Thermo Fisher SCIENTIFIC

Tomography

for TEM and EFTEM mode
User Manual

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1 Introduction

1.1 Introduction to Tomography

The Thermo Scientific Tomography software provides the functionalities to prepare and execute the automated acquisition of one or more *Tilt Series*. A Tilt Series is a set of images of the same specimen feature, viewed at different tilt angles. The Tilt Series images are processed by reconstruction software to create a 3D reconstruction of the specimen feature.

The Tomography software aims to:

- Enable fast, reliable, automated Tilt Series acquisition.
- Produce high quality Tilt Series images for successful reconstruction of the largest achievable volume.

  This requires maximum stability of the position and focus of the feature of interest at each Tilt Series angle.

Tomography facilitates the following steps in the data acquisition procedure:

- Screening of multiple specimens to select the specimens and features with the highest potential for high quality data acquisition.
- High throughput, high resolution data collection.

1.2 Audience

This manual is aimed at users of Thermo Scientific Transmission Electron Microscopes. In particular, you should be able to successfully identify issues with the microscope. Morover, you should be able perform the necessary corrective actions to resolve the identified issues, provided that these actions are available. For higher level corrective actions, the assistance of a Thermo Fisher Scientific service engineer may be required.

For online training materials to help you improve your Cryo-EM skills, visit the EM-learning.com website.

1.3 System and software compatibility

The Thermo Scientific Tomography software is available for Thermo Scientific TEM systems and FEI TEM systems that run up-to-date microscope software. For detailed system and software version compatibility information, see the Tomography Release Notes.

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

2 Getting Started

Start

Verify that the TEM server is running before starting Tomography software.

Tomography software can be started from the Windows Start menu (**All programs** > **Tomography**).

2.1 Prepare for an Tomography session

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

1. Verify that an **Alignments File** and a **FEG Register** are loaded that match with the High Tension and Extractor settings of the system.
2. Verify that the system is in **TEM mode**.
3. Verify that all **cameras** are cooled and at a stable temperature.

For an extensive preconditions check, see Detailed Preconditions for Successful Tomography Usage on page 167.

Detailed instructions for the individual alignments and calibrations are available in the online help of the TEM User Interface.

2.2 Recommendations for loading the specimen on a holder for a Side Entry system

For most Tomography experiments, a flat specimen with a diameter of 3 mm is used. This specimen is often mounted on a copper grid. In such flat samples, the tilt angle at which high quality Tomography data can be acquired is limited by the orientation of the copper grid and the holder geometry. At higher tilt angles, the holder tip and the grid bars may cast a shadow.

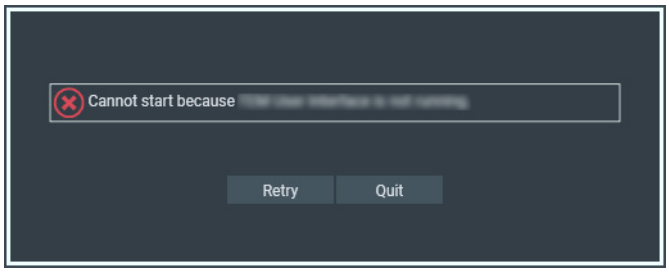
To minimize or prevent shadowing:

- Load the specimen onto the holder with its grid bars at a 45 degree angle relative to the tilt axis. This increases the angle at which the shadow of the grid bars reaches the center of the GridSquare, so the usable tilt angle range is enlarged.
- Use a rod shaped specimen with a Fischione 2040/2045 or similar holder.

2.3 Start the Tomography software

1. Verify that the system is in **TEM mode** or in **EFTEM mode**.
2. Start **Tomography**
   Shortcuts can be found on the desktop and in the Windows Start menu.

   Tomography starts the user interface for the current optics mode.
3. A splash screen appears while Tomography runs the startup checks.
   If one or more checks fail, the pop-up displays the related messages.



   - Solve the reported issue(s).
   - Select **Retry**

2.4 Log in on Thermo Scientific Athena

Note The use of Tomo Live and Athena requires a separate license.

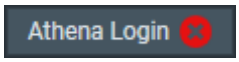
Thermo Scientific **Athena** software is part of the Data Management Platform (DMP) services. Logging in to Athena grants your Tomography software access to a number of services on the DMP Server. The services and their respective functions are:

- *Athena* itself; used for the automated upload of acquired data into a selected dataset.
- *Tomo Live*, which offers:
  - automatic reconstruction of the tomogram during batch acquisition for the purpose of review and quality control.

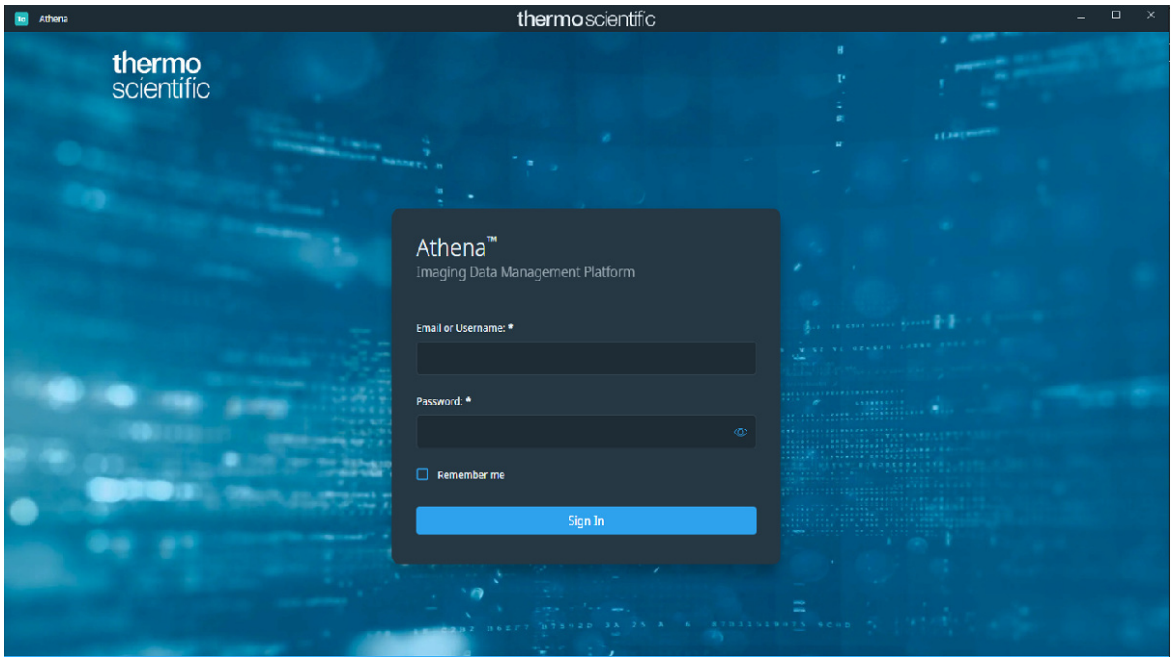
- reconstructed datasets which can be viewed anywhere using our web-based portal.
- filtering based on certain parameters and/or manual rating.
- exporting in batches ready for segmentation and processing.

To connect to Athena, follow the instructions below:

1. In the lower-right corner of the Tomography user interface, select **Athena Login**

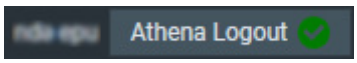
   

   The red dot indicates that there is no active connection to Athena yet.
2. The *Thermo Scientific Athena* login screen appears.

3. Enter the **username** and **password** for Athena.

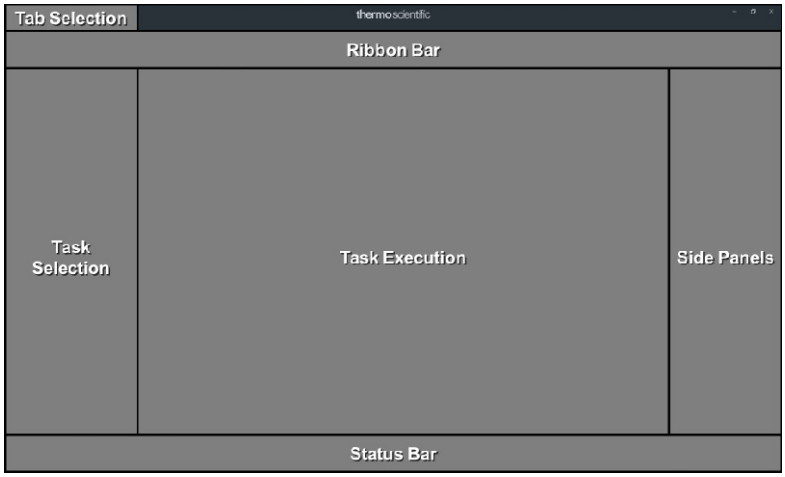
   If the login is successful, the Athena connection indicator turns green and the username is displayed.

3 The User Interface

3.1 User interface panels

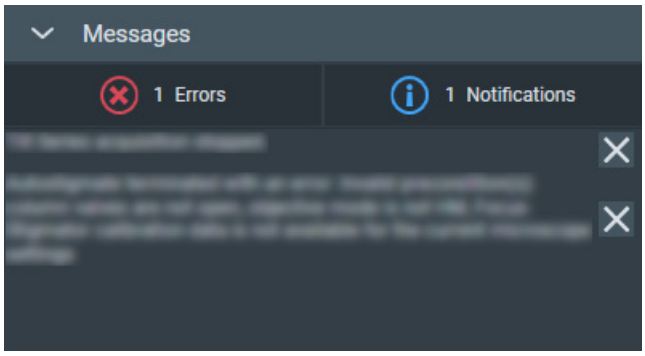
The Tomography user interface guides the user through all actions that are needed to prepare and execute an Automated Acquisition run.



