appears in the Grid Square image.

---
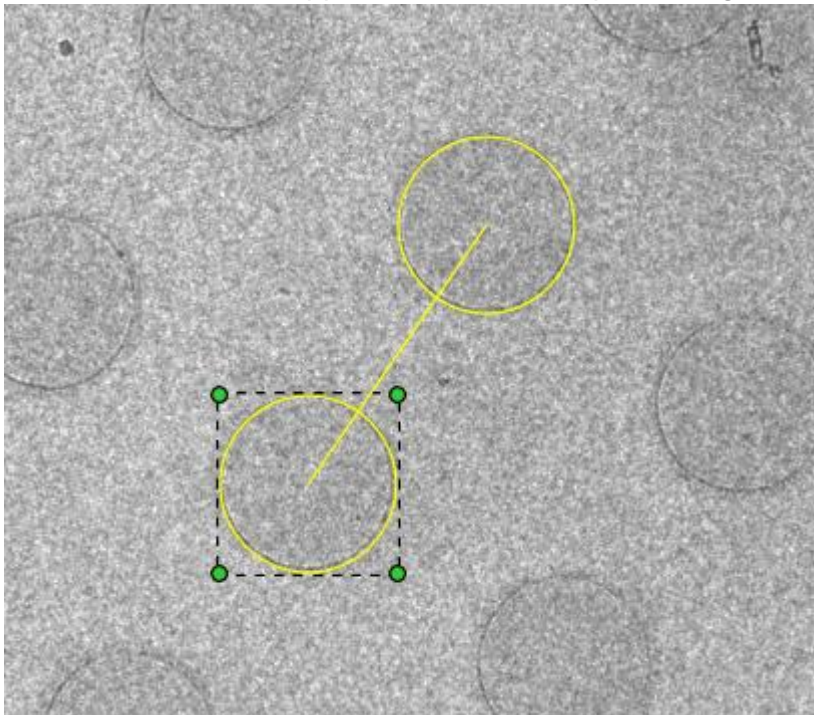
    b. Zoom in on the location of the measurement tool.

    c. Drag **one of the yellow circles** to the center of a Foil Hole.

    d. Drag **one of the green corner handles**, so that the circle diameter accurately matches with the diameter of the physical Foil Hole.

    e. Drag **the other yellow circle** to the center of a nearest neighboring Foil Hole define the interspacing.

3. Identify and select all Foil Holes in the Grid Square:

    a. If the Foil Holes appear darker than the surrounding carbon foil, then first select **Allow Dark Foil Holes**.
By default, the Hole Detection algorithm searches for bright circles in a darker environment. If the *Allow Dark Foil Holes* toggle in active, then the algorithm searches for dark circles in a brighter environment.

Allow Dark Foil Holes is not active:
EPU does not recognize the real Foil Holes.

Allow Dark Foil Holes is active:
EPU finds the real Foil Holes.

    b. Select **Find Holes**.

The Hole Detection algorithm searches for Foil Holes that match with the specified diameter and interspacing, and generates a pattern of Foil Hole outlines in the Grid Square image.

    c. Verify that the generated pattern matches with the physical Foil Holes in the Grid Square image.
If necessary, adjust the diameter and interspacing and select **Find Holes** again.

4. Set or adjust the Ice Quality filter:

a. Select **Preset Ice Filter** to reset the Ice Quality filter boundaries.

b. In the **Filter Ice Quality** histogram:

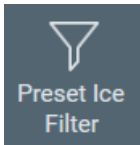*   Drag the red lower boundary marker to exclude Foil Holes that are too dark.
*   Drag the red upper boundary marker to exclude Foil Holes that are too bright.

After every change, the Ice Quality filter updates the target Foil Holes selection immediately. Depending on the number of Foil Holes in the Grid Square image, this may take some time.

5. Select **Remove Holes Close To Grid Bar** to exclude Foil Holes near the edge of the Grid Square.

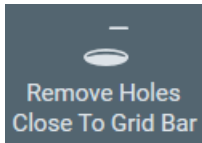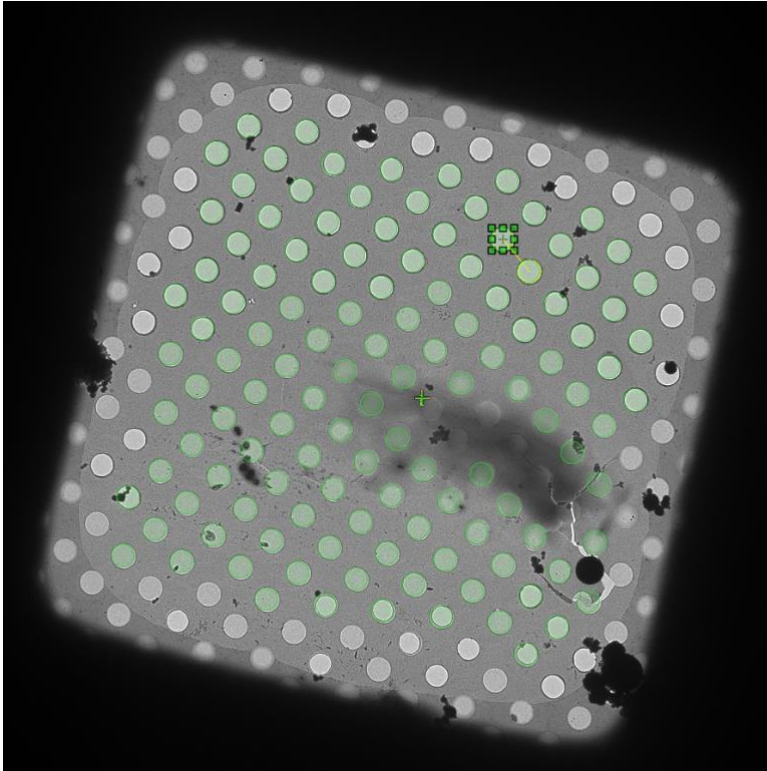Foil Holes that are near the Grid Bars are excluded from the selection.

If the result of the *Remove Holes Close to Grid Bar* filter is not satisfactory, then select the filter button again to de-activate it. This will automatically run the Find Holes function and apply the Ice Quality filter.

7.4.2 Handpick the Foil Holes for a Manual Selection Session

If *Session Setup* > *Type* is *Manual*, then each selected Grid Square must be visited to handpick all target Foil Holes.

For each target Foil Hole, the X, Y and Z position of the Foil Hole center is stored. During the Automated Acquisition Run:

- EPU performs the *Hole Position Refinement* function. This function acquires a new *just-in-time* Grid Square image and updates the stored coordinates for each target Foil Hole, so that the Foil Holes can be centered with better accuracy for execution of the Foil Hole Template.

- EPU does not re-evaluate the suitability of the selected Foil Holes.

After the suitable Grid Squares are selected in the *Square Selection* task, the procedure to handpick the target Foil Holes is as follows:

1. In the **Square Selection** task:
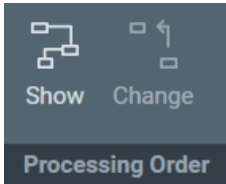
   a. Select **Processing Order** > **Show**

   b. If desired, change the Grid Square processing order.
      For instructions, refer to Change the Grid Square Processing Order on page .

   c. Right-click on **Grid Square 1** and select **Select Holes**
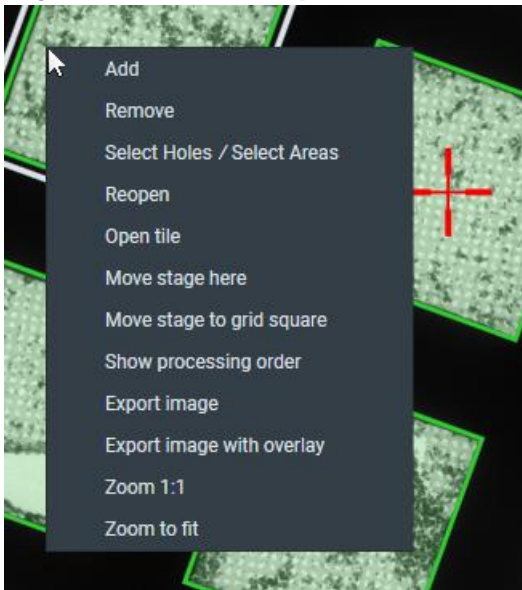
   EPU switches to the *Hole Selection* task for the selected Grid Square. The lower-left corner of the Hole Selection Image Display displays the processing order number of the Grid Square and Z-position of the selected Grid Square.

   d. Select **Auto Eucentric**

- If the specimen is at a different location, then EPU first moves the specimen to the center of the selected Grid Square.

- The Auto Eucentric function includes the acquisition of a new Grid Square image.

- While the Auto Eucentric function runs, the *Auto Eucentric* button changes to *Stop Auto Eucentric*. If selected, the Auto Eucentric function is aborted and the specimen returns to its previous Z position.

- If the specimen is already at eucentric height, or if there is a different reason to skip the Auto Eucentric Height function, then select **Acquire** to only acquire a new Grid Square image.

If an Atlas is loaded from the Screening task, then there is a chance that the orientation of the specimen does not match well enough with the orientation of the loaded Atlas. If the orientation of the grid, or the content of the imaged area does not match with the Atlas, then refer to Correct a Mismatch in the Orientations Between a Re-used Atlas and a Reloaded Specimen on page .
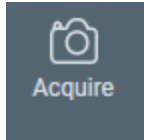
e. Generate the Foil Hole pattern.
   For instructions, refer to Create the Initial Foil Hole Selection for a Manual Selection Session on page .

f. Customize the selection of target Foil Holes.
   For instructions, refer to Customize the Selection of Foil Holes on page .

g. Select **Navigate** > **Next Square** to move to the next Grid Square.

   In the Square Selection image display, the white border moves to the next Grid Square. The specimen does not move until *Auto Eucentric* or *Acquire* is selected.

h. Repeat steps *a – e* above until all Grid Squares are prepared.

2. If desired, inspect or adjust the target Foil Hole selection for a prepared Grid Square:

   a. Navigate to the desired Grid Square.
      Either:

      - Select **Navigate** > **Previous Square** until the desired Grid Square is reached.
        In the Square Selection image display, the white border moves to the previous Grid Square. The specimen does not move until *Auto Eucentric* or *Acquire* is selected.

      - Select the **Square Selection** task
        > right-click on the desired **Grid Square**
        > select **Select Holes**

      EPU displays the Grid Square image for the selected Grid Square.

Note	Unless the current selection of target Foil Holes must be cleared:
	• Do *not* select *Acquire*
	• Do *not* select *Find Holes*
	• If *Remove Holes Close to Grid Bar* is active,
	then do *not* select *Remove Holes Close to Grid Bar*

   b. If a new Grid Square image is acquired, then create a new initial Foil Hole selection. For instructions, refer to Create the Initial Foil Hole Selection for a Manual Selection Session on page .

   c. Customize the selection of target Foil Holes.
      For instructions, refer to Customize the Selection of Foil Holes on page .

7.4.2.1 Create the Initial Foil Hole Selection for a Manual Selection Session

In a Manual Selection Session, the actions below must be repeated for each selected Grid Square.

1. Specify the Foil Hole diameter and center-to-center interspacing:

   a. Select **Measure Hole Size**.

A measurement tool appears in the Grid Square image.
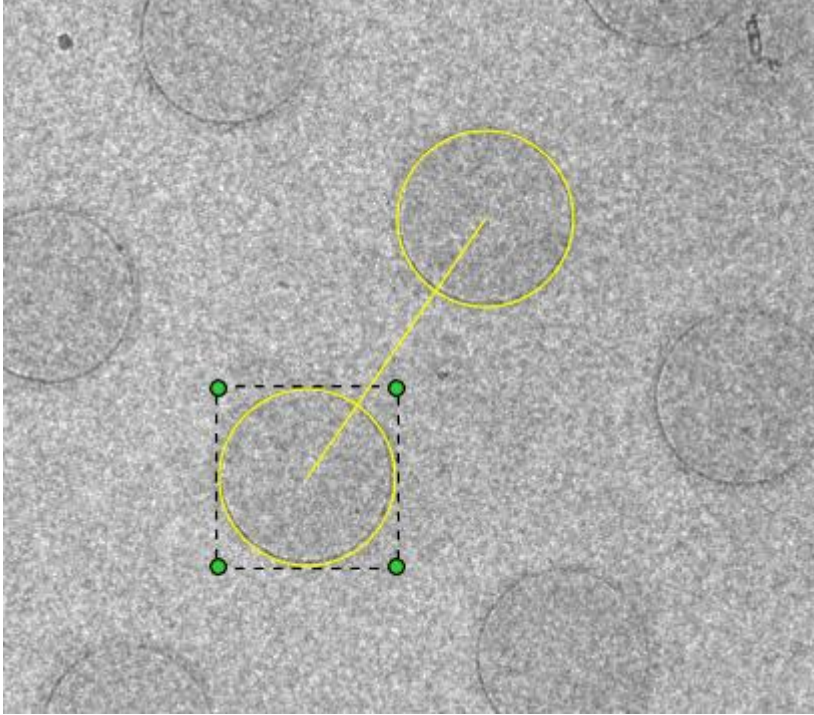
b. Zoom in on the location of the measurement tool.

c. Drag **one of the yellow circles** to the center of a Foil Hole.

d. Drag **one of the green corner handles**, so that the circle diameter accurately matches with the diameter of the physical Foil Hole.

e. Drag **the other yellow circle** to the center of a nearest neighboring Foil Hole define the interspacing.

2. Identify and select all Foil Holes in the Grid Square:

   a. If the Foil Holes appear darker than the surrounding carbon foil, then first select **Allow Dark Foil Holes**.
   By default, the Hole Detection algorithm searches for bright circles in a darker environment. If the *Allow Dark Foil Holes* toggle in active, then the algorithm searches for dark circles in a brighter environment.

Allow Dark Foil Holes is not active:
EPU does not recognize the real Foil Holes.

Allow Dark Foil Holes is active:
EPU finds the real Foil Holes.

   b. Select **Find Holes**.

   The Hole Detection algorithm searches for Foil Holes that match with the specified diameter and interspacing, and generates a pattern of Foil Hole outlines in the Grid Square image.

   c. Verify that the generated pattern matches with the physical Foil Holes in the Grid Square image.
   If necessary, adjust the diameter and interspacing and select **Find Holes** again.

3. Set or adjust the Ice Quality filter:

    a. Select **Preset Ice Filter** to reset the Ice Quality filter boundaries.

    b. In the **Filter Ice Quality** histogram:

    - Drag the red lower boundary marker to exclude Foil Holes that are too dark.

    - Drag the red upper boundary marker to exclude Foil Holes that are too bright.

    After every change, the Ice Quality filter updates the target Foil Holes selection immediately. Depending on the number of Foil Holes in the Grid Square image, this may take some time.

4. Select **Remove Holes Close To Grid Bar** to exclude Foil Holes near the edge of the Grid Square.
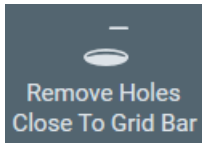
Foil Holes that are near the Grid Bars are excluded from the selection.

If the result of the *Remove Holes Close to Grid Bar* filter is not satisfactory, then select the filter button again to de-activate it. This will automatically run the Find Holes function and apply the Ice Quality filter.

Note	When the Find Holes function is performed, all Foil Holes that match with the Ice Quality Filter are automatically included in the target Foil Hole selection. Any previous customization of the target Foil Hole selection is cleared.

If desired, customize the initial selection of target Foil Holes as described in Customize the Selection of Foil Holes on page 154.

7.4.2.2 Customize the Selection of Foil Holes

Before starting the manual selection, check if the manual selection tools are available in the Ribbon Bar.

If not, set the **EPU** tab > **Session Setup** task > **General session setting** > **Type** to **Manual**.

Note	The following steps must be repeated for *each* individual selected GridSquare.

1. Perform all the steps that are described in Define the Acquisition Area Pattern and Filter Settings for an Automated Selection Session on page 128.

Note	Make sure the specimen is accurately set to eucentric height.

The positions of the selected areas are stored as (X,Y,Z) coordinates. If the specimen is not at eucentric height during manual selection, the X and Y coordinates of the selected areas may have an offset. This may cause failure of the Autofocus and/or Eucentric Correction functions during the Automated Acquisition run, which may negatively impact the amount and quality of the acquired data.

2. Create a selection of Foil Holes.
    Either:

    - Select the suitable Foil Holes in the generated pattern.

    - Unselect the bad Foil Holes in the generated pattern.

    - Select the bad Foil Holes in the generated pattern, then invert the selection.