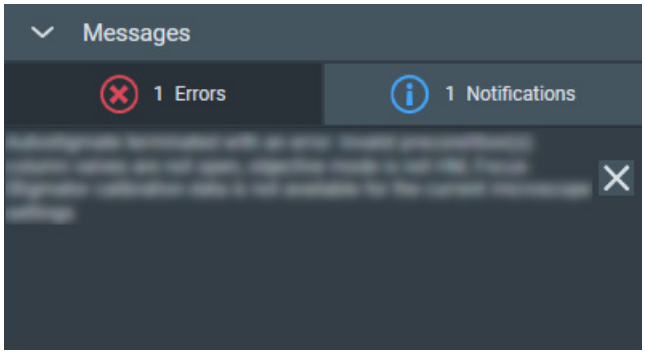
- **Tab Selection**
  The tabs are typically worked through from left to right. Each tab provides a set of tasks.
- **Task Selection**
  Tasks are typically executed from top to bottom. The set of available tasks depends on the selected tab.
- **Task Execution**
  The content of the Task Execution pane 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.
- **Status Bar**
  The Status Bar displays the Athena login and status. The Status Bar is only visible when Athena is available.

3.2 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.

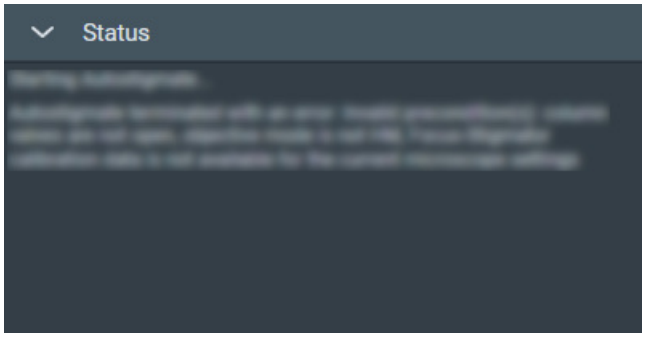


The same applies to the Error messages.

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 Status side panel



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

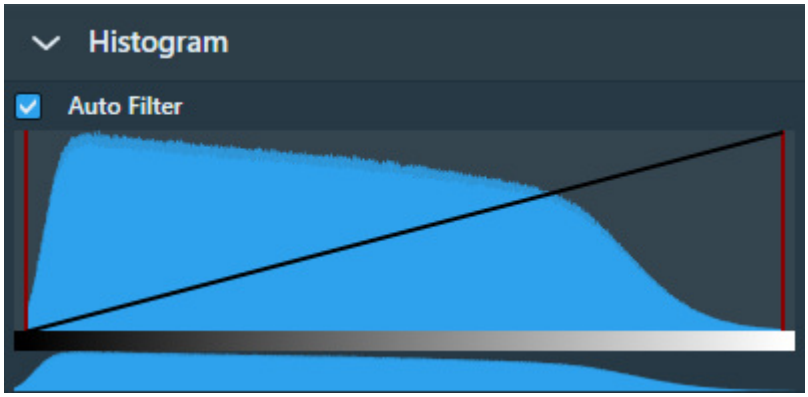
- Errors and Notifications.
- Progress messages for ongoing automated procedures.
- Intermediate and final results of automated procedures.
- Instructions and recommendations to the user.

3.4 Histogram side panel

If two or more images are present in the Task Execution panel, then the Histogram applies only to the selected image. The selected image is recognizable by the highlighted image title and frame.

3.4.1 Histogram side panel

The Histogram side panel shows a histogram of image-pixel intensities for the selected image in the Image Display.



The Histogram side panel offers the following functionalities:

Auto Filter:

- Ticked:
  Tomography 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:

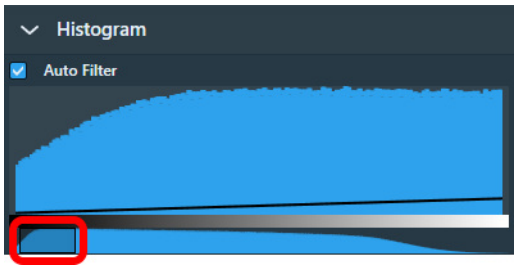
Drag the **red line at the right-side** of the main histogram to adjust the white level. Pixels with an intensity above the white level value are displayed with maximum intensity (white).

Gamma:

Drag the **diagonal black line** up or down to adjust the Gamma curve.

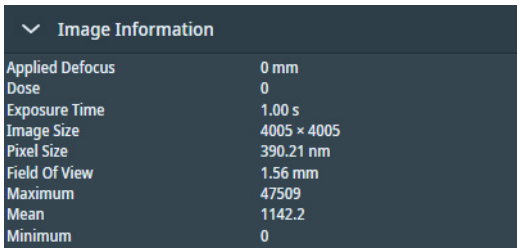
If the Black Level and/or White Level are adjusted, then the Gamma curve is scaled proportionally in the range between the Black Level and White Level values.

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 Image Information side panel

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



Example for a camera image.
For STEM images, different parameters can be displayed.

3.6 Image and plot display area

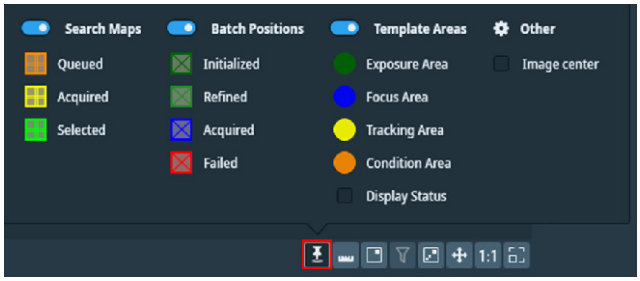
The availability of functionalities for the Image Display depends on the active task, the applied Acquisition and Optics Preset and the selected image.

Zoom slider

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

Image Markers menu

Specify which image markers are displayed on an image.



Measurement tool

Enables you to measure real distances on an acquired image by drawing rulers.

Show/hide tiles locations

Shows or hides the outlines of the tiles in the image.

Show/hide tilt axis

Shows or hides the Tilt Axis in the image.

Show/hide template areas

Shows or hides the Template Areas in the image.

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 image (FFT)

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

Filter the FFT image

The FFT filter optimizes the contrast and brightness of the FFT, so that for example Thon Rings are shown clearer. The FFT filter does not change the acquired image data 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 the inset and main image

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 main 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 to 100%

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 the panning window

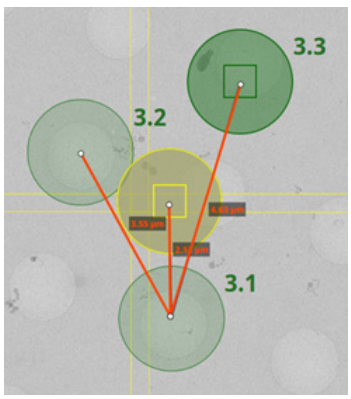
Show or hide the panning inset window.

3.6.1 Zoom in/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 The Measurement tool

The measurement tool enables you to measure real distances on an acquired image by drawing rulers.



Basic use: To draw a single ruler:

1. Click on the Measurement tool icon .
2. Click on a point of interest on the image.

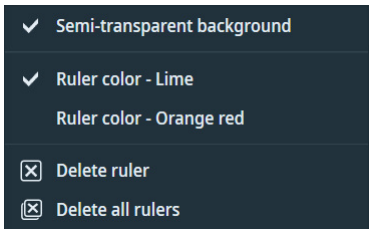
3. Move the mouse to a second point of interest on the image and click to place the second end of the ruler.

Multiple rulers: To draw more rulers, repeat the steps above.

Measurement: The length of a ruler is displayed next to it. This measurement is the real distance on the sample, e.g. measured in µm.

Edit: Click and drag any of the two endpoints of a ruler to change its position. Click and drag the body of the ruler to change its position.

Delete: Right-click a ruler to display the following context menu, then click Delete ruler.



The right-click menu also allows you to:

- Delete all rulers on this image.
- Choose between two colors for all rulers on all images.

Persistence: The rulers drawn on an image remain there until you either:

1. Quit Tomo.

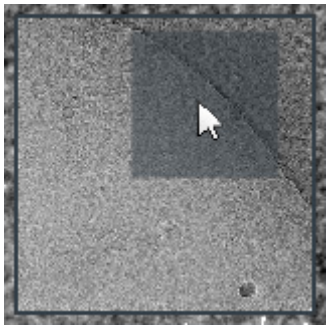
2. Perform a new acquisition, a new search image, etc.

3.6.3 Navigate and pan in a zoomed image

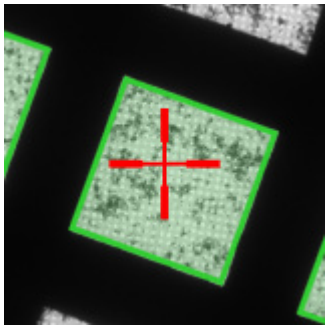
To navigate and pan in an image, either drag the image with the mouse, or use the panning inset.

1. Select the **panning inset**

2. Drag the **dark gray square** across the **panning inset**



3.6.4 The red crosshair

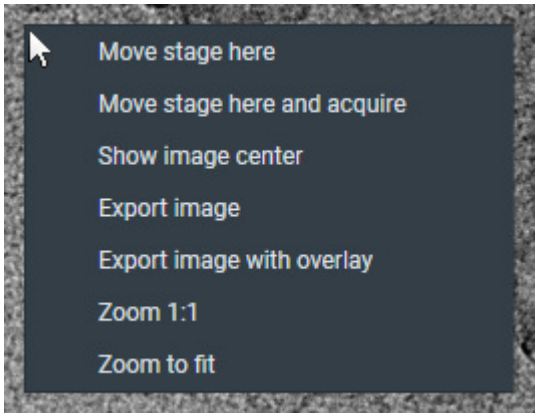


The red crosshair is commonly referred to as the *stage position*. Although the red crosshair moves when the stage moves, it does not mark the stage position itself. The red crosshair marks the *center of the field of view* if a new image were acquired.

3.6.5 Export an image to file

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 resolution of the image file is the same as as displayed in Tomography. This may be less detailed than the original image.

4 Preparation Tab

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

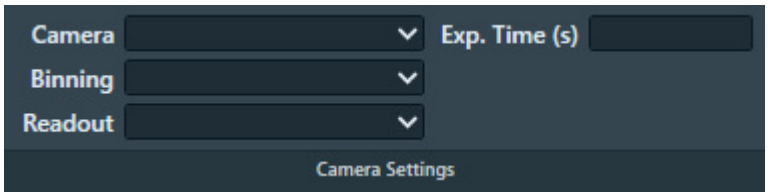
4.1 Acquisition and Optics Settings task

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:

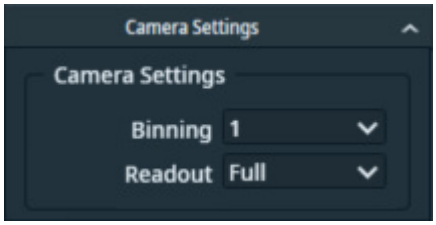
- Camera Settings.
- Advanced camera and/or exposure settings
  (not for all camera types).
- Optics Settings.

4.1.1 Description of the Camera Settings

4.1.1.1 Camera Settings for all camera types



The Binning and Readout parameters may be found in the tray of Camera Settings.



For the Thermo Scientific Falcon 3EC and Falcon 4(i) 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.
  For the exposure preset, the Binning mode is always set to 1.
- *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 4096x4096 sensor, all the signal of all pixels is used.
- *Half*: on a 4096x4096 sensor, the signal of a 2048x2048 area around the center is used.
- *Quarter*: on a 4096x4096 sensor, the signal of a 1024x1024 area around the center is used.

A smaller Readout value:

- Decreases the field of view.
- Does not affect the image resolution.
- Does not affect the signal strength of the image pixels.

Exp. time (s)

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

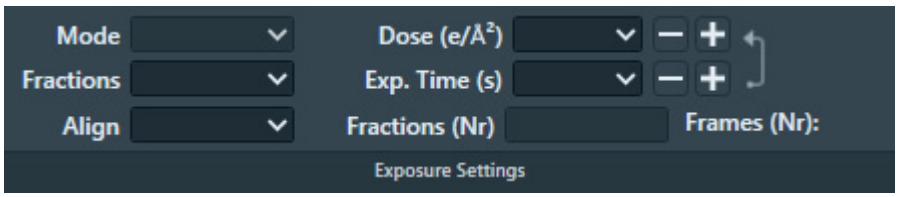
Specify the time during which the camera sensor is exposed to the electron beam.

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.

Tomography validates the specified value. If necessary, Tomography adjusts the specified value to the nearest valid value and shows a message.

4.1.1.2 Exposure Settings for Thermo Scientific Falcon 3EC and Falcon 4(i) cameras



Mode

Select the integration mode.

The following options are available:

- *Linear*: the normal integrating mode.
  For the Falcon 4(i) camera, the Linear mode is not available in the Data Acquisition preset.
- *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.

Note For the Falcon 4(i) camera, The *Counted* mode is selected default.

Mode

Select the integration mode.

The following options are available:

- *Linear*: the normal integrating mode.
  For the Falcon 4(i) camera, the Linear mode is not available in the Data Acquisition preset.
- *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.

Note For the Falcon 4(i) camera, The *Counted* mode is selected default.

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 Dose Fractions can be saved as separate images, next to the integrated image. The total number of frames depends on the exposure time and the internal frame rate of the camera.

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.

  Only for the Thermo Scientific Falcon 4(i) camera.

  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 the coordinates and a Gain Reference image.
  - An integrated image of the recorded specimen area.
  - A copy of the gain reference is supplied in the same folder containing the movies when using EER or TIFF as output for the data acquisition preset.

  In EER mode, the size of the acquired data 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 view and process `*.eer` files, specialist software is required.

Fraction settings are not applied when acquiring images for focusing and other preparatory actions.

Compression

Select whether or not the saved Dose Fraction images are compressed into TIFF LZW format. The compression is applied on acquired images stored in TIFF at native 4k x 4k resolution. To activate compression, set **Fractions** to **manual** or **max**, specify the number of fractions desired, and set **Compression** to **Yes**.

Note The Compression option is only available with Falcon 4(i) and TEM Software version 7.17 and higher.

Compression and Native Resolution

When Compression is enabled, the images are stored in their native 4k x 4k resolution. This ensures that the highest level of detail is preserved in the compressed images. The compression is non-gain-normalized, meaning that it does not alter the gain of the image during the compression process. This allows for a more accurate representation of the original image in the compressed format.

Align

Select whether or not the camera frames are aligned before summing.

This parameter is found in the tray of Camera Settings.

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:

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

Dose and **Exp. Time**

The values of the Dose and Exposure Time 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.

If the Dose Rate is known, then Tomography automatically calculates Dose or Exposure Time. Tomography uses the *leading* parameter as the basis to calculate the value of the other parameter. In the 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, either:

- Select the **arrow** to reverse it.
- Select the **Exp. Time** input field.
- Select **[+]** or **[–]** to adjust the Exposure Time value.

Fractions (Nr)

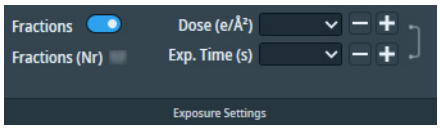
- *Manual*: manually specify the number of Dose Fractions.
- *Auto*: Tomography calculates the number of Dose Fractions.
  - The average dose in each Dose Fraction must be at least 1 e/px. This requires that the the Dose Rate is known.
  - For Falcon 3EC cameras there are additional conditions.
    See: The automatic Dose Fractions calculation for Falcon 3EC and Falcon 4(i) cameras on page 22.

Frames (Nr)

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

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

4.1.1.3 Exposure Settings for the Thermo Scientific Ceta-F camera



Fractions

- *Enabled*: acquire and store Dose Fractions.
- *Disabled*: acquire and store only integrated images.

Fractions can only be enabled for the Data Acquisition preset.

Frames (Nr)

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

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

Dose and **Exp. Time**

The values of the Dose and Exposure Time 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.

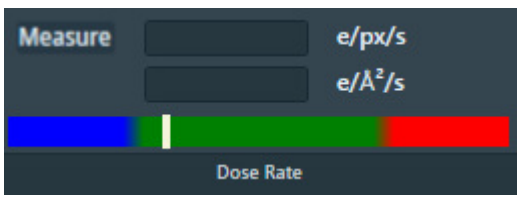
If the Dose Rate is known, then Tomography automatically calculates Dose or Exposure Time. Tomography uses the *leading* parameter as the basis to calculate the value of the other parameter. In the image below, *Dose* is the leading parameter.

- The arrow points from Dose to Exposure Time.
- Exposure Time is grayed out.

To make *Exposure Time* leading, either:

- Select the **arrow** to reverse it.
- Select the **Exp. Time** input field.
- Select **[+]** or **[–]** to adjust the Exposure Time value.

4.1.1.4 Dose Rate for Thermo Scientific cameras



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.

The acceptable Dose range in Tomography can be different than the Dose range that is used in Velox. The range in Velox is based on the technical range of the camera. The value in Tomography is based on what a typical life-science specimen can handle before it becomes severely damaged.

4.1.1.4.1 The automatic Dose Fractions calculation for Falcon 3EC and Falcon 4(i) cameras

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.
  - For the Falcon 3EC camera, when *Align* is *Yes*, the number of frames per Dose Fraction in the initial distribution must be a multitude of 6.

    For the Falcon 4(i) camera there is no additional condition when Align is Yes.

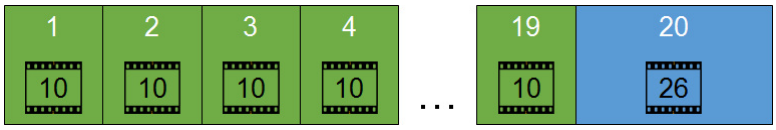
- Remaining frames are added to the last Dose Fraction.

The rules above may lead to unexpected fractionation schemes. Run a test acquisition to check the distribution. For the 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:

- Falcon 4(i), or Falcon 3EC with *Align* is *No*

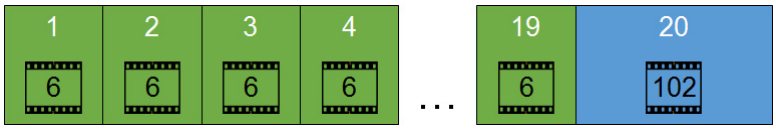


  - `216 / 20 = 10.8`

    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.

- Falcon 3EC with *Align* is *Yes*

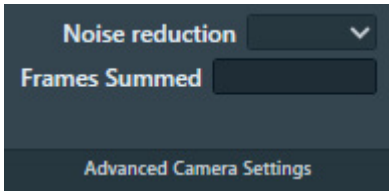


  - `216 / 20 = 10.8`

    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.