    - Create a selection of suitable Foil Holes without using a generated pattern.

    Instructions for each of these selection procedures are described below.

If the Ice Quality Filter boundaries are changed after a manual selection has been made, then the selected set is not updated. Selected areas that fall outside the new boundaries will not be removed from the selection. Likewise, areas that are not part of the selection will not be added to selection if their average gray scale value now falls inside the new boundaries.

Select the Suitable Foil Holes in the Generated Pattern

When the number of bad Foil Holes is larger than the number of suitable Foil Holes:

1. Select **Unselect All** to clear the selected set.

2. Add the suitable Foil Holes to the selection:

   - To add a single Foil Hole to the selected set, right-click on the Foil Hole and select **Add** from the context menu.

   - To add one or more separate Foil Holes to the selected set, hold the **Control** key and select the individual suitable Foil Holes.

   - To add multiple adjacent Foil Holes, select **Selection Brush**, hold the **Control** key and swipe across the region of suitable Foil Holes.

Unselect the Bad Foil Holes in the Generated Pattern

When most Foil Holes in the generated pattern are suitable for high quality data acquisition, it can be more efficient to remove the ones that are *not* suitable:

1. Select **Select All** to add all Foil Holes to the selected set.

2. Remove the bad Foil Holes from the selection:

   - To remove a single Foil Hole from the selected set, right-click on the Foil Hole and select **Remove** from the context menu.

   - To remove one or more separate Foil Holes from the selected set, hold the **Shift** key and select the individual bad Foil Holes.

   - To remove multiple adjacent Foil Holes, select **Selection Brush** and swipe across the region of bad Foil Holes.

Select the Bad Foil Holes in the Generated Pattern, then Invert the Selection

When the bad Foil Holes in the generated pattern are easier to identify than the suitable Foil Holes:

1. Select **Unselect All** to clear the selected set.

2. Select the bad Foil Holes as if they were added to the selection:

   - To add a single Foil Hole to the selected set, right-click on an Foil Hole and select **Add** from the context menu.

   - To add one or more separate Foil Holes to the selected set, hold the **Control** key and select the individual Foil Holes.

   - To add multiple adjacent Foil Holes, select **Selection Brush** , hold the **Control** key and swipe across the region of bad foil holeFoil Holes.

3. Select **Invert**.

Add and Remove Foil Holes with the Selection Brush

1. From the **Selection** section of the the Ribbon Bar, select **Selection Brush**.
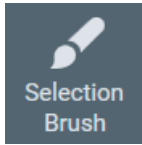
2. Move the mouse to the Image Display.
   The Selection Brush is displayed as a dark yellow circle.

3. If desired, change the **Selection Brush size**:
   Hold down the **Shift** key and scroll with the **mouse wheel**

4. To remove Foil Holes from the selection:

   a. Hold down the **left mouse button**

   b. Drag the **Selection Brush** across the image.
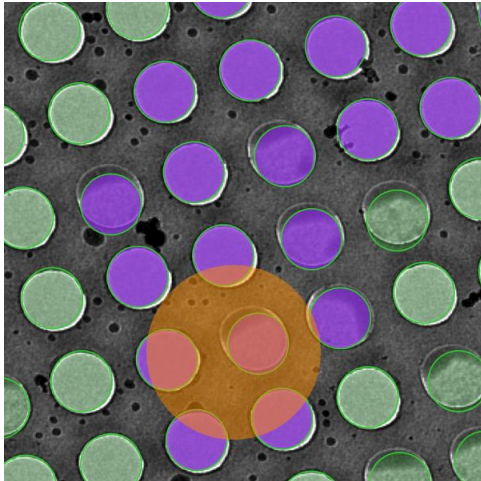
   Foil Holes that are touched by the brush have a purple highlight.

   c. Release the **left mouse button**
   The purple Foil Holes are removed from the selection.

5. To add Foil Holes to the selection:

   a. Hold down the **Control** key.

   b. Hold down the **left mouse button**

   c. Drag the **Selection Brush** across the image.

   d. Release the **left mouse button**
   The purple Foil Holes are added to the selection.

Create a Selection of Foil Holes Without Using a Generated Pattern

When it not possible to let the Hole Detection algorithm generate a pattern that matches the specimen, it is possible to freely create Foil Hole locations at any position on the specimen.

1. Select **Acquire** to start with a fresh Grid Square image.
   Do *not* select Find Holes.

2. Create Foil Holes.
   Either:

   - Right-click in the center of a Foil Hole, then select **Add** from the context menu.

   - Hold the **Control** key, then select the center of one or more suitable Foil Holes.

7.4.2.3 Inspect a Foil Hole to Assess Suitability

To decide whether or not to include a Foil Hole in the selection, a closer look may be needed to assess particle content, ice thickness, charging, etcetera.

1. In the Grid Square image:

   a. Right-click on the Foil Hole you wish to inspect.

   b. Select one of the **Move stage...** options
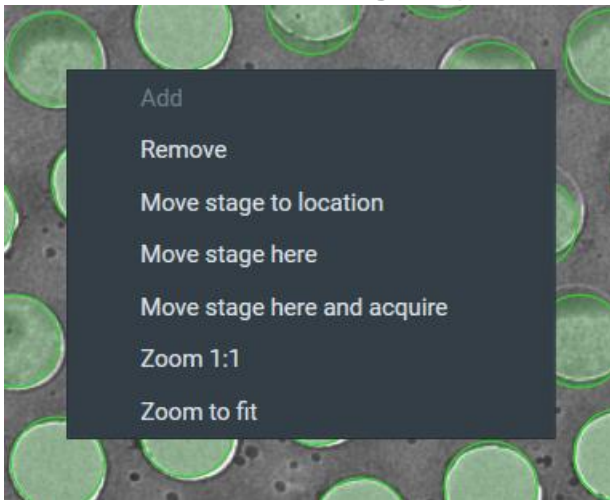
   - **Move stage to location**: centers the Foil Hole in which the right mouse button was clicked.

   - **Move stage here**: centers the exact position at which the right mouse button was clicked.

   - **Move stage here and acquire**: centers the exact position at which the right mouse button was clicked, and acquires a new Grid Square image.
     To prevent clearing the current selection, this option is only available when no Foil Holes have been selected.

2. Select **TEM User Interface** > **CCD/TV Camera** control panel > **Acquire** to acquire a single image.
   To avoid unnecessary exposure:

   - Do not select Search.

   - Do not use the FluScreen to view the specimen.

3. If necessary, change magnification and/or illumination, and acquire a new image.

Note	Every exposure causes damage to the specimen. To maintain the highest quality, try to keep the accumulated dosage to a minimum.

In an *EPU > Session Setup > Type*: *Manual* session, the *Hole Selection > Find Holes* function identifies Foil Holes in a Grid Square image, and stores the XYZ coordinates of each Foil Hole center. When the **Move stage to location** function is used during preparation for the Automated Acquisition run, the red crosshair in the Image Display will end up in the center of the Foil Hole.
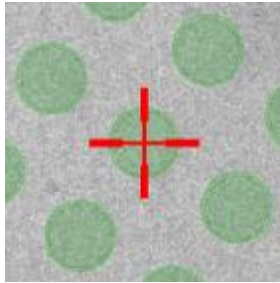
Between the acquisition of a Grid Square image in the Hole Selection task and the time at which the Automated Acquisition run reaches that same Grid Square, a small positioning offset may have accumulated. To prevent skipped Foil Holes due to such offsets, the Automated Acquisition procedure features an automatic **Hole Position Refinement** function. This function acquires a new *just-in-time* Grid Square image and updates the stored coordinates for each Foil Hole, so that the Foil Holes can be centered with better accuracy for execution of the Foil Hole Template.

During the Automated Acquisition run it is possible to fine-tune the Foil Hole Template. To do so, return to the **Hole Selection** task, use the **Move stage to location** function to center a Foil Hole, and then select the *Template Definition* task to revise the Foil Hole Template.
In the Hole Selection task, the image display uses the initial Grid Square image, but the *Move stage to location* function uses the updated Foil Hole coordinates. As a result, the crosshair in the image display may end up at a small offset from the uncorrected Foil Hole center in the Grid Square image.

7.5 The Template Definition Task for Quantifoil Specimens

The purpose of the Template Definition task is to assign the areas in and around a Foil Hole where the Autofocus function, the (optional) Drift Measurement function, and one or more Data Acquisitions are executed. The positions of the assigned areas are relative to the Foil Hole center, so the specified area positions can be applied to every Foil Hole during the Automated Acquisition run.

7.5.1 Define the Foil Hole Template

To set up a Foil Hole Template, follow the procedure below:

1. Acquire a Foil Hole image:

    a. Select the **EPU** tab > **Hole Selection** task.

    b. Select **Auto Eucentric**.
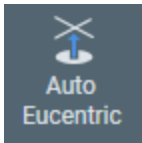
    c. Select **Acquire** to acquire a Grid Square image.
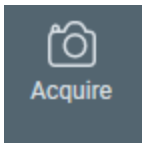
    d. In the Grid Square image, right-click on a suitable Foil Hole and select **Move stage to location**.
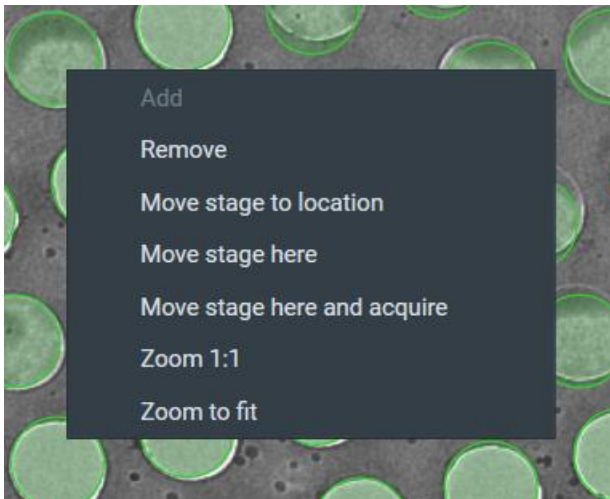
e. Select the **EPU** tab > **Template Definition** task.
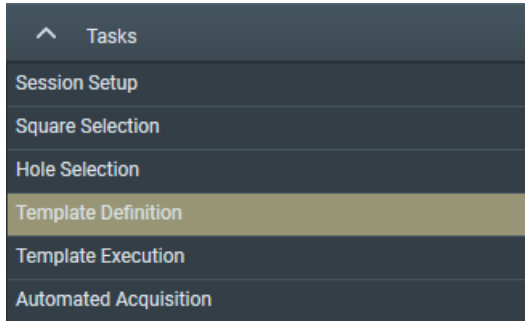
f. Select **Acquire** to acquire a Hole/EucentricHeight image.

g. Select **Find And Center Hole**.
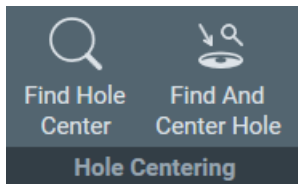
2. Use the **Template Definition** functions to create a new Foil Hole Template.
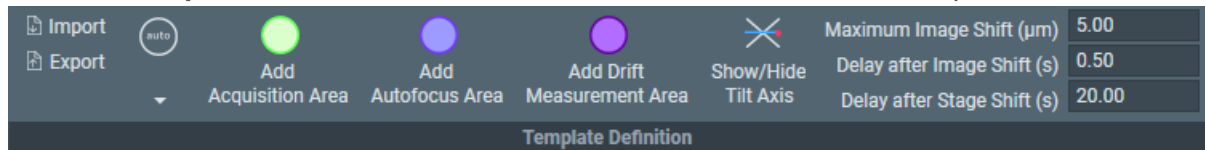
a. Create one or more Acquisition Areas as described in
    Manually Create One or More Acquisition Areas on page 161.

b. Create an Autofocus Area as described in
    Create an Autofocus Area on page 164.

c. Set the shift and delay parameters as described in
    Specify the Maximum Image Shift and Delay Timers on page 170.

d. Optionally, create a Drift Measurement Area as described in
    Create a Drift Measurement Area on page 166.

When an Acquisition, Autofocus or Drift Measurement Area is selected in the image display, additional Ribbon Bar sections appear to specify the values for the selected area. The Template Definition section may change to a more compact layout to make room for the additional Ribbon Bar sections.

Instead of creating a new Foil Hole Template template, it is also possible to import the Template Areas and their settings from an earlier session. Refer to Import and Export a Foil Hole Template on page 171.

7.5.1.1 Create One or More Acquisition Areas

Acquisition Areas can be placed manually at specifically selected locations. It is also possible to let EPU generate a pattern of Acquisition Areas inside the Foil Hole perimeter.

Manually Create One or More Acquisition Areas

1. Select **Template Definition** > **Add Acquisition Area**

2. In the image display, select the **location for the first Acquisition Area**.
    A green circle appears in the Foil Hole Template with its center at the selected position.
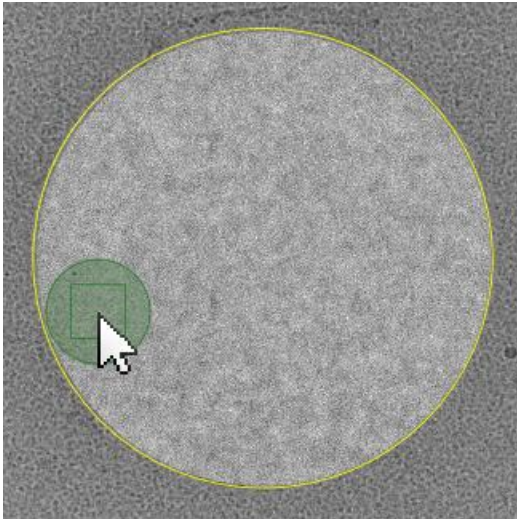
   - On microscopes with a Three Lens Condenser system, the size of the Acquisition Area is defined by the Illuminated Area which is specified in the Data Acquisition Preset.

   - On microscopes with a Two Lens Condenser system, the Acquisition Area is defined as the smallest circle that fully covers the camera field of view at the Data Acquisition Preset magnification.

3. In the image display, select the **Acquisition Area**.
    The Data Acquisition Area section appears in the Ribbon Bar.
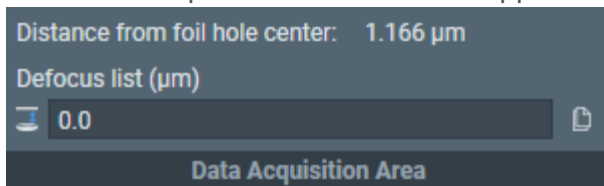
4. By default, the image acquisition is executed with zero Defocus. The Defocus value that is specified in the Data Acquisition Preset is ignored.
    To apply a non-zero Defocus value, define a **Defocus List** as explained in Modify the Defocus List on page 167.

5. If desired, add more Acquisition Areas in the Foil Hole Template:

   a. Optionally select the first Acquisition Area.
      If the first Acquisition Area is selected while adding new Acquisition Area(s), then the Defocus List of the selected Acquisition Area will be copied to the new Acquisition Area(s).

      • The Defocus List of the selected Acquisition Area is not *shared* with the new Acquisition Area(s). Each Acquisition Area has its own copy.
        Modifications to the Defocus List of one Acquisition Area are not applied to other Acquisition Areas.

      • It is not possible to copy the Defocus List from one Acquisition Area to another Acquisition Area.

   b. Select **Add Acquisition Area** and place a new Acquisition Area.

6. If desired:

   • Adjust the **location** of a individual Acquisition Area by dragging it with the mouse.

   • Modify the **Defocus List** for one or more individual Acquisition Areas.