4.1.1.5 Advanced Camera Settings for Thermo Scientific Ceta cameras



Noise reduction

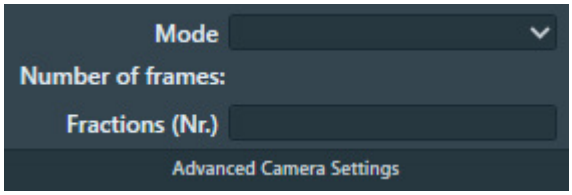
Select **Yes** to decrease the readout noise at low dose. Enabling Noise reduction decreases the maximum frame rate by up to 50%.

Noise reduction is only available when *Frames Summed* is *1*.

Frames Summed

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

4.1.1.6 Advanced Camera Settings for Gatan K2 and K3 cameras



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. The number of frames depends on:

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

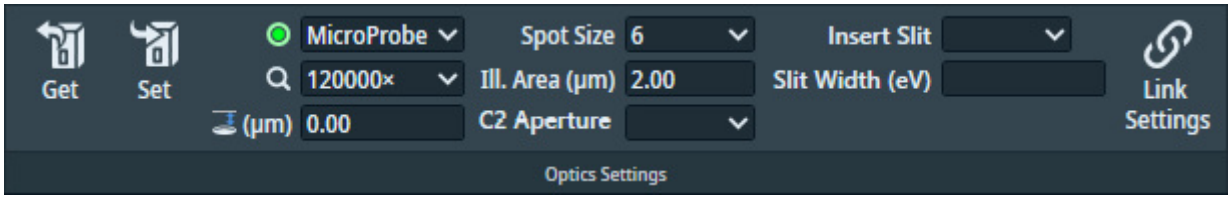
Fractions (Nr)

Specify the number of Dose Fraction images.

The specified value is only applied when acquiring images with the Data Acquisition Preset. When acquiring a preview image, the Dose Fraction images are saved in the `Preparation Preview` sub-folder of the camera root folder.

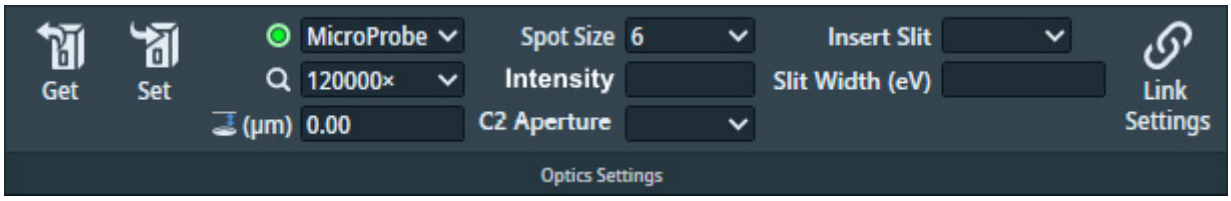
4.1.2 Description of the Optics Settings

Optics Settings for microscopes with a C3 lens.
These are typically the High End systems with Titan software.



The beam diameter and magnification are used to calculate the illuminated area on the specimen.

Optics Settings for microscopes without a C3 lens.
These are typically the Mid Range systems with Talos software.



The beam diameter is displayed as an *Intensity* value. On a microscope with two condenser lenses it is not possible to calculate the beam diameter or the illuminated area on the specimen.

Note *Insert Slit* and *Slit Width* are only available in EFTEM mode on a system with an energy filter.

Get

Imports the Optics Settings parameter values from the microscope.

Set

Applies the Optics Settings parameter values to the microscope.

Link Settings

- Link the Optics settings of high SA presets to the Exposure Preset. You can link any of the following presets: Tracking, Focus, Thon Ring and Zero Loss.
- Certain parameters from the Exposure Preset are linked, i.e., ProbeMode, Magnification, Spot Size, Illuminated Area, Intensity, Insert Slit and Slit Width
- You can quickly recognize which presets are linked through the icon displayed next to the linked preset in the dropdown selection menu.
- The Exposure Preset itself cannot be linked.

Probe mode

The microscope software remembers the last used defocus value for MicroProbe and for NanoProbe mode. When returning to a probe mode, the last used defocus value for that probe mode is automatically restored. Because of this behavior, some of the Presets must use the same Probe mode.

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

- All High SA Presets: Tracking, Focus, Drift, Eucentric Height
- Autofunctions: Eucentric height by beam tilt, Eucentric height by stage tilt, Autofocus

Magnification

The Magnification determines the field of view, and therefore also the dimensions of the imaged area per pixel (or pixel size).

Defocus

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

Tomography applies the specified defocus relative to the current focus, not relative to eucentric focus.

Spot Size

The Spot Size determines the beam current. A higher Spot Size value corresponds to a lower beam current, and therefore a lower Dose Rate.

Illuminated Area (Ill. Area)

Only available on microscopes with three condenser lenses.

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 ideal beam has the following properties:

- The beam is parallel.
- The beam diameter is just large enough to fully cover the camera field of view.
- The beam has no distortions or aberrations along the edges.

Intensity

Only on microscopes with two condenser lenses.

The Intensity value determines the spreading of the beam. On a system without a C3 lens, the beam diameter can not be locked to a specific size. If the Probe, Magnification or Spotsize value changes, then the Intensity value may require adjustment as well.

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 ideal beam has the following properties:

- The beam is parallel.
- The beam diameter is just large enough to fully cover the camera field of view.
- The beam has no distortions or aberrations along the edges.

C2 Aperture

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

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

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

Insert Slit

Only available in EFTEM mode on a system with an energy filter.

Select **Yes** to insert the slit.

Slit Width (eV)

Only available in EFTEM mode on a system with an energy filter.

Specify the electron energy bandwidth that can pass through the slit.

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 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 most efficient sequence to define the Presets is slightly different from their order in the Presets list:

1. Exposure
2. Presets that use (almost) identical Optics Settings as the Exposure Preset:
   a. Tracking
   b. Focus
   c. Thon Ring
3. Eucentric Height
4. Search / Template

5. Overview / Positioning
6. Atlas

In Data Acquisition run, the data acquisition step is the value-creating action. To get the best quality images for 3D reconstruction, the Exposure 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 Exposure Preset as the basis for all other Presets that are used during the Data Acquisition run.

4.1.4 Define the Exposure Preset

The Exposure Preset is used to acquire the images that will be used for 3D reconstruction of the imaged feature. The parameter values of the Exposure Preset depend only on the requirements for successful 3D reconstruction.

Parameters	Typical Settings and Recommendations

Optics Settings	• Probe Mode: Nanoprobe.
	Nanoprobe is well suited for a narrow parallel beam at high magnifications.
	• Magnification must match feature size and the required resolution.
	• Defocus: apply a small amount of defocus if necessary.
	• Illuminated Area / Intensity:
	  • A small Illuminated Area prevents double exposure when the features of interest are close together.
	  • A larger Illuminated Area is preferred when the features of interest 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.
	• Parallel beam.
	• Intensity Zoom is Off.
	When using Phase Plates, the Optics Settings must be defined with on-plane conditions. For instructions, see:
	• Guidelines for the Exposure Preset when using Phase Plates on a microscope with a C3 lens on page 32
	• Guidelines for the Exposure Preset when using Phase Plates on a microscope without a C3 lens on page 35

Camera Settings	• Binning: 1 • Readout: Full These values for the Binning and Readout parameters cannot be modified in the Exposure Preset.
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.

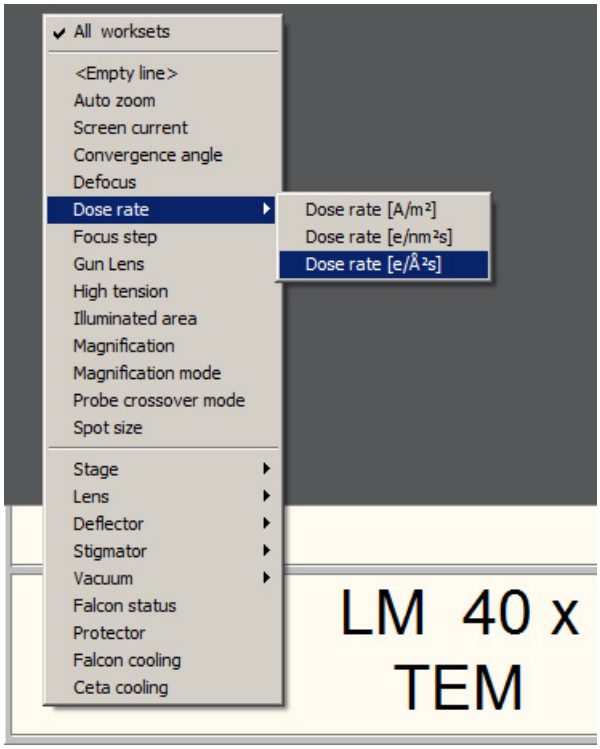
Use the procedure below to set the Exposure Preset.

1. Select the **Preparation** > **Acquisition and Optics Settings** task.
2. Select **Preset Selection** > **Presets**: **Exposure**
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.*
4. Use the handpanels to create an image that meets the requirements.
   If desired, use the FFT of the live image to assess the image quality.
5. 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 and Ceta-F cameras, use Tomography 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]**



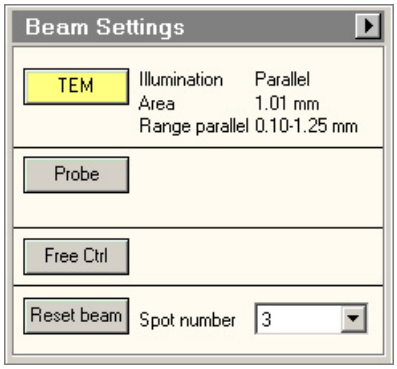
c. Note the current **Dose Rate** value.