Automatically Generate a Pattern of Acquisition Areas

To save time, EPU can automatically generate a pattern of Acquisition Areas in the Foil Hole Template.
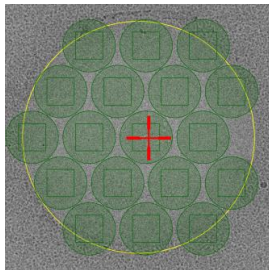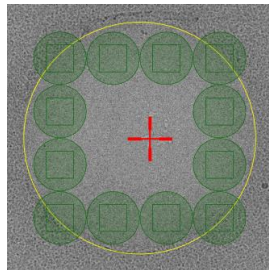
Hexagonal pattern   *Rectangular pattern*   *Circular pattern*   *M X N Matrix*
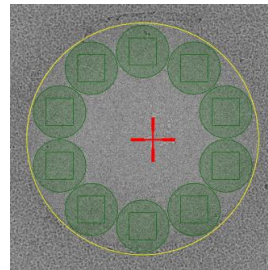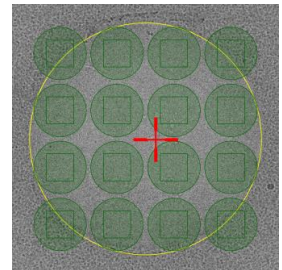
1. If desired, create a Defocus List for the generated Acquisition Areas:

   a. Select **Template Definition** > **Add Acquisition Area**

   b. In the image display, select a location *outside* the Foil Hole.
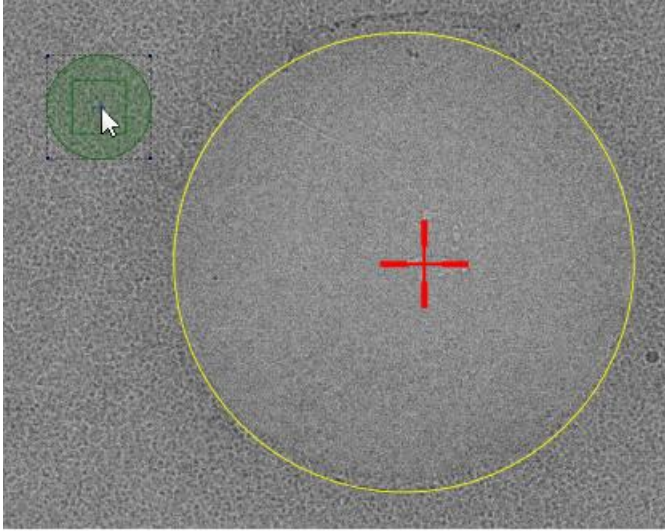
   This Acquisition Area will *only* be used to define the Defocus List. It will be removed again after a pattern of Acquisition Areas has been created.

   c. In the image display, select the Acquisition Area.
      The Data Acquisition Area section appears in the Ribbon Bar.
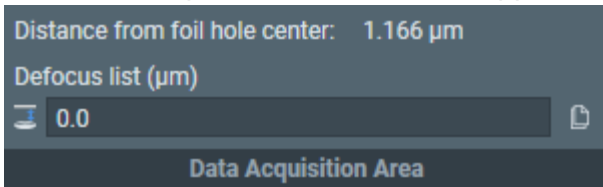
   d. Define a **Defocus List** as explained in Modify the Defocus List on page 167.

2. Select Auto, and select the desired pattern:
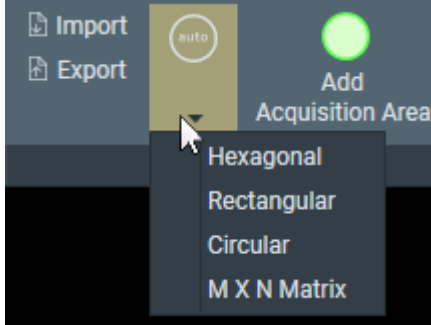
EPU adds as many Acquisition Areas as can be fit in the Foil Hole, arranged according to the selected pattern.

3. Right-click on the **initial Acquisition Area** that is placed outside the Foil Hole, and select **Remove**
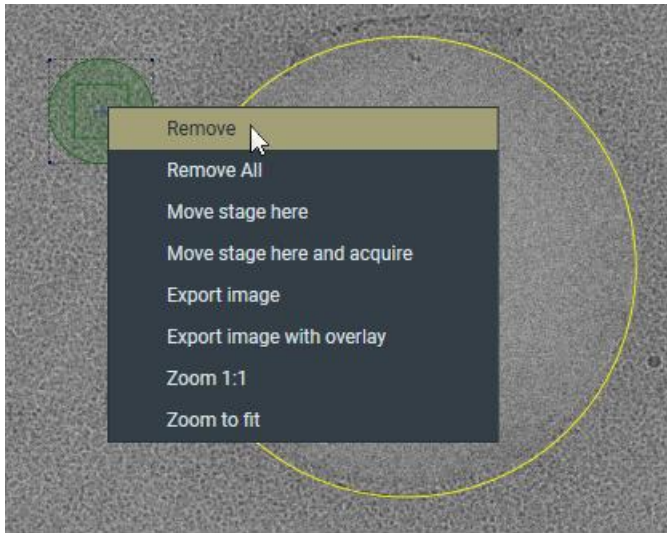
7.5.1.2 Create an Autofocus Area

A Foil Hole template contains one Autofocus Area. The Autofocus Area is best placed outside the Foil Hole perimeter, on the carbon foil. The Autofocus function works better on an area of only carbon foil, and no valuable specimen material is exposed while performing the Autofocus function.

1. Add an Autofocus Area to the Foil template:

    a. Select **Template Definition** > **Add Autofocus Area**

    b. In the image display, select the location for the Autofocus Area.
    A blue circle is placed in the Foil Hole Template with its center at the selected position.
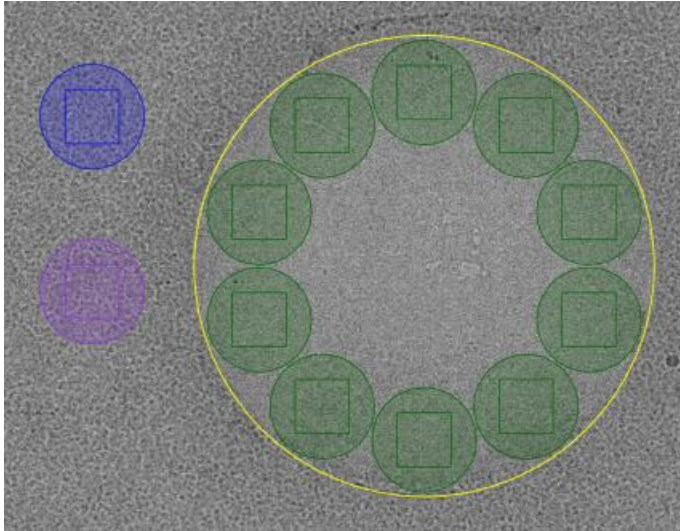
   - On microscopes with a Three Lens Condenser system, the size of the Autofocus Area is defined by the Illuminated Area which is specified in the Autofocus Preset.

   - On microscopes with a Two Lens Condenser system, the Autofocus Area is defined as the smallest circle that fully covers the camera field of view at the Autofocus Preset magnification.

2. In the image display, select the **Autofocus Area**

3. In the **Autofocus Area Settings** section of the Ribbon Bar:
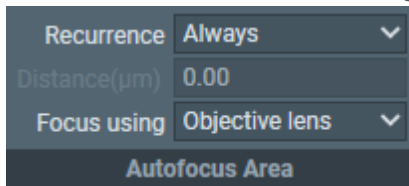
    a. Select **Recurrence**:

      - **Always**: Autofocus is performed at every Foil Hole.

      - **Never**: Do not run the autofocus function at all.

      - **After distance**: only perform the autofocus function when the acquisition has moved on the specimen by more than a specified distance.

    b. If **Recurrence** is set to **After Distance**, specify **Distance**.

    c. Select **Focus using**:

      - **Objective Lens**: EPU adjusts the focal plane to the Z-position of the specimen.

      - **Stage Z axis**: EPU adjusts the Z-position of the specimen to the focal plane.

7.5.1.3 Create a Drift Measurement Area

The Foil Hole Template contains at most one Drift Measurement Area. If a Drift Measurement Area is present, then EPU executes the Drift stabilization function instead of counting down the Delay after Stage Shift timer. The Drift stabilization function pauses the Automated Acquisition run as long as the actual drift speed is higher than a specified threshold value. This threshold value is specified in **Auto Functions** tab > **Auto Functions (TEM): Drift stabilization** task > **Max. Remaining Drift**

1. Add an Drift Measurement Area to the Foil Hole Template:

    a. Select **Template Definition** > **Add Drift Measurement Area**

    b. In the image display, select the location for the Drift Measurement Area.
        A purple circle is placed in the Foil Hole Template with its center at the selected position.
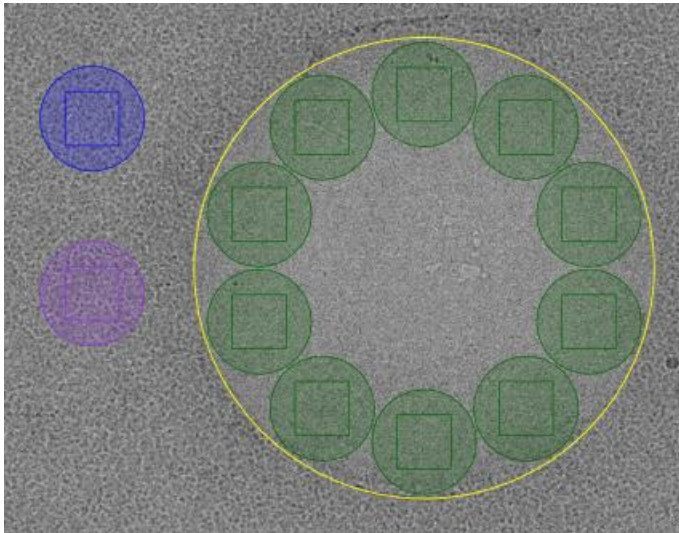
The Drift Measurement Area is best placed outside the Foil Hole perimeter, on the carbon foil. This way no valuable specimen material is exposed.

- On microscopes with a Three Lens Condenser system, the size of the Drift Measurement Area is defined by the Illuminated Area which is specified in the Drift Measurement Preset.

- On microscopes with a Two Lens Condenser system, the Drift Measurement Area is defined as the smallest circle that fully covers the camera field of view at the Drift Measurement Preset magnification.

2. In the image display, select the **Drift Measurement Area**.

3. In the **Drift Measurement** section of the Ribbon bar:
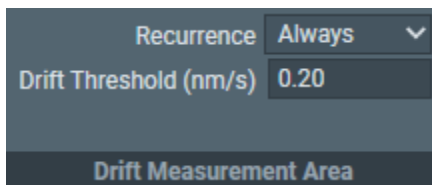
a. Specify the **Threshold**.
This is the maximum allowed drift speed for data acquisition.
If the specified threshold is not achieved in 600 seconds or less, the Foil Hole will be skipped and the Automated Acquisition run will move on to the next Foil Hole.

b. Specify the **Recurrence**.

- **Always**: the Drift Measurement function is executed after every CompuStage move. In practice this means every time the Automated Acquisition run moves to the next Foil Hole.

- **Once per GridSquare**: the Drift Measurement function is executed only after moving to another Grid Square.
For long moves like this, the actual drift speed can vary more than for the relatively short moves between Foil Holes within the same Grid Square. For the short hole-to-hole movements, the Delay after Stage Shift timer is used. If the **Maximum Image Shift** is set smaller than the distance between the Acquisition Areas, then the **Once per GridSquare** option is the only valid choice.

7.5.2 Modify the Defocus List

EPU acquires one image per Acquisition Area. By default, this image is acquired with zero defocus. The defocus value that is specified in the Data Acquisition Preset is not used during the Automated Acquisition run.

To acquire images with a non-zero defocus, specify one or more defocus values in the Defocus List. During the Automated Acquisition run, EPU cycles through the Defocus List to acquire images with different defocus values. On Quantifoil specimens, the Foil Hole Template can contain multiple Acquisition Areas. Each Acquisition Area has its own Defocus List.

The example below describes a Foil Hole Template with two Acquisition Areas:

- Acquisition Area *A* has a Defocus List with three values.
- Acquisition Area *B* has a Defocus List with two values.

As the Foil Holes are processed one after the other, EPU steps through the Defocus Lists for each of the Acquisition Areas.
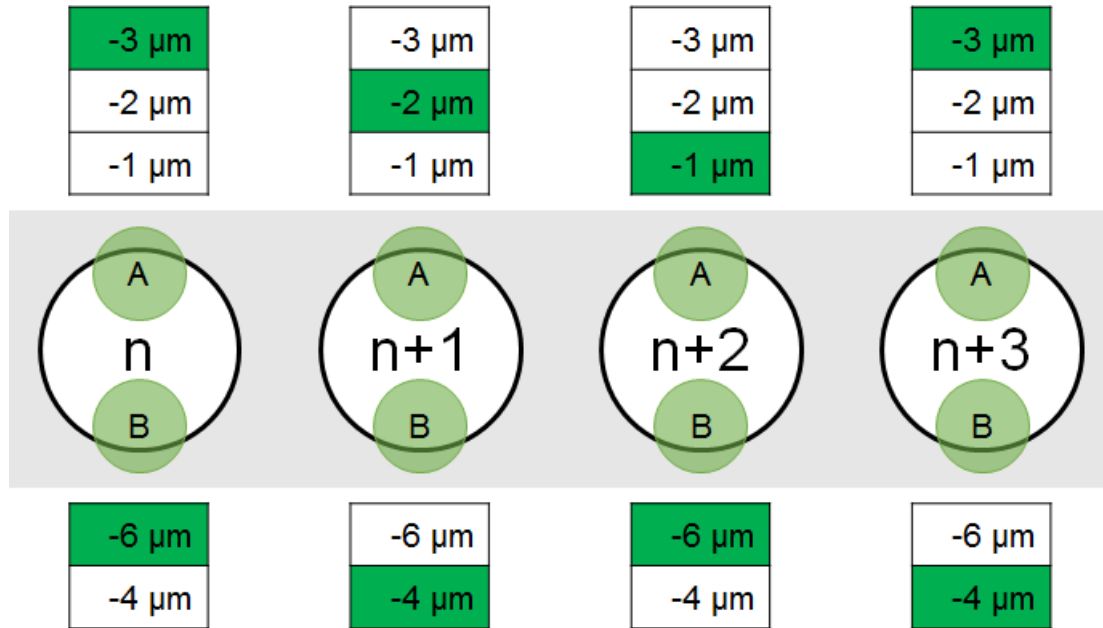
If a Foil Hole is skipped by EPU or by the user, then the defocus values that were planned for the acquisitions on that Foil Hole are also skipped.
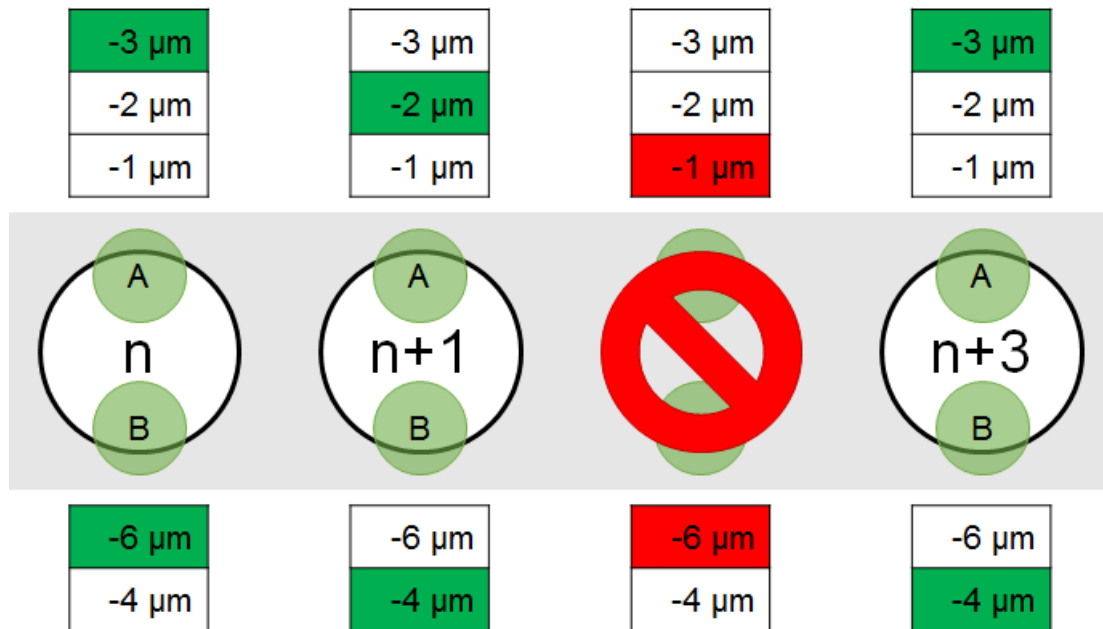
To add or remove Defocus values for the Acquisition Area, follow the instructions below:

1. In the image display, select an **Acquisition Area**
   The Data Acquisition Area section appears in the Ribbon Bar.

   Distance from foil hole center: 1.166 µm
   Defocus list (µm)
   0.0
   Data Acquisition Area
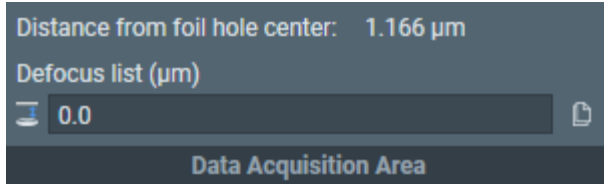
2. Select the **Defocus list**

3. Use the **arrow keys** or the **mouse** to move the cursor to the desired location in the list.

4. Add, modify, or remove values from the list.

   Distance from foil hole center: 1.166 µm
   Defocus list (µm)
   0.0, -2.0, -3.0
   Data Acquisition Area
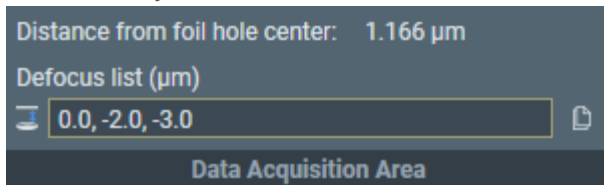
   Use a **comma** or **space** to separate multiple values.

5. Select **Enter** or click outside the list to store the updated Defocus list.
   EPU validates the list. If invalid values are present, then EPU corrects or removes the invalid values.

6. If desired, select **Copy defocus list to all areas** to apply the updated defocus to all Acquisition Areas in the Template.

   Distance from foil hole center: 1.166 µm
   Defocus list (µm)
   0.0, -2.0, -3.0
   Data Acquisition Area
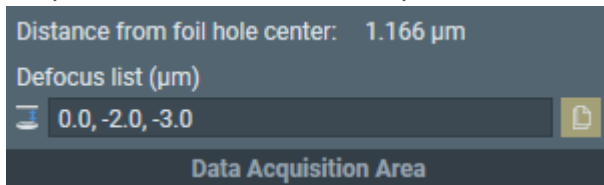
   After the Defocus is applied to all Acquisition Areas, it is still possible to modify the Defocus list for an individual Acquisition Area.

It is not possible to modify the Defocus list while an Automated Acquisition run is ongoing or paused. To modify the Defocus list, the run must first be stopped.

7.5.3 Specify the Maximum Image Shift and Delay Timers

The use of Image Shift to navigate between Template Areas is trade-off between accuracy and throughput. Image Shift is typically faster than moving the specimen with the stage, but it also induces coma and astigmatism. The required resolution for the 3D reconstruction puts a restriction on the maximum acceptable coma.

The Maximum Image Shift depends on:

- The presence and calibration status of Aberration Free Image Shift (AFIS).
  AFIS drastically reduces coma at large Image Shift values.

- The presence of an Image Corrector.
  An Image Corrector significantly improves image resolution near the optical axis, but it also limits the available Image Shift range.

- The use of Phase Plates.
  With Phase Plates, Image Shift must be limited so the beam does not shift outside the activated Phase Plate area.

- The Session Setup > Acquisition mode.
  When **Session Setup** > **Acquisition Mode** is **Faster acquisition** and the conditions for Foil Hole navigation by Image Shift are met, then EPU locks the Maximum Image Shift value at a predetermined value, and disables the Maximum Image Shift input field.

Follow the steps below to specify the Image Shift and delay values.

1. If not disabled, specify the **Maximum Image Shift** distance.
   The *Maximum Image Shift* distance determines if a Template Area can be visited by applying an Image Shift, or if it is necessary to move the stage.

2. Specify the **Delay after Image Shift** time.
   The *Delay after Image Shift* timer postpones image acquisition until the image shift deflectors have settled at their new values.

3. Specify the **Delay after Stage Shift** time.
   The *Delay after Image Shift* timer postpones image acquisition until:

   - The stage mechanics have relaxed and are settled at their new positions.

   - Thermal expansion due to heat from the motors has stabilized or disappeared.

   The *Delay after Stage Shift* timer is ignored when the Foil Hole Template contains a Drift Measurement Area. The delay after a stage move is then no longer a fixed time.

The values for the delay timers can be determined in the **Auto Functions** tab > **Drift stabilization** task.

7.5.3.1 Guidelines for the Drift Measurement Function and the Delay After Stage Shift Time

There are circumstances in which Drift Measurement function may not always be as accurate and fast as desired. The appearance of the specimen can change due to the accumulating damage that is caused by repetitive exposure. This may make it difficult to accurately determine the image shift between consecutive images. In extreme cases, this could even cause the Drift Measurement function to time out at 10 minutes.

Under these circumstances:

- Adding a **Drift Measurement Area** and selecting **Recurrence**: **Always** would significantly slow down the Automated Acquisition run, and make the predictions in the **Progress** panel less reliable. It may also result in a relatively large number of skipped Foil Holes due to failed Drift Measurements.

- Adding a **Drift Measurement Area** and selecting **Recurrence**: **Once per Grid Square** decreases the chance of occurrence and impact of a time out. At the same, the Automated Acquisition run is not paused longer than necessary after moving on to the next Grid Square.

- If a predictable throughput is most important, it can be better to *not* add a Drift Measurement Area to the Foil Hole Template, and use the **Delay after Stage Shift** timer for all moves.

A usable value for the **Delay after Stage Shift** can be determined with the **Auto Functions** tab > **Drift stabilization** task (see Run the Drift Stabilization Auto Function on page 88).

7.5.4 Import and Export a Foil Hole Template

With the Import and Export functions it is possible to archive the current Template Definition as a file, and to load a Template Definition that has been created in an earlier EPU session. This way, setting up an Automated Acquisition run for a regularly used specimen type can be done much easier and much faster.
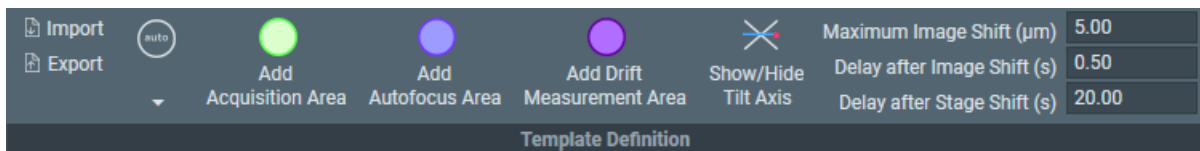
Export

Select **Export** to write the current Foil Hole Template to a file (`*.epuTemplate`).
The Template file contains:

- The type, location and *all* settings of *all* Template Areas.
  The Template file does not contain the size of the Template Areas. The size is determined by the pertaining Presets.

- The Maximum Image Shift, Delay after Image Shift and Delay after Stage Shift values.

- The Defocus list for the Acquisition Area(s).

The Template file also contains the Foil Hole diameter. The Foil Hole diameter is only used as a reference value for the Import functionality.

Import

Select **Import** to load all Template Areas and their settings from a previously stored Template file.

EPU determines the ratio of the Foil Hole diameter of the loaded specimen and the diameter that is stored in the Template file. The positions of the Template Areas are scaled according to the ratio. If the ratio is too large, then EPU shows a warning. In that case, the Focus and/or Drift Area may be located outside the field of view of the Hole/Eucentric Preset.

If necessary or desirable, the reposition the Template Areas and/or adjust their settings.

7.6   The Template Execution Task for Quantifoil Specimens

The purpose of the Template Execution task is to validate the Foil Hole Template, including finding and centering the Foil Hole that is nearest to the center of the camera field of view.

7.6.1   Validate the Foil Hole Template

To validate the Foil Hole Template, follow the procedure below. First perform this procedure at an ideal Foil Hole. If the result is good, also perform the procedure at one or two Foil Holes that are not ideal, but still good enough for high quality data acquisition.

1. Select a Foil Hole:

   a. Select the **EPU** tab > **Hole Selection** task.

   b. Select **Acquire**.

   c. Right-click on a Foil Hole and select **Move stage to location**.

2. Prepare the starting conditions for Template Execution:

   a. Insert the **FluScreen**.

   b. Accurately set the specimen to eucentric height.

   c. Accurately focus the image, then select the appropriate **Handpanel User Button** to **Reset Defocus**.

   d. Change the **XY-position** of the specimen so that the Foil Hole is still fully visible, but is not exactly centered in the camera field of view.
   This will test the **Find And Center Hole** function during Template Execution.

   e. Change the **Defocus** a little.
   This will test the **Autofocus** function during Template Execution.

3. Select the **EPU** tab > **Template Execution** task.
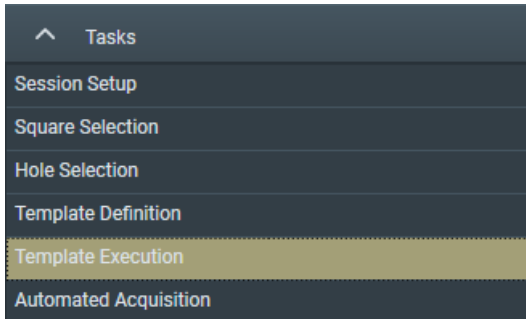
4. Select **Preview**.
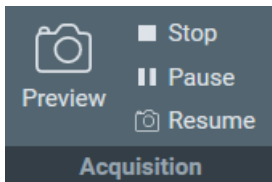
   This runs the template, including finding and centering the hole nearest to the center of the cameras field of view at the current stage position.

5. If problems are found during the Template Execution:

   a. Solve the problems.

   b. Repeat this procedure from step 1.

The time it takes to complete a **Preview** is a good estimate for the **Session Information** panel > **Exposures per hour**. Keep in mind that the a Foil Hole Template can contain multiple Acquisition Areas.

7.7   The Automated Acquisition Task

7.7.1   How to Plan for High Microscope Utilization

The Session Information panel indicates how efficient the available time is utilized. To maximize utilization, follow these guidelines:

1. Specify the **End of tool time**.

2. Specify the **Exposures per hour**.
   This number is an estimate. It can be based on experience with similar Automated Acquisition runs.
   On Quantifoil specimens, the time that is needed to complete a **Template Execution** task > **Preview** also provides a good indication. Keep in mind that the a Foil Hole Template can contain multiple Acquisition Areas.

3. Check the **Holes / Exposures** numbers.

   - **Holes**: the number of Foil Holes (Quantifoil) or Acquisition Areas (Lacey Carbon).

     - For an Automatic Session type, this number is an estimation. Among others, it is based on the number of Grid Squares that is selected in the Square Selection task, and the spacing between the Foil Holes or Acquisition Areas.

     - For a Manual Session type, the number of selected holes is counted.

   - **Exposures**: the number of Acquisition Areas.

     - On Lacey Carbon specimens the number of exposures is the same as the number of holes.

     - On Quantifoil specimens, the number of holes is multiplied by the number of Acquisition Areas in the Foil Hole Template.

   The estimated number of exposures assumes that no Grid Squares and no holes are skipped.

4. Check the **Total time**.
   This is the duration of the Automated Acquisition run. It is calculated by dividing the number of exposures by the number of Exposures per hour.

   The color indicates whether or not the available tool time is fully used, assuming that no Grid Squares or holes are skipped:

   - **Red**: poor utilization.
     When the Total time is spent, there is still a significant amount of tool time left.

   - **Yellow**: adequate utilization, there is room for improvement.
     When the Total time is spent, there is a small amount of tool time left. If the **Session setup** > **Type** is Manual, add a few more holes to the selection so all available tool time is used for data acquisition.

   - **Green**: maximum utilization.
     When the Total time is spent, there is no tool time left.

7.7.2 Last Checks Before Starting the Automated Acquisition Run

Before starting the Automated Acquisition run, check the following:

1. Make sure there is sufficient available **disk space**.

   - On the **Microscope PC**.
     Session related data and images are stored in the **Session Setup** > **Storage folder**. In case no more data can be stored in this location, EPU pauses the Automated Acquisition run and requests another storage location. If this happens, select a new storage location and resume the Automated Acquisition run.

   - On the **Storage Server** or **Gatan PC**.
     Dose Fractions are stored on the StorageServer or Gatan PC. Data volumes of several hundreds of Gigabytes per day are no exception. If there is no more space to store Dose Fraction images, the Automated Acquisition run will stop.

2. Make sure the **Liquid Nitrogen** (LN2) dewars are full.
   To avoid failure of an unattended run, make sure that the LN2 supply is sufficient to finish the run. EPU frequently checks if a refill of the dewars is required and will request a refill if needed. EPU cannot detect if the main LN2 supply runs out. If the LN2 is not refilled, the Automated Acquisition run keeps going while the sample temperature slowly increases.

7.7.3 Activate a New Phase Plate Before Starting the Automated Acquisition Run

The instructions below are only applicable when **EPU** tab > **Session Setup** task > **Use Phase Plate** is ticked.

To prepare a fresh Phase Plate, follow the instructions below:

1. In the Phase Plate section of the Ribbon Bar:
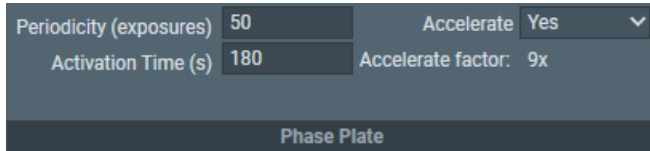
   a. Specify **Periodicity (exposures)**:
   The number of acquisitions after which the Objective aperture mechanism moves to the next Phase Plate position to start the activation of a new Phase Plate area. The interval is typically between 50 and 80 exposures.

   b. Specify **Activation Time (s)**:
   The time that is needed to activate a new Phase Plate.

   c. If desired, select **Accelerate**: **Yes**
   The Accelerate function temporarily moves the specimen to an area that has been exposed already, and selects the largest C2 aperture to increase the beam current. After the phase plate activation is completed, the C2 aperture mechanism and the specimen are returned to their initial positions. The Accelerate function does not change any optics settings.
   The Accelerate factor is the area ratio between the initial C2 aperture and the largest C2 aperture.

2. Retract the **FluScreen**.
   The beam will be blanked now.

3. Select the **Preparation** tab > **Activate Phase Plate** task.
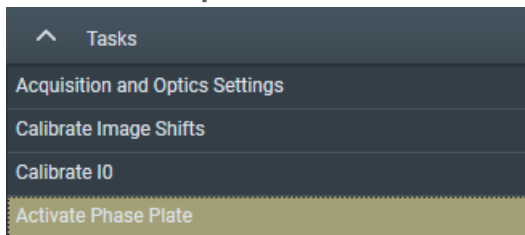
4. Select **Next Position** to move the Objective aperture mechanism to a fresh area on the phase plate.
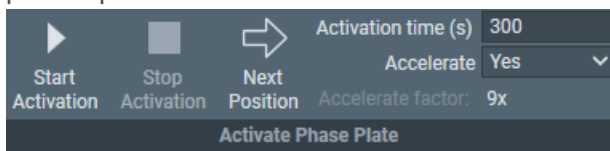
5. Wait for the drift of the aperture mechanism to settle.
   For a small move, this takes about 30 seconds.

Note	When making a large move with the aperture mechanism, such as changing to a different Phase Plate, or re-inserting after the mechanism has been retracted, it may take up to 5 minutes for drift to settle.
	Please refer to the Phase Plate documentation for the latest specifications.

6. Specify the **Activation time (s)** value.

7. If desired, select **Accelerate**: **Yes**

8. Select **Start Activation**.
   An image is acquired and displayed in the top left section of the Task Execution panel.

9. Wait for the Activation time to expire.
   EPU acquires a new image and displays it in the top right section of the Task Execution panel.
   The FFT of both images are displayed side-by-side in the bottom section of the Task Execution panel, so they are easy to compare. If the Thon rings are shifted inward by half a period, then Phase Plate activation is completed successfully.
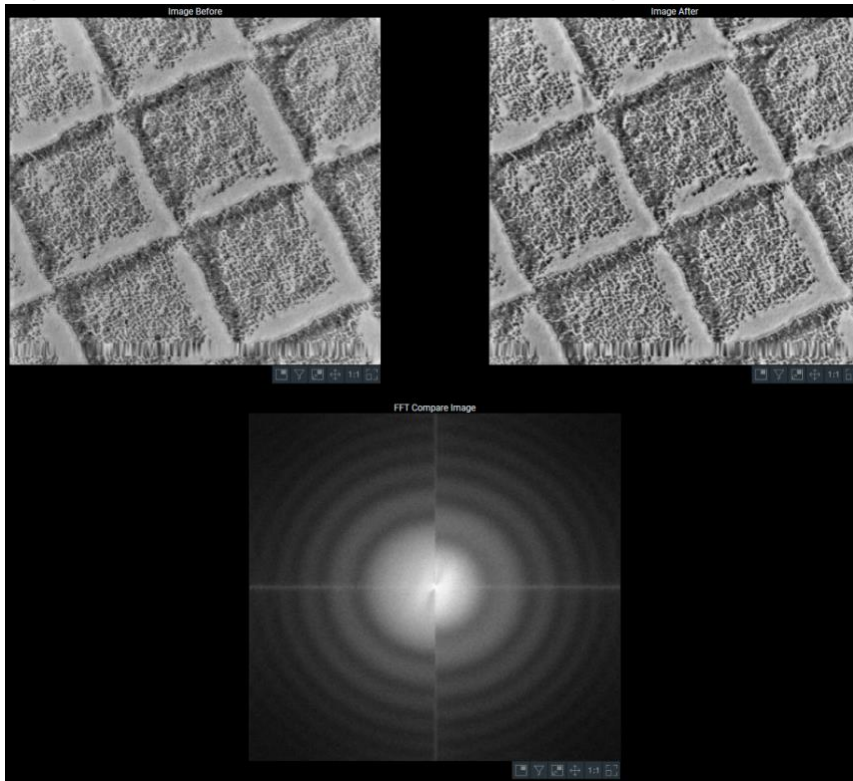
Left half: FFT of an image at the beginning of the activation.
Right half: FFT of an image at end of the activation.