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

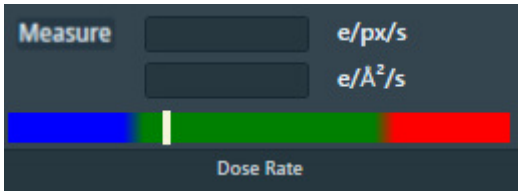
e. Verify that the **Dose Rate** value is unchanged.

f. Decrease the magnification by two index steps, back to the initial value.
g. Verify that the **Dose Rate** value is unchanged.

6. Verify that the beam is parallel.
   Depending on the system type, either:
   - On a system with a C3 lens (typically High End systems with Titan software), verify that **Beam Settings** control panel > **Illumination** is **Parallel**.



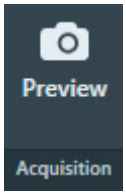
   - On a system without a C3 lens (typically Mid Range systems with Talos software), verify that the **specimen** and the **Objective aperture** are both focused.
     If no Objective aperture is inserted, then:
     - Select a small Objective aperture.
     - Verify that the aperture is focused.
     - Return the Objective mechanism to its initial position.
7. Select **Optics Settings** > **Get** to import the current optical settings from the microscope.
8. In **Camera Settings**:
   a. Select the **Camera**.
   b. If the camera is *not* a Thermo Scientific Falcon 3EC or Falcon 4(i), then specify the **Exp. Time (s)**.
      For Thermo Scientific Falcon 3EC and Falcon 4(i) cameras, this parameter is specified in *Exposure Settings*, after the Dose Rate has been measured.
9. If a Thermo Scientific Falcon 3EC or Falcon 4(i) 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.

10. Depending on the selected Camera, also specify the additional camera-specific parameters:
    - Thermo Scientific Falcon 3EC / Falcon 4(i): **Exposure Settings** with the **Exp. Time (s)** and **Dose** parameters.
    - Thermo Scientific Ceta: **Advanced Camera Settings**
    - Gatan K2 / K3: **Advanced Camera Settings**
11. Select **Acquisition** > **Preview**



12. Verify that the acquired image meets the requirements above.

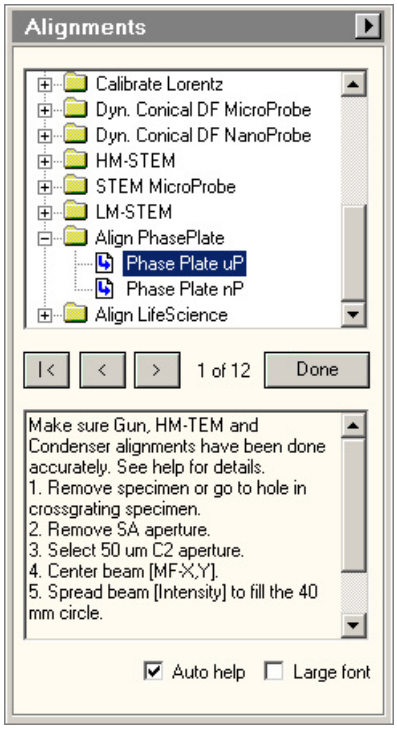
4.1.4.1 Guidelines for the Exposure Preset when using Phase Plates on a microscope with a C3 lens

Microscopes with a C3 lens are typically the High End systems with Titan software.

4.1.4.1.1 Perform the Phase Plate Microprobe (uP) Alignment

On systems with Titan software, 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.

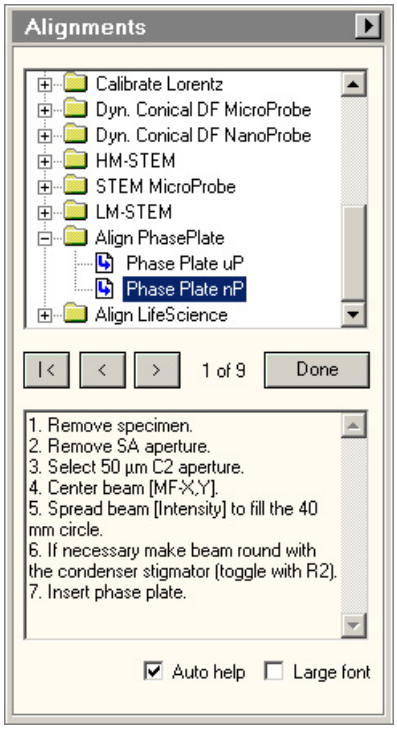
After the Phase Plate Microprobe (uP) alignment procedure is completed, continue with the Phase Plate Nanoprobe (nP) alignment procedure.

4.1.4.1.2 Perform the Phase Plate Nanoprobe (nP) Alignment

Perform the Phase Plate Nanoprobe (nP) alignment procedure.

Note On systems with Titan software,
the Phase Plate nP Alignment must be preceded by the Phase Plate uP Alignment.

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.

4.1.4.1.3 Verify the on-plane illumination on a microscope with a C3 lens

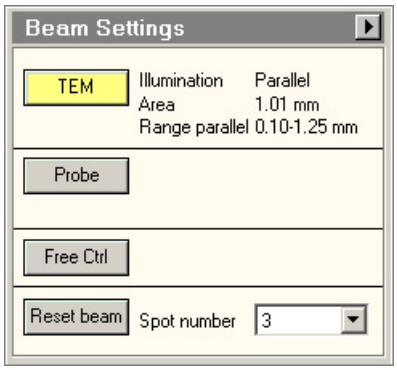
When the beam is focused onto the Phase Plate, the illumination is called *on-plane*. In all other cases, the illumination is called *off-plane*.

For the desired illumination conditions, the on-plane conditions must be verified. On-plane illumination of the phase plate implies parallel illumination of the specimen.

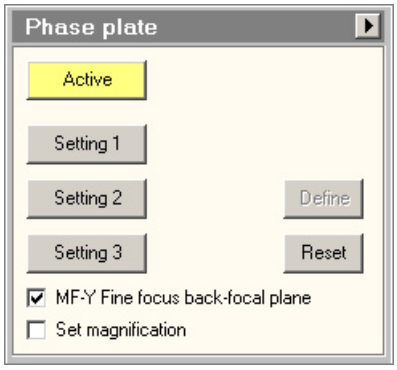
Follow the steps below to attain on-plane illumination:

1. If the *Exposure Preset* is already completed, then:
   a. Select **Tomography** > **Preparation** > **Acquisition and Optics Settings** > **Presets** > **Preset**: **Exposure**
   b. Select **Optics Settings** > **Set**
2. If the *Exposure 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 Exposure Preset when using Phase Plates on a microscope without a C3 lens

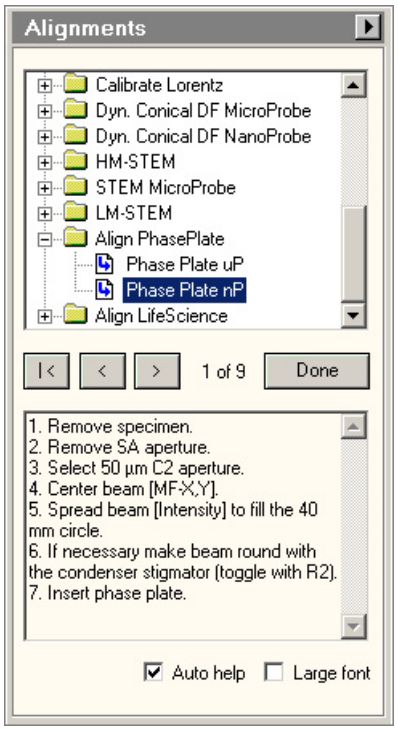
Microscopes without a C3 lens are typically the Mid Range systems with Talos software.

4.1.4.2.1 Perform the Phase Plate Nanoprobe (nP) Alignment

Perform the Phase Plate Nanoprobe (nP) alignment procedure.

Note On systems with Titan software,
the Phase Plate nP Alignment must be preceded by the Phase Plate uP Alignment.

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.

4.1.4.2.2 Verify the on-plane illumination on a microscope without a C3 lens

Follow the steps below to achieve on-plane illumination:

1. If the *Exposure Preset* is already completed, then:
   a. Select **Tomography** > **Preparation** >
      Acquisition and Optics Settings > **Presets** > **Preset**: **Exposure**
   b. Select **Optics Settings** > **Set**
2. If the *Exposure 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 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 illuminated area are changed, the on-plane condition needs to be verified again.

4.1.5 Define the Tracking Preset

The Tracking Preset is used to center the feature of interest after a tilt angle step.

To minimize the overhead time due to lens normalizations it is best to use the same Optics Settings as the Exposure Preset. If these settings do not result in a good tracking performance, the following adjustments can help:

- Increase the exposure time obtain a stronger signal.
  This will not affect the lens currents.
- Adjust the optics settings only when necessary:
  - Increase defocus for better contrast, so that the detection of usable features is faster and more reliable.
  - Decrease the magnification if the feature of interest moves more than half of the field of view after a tilt step.

In a Low Dose Tomography session, the Tracking function is performed on an area near the feature of interest, so that the feature itself is not exposed. The location of the Tracking Area is defined in the *Tomography* > *Batch Position* or *Sample Navigation* task. If the feature of interest moves more than half of the field of view after a tilt step, then move the Tracking Area a bit further away from the Exposure Area, so that the feature of interest is not exposed.

The Tracking function uses the same *High SA* filter settings as the Exposure and Focus Presets.

Parameters	**Typical Settings and Recommendations**
Optics Settings	Same as the Exposure Preset. Only when necessary, increase defocus or decrease the magnification.

Camera Settings	• Mode: Counted.
	• 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, so Binning 1 does not increase the accuracy of the Autofocus function, but the image acquisition may take more time.
	  • CMOS cameras:
	  A higher Binning value 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 Exposure Preset.

Use the procedure below to set the Tracking Preset. In place of steps 1 to 3 below, we recommend using the **Link** option to link the values of the Tracking Preset to those of the Exposure preset.

1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   c. Select **Optics Settings** > **Set**
2. Select **Preset Selection** > **Presets**: **Tracking**
3. Select **Optics Settings** > **Get**
4. In **Camera Settings**, select the same **Camera** as used in the Exposure Preset.
5. Select **Binning** and **Readout**.
6. Depending on the selected camera, duplicate the camera-specific values from the Exposure Preset:
   - Falcon 3EC / Falcon 4(i): duplicate the **Exposure Settings** values.
   - Ceta: duplicate **Advanced Camera Settings** values.
   - Gatan K2 / K3: duplicate the **Advanced Camera Settings** values.

   Settings that are not used in the Tracking Preset can be duplicated without negative consequences. When a value is not applicable to the Tracking Preset, it will be ignored.
7. Select **Acquisition** > **Preview**
8. Verify that the acquired image meets the requirements above.

4.1.6 Define the Focus Preset

The Focus Preset is used for:

- Calibration of the Autofocus function.
- Execution of the Autofocus step during the automated run.
- Execution of the manual focus action in case the Autofocus function fails to find the optimal focus.

In a Low Dose Tomography session, the Autofocus function is performed on an area near the feature of interest, so that the feature itself is not exposed. The location of the Focus Area is defined in the *Tomography* > *Batch Position* or *Sample Navigation* task. If the feature of interest moves more than half of the field of view after a tilt step, then move the Focus Area a bit further away from the Exposure Area, so that the feature of interest is not exposed.

The Autofocus function uses the same *High SA* filter settings as the Exposure and Tracking Presets.

The Focus Preset must meet the following requirements:

- The Optics Settings for the Focus Preset must be identical to the Exposure Preset, or at least as close as possible to the Exposure Preset.
- 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. The Autofocus function measures the image shift that is induced when the beam is tilted. After calibration, this shift can be related to a defocus. Focus is defined by zero beam tilt induced image shift.

Recommendations for beam diameter and field of view, relative to the Exposure Preset:

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

Parameters	Typical Settings and Recommendations
Optics Settings	Same as the Exposure 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, so Binning 1 does not increase the accuracy of the Autofocus function, but the image acquisition may take more time.
	  ● CMOS cameras:
	  A higher Binning value 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 Exposure Preset.

Use the procedure below to set the Focus Preset. In place of steps 1 to 3 below, we recommend using the **Link** option to link the values of Focus Preset to those of the Exposure preset.

1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   c. Select **Optics Settings** > **Set**
2. Select **Preset Selection** > **Presets**: **Focus**
3. Select **Optics Settings** > **Get**
4. In **Camera Settings**, select the same **Camera**, **Binning** and **Readout** as used in the Exposure Preset.
5. Depending on the selected camera, duplicate the camera-specific values from the Exposure Preset:
   - Falcon 3EC / Falcon 4(i): duplicate the **Exposure Settings** values.
   - Ceta: duplicate **Advanced Camera Settings** values.
   - Gatan K2 / K3: duplicate the **Advanced Camera Settings** values.

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

6. Select **Acquisition** > **Preview**
7. Verify that the acquired image meets the requirements above.

The Focus 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 Focus 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 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 requirement:

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

The recommendations below are specified relative to the Exposure Preset.

Parameters	Typical Settings and Recommendations

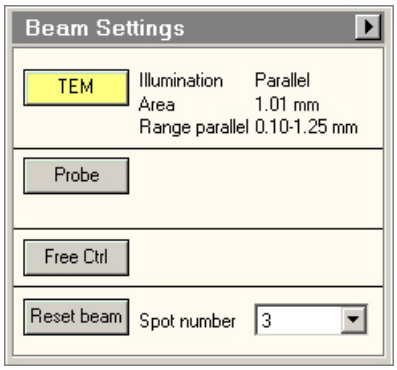
Optics Settings	If Phase Plates are used, then the Optics Settings must be the same as the Exposure Preset to prevent accidental overexposure of an activated Phase Plate.
	If Phase Plates are *not* used, then the recommendations below are applicable.
	● Defocus: typically between -1 μm and -3 μm.
	● 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.

Camera Settings	Binning: 2
Apertures	Same as the Exposure Preset.

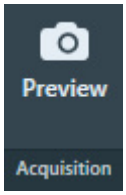
Use the procedure below to set the Thon Ring Preset. In place of steps 1 to 3 below, we recommend using the **Link** option to link the values of the Thon Ring Preset to those of the Exposure preset.

1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   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 **Integration time**, **Magnification**, **Intensity** and/or **Spot Size** to improve the sharpness of the Thon Rings.

6. Verify that the beam is parallel.
   Depending on the system type, either:
   - On a system with a C3 lens (typically High End systems with Titan software), verify that **Beam Settings** control panel > **Illumination** is **Parallel**.



   - On a system without a C3 lens (typically Mid Range systems with Talos software), 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 **Tomography**:
   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. Inspect the quality of the Thon Rings.
    Select **Filter FFT** to improve the contrast and brightness of 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**

4.1.8 Define the Eucentric Height Preset

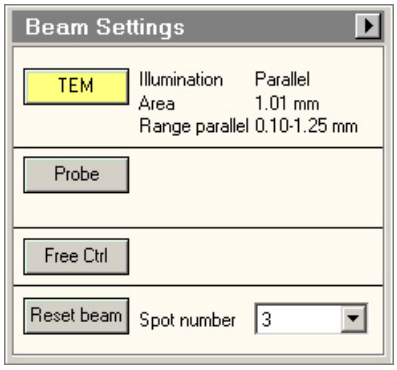
The Eucentric Height Preset is used to perform the Auto-eucentric Height function during the automated run. The Eucentric Height Preset must result in an image with sufficient contrast and brightness to clearly identify a contrast-rich feature on the specimen.

Parameters	Typical Settings and Recommendations
Optics Settings	• Probe Mode: the same as the Exposure Preset. • Magnification: low SA range, so the Illuminated Area matches the field of view. • Defocus: 10-20 µm. Defocus helps to enhance contrast. • Illuminated Area / Intensity: the beam diameter should be typically 10–15 µm. • Parallel beam. • Dose Rate must be smaller than 0.1 e-/Å^{2}s When using Phase Plates, the Eucentric Height Preset must be defined with an off-plane illumination, so that an activated Phase Plate is not harmed.
Camera Settings	• Binning: 1 • Readout: Full (This parameter may be hidden, but it is always set to *Full*).
Apertures	Same as the Exposure Preset.

Use the procedure below to define the Eucentric Height Preset.

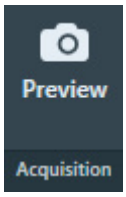
1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   c. Select **Optics Settings** > **Set**
2. Select **Preset Selection** : **Presets**: **Eucentric Height**
3. Select **Optics Settings** > **Get**

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.*
5. Move the **specimen** to an area that can be sacrificed for tuning the optics settings.
6. Adjust the optics settings, so that the image meets the Field of View requirements above.
   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 beam is parallel.
   Depending on the system type, either:
   - On a system with a C3 lens (typically High End systems with Titan software), verify that **Beam Settings** control panel > **Illumination** is **Parallel**.



   - On a system without a C3 lens (typically Mid Range systems with Talos software), 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. 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 **Tomography**:
    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**

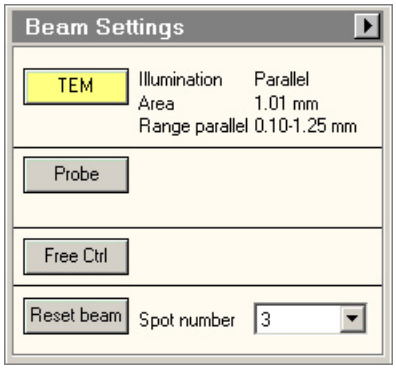


12. Verify that the **image quality** is identical to the previously acquired image.

4.1.8.1 Guidelines for the Eucentric Height Preset when using Phase Plates

When using Phase Plates, the Eucentric Height Preset must be defined with an off-plane illumination, so that 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 Flucam Viewer.

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 Tomography is started. The Zero Loss Preset is used for the Auto Zero Loss function, which compensates for drift in the energy filter.

In a Low Dose Tomography session, the Auto Zero Loss function uses the Focus Area. In a regular session the Auto Zero Loss function uses the Exposure Area.

Parameters	Typical Settings and Recommendations
Optics Settings	If Phase Plates are used, then the Optics Settings for the Zero Loss Preset must be identical to the Exposure Preset, including the on-plane illumination conditions. If Phase Plates are not used, then the below recommendations are applicable. ● Illuminated Area / Intensity: Decrease the beam diameter if possible, but make sure the entire field of view is illuminated. ● All others: same as the Exposure Preset.
Camera Settings	● CMOS cameras: same as the Exposure Preset. ● CCD cameras: To increase the readout speed, select: ● Binning: 2 ● Readout: Half
Filter Settings	Slit width: 3 eV
Apertures	Same as the Exposure Preset.