7.7.4 Start the Automated Acquisition Run

To start the Automated Acquisition, follow the procedure below:

1. Select the **EPU** tab > **Automated Acquisition** task.
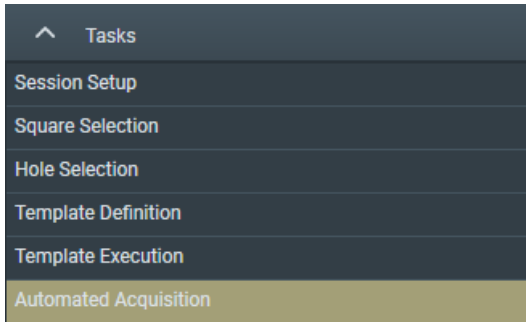
2. If the microscope is in EFTEM mode,
   then specify the *Auto Zero Loss* settings:
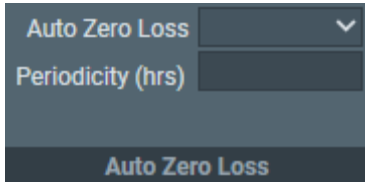
   a. Select **Auto Zero Loss**: **Yes** to enable periodic execution of the Auto Zero Loss function during the Automated Acquisition run.

   b. Specify the **Periodicity**

3. Select **Start Run**.
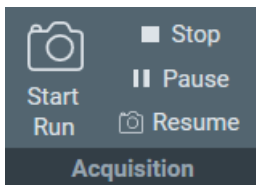
   Before the automated acquisition starts, EPU runs a preconditions check.

   • If no issues are found, then the automated acquisition run starts.

   • If issues are found, then EPU displays these in the *Messages* side panel. The automated acquisition run is not started.

4. Monitor the Automated Acquisition run during the first few data acquisitions.

5. If there are no irregularities, select the desired *Options*:
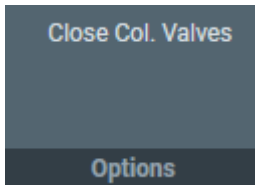
a. Select **Close Col. Valves**.
   When active, EPU closes the Column Valves after the Automated Acquisition run is completed.

b. On systems with a thermionic gun, select **Switch Off Emission** *(not shown in the image above).*
   When active, EPU switches off the emission, or brings the electron source to a safe standby state after the Automated Acquisition run is completed.

7.7.5 Monitor the Progress of the Automated Acquisition Run

EPU provides two to monitor the progress of an Automated Acquisition run:

- The **EPU** tab > **Automated Acquisition** task > **Progress panel**
  The contents of the Progress panel are described below.

- The tooltip that appears when hovering over a Grid Square in the **EPU** tab > **Square Selection** task

On a Lacey Carbon specimen, the counters apply to the number of defined and processed Acquisition Areas.

The Progress panel displays the following information:
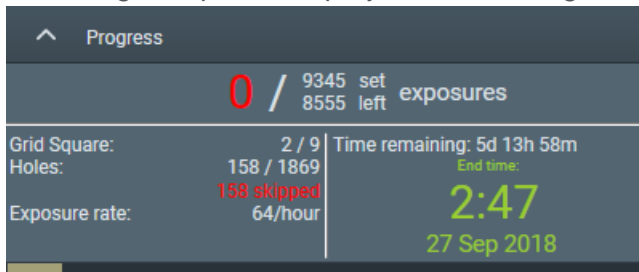
Exposures

- Number of completed data acquisitions.
  If too many acquisitions are skipped, the number of exposures will appear yellow or red.

- Number of planned data acquisitions (set).

- Number of remaining data acquisitions (left).

Grid Square

- Number of processed Grid Squares.
  The processing status of each Grid Square is also indicated by color coding in the Atlas.

- Number of planned Grid Squares.

Holes

Quantifoil only

- The number of processed Foil Holes.

- The number of planned holes.

- The number of skipped holes.
  When too many holes are skipped, the counter will turn red.

Exposure rate

The actual number of data acquisitions per hour, based on the statistics of the current session, including the skipped areas.

Time remaining

The estimated remaining duration, based on the actual Exposure rate and the number of remaining data acquisitions.

End time

The estimated end time of the run.

> The color indicates whether or not the available tool time is fully used, assuming that no Grid Squares or holes are skipped:

- **Red**: poor utilization.
  When the Total time is spent, there is still a significant amount of tool time left.

- **Yellow**: adequate utilization, there is room for improvement.
  When the Total time is spent, there is a small amount of tool time left. If the **Session setup** > **Type** is Manual, add a few more holes to the selection so all available tool time is used for data acquisition.

- **Green**: maximum utilization.
  When the Total time is spent, there is no tool time left.

A **progress bar** at the bottom side of the Progress panel indicates the overall progress.

7.7.6 Pause and Resume the Automated Acquisition Run

There can be multiple reasons to pause an Automated Acquisition run, for example to refill an LN2 Dewar.

While a run is paused, it is not possible to adjust any settings, such as the Acquisition and Optics Presets, the size and spacing of the Foil Holes on a Quantifoil specimen, or the spacing of the Acquisition Area pattern on a Lacey Carbon specimen. If a setting needs to be adjusted, the run must be stopped and restarted after the adjustments are done.

1. Select **Pause**.
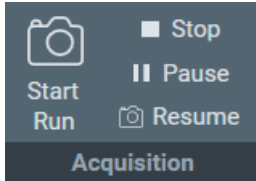
2. Perform the activities for which the Automated Acquisition run needed to be paused.

3. Select **Resume**.

When the **Session Setup** > **Acquisition Mode** is **Faster acquisition**, then EPU recalculates the Foil Hole groups before automated acquisition starts again.

7.7.7 Skip a Grid Square, Foil Hole or Acquisition Area

Various automatic functions are executed during the Automated Acquisition run. Depending on their results, these functions can decide to:

- Skip a Grid Square.
- Skip a Foil Hole on a Quantifoil specimen.
- Skip an Acquisition Area on a Lacey Carbon specimen.

The number of skipped locations is reported in the Progress panel.

The **Skip Options** section of the Ribbon Bar provides functions to manually skip the current location.
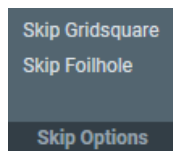
- If the quality of the current Grid Square results in too many skipped Foil Holes or Acquisition Areas, select **Skip Gridsquare** to move on to the next Grid Square.

- If the quality of the current Foil Hole or Acquisition Area causes a delay, or will surely result in failure of an automated function, select **Skip Foilhole** or **Skip Area**.

When the **Session Setup** > **Acquisition Mode** is **Faster acquisition** then EPU recalculates the Foil Hole grouping.

7.7.8 Troubleshoot an Ongoing Automated Acquisition Run

If the automated run does not perform as expected, it is possible to adjust the settings:

1. Select **Stop**.
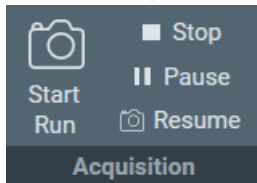
    The EPU Session file contains the processing status of the Grid Squares and the selected areas.

2. Depending on the type of failures, adjust the **Preparations** tab > **Acquisition and Optics Presets**.

Note	Be very careful! Some adjustments may invalidate certain calibrations. Refer to the descriptions of the Presets, Calibrations and Auto Functions to check if additional updates are necessary.

3. For Quantifoil specimens:

    *   If execution of the Foil Hole Template fails often, then return to the **Hole Selection** task, right-click on a Foil Hole in the Image Display and select **Move stage to location** to center a Foil Hole. Then select the **Template Definition** task to adjust the Foil Hole Template.
        If the Grid Square has been (partially) processed already, then the crosshair that marks the stage position may not move the exact center of the Foil Hole. Refer to   on page 158 for an explanation of such a possible offset.

    *   If the *Find And Center Hole* function fails often, use the **Hole Selection** task > **Measure Hole Size** tool to check the Foil Hole diameter and spacing.

        *   In a *Session Setup > Type*: *Manual* session, the Foil Hole positions are already defined by their XYZ-coordinates. Changing the Foil Hole spacing will have no effect: the coordinates of the Foil Holes that are selected in the Hole Selection task will not be updated.
            To update the Foil Hole positions, return to the Square Selection task and Hole Selection task to create a new selection of Foil Holes with more accurate coordinates.

        *   In a *Session Setup > Type*: Automatic session, adjusting the Foil Hole diameter and/or spacing will have an impact on the Find And Center Hole function, but it may also have an impact on the Find Holes detection algorithm.
            These functions use the Hole/EucentricHeight Preset and the GridSquare Preset. If these Presets do not have a parallel beam and/or do not use little to no defocus, then the hole diameters in the acquired images may deviate significantly from the physical specimen.

4. If desired, change the processing status of one or more Grid Squares from *Processed* (blue) back to *Open* (green).

    a. Select the **EPU** tab > **Square Selection** task.

    b. Right-click on a processed Grid Square and select **Reopen**.
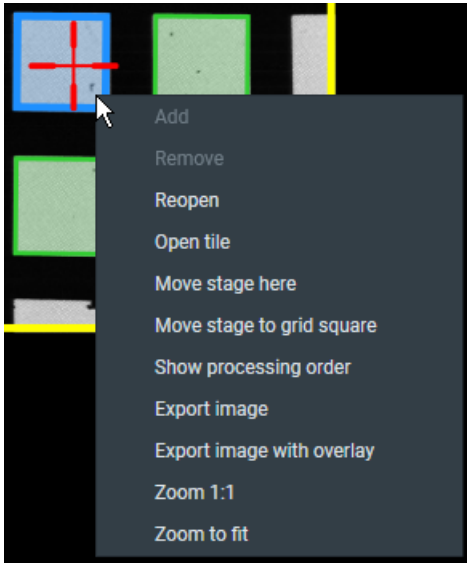
5. Select **Start**.
    Because the processing status and progress are stored in the EPU Session file, the Automated Acquisition run continues from where it was stopped. Even if the EPU software has been closed, the Automated Acquisition run will continue from where it was stopped.

7.7.9 Reopen a Processed Grid Square

When a Grid Square is skipped or when data acquisition on a Grid Square is completed, it is marked as *Processed*. In the Square Selection task, processed Grid Squares are marked blue. Processed Grid Square are excluded from future data acquisition in the current session.

It is possible to reset the Grid Square's status to *Open* without creating a new session.

1. Select the **EPU** tab > **Square Selection** task.

2. Right-click on a processed Grid Square and select **Reopen**.
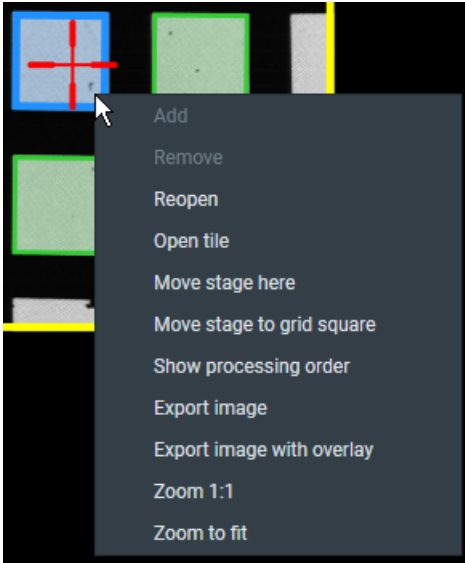
7.7.10 Phase Plate Position Logging

For each acquisition, the used Phase Plate positions are logged in the XML files that are associated with that acquisition.

This logging facilitates Phase Plate mapping in the post-processing pipeline at customers, where the measured phase shift from the CTF estimation can be correlated with the used Phase Plate positions. The Phase Plate position logging itself does not qualify the used positions as 'good' or 'bad'.

7.8 Correct a Mismatch in the Orientations Between a Re-used Atlas and a Reloaded Specimen

The orientation of a reloaded specimen is often very close to the orientation at which the Atlas is acquired. Experience has shown that the difference is often so small that EPU can compensate for it during the preparation and execution of the Automated Acquisition run.
It can happen that the difference between the orientations of the re-used Atlas and the reloaded specimen is too large.

- For Quantifoil specimens this becomes noticeable in the *EPU > Hole Selection* task.

- For Lacey Carbon specimens this becomes noticeable in the *EPU > Area Selection* task.

In both cases, the orientation and/or field of view of the imaged area of a new GridSquare image does not match with the selected Grid Square in the Atlas.

To correct mismatching orientations:

1. Acquire a new Atlas for the currently loaded specimen.
   Either:

   - As described in The Atlas Acquisition Task on page 102.

   - As described in The Screening Task on page 95.
     In the acquired Atlas, tick the high potential **Grid Square categories**,
     then select **Atlas Acquisition** task > **Load from Screening**

2. In the **EPU** > **Square Selection** task, customize the selection of Grid Squares as described in Select Grid Squares for High Quality Data Acquisition on page 123.
   If the new Atlas is acquired via the Screening task, then the default selection already contains the selected Grid Square categories.

3. Continue with the *Hole Selection* or *Area Selection* task.

8   Inspect the Acquired Images

8.1   View the Images in Windows Explorer and Photo Viewer

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

- For the Atlas, EPU stores a lower resolution JPEG file with compressed image data for each tile.

- A high quality MRC or TIFF file.

- Additional MRC file(s) with the Dose Fraction images.
  (Only in case the Intermediate Images functionality from a Direct Detection camera is used).

- An XML file with meta data.

8.1.1   View the JPEG Images

To view the JPEG files on any computer:

1. In **Windows Explorer**, go to the directory where the images are saved.
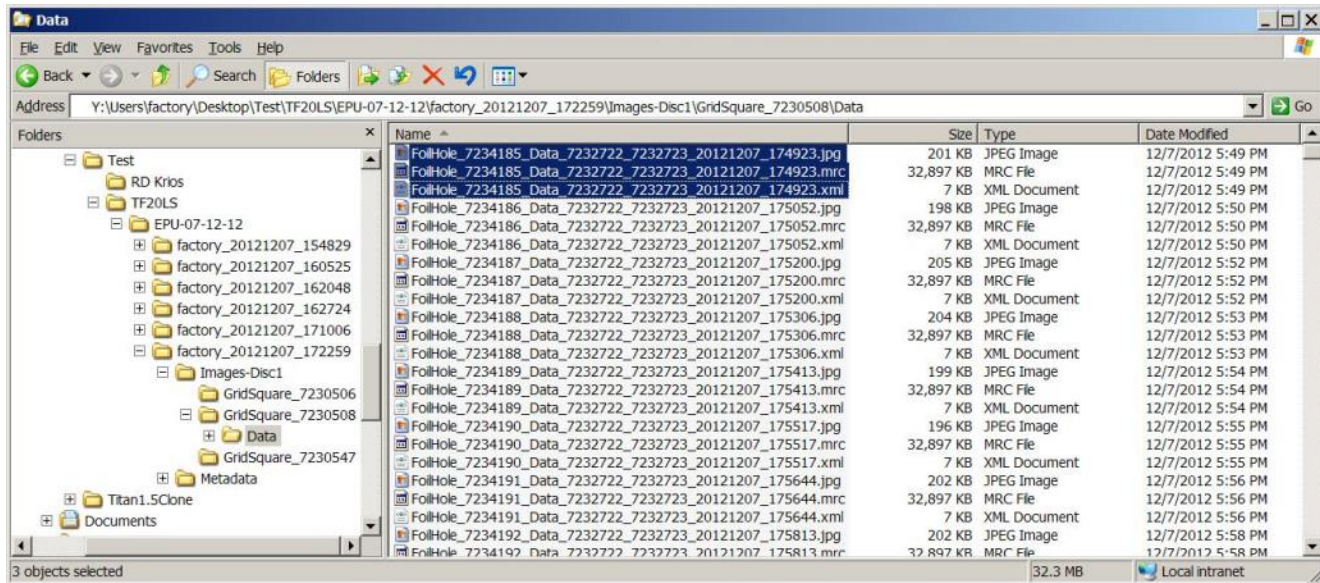
2. Select a **JPEG image file**.

Although TIFF images can be supported by Window's native image viewer, the TIFF format variation that is used by the microscope may not be readable without installing a codec or an image viewer with a broad TIFF format support.

8.1.2 View the Microscope Images

The high resolution MRC files can be viewed and post-processed with most electron microscopy post-processing applications.

It is also possible to view the acquired images with the built-in Windows Photo Viewer:

1. Install the **Thermo Scientific Imaging Codec Pack** on the computer.
   With the Imaging Codec Pack, Windows Explorer and Windows Photo Viewer support many image formats that are commonly used by FEI and Thermo Scientific systems and software, such as `*.mrc` files, `*.emi` files (TIA) and `*.dmx` files (Gatan Digital Micrograph).

2. Reboot the computer.

3. In **Windows Explorer**,

   a. Go to the directory where the images are saved.

   b. Open an image file in the default image viewer.
      If an `*.mrc` file contains a stack of images, all the individual images can be viewed.

8.2 View and Post-process MRC Images with Velox

MRC images can be viewed and post-processed:

- On the Microscope PC with the Thermo Scientific Velox Online Processing software.

- On any other computer with the Thermo Scientific Velox Offline software.

To open an MRC image in Velox:

1. Open **Velox Online Processing** or **Velox Offline**.

2. Drag and drop the **MRC image file** in the Velox window.

Refer to the Velox User Manual for detailed descriptions and instructions.
The Velox software does not provide 3D reconstruction functionalities.

9   Detailed Preconditions

This chapter describes the preconditions that must be fulfilled for high quality data acquisition. Most preconditions involve microscope alignments and calibrations.

Unless the alignment or calibration procedure describes otherwise, make sure all alignments and calibrations are done:

- At a central stage position.
- At eucentric height.
- In focus.
- Preferably with a parallel beam.

The instructions and verifications in this chapter typically use the *TEM User Interface* > *CCD/TV Camera* control panel and *TIA* for image acquisition and inspection. Depending on the camera type, other applications may be more suitable to acquire and inspect images:

- Thermo Scientific Falcon 4: use *Velox*.
- Stand-alone Gatan cameras: use Gatan Digital Micrograph.

9.1   Preconditions for the Specimen and Specimen Holder

Side entry holders should be cleaned and pumped before use. After insertion into the microscope:

1. Manually adjust the eucentric height of the specimen, preferably at or near the center of the specimen.
2. Roughly focus the specimen.

9.2   Preconditions for the Microscope

The microscope must meet the following conditions:

- The column alignments must be completed.
- Magnification Calibrations for LM and SA ranges must be completed.
- High Tension is stable.
- For Thermionic instruments, the gun saturation (heating) must be optimized and the emission chosen.
  If necessary, optimize the gun settings via the Gun Tilt and Gun Shift Direct Alignments.
- FEG registers, Alignment files and calibrations should form a consistent set.
- Apertures are properly centered.

- Focus calibration must be completed.
  This task is performed in EPU.

Note	To work in both Nanoprobe and Microprobe modes, the focus calibration must be done separately for both modes.

- Direct alignments should be checked and, if necessary, adjusted in the modes that are used for EPU.
  These include, but may not be limited to the LM and SA range, Nanoprobe and Microprobe modes.

- Bias/Dark and Gain Reference images are available and well averaged for all cameras.

- The camera is at constant temperature.

- After any actions that may have introduced exceptionally strong drift, such as inserting a holder, enough time should be allowed for settling .

9.3 Preconditions for the Microscope Alignments

High quality data acquisition relies on an accurate lens series alignment. The system alignments are generally quite stable and do not require frequent adjustment. Only adjust the system alignments when absolutely necessary.

9.3.1 Gun and Condenser

When switching between Acquisition and Optics Presets, 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 tests described below.

9.3.1.1 Test the Gun Alignment

1. Select **Spot Size**: **2**

2. Adjust the **Intensity**, so that the beam diameter matches the smallest circle on the FluScreen.

3. Center the beam on the FluScreen.

4. Verify the Gun Alignment for Spot Sizes 2 to 11:

   a. Increase the **Spot Size** number by one step.

   b. Verify that the beam stays centered.

   If the verification in step 4 fails:

- Perform all **Alignments** control panel > **Gun** alignments.

- Repeat steps 1 to 4.

5. When Spot Size 11 is checked, verify the Gun Alignment in reverse sequence:

    a. Decrease the **Spot Size** number by one step.

    b. Verify that the beam stays centered.

    If the verification in step 5 fails:

    - Perform all **Alignments** control panel > **Gun** alignments.

    - Repeat steps 1 to 5.

9.3.1.2 Test the Condenser Alignment

1. Select the **highest SA magnification**.

2. Adjust the **Intensity**, so that the beam diameter matches the smallest circle on the FluScreen.

3. Center the beam.

4. In the **TEM User Interface** > **Beam Settings** control panel:

    - Titan: enable **Auto Zoom**

    - Talos:enable **Intensity Zoom**

5. Verify the Condenser Alignment for each SA magnification:

    a. Decrease the magnification by one step.

    b. Normalize all lenses.

    c. Verify that the beam stays centered.

    d. Verify that:

        - Titan: the beam size does not change.

        - Talos: the beam intensity does not change.

    If the verifications in steps 5c and 5d fail, perform the **Align HM-TEM** > **All SA Magnifications** alignment.

6. In the **TEM User Interface** > **Beam Settings** control panel:

    - Titan: disable **Auto Zoom**

    - Talos:disable **Intensity Zoom**

9.3.1.3 Test the Parallel Beam Range on Titan Systems

Perform this test in the following optical modes:

- TEM nP

- TEM uP

- EFTEM nP (if a filter is present)

- EFTEM uP (if a filter is present)

1. Insert the **FluScreen**.

2. If the system is not in Diffraction mode, select **Handpanels** > **Diffraction** .

3. Select **Camera Length**: **800 mm**

4. Select **Handpanels** > **Eucentric Focus**

5. Decrease the **Intensity** to the smallest value at which the **Beam Settings** control panel > **Illumination** is **Parallel**.
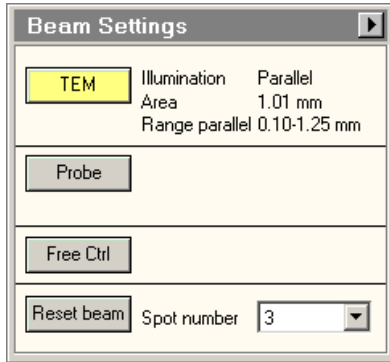
6. On the **FluScreen**, check that the **diffraction pattern circles** for gold are sharply defined.

7. Take note of the **Area** value.

8. Slowly increase the Intensity until the **Beam Settings** control panel > **Illumination** is no longer **Parallel**.

9. Take note of the **Area** value.

10. Decrease the **Intensity**, so that the **Area** value is the average of the lower and upper Area values that are noted in steps 7 and 9.

11. On the **FluScreen**, check that the **diffraction pattern circles** for gold are sharply defined.

If the diffraction pattern circles are not sharply defined at step 11, perform the **Align HM-TEM** > **Basic SA** alignment.

9.3.1.4 Test the Condenser Zoom on Titan Systems

Perform this test in the following optical modes:

*   TEM nP
*   TEM uP

---

1. Select the **lowest SA magnification**.

2. With the **Handpanels** > **Intensity** knob, decrease the **Beam Settings** control panel >**Area**, so that the **Illumination** changes from **Parallel** to **Condensing**.

3. Center the beam.

4. With the **Intensity** knob, slowly increase the illuminated area so that the **Illumination** is **Parallel** again.

5. Verify that the beam is still centered.

6. With the **Intensity** knob, slowly increase the illuminated area further.

7. Check that the beam stays centered for as long as **Illumination** is **Parallel**.

If the check at step 7 fails, perform the **Direct Alignments** control panel > **Condenser Zoom** alignment and repeat steps 1 to 7.

8. Center the beam on a feature of known size.

9. With the **Intensity** knob, adjust the beam diameter so that it matches the size of the feature.

10. Verify that the **Area** value corresponds with the known feature size.

If the check at step 10 fails, perform the **Direct Alignments** control panel > **Condenser Zoom** alignment and repeat steps 1 to 10.

9.3.1.5 Test the Condenser Focus Alignment

Perform this test in the following optical modes:

- TEM nP
- TEM uP

1. With the **Handpanels** > **Intensity** knob, condense the beam to the smallest achievable spot.

2. Verify that the **Beam Settings** control panel >**Area**, is **0±100 nm**
   If this check 2 fails:

   a. Perform the **Condenser** > **Condenser Preparation** alignment.

   b. Depending the used optical mode, either:

      - Perform the **Focus + calib nP** alignment.
      - Perform the **Focus + calib nP** alignment.

9.3.2 Deflectors

The image/beam shift calibration must have been done carefully:

- To keep the illumination centered when some image shift is added.

- 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 so the illumination conditions (coma, rotation center) and crossover shifts (GIF energy selection) do not change when applying image (beam) shift.

9.3.2.1 Test the Image/Beam Shift Calibration

1. Select a **high SA magnification**

2. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it matches the largest circle on the FluScreen.

3. In the **TEM User Interface** > **Image Settings** control panel, select **MF knobs**

4. With the **Handpanels** > **MF-X** and **MF-Y** knobs, shift the image roughly 50% of the beam diameter in the positive and negative **X** and in **Y** directions.

5. Check that the beam stays centered.

6. Select an **SA magnification** of approximately **50 kX**, so that the field of view spans approximately 15 grid squares.

7. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it matches the largest circle on the FluScreen.

8. In the **TEM User Interface** > **Image Settings** control panel, select **MF knobs**

9. With the **Handpanels** > **MF-X** and **MF-Y** knobs, shift the image roughly 50% of the beam diameter in the positive and negative **X** and in **Y** directions.

10. Verify that the beam stays centered.

If the check at step 5 and/or step 10 fails, perform the **Calibrate HR-TEM** > **Image/Beam Calibration** alignment.

9.3.3 Projector

9.3.3.1 Test the Lens Series Magnification Center Alignment

The LM and HM lens series must be aligned accurately to ensure that a centered feature stays centered when switching to another magnification. The alignment must be performed accurately on the camera that is used for data acquisition.

1. With the **Handpanels** > **Magnification** knob, select the **highest Acquisition and Optics Presets magnification**

2. In the **TEM User Interface** > **Image Settings** control panel, verify that no image shift is applied.

3. In the **TEM User Interface** > **CCD/TV Camera** control panel:

   a. Select the **Camera** that is used for data acquisition.

   b. Select **Search**

4. Move the **specimen**, so that a feature is visible in the image center that is also recognizable on the camera at the Atlas Preset magnification.

5. Place a **marker** on the recognizable feature.

6. With the **Handpanels** > **Magnification** knob:

   a. Step down through all Acquisition and Optics Presets magnifications and check if the feature stays aligned with the marker.

   b. Step up again through all Acquisition and Optics Presets magnifications and check if the feature stays aligned with the marker.

This alignment is often done on the FluScreen, which might have a different magnification center. Perform the **Preparation** tab > **Calibrate Image Shifts** task to compensate for magnification center offsets.

9.3.3.2 Test the Eucentric Focus

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

1. If not visible yet, add the **Defocus** value to the **TEM User Interface** > **Information Panels**.

2. Accurately bring the specimen to eucentric height by using the stage wobbler.

3. Select a **higher SA magnification** (approx. 150kX).

4. Select **Handpanels** > **Eucentric Focus**.

5. Reset Defocus.
   Either:

   • Select the **Handpanels** > **User button** that is assigned to **Reset Defocus**.
     This is often **R2**.

   • Select **TEM User Interface** > **Image Settings** control panel > **Reset Def**.

6. Use the **Handpanels** > **Focus** knob to accurately focus the image.

7. Verify that the **Information Panel** > **Defocus** value is **0±500 nm**.

If the check at step 7 fails, either:

• Perform the **Align HM-TEM** > **Basic SA** alignment

• Perform the **Auto Functions** tab > **Calibrations: Eucentric Correction** task to compensate for the eucentric offset

9.3.3.3 Test the Magnification-Dependent Focus

The lens series alignment should be parfocal as much as possible, so switching magnification does not significantly affect (de-)focus. For the LM range and the lower magnifications of the HM series, this alignment is often not performed with maximum achievable accuracy.

1. Insert the **FluScreen**.

2. Select a **low LM magnification**.

3. With the **Handpanels** > **Intensity** knob, condense the beam to the smallest achievable spot.

4. Select **Handpanels** > **Wobbler**.

5. With the **Handpanels** > **Focus** knob, adjust focus so that the spot does not move.

6. Reset Defocus.
   Either:

   - Select the **Handpanels** > **User button** that is assigned to **Reset Defocus**.
     This is often **R2**.

   - Select **TEM User Interface** > **Image Settings** control panel > **Reset Def**.

7. For all LM magnifications, check the magnification-dependent focus:

   a. Increase the **magnification** one step.

   b. Reset **Defocus**.

   c. Adjust **focus** so that the spot does not move.

   d. Verify that the **defocus** value is **less than 1 mm**.

If the check at step 7d fails, perform the **nP calibrations** and/or **LM uP calibrations**.

8. For the **lower HM magnifications**, check the magnification-dependent focus:

    a. Increase the **magnification** one step.

    b. Reset **Defocus**.

    c. Adjust **focus** so that the spot does not move.

    d. Verify that the **defocus** value is **less than 2 um**.

9. Select **Handpanels** > **Wobbler** to stop the beam wobbler.

10. For the **lower HM magnifications**, check the magnification-dependent focus:

    a. Increase the **magnification** one step.

    b. Reset **Defocus**.

    c. Adjust **focus** so that the image has minimum contrast.

    d. Verify that the **defocus** value is **less than 2 um**.

If the checks at step 8d and/or 10d fail, perform the **nP calibrations** and/or **HM uP calibrations**.

9.3.3.4 Test the Magnification-Dependent Beam and Image Shift - TEM mode

1. Insert the **FluScreen**

2. Switch to **TEM MicroProbe**

3. Select the **highest LM magnification**

4. Move the **specimen**, so that an **easily recognizable feature** is located in the center of the FluScreen.

5. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it covers approximately 80% of the FluScreen.

6. Assign the **Multifunction X** and **Y** knobs to **Image Shift**

7. Use the **Multifunction X** and **Y** knobs to shift the recognizable feature away from the center, to a location near the smallest circle on the FluScreen.

8. In the FlucamViewer:

    a. Place a **marker** on the **recognizable feature**

    b. Place a **circle** around the **circumference of the beam**

9. Increase the **magnification** one step to the **lowest SA magnification**

10. In the FlucamViewer:

   a. Measure the **distance** between the recognizable feature position and the marker.

   b. Measure the beam shift:
       Measure the largest **distance** from the circle to the beam circumference.

   The image shift *and* the beam shift from highest LM to lowest SA magnification must be less than **2 um**

If the check at step 10 fails, perform the **nP calibrations** and/or **LM uP calibrations**, and repeat steps 1 to 10.

11. Select the **highest SA magnification**

12. Move the **specimen**, so that an **easily recognizable feature** is located in the center of the FluScreen

13. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it covers approximately 80% of the FluScreen.

14. Assign the **Multifunction X** and **Y** knobs to **Image Shift**

15. Use the **Multifunction X** and **Y** knobs to shift the recognizable feature away from the center, to a ocation near the smallest circle on the FluScreen.

16. In the FlucamViewer:

   a. Place a **marker** on the recognizable feature.

   b. Place a **circle** around the beam.

17. Select the **lowest HM magnification**

18. In the FlucamViewer:

   a. Measure the **distance** between the recognizable feature position and the marker.

   b. Measure the beam shift:
       Measure the largest **distance** from the circle to the beam circumference.

   The image shift *and* the beam shift from highest SA to lowest HM magnification must be less than **50 nm**

If the check at step 18 fails, perform the **nP calibrations** and/or **HM uP calibrations**, and repeat steps 1 to 18.

9.3.3.5   Test the Magnification-Dependent Beam and Image Shift - TEM mode

1. Insert the **FluScreen**

2. Switch to **EFTEM MicroProbe**

3. Select the **highest LM magnification**

4. Move the **specimen**, so that an **easily recognizable feature** is located in the center of the FluScreen.

5. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it covers approximately 80% of the FluScreen.