Use the procedure below to define the Zero Loss Preset. In place of steps 1 to 3 below, we recommend using the **Link** option to link the values of the Zero Loss Preset to those of the Exposure preset.

1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   c. Select **Optics Settings** > **Set**
2. Select **Preset Selection** > **Presets: Zero Loss**
3. Select **Optics Settings** > **Get**
4. In **Camera Settings**, select the same **Camera**, **Binning** and **Readout** as used in the Exposure Preset.
5. In **Advanced Camera Settings**, duplicate the values from the Exposure Preset.
6. In the filter control software, select a **Slit width** of **3 eV**
7. Move the specimen to an area that has a similar thickness as the rest of the specimen, but that can be sacrificed for defining the presets.
   Either:
   - Select **Acquisition** > **Preview**, then right-click in the **Image Display** and select **Move stage here**
   - Use the **Handpanels** > **Joystick** and the **FluScreen** or **Flucam Viewer**
8. Select **Acquisition** > **Preview**
9. Verify that the **counts** value is sufficient for high quality data acquisition.

4.1.10 Define the Search / Template Preset

The Search / Template Preset is used:

- To acquire the Search Map.
- To acquire the Search image, in which:
  - The feature of interest is identified and centered
  - The template areas are assigned to their locations.

The Search / Template Preset must fulfill the following requirements:

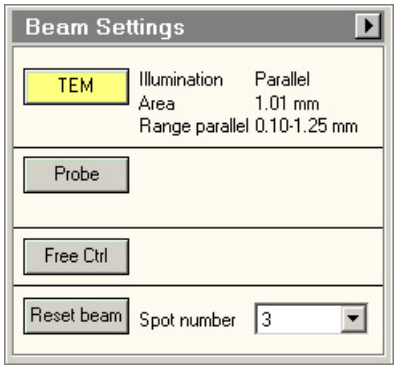
- The magnification and contrast must be high enough to reliably identify features of interest.
- The field of view must be at least 3 µm.
  On a Quantifoil specimen, the field of view should be at least 3 Foil Holes wide.
- The beam must be parallel, so that the actual magnification does not change when the specimen is out of focus.
- On Life Science specimens, the Dose Rate should not exceed 0.1 $e^-/Å^2s$.

Parameters	Typical Settings and Recommendations
Optics Settings	• Probe Mode: Nanoprobe. Nanoprobe is well suited for a narrow parallel beam at SA magnifications. • Magnification: typically in the low SA range. • Defocus: –10 to –20 µm • Spot Size in combination with the Illuminated Area / Intensity must result in a Dose Rate that is equal or lower than 0.1 $e^-/Å^2s$. • Illuminated Area / Intensity: the beam diameter must be typically 10-15 µm, or at least as large as the field of view. • Parallel beam. • Intensity Zoom is Off. When using Phase Plates, the Optics Settings must be defined with on-plane conditions. How to do this is described in Define the Exposure Preset on page 28.
Camera Settings	• Binning: 1 • Readout area: Full (This parameter may be hidden, but it is always set to *Full*).
Apertures	Same as the Exposure Preset.

Use the procedure below to set the Search / Template Preset.

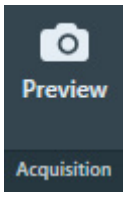
1. Apply the Exposure Preset values to the microscope:
   a. Select the **Preparation** > **Acquisition and Optics Settings** task.
   b. Select **Preset Selection** > **Presets**: **Exposure**
   c. Select **Optics Settings** > **Set**
2. Select **Preset Selection** > **Presets**: **Search / Template**
3. Select **Optics Settings** > **Get**

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.*
5. Move the **specimen** to an area that can be sacrificed for tuning 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 **Illuminated Area** of 10–15 μm across.
7. Adjust the illumination parameters to meet the contrast and optional Dose Rate requirements.
8. Verify that the beam is parallel.
   Depending on the system type, either:
   - On a system with a C3 lens (typically High End systems with Titan software), verify that **Beam Settings** control panel > **Illumination** is **Parallel**.



   - On a system without a C3 lens (typically Mid Range systems with Talos software), 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. 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 **Tomography**:
    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.

4.1.11 Define the Overview / Positioning Preset

The Overview / Positioning Preset is used during target selection to view an area of interest: an area on the specimen where features of interest are located. This Preset is not used during the automated Data Acquisition run, so there are no strict requirements for the Overview / Positioning Preset.

For a slab-like specimen with Grid Squares it is common practice that the area of interest contains an entire Grid Square.

Parameters	Typical Settings and Recommendations
Optics Settings	• Probe Mode: Microprobe (This parameter may be hidden, but it is always set to *Microprobe*). Microprobe is better suited for a wide parallel beam at lower magnifications. • Defocus: –1 mm to –3 mm • Magnification: depends on the specimen type, and the size and interspacing of the features of interest. On a flat carbon foil specimen with Grid Squares: typically LM 400X - LM 600X • Illuminated Area / Intensity: depends on the magnification. The beam diameter must be slightly larger than the field of view of the camera. On a flat carbon foil specimen with Grid Squares: typically 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 area: Full (This parameter may be hidden, but it is always set to *Full*).
Apertures	Same as the Exposure Preset.

Use the procedure below to set the Overview / Positioning Preset.

1. Select **Preset Selection** > **Presets**: **Overview / Positioning**

2. 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.*
3. Move the **specimen** to an area that can be sacrificed for tuning the optics settings.
4. Use the handpanels to create an image that meets the field of view requirements.
5. Select **Optics Settings** > **Get** to import the current optics values from the microscope.
6. In **Camera Settings**, select the same **Camera**, **Binning**, **Readout** and **Exp. time (s)** values as used for the *live image view*.

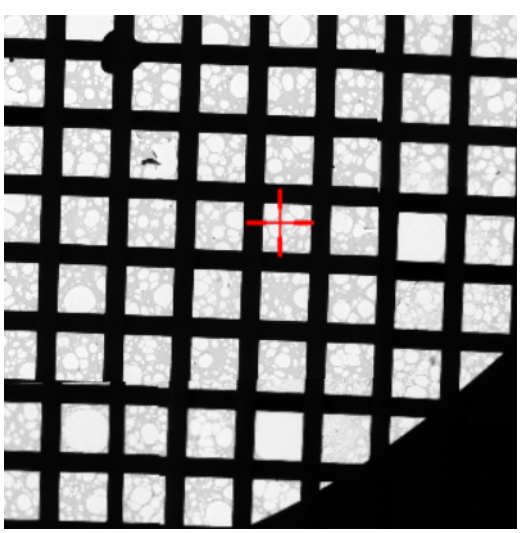
4.1.12 Define the Atlas Preset

An automated run starts by acquiring an Atlas. The Atlas is an overview of the specimen. 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 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.
- For Cryo Tomography experiments, the contrast and brightness must be good enough to assess the ice thickness.

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



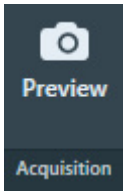
Parameters	Typical Settings and Recommendations
Optics Settings	● Probe Mode: Microprobe (This parameter may be hidden, but it is always set to *Microprobe*). Microprobe is better suited for a wide parallel beam at lower magnifications. ● Magnification: LM 60X - LM 200X. ● Illuminated Area / Intensity: the entire field of view must be illuminated. ● 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 (This parameter may be hidden, but it is always set to *Full*).
Apertures	● Objective: retracted ● Condenser 2: largest aperture Tomography can not automatically change the selected aperture. After the Atlas Preset is defined, return the Objective and the Condenser 2 aperture mechanisms to the positions that are used for the Exposure Preset.

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

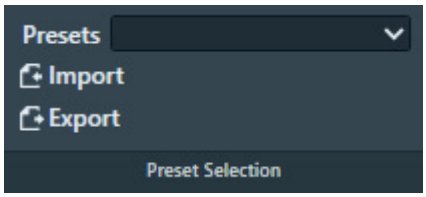
7. If the system has a **Cryobox** and the image still has cut-offs at a magnification of LM 200X or higher, then the center of the Cryobox aperture may have an offset relative to the system's optical axis. To compensate for this offset, continue as follows:
   a. Complete the **Atlas Preset** procedure 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.
      See chapter Perform the Atlas Optics Alignment on page 67.
   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.
8. 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.
9. 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.
10. In **Tomography**:
    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**



11. Verify that the **image quality** is identical to the previously acquired image.
12. In the **TEM User Interface** > **Apertures** control panel:
    - **Objective**: select the position that is used for the Exposure Preset.
    - **Condenser 2**: select the aperture that is used for the Exposure Preset.

4.1.13 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.



Export

Writes the current parameter values for all Presets to an XML file. The Presets file contains the following values for all Presets:

- The Optics Settings.
- The Camera Settings.

  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 Tomography 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 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 could 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 must compensated.

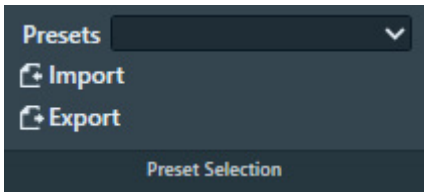
All shifts are relative to the Exposure Preset, so reconstruction is always based on zero-shift images.

4.2.1 Prepare for Image Shift Calibration

The Image Shift Calibration acquires images for each magnification that is used in the Presets. 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**: **Exposure**

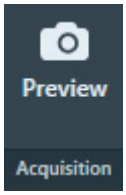


3. Select **Optics Settings** > **Set**
4. Insert the **FluScreen**
5. Move the specimen to find an **easily recognizable feature**, and center it in the image on the FluScreen.