6. Assign the **Multifunction X** and **Y** knobs to **Image Shift**

7. Use the **Multifunction X** and **Y** knobs to shift the recognizable feature away from the center, to a ocation near the smallest circle on the FluScreen.

8. In the FlucamViewer:

    a. Place a **marker** on the **recognizable feature**

    b. Place a **circle** around the **circumference of the beam**

9. Increase the **magnification** one step to the **lowest SA magnification**

10. In the FlucamViewer:

    a. Measure the **distance** between the recognizable feature position and the marker.

    b. Measure the beam shift:
       Measure the largest **distance** from the circle to the beam circumference.

    The image shift *and* the beam shift from highest LM to lowest SA magnification must be less than **2 um**

If the check at step 10 fails, perform the **nP calibrations** and/or **LM uP calibrations**, and repeat steps 1 to 10.

11. Select the **highest SA magnification**

12. Move the **specimen**, so that an **easily recognizable feature** is located in the center of the FluScreen

13. With the **Handpanels** > **Intensity** knob, adjust the beam diameter, so that it covers approximately 80% of the FluScreen.

14. Assign the **Multifunction X** and **Y** knobs to **Image Shift**

15. Use the **Multifunction X** and **Y** knobs to shift the recognizable feature away from the center, to a ocation near the smallest circle on the FluScreen.

16. In the FlucamViewer:

    a. Place a **marker** on the recognizable feature.

    b. Place a **circle** around the beam.

17. Select the **lowest HM magnification**

18. In the FlucamViewer:

    a. Measure the **distance** between the recognizable feature position and the marker.

    b. Measure the beam shift:
        Measure the largest **distance** from the circle to the beam circumference.

    The image shift *and* the beam shift from highest SA to lowest HM magnification must be less than **50 nm**

    If the check at step 18 fails, perform the **nP calibrations** and/or **HM uP calibrations**, and repeat steps 1 to 18.

9.4 Aperture Alignments

9.4.1 Check C2 Apertures Centering on Three Lens Condenser Systems

Three Lens Condenser systems include all Titans, except early Metrios systems.

Not all apertures are used for the acquisition of the Atlas and during the Automated Acquisition run. Nonetheless, all C2 apertures must be properly centered in Microprobe and Nanoprobe mode. When the condenser is in Two Lens Condenser mode, a centered beam should expand symmetrically without moving.

1. In the **TEM User Interface** > **Beam Settings** control panel:
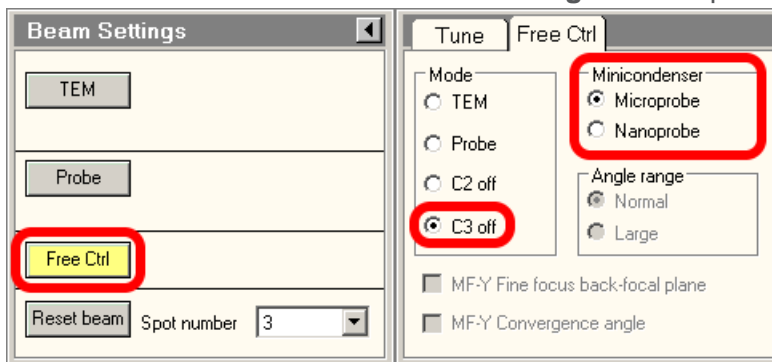
    a. Select **Free Ctrl**.

    b. Select **C3 off**.

    c. Select **Microprobe**.

2. Use the **Handpanels**> **Multifunction** knobs or **Trackball** to center the beam.

3. In the **TEM User Interface** > **Apertures** control panel:

    a. Select the **largest C1 aperture**.

    b. Select the **largest C2 aperture**.

    c. If present, select the **largest C3 aperture**.

    d. Retract the **Objective** and **Selected Area** aperture mechanisms.

4. Turn the **Handpanels**> **Intensity** knob on the handpanel in both directions, and:

    - Check that the beam expands symmetrically.

    - Check that the beam remains centered.

    If the beam does not expand symmetrically or does not stay centered, **adjust** the C2 aperture position.
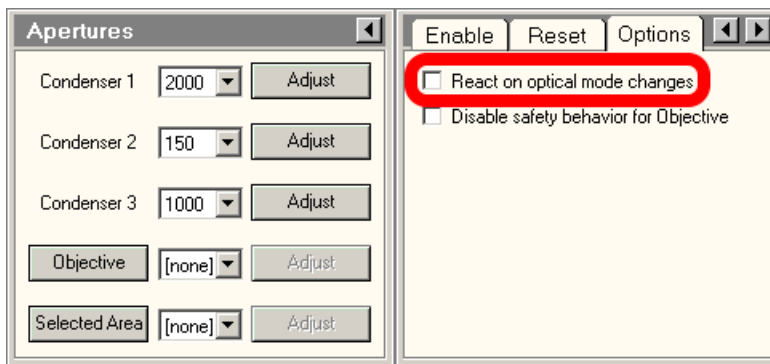
5. Repeat step 4 for **every C2 aperture**.

6. Select **TEM User Interface** > **Beam Settings** > **Nanoprobe**.

7. Repeat steps 4 and 5.

9.4.2   Check C2 Apertures Centering on Two Lens Condenser Systems

Two Lens Condenser systems include all Talos, Tecnai, and early Metrios systems.

Not all apertures are used for the acquisition of the Atlas and during the Automated Acquisition run. Nonetheless, all C2 apertures must be properly centered in Microprobe and Nanoprobe mode. A centered beam should expand symmetrically without moving.

1. Switch to **Microprobe** mode.

2. Use the **Handpanels**> **Multifunction** knobs or **Trackball** to center the beam.

3. In the **TEM User Interface** > **Apertures** control panel:

    a. Select the **largest C1 aperture**.

    b. Select the **largest C2 aperture**.

    c. Retract the **Objective** and **Selected Area** aperture mechanisms.

4. Turn the **Handpanels**> **Intensity** knob on the handpanel in both directions, and:

    - Check that the beam expands symmetrically.

    - Check that the beam remains centered.

    If the beam does not expand symmetrically or does not stay centered, **adjust** the C2 aperture position.

5. Repeat step 4 for **every C2 aperture**.

6. Select **TEM User Interface** > **Beam Settings** > **Nanoprobe**.

7. Repeat steps 4 and 5.

9.4.3   Check Objective Apertures Centering

Not all apertures are used for the acquisition of the Atlas and during the Automated Acquisition run. Nonetheless, all Objective apertures must be properly centered in Microprobe and Nanoprobe mode.

1. Switch to **HM diffraction** mode.

2. Verify that all Objective apertures are accurately centered around the beam.

3. Switch to **LM mode**

4. Select a **lower LM magnification**.

5. Verify that all Objective apertures are accurately centered around the beam.

6. Select a **lower SA magnification**.

7. Verify that all Objective apertures are accurately centered around the beam.

Note	**Do not use the** *React on optical mode changes* **function to change the apertures automatically.**

Among others, the **React on optical mode changes** function automatically applies the C2 aperture that was selected the previous time that the optical mode was used. This may conflict with the *C2 Aperture* value in the *Acquisition and Optics Presets*.

The **React on optical mode changes** function may also retract the Objective aperture mechanism. If Phase Plates are used in the experiment, then retracting the Objective aperture mechanism means that the Phase Plates are moved away from the beam.

9.5 Direct Alignments and Astigmatism

Direct alignments must be checked regularly.

The preconditions below describe the direct alignments for uncorrected systems. Systems with image and/or probe correctors are not discussed here. Fine-tuning of the corrector eliminates coma, astigmatism, and other aberrations.

*   **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. This is important for Autofocus and astigmation.

- **Beam Shift**
  Center the beam when no user beam shift is applied.
  Perform this Direct Alignment at the highest magnification that will be used during the Automated Acquisition run.

- **Rotation Center**
  When focusing, the central image features should stay on the optical axis and should not move.
  Perform this Direct Alignment at the highest magnification that will be used during the Automated Acquisition run.

- **Coma-free Alignment X and Y**
  For acquiring high resolution images, it is important that the illumination is coma-free.
  This procedure can only be done properly:

  - On a thin carbon foil.

  - With the camera in Search mode.

  - With live FFT.

  Image contrast and the FFT should not change when the beam is tilted.

Note	**Coma free overwrites rotation center, but the difference should not be critical.**

- **Astigmatism**
  In a simple FFT it is very difficult to distinguish astigmatism from beam tilt induced coma.
  Make sure that the system is nearly coma-free before correcting astigmatism.
  Use the objective stigmator to adjust astigmatism. This procedure can only be done properly:

  - On a thin carbon foil.

  - With the camera in Search mode.

  - With live FFT.

9.6 Calibrations

9.6.1 Magnification Calibrations

The Magnification 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. The Magnification Calibrations are generally very stable. They only need to be renewed when the system alignments have changed significantly.

The calibrations must be present:

- For all cameras.
- For LM, SA and, if used, Mi ranges.

Use the **TEM User Interface** > **Calibrations** or **Magnification Calibration** control panel to perform the Magnification Calibrations and follow the instructions in the control panel.

9.6.2 Focus Calibration

The Focus calibration is executed in the **EPU** > **Auto Functions** tab > **Calibrations**: **Autofocus** task.

This calibration may need to be redone more frequently, as it may depend on changes in the direct alignments. Redo this calibration if the Autofocus function does not converge nicely any more. Follow the instructions in The Autofocus Calibration Task on page 75.

9.6.3 Eucentric Correction Calibration (Optional)

The Eucentric Correction Calibration is executed in the **EPU** > **Auto Functions** tab > **Calibrations**: **Eucentric Correction** task. Follow the instructions in The Eucentric Correction Calibration Task on page 79.

10 Troubleshooting: Symptoms and Solutions

10.1 Troubleshooting: Atlas

Symptom	Possible Cause	Possible Solution
Atlas does not look consistent: • Tiles do not match. • Grid bars do not continue across neighboring tiles. • Atlas contains a regular pattern of black areas.	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.	Refocus.
	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?)	Recalibrate.
	Distortion can be inherent to images at very low magnification.	Acquire an atlas at increased magnification.
	The view is partly blocked (e.g., by an aperture) at the selected magnification.	Acquire an atlas at increased magnification; confirm with a preview (or TIA) that the field–of–view of a single tile is unobstructed.
	The system's beam and image shift are not well–aligned, causing loss of illumination.	Re–align the beam and image shifts for LM mode using the TEM User Interface (Alignments Control, Calibrate LM–Image/Beam calibration).

Symptom	Possible Cause	Possible Solution
"Move to" command does not center feature or grid square. Check this at the magnification at which the Atlas is acquired. Use the camera, not the FlucamViewer.	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.	Refocus.
	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?)	Recalibrate.
	Distortion can be inherent to images at very low magnification.	Acquire an Atlas at increased magnification.
	The Image Shift calibration is not performed accurately.	Accurately perform the Image Shift Calibration.

10.2 Troubleshooting: GridSquare

Symptom	Possible Cause	Possible Solution
"Move to" command (in Atlas) does not center the Grid Square. Atlas looks OK, but navigation to the grid square is inaccurate. Grid square is not centered when acquiring an image in the Location / Area Selection view.	Some image shift is applied (check in the 'Image Settings' control panel in the TEM User Interface).	Reset it.
	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.	Focus and/or redo the calibrations.
	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.	Adjust rotation center.
	Nonparallel beam or excessive defocus is used. (This results in small centering errors.)	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.
	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.	Repeat EPU image shift calibration or when shifts are large, adjust the LM lens series alignment.

Symptom	Possible Cause	Possible Solution
"Move Stage to" command does not center Foil Hole or feature. If you see this while staying at the magnification used for imaging the grid square, check the causes and solutions in this table. 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 holeFoil Hole or feature in Template Definition view."	Too much defocus can change image rotation. A non-parallel beam will lead to additional change in magnification.	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.
	Mechanical play in stage.	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).
	Calibrations are no longer valid (microscope alignments have changed?).	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.

Symptom	Possible Cause	Possible Solution
Measure Foil Holes: the original size of yellow glasses does not match at all.	Correct Quantifoil type was not selected at start of session or the size of the holes deviates from specification.	Set the size correctly in this view.
	Size of the holes deviates from specification.	Set the size correctly in this view.

10.3 Troubleshooting: Template Definition

Symptom	Possible Cause	Possible Solution
"Move Stage to …" command in Location Selection / Area Selection view does not center foil holeFoil Hole or feature in Template Definition view. 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):".	Some image shift is applied (check this in 'Image Settings' control panel in the TEM User Interface).	Reset it.
	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 in the acquired images. • 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.	Repeat EPU image shift calibration or when the shifts are large, adjust LM lens series alignment.

Symptom	Possible Cause	Possible Solution
"Move Stage here..." command in Template Definition view does not center feature.	Mechanical play in stage.	Try at least twice to make sure that a backlash correction was performed.
	Working far from eucentric focus will render the magnification calibrations invalid. Large defocus will slightly rotate the image and (with a nonparallel beam) magnification changes.	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 um.
	Calibrations are invalid (maybe the microscope alignment changed?).	Redo calibrations
	Stage may have a hardware problem and does not move correctly over these smaller distances	Call Service

Symptom	Possible Cause	Possible Solution
Yellow circle does not match Foil Hole size.	Measure Foil Holes/Find Foil Holes in the Location Selection view was not performed.	Return to Location Selection and do it.
	Specimen is not at eucentric height for the current sample position and not focused.	Bring specimen to eucentric height and focus it.
	Too large defocus is used and beam is not parallel. (The apparent magnification of the image may change under these circumstances.)	Select a defocus selected in the Template Optics settings of EPU of less than 20 µm. If possible, choose a parallel beam.
	The Measure Foil Hole step was inaccurate.	Repeat this step (Location Selection view) with a slight change in the size of the yellow glasses.
	Calibrations are invalid (maybe the microscope alignment changed?).	Redo calibrations

Symptom	Possible Cause	Possible Solution
"Find Foil Hole" does not succeed.	Does the yellow circle match the hole size? If not, see above, Yellow circle does not match Foil Hole size.	-
	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.)	Switch to the Location Selection view and perform Measure Foil Holes and Find Foil HoleFoil Holes.
	The circle size is wrong. (The algorithm of finding holes may be quite sensitive to the exact hole size.)	Repeat the Measure Foil Holes step (Location Selection view). Change the size of the yellow glasses slightly.
	Magnification is too high.	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 holeFoil Hole pattern can be exploited.
	Magnification is too low. (The current algorithm does not work with too many Foil Holes visible.)	Increase the magnification such that only the Foil Hole and its nearest neighbors are visible on the acquired image.

Symptom	Possible Cause	Possible Solution
	The current specimen location contains too many features (crystalline ice, etc.) that cause the algorithm to fail.	Find a cleaner sample area.
	Contrast is too low.	Add or increase the defocus in the optics settings. (Make sure to start from focused specimen, specimen at approximately eucentric height).
	Image quality too bad (noise). The human eye is very good at integrating over features, so noise may easily be underestimated at this magnification	Take image with higher dose.
	Foil Holes appear dark when the Allow Dark Foil Holes option is not selected.	If the Foil Hole appears darker than the foil itself, select the option Allow Dark Foil HoleFoil Holes (in ribbon of Location Selection view).

Symptom	Possible Cause	Possible Solution
Foil Hole centering fails (or hole is not correctly centered). Tracing this problem is a combination of tracing problems in: • "Find Foil Hole": see "'Find Foil HoleFoil Hole' does not succeed". Can be tested separately. • "Move stage here": see "'Move Stage here...' command in Template Definition view does not center feature". Can be tested separately.	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.	Reduce the magnification.

Symptom	Possible Cause	Possible Solution
Pattern acquisition fails.	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.	See above, Foil Hole centering fails (or hole is not correctly centered).
	Auto Focus fails.	See below, "Pattern acquisition: Auto Focus fails.".

Symptom	Possible Cause	Possible Solution
Pattern acquisition: Auto Focus fails.	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	Adjust eucentric height
	Cause: Auto focus does not converge because the beam is visible in the images. There maybe 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 the beam shift was not aligned.	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.
	Beam tilt pivot point misaligned.	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.

Symptom	Possible Cause	Possible Solution
	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?	Perform the Calibrate HM-TEM > Image/Beam calibration (use the 'Alignments' control panel in the TEM User Interface).
	Auto focus does not converge, although the beam edge is not visible; invalid focus calibration. Test with the standalone Auto Focus routine.	Redo focus calibration

Symptom	Possible Cause	Possible Solution
Pattern acquisition: beam edge visible in data acquisition	Acquire a single image with the Data Acquisition presets (Preparation tab). If the beam edge is visible on this image, this means Beam shift was not aligned correctly in the first place.	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.
	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 Image/beam shift calibration is not done.	Perform HM-TEM > Image/Beam calibration ('Alignments' control panel of the TEM User Interface).

Symptom	Possible Cause	Possible Solution
Pattern acquisition: acquisition areas are not placed correctly.	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.	Try again. This time the Z adjustment should be small.
	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.	Use the Direct Alignments panel to align the rotation center.

Symptom	Possible Cause	Possible Solution
	EPU image shift calibration no longer valid or not done. Use the Preparation tab to acquire images with the Hole/EucentricHeight 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.	Redo the EPU image shift calibrations. If the shift is very large, redo the microscope HM image shift alignment first.
	Image beam shift calibration not valid. (If you use image/beam shift only to place the acquisition areas, placement should generally be quite accurate.)	Perform HM-TEM > Image Beam calibration ('Alignments' control panel of TEM User Interface).
	Magnification calibration not valid. Test: Check 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.	Redo the magnification calibrations.

10.4 Troubleshooting: Automated Acquisition
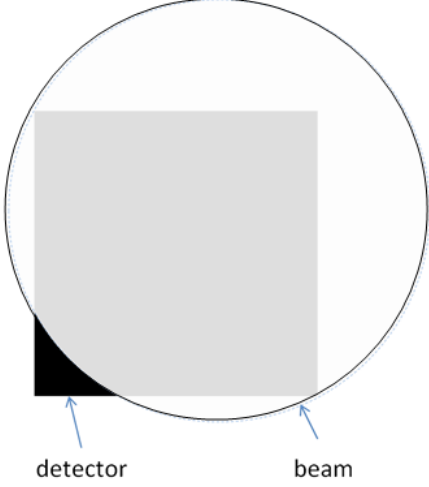
Symptom	Possible Cause	Possible Solution
Grid squares are all skipped.	The eucentric height procedure is failing. The performance of the auto-eucentric height procedure can be tested standalone in the Auto Functions tab. During automated data acquisition, the procedure is performed with the Hole/EucentricHeight 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.	Increasing the number of counts per exposure may help if the images are very noisy. The focus calibration may have to be redone.
	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.	Redo the "Measure Foil Holes" procedure that should have been done during the setup phase (Location Selection task).

Symptom	Possible Cause	Possible Solution
	No Foil Holes are selected. A grid square image is acquired, holes are found, but none is selected by the ice thickness filters.	Redefine the filters (go to the Location/Area Selection). If the intensity changes due to an instability of the gun, use the I0 calibration in EPU in addition. It will periodically measure the intensity in a hole and correct the ice filters accordingly.

Symptom	Possible Cause	Possible Solution
Too many Foil Holes: target areas are skipped.	They are not found in the grid square image in the first place because step(s) were omitted in the setup phase.	Repeat setup steps
	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.	Go to the Location/Area Selection view and adjust the ice filter settings
	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	For troubleshooting, see "'Find Foil Hole' does not succeed." and "Foil Hole centering fails (or hole is not correctly centered)".

Symptom	Possible Cause	Possible Solution
	Auto focus fails. A Foil Hole is skipped if the focus could not be determined.	For trouble shooting, see "Pattern acquisition: Auto Focus fails.". 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).

Symptom	Possible Cause	Possible Solution
Acquisition areas are not placed correctly.	See "Pattern acquisition: acquisition areas are not placed correctly"	
	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 to nothing can be done to fix this.

Symptom	Possible Cause and Solution

Beam is shifted in Autofocus routine.	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 one or both of the following causes:
	
	• Beam tilt pivot points not set correctly
	• 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).
	• Select 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.
	• Select Reset defocus.
	  Defocus value in the TEM User Interface 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,
	  • Reduce the camera area to Half or Quarter,
	  • Increase the beam diameter.

11 The MRC2014 Image Format

11.1 MRC2014 Main and Extended Headers

MRC files have a generic main header and an optional extended header.

- The main header contains generic image information, such as the image dimensions and the pixel format. For the specification of the main header,
  refer to MRC format specification (http://www.ccpem.ac.uk/mrc_format/mrc_format.php).

- The extended header can be used for storing application specific metadata.
  For Thermo Scientific and FEI products, the extended header contains information about:

  - The microscope state at acquisition time, such as magnification, accelerating voltage, stage position, beam shift.

  - Image acquisition information, such as binning and exposure time.

The space that is reserved for the extended header is specified by the NSYMBT field in the main header. The type of the extended header is specified by the EXTTYP field. Thermo Scientific and FEI applications use the FEI1 format, which is described below.

For every frame in the file, the extended header contains one block of metadata. The first element of each block contains the size of the block.
The NZ field in the main header specifies the number of frames in the file. This number has to be multiplied with the number of frames to get the total size of the FEI1 metadata. This total size fits within the size of the reserved extended header as shown below.
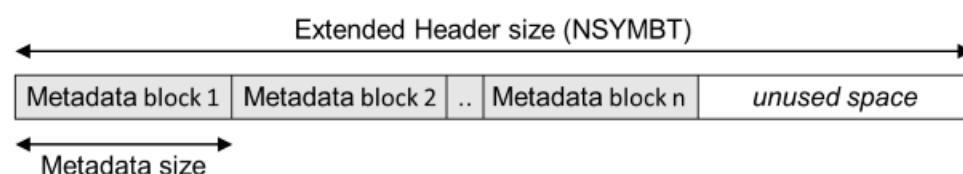
11.2 FEI1 Specification for the Extended Header

The tables below specify the content of the extended header for the FEI1 MRC2014 file format. In these tables, the **Format** and **'IsPresent' flag** columns have to the following values:

- **Format:**

  - 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

- **'Is Present' flag:**
  The bitmask is an unsigned integer. The integer value is calculated as:
  $[\texttt{byte 1}] + ([\texttt{byte 2}] \times 256) + ([\texttt{byte 3}] \times 2562) + ([\texttt{byte 4}] \times 2563)$

Image, System and Application

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Metadata size	0	0x0000	Int32	NA	Metadata size [bytes]
Metadata version	4	0x0004	Int32	NA	Version ID of the metadata format. 0 = Initial version which is described here
Bitmask 1	*8*	*0x0008*	*UInt32*	*NA*	*Individual bits indicate which metadata fields are set.*
Timestamp	12	0x000C	Float64	Bitmask 1 – #0	Time when the image was taken. The used format is the DATE data type that is used in OLE automation by Microsoft: Microsoft OLE DATE data type specification (https://msdn.microsoft.com/enus/llibrary/38wh24td.aspx)
Microscope type	20	0x0014	16 chars	Bitmask 1 – #1	Identifier for microscope type (Krios, Talos, Titan, Metrios, etc.)
D-Number	36	0x0024	16 chars	Bitmask 1 – #2	Microscope identifier
Application	52	0x0034	16 chars	Bitmask 1 – #3	Application name
Application version	68	0x0044	16 chars	Bitmask 1 – #4	

Gun

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
HT	84	0x0054	Float64	Bitmask 1 – #5	High tension [Volt]
Dose	92	0x005C	Float64	Bitmask 1 – #6	Dose [electrons/m²]

Stage

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Alpha tilt	100	0x0064	Float64	Bitmask 1 – #7	Holder Alpha tilt along axis [degr.]
Beta tilt	108	0x006C	Float64	Bitmask 1 – #8	Holder Beta tilt along axis [degr.]
X-Stage	116	0x0074	Float64	Bitmask 1 – #9	Stage X position [m]
Y-Stage	124	0x007C	Float64	Bitmask 1 – #10	Stage Y position [m]
Z-Stage	132	0x0084	Float64	Bitmask 1 – #11	Stage Z position [m]
Tilt axis angle	140	0x008C	Float64	Bitmask 1 – #12	Angle of tilt axis in image [degr.]
Dual axis rotation	148	0x0094	Float64	Bitmask 1 – #13	Measured rotation angle after b flip [degr.] (Tomography only)

Pixel Size

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Pixel size X	156	0x009C	Float64	Bitmask 1 – #14	Pixel size X [m]
Pixel size Y	164	0x00A4	Float64	Bitmask 1 – #15	Pixel size Y [m]

Optics

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Defocus	220	0x00DC	Float64	Bitmask 1 – #22	Defocus [m]
STEM Defocus	228	0x00E4	Float64	Bitmask 1 – #23	STEM defocus [m]
Applied defocus	236	0x00EC	Float64	Bitmask 1 – #24	Relative defocus applied by application [m]
Instrument mode	244	0x00F4	Int32	Bitmask 1 – #25	• 1: TEM • 2: STEM
Projection mode	248	0x00F8	Int32	Bitmask 1 – #26	• 1: Diffraction • 2: Imaging
Objective lens mode	252	0x00FC	16 chars	Bitmask 1 – #27	• LM • HM • Lorentz
High magnification mode	268	0x010C	16 chars	Bitmask 1 – #28	• Mi • SA • Mh
Probe mode	284	0x011C	Int32	Bitmask 1 – #29	• 1: NanoProbe • 2: MicroProbe
EFTEM On	288	0x0120	Bool	Bitmask 1 – #30	TRUE when the magnifications are adapted to the energy filter
Magnification	289	0x0121	Float64	Bitmask 1 – #31	Nominal magnification
Bitmask 2	***297***	***0x0129***	***UInt32***	***NA***	***Individual bits indicate which metadata fields are set.***
Camera length	301	0x012D	Float64	Bitmask 2 – #0	Nominal camera length [m]
Spot index	309	0x0135	Int32	Bitmask 2 – #1	-
Illuminated area	313	0x0139	Float64	Bitmask 2 – #2	• TEM: beam diameter in meters • STEM: not used • Undefined on 2 lens condenser systems
Intensity	321	0x0141	Float64	Bitmask 2 – #3	Uncalibrated measure of beam diameter on 2 lens condenser systems
Convergence angle	329	0x0149	Float64	Bitmask 2 – #4	[degr.] Undefined on 2 lens condenser systems

| Illumination mode | 337 | 0x0151 | 16 chars | Bitmask 2 – #5 | • None
• Parallel
• Probe
• Free
• Undefined on 2 lens condenser systems |
| Wide convergence angle range | 353 | 0x0161 | Bool | Bitmask 2 – #6 | Undefined on 2 lens condenser systems |

EFTEM Imaging

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Slit inserted	354	0x0162	Bool	Bitmask 2 – #7	-
Slit width	355	0x0163	Float64	Bitmask 2 – #8	Slit width [eV]
Acceleration voltage offset	363	0x016B	Float64	Bitmask 2 – #9	[Volt]
Drift tube voltage	371	0x0173	Float64	Bitmask 2 – #10	[Volt]
Energy shift	379	0x017B	Float64	Bitmask 2 – #11	[eV]

Image Shifts

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Shift offset X	387	0x0183	Float64	Bitmask 2 – #12	Corrective image or beam shift relative to exposure preset (in logical units) • TEM: pure image shift • STEM: image-beamshift-
Shift offset Y	395	0x018B	Float64	Bitmask 2 – #13	
Shift X	403	0x0193	Float64	Bitmask 2 – #14	Applied shift due to optimized position and tracking (in logical units) • TEM: image beam shift • STEM: beam shift-
Shift Y	411	0x019B	Float64	Bitmask 2 – #15	

Camera

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
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 name	435	0x01B3	16 chars	Bitmask 2 – #19	Name of the camera
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	-
Ceta frames summed	468	0x01D4	Int32	Bitmask 2 – #25	Number of frames summed for dynamic range
Direct detector electron counting	472	0x01D8	Bool	Bitmask 2 – #26	-
Direct detector align frames	473	0x01D9	Bool	Bitmask 2 – #27	-
Camera param reserved 0	474	0x01DA	Int32	Bitmask 2 – #28	-
Camera param reserved 1	478	0x01DE	Int32	Bitmask 2 – #29	-
Camera param reserved 2	482	0x01E2	Int32	Bitmask 2 – #30	-
Camera param reserved 3	486	0x01E6	Int32	Bitmask 2 – #31	-
Bitmask 3	*490*	*0x01EA*	*UInt32*	*NA*	*Individual bits indicate which metadata fields are set.*
Camera param reserved 4	494	0x01EE	Int32	Bitmask 3 – #0	-
Camera param reserved 5	498	0x01F2	Int32	Bitmask 3 – #1	-

Camera param reserved 6	502	0x01F6	Int32	Bitmask 3 – #2	-
Camera param reserved 7	506	0x01FA	Int32	Bitmask 3 – #3	-
Camera param reserved 8	510	0x01FE	Int32	Bitmask 3 – #4	-
Camera param reserved 9	514	0x0202	Int32	Bitmask 3 – #5	-
Phase Plate	518	0x0206	Bool	Bitmask 3 – #6	Indicates whether phase plate was used for data acquisition

STEM

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
STEM Detector name	519	0x0207	16 chars	Bitmask 3 – #7	-
Gain	535	0x0217	Float64	Bitmask 3 – #8	-
Offset	543	0x021F	Float64	Bitmask 3 – #9	-
STEM param reserved 0	551	0x0227	Int32	Bitmask 3 – #10	-
STEM param reserved 1	555	0x022B	Int32	Bitmask 3 – #11	-
STEM param reserved 2	559	0x022F	Int32	Bitmask 3 – #12	-
STEM param reserved 3	563	0x0233	Int32	Bitmask 3 – #13	-
STEM param reserved 4	567	0x0237	Int32	Bitmask 3 – #14	-

Scan settings

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Dwell time	571	0x023B	Float64	Bitmask 3 – #15	Dwell time per pixel [sec]
Frame time	579	0x0243	Float64	Bitmask 3 – #16	Frame time [sec] (currently it will not be used)
Scan size left	587	0x024B	Int32	Bitmask 3 – #17	-
Scan size top	591	0x024F	Int32	Bitmask 3 – #18	-
Scan size right	595	0x0253	Int32	Bitmask 3 – #19	-
Scan size bottom	599	0x0257	Int32	Bitmask 3 – #20	-
Full scan FOV X	603	0x025B	Float64	Bitmask 3 – #21	Field of view [m]
Full scan FOV Y	611	0x0263	Float64	Bitmask 3 – #22	-

EDX Elemental maps

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Element	619	0x026B	16 chars	Bitmask 3 – #23	-
Energy interval lower	635	0x027B	Float64	Bitmask 3 – #24	-
Energy interval higher	643	0x0283	Float64	Bitmask 3 – #25	-
Method	651	0x028B	Int32	Bitmask 3 – #26	-

Dose fractions

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Is dose fraction	655	0x028F	Bool	Bitmask 3 – #27	-
Fraction number	656	0x0290	Int32	Bitmask 3 – #28	-
Start frame	660	0x0294	Int32	Bitmask 3 – #29	-
End frame	664	0x0298	Int32	Bitmask 3 – #30	-

Reconstruction

Name	Offset (dec)	Offset (hex)	Format	'Is Present' flag	Description
Input stack filename	668	0x029C	80 chars	Bitmask 3 – #31	-
Bitmask 4	***748***	***0x02EC***	***UInt32***	***NA***	***Individual bits indicate which metadata fields are set.***
Alpha tilt min	752	0x02F0	Float64	Bitmask 4 – #0	
Alpha tilt max	760	0x02F8	Float64	Bitmask 4 – #1	

11.3 Pixel Sequence in the MRC2014 Format

In the MRC2014 files, the image pixel data is stored as rows from top to bottom, where each row is stored from left to right.
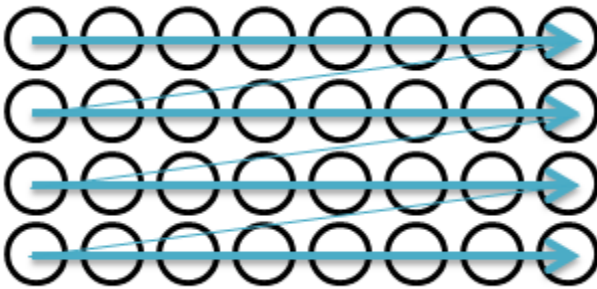
Most image viewers and image processing applications use the same pixel position sequence as the MRC file. Some image viewing and processing applications such as IMOD and Fiji/ImageJ use a pixel position sequence. In these applications, the image display may be mirrored and/or rotated.

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Revision Table

Revision	Date	Description of Changes
A - K		EPU 1.X User Manual revisions
L	12-APR-2018	Last version for EPU 1.X
M	12-OCT-2018	Update for EPU 2.0 - 2.1
N	14-JAN-2019	Update for EPU 2.2
O	APR-2019	Update for EPU 2.3
P	OCT-2019	Update for EPU 2.4 - 2.5
Q	JAN-2020	Update for EPU 2.6
2.7	APR-2020	Update for EPU 2.7

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