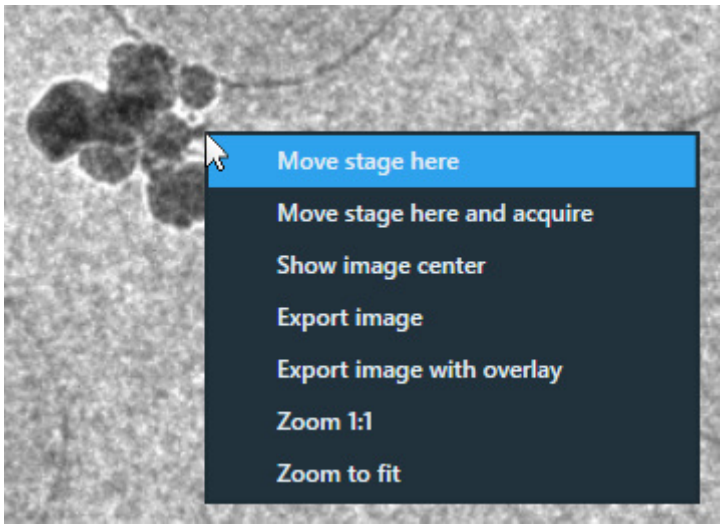
   Select an asymmetric feature that is easily recognizable at LM magnifications. If you are not sure about the recognizability of the feature details at low magnifications, switch to LM magnifications for a quick check.

   Note that on some system types, the image orientation can flip when the magnification switches between LM and HM modes.

6. Select **Preset Selection** > **Presets**: **Overview / Positioning**
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.

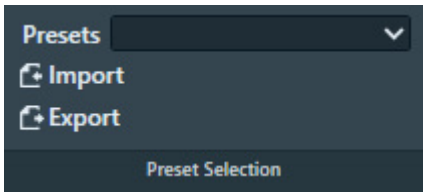


9. Select **Preset Selection** > **Presets**: **Eucentric Height**
10. Select **Acquisition** > **Preview**
11. In the acquired image, check if the selected feature is easily recognizable.
    It doesn't have to be centered, but on a well-aligned system it should be well within the field of view.
    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**: **Exposure**
14. Select **Acquisition** > **Preview**
15. In the acquired image, check if the selected feature is visible near the image center.

4.2.2 Perform the Image Shift Calibration

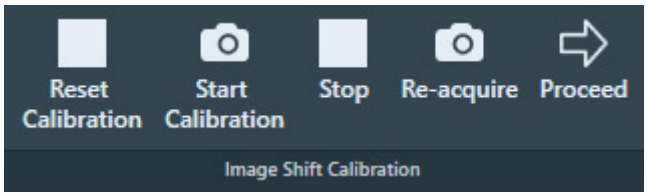
1. If the Prepare for Image Shift Calibration has not been performed yet, do so first.
2. Insert the **FluScreen**
3. In the **Preparation** tab > **Acquisition and Optics** task:

a. Select **Preset Selection** > **Presets**: **Exposure**

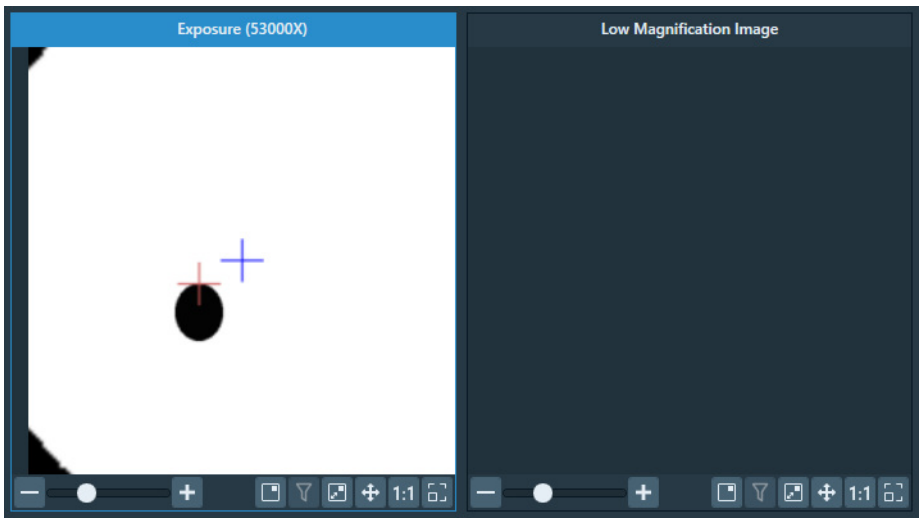


b. Select **Optics Settings** > **Set**

4. Roughly center a **recognizable feature**.
5. Select **Preparation** > **Calibrate Image Shifts** task.
6. Select the **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. Accurately 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.
      Optionally zoom in for better accuracy.
   b. Select **Re-acquire**
      Tomography 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**

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

b. In the **new right-side image**, 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 left-side image serves as a reference.
   - Use the new right-side image 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.

4.2.3 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 or 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.

4.3 The Image Filters Settings Task

The image shift calculation uses the cross-correlation of the Fourier transforms of two images. Depending on the specimen and the required imaging conditions, there are multiple factors that can decrease the reliability and accuracy of the image shift calculation.

- A Fourier transform is based on the assumption that the image can be extended periodically in all directions. In reality however, the image is finite and has edges. This results in artifacts in the Fourier transform, such as horizontal and vertical lines. These artifacts can have a negative impact on the accuracy of the image shift calculation.
- The signal-to-noise ratio of the images influences the quality of the cross-correlation. Especially in low dose conditions, the signal counts can be so low that recurring patterns and noise in the dark and gain reference images can be interpreted as features of the specimen. Because the reference images are identical in all acquired images, the cross-correlation image may show a peak at zero shift.
- In low dose conditions, spurious X-rays may cause *hot pixels*, which can be interpreted as features of the imaged specimen.

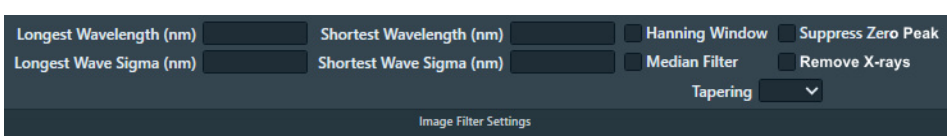
To overcome these factors, the following preventive and corrective actions are available to improve the accuracy of the image shift measurement:

- Specify a large *Reference Image Manager* > *# Images to average* value during reference image acquisition on a Ceta or Falcon camera.

  When averaged across a large number of images, a spurious noise peak in one of the acquired images is reduced to a near-average value in the reference image.

  For Gatan cameras, Digital Micrograph features a similar parameter to specify the number of averaged images.
- Specify well-chosen values in the *Tomography* > *Preparation* > *Image Filters Settings* task to:
  - Decrease or eliminate the false peak at zero shift.
  - Improve the sharpness and relative height of the peak in the cross-correlation.

The following Image Filters are available:



- The *Band Pass* filter.

  This filter is defined by:
  - *Longest Wavelength (nm)* and *Shortest Wavelength (nm)*
  - *Longest Wave Sigma (nm)* and *Shortest Wave Sigma (nm)*
- The *Hanning Window*
- The *Median Filter*
- The *Tapering* filter
- The *Suppress Zero Peak* filter
- The *Remove X-rays* filter

4.3.1 Description of the image filters

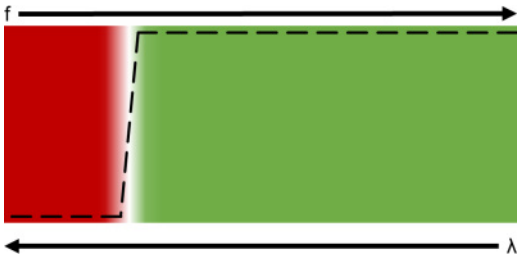
4.3.1.1 The Band Pass filter

The Band Pass filter is a combination of a High Pass filter and a Low Pass filter.

Although the terms High Pass and Low Pass apply to the passed-through *frequency* ranges, the filter parameters are specified as *wavelengths [nm]*. These wavelengths are directly related to feature sizes in nanometers, so that they remain valid when a Preset is changed.

High Pass filter

In the frequency domain, the High Pass filter suppresses frequencies below the cut-off frequency.



In the wavelength domain, the High Pass filter suppresses features that are larger than the cut-off wavelength.

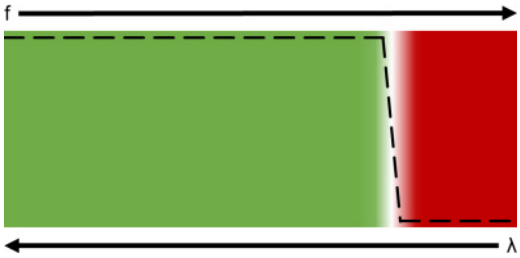
In the Tomography software, the High Pass filter is specified by:

- **Longest Wavelength**
  The filter suppresses features that are larger than the specified value.
- **Longest Wave Sigma**
  The abruptness (Gaussian width) of the filter's cut-off. A low value corresponds to a sharp cut-off.

  In the illustrations above, the Sigma value corresponds to the steepness of the cut-off, where 0.0 would be a vertical cut-off.

Low Pass filter

In the frequency domain, the Low Pass filter suppresses frequencies above the cut-off frequency.



In the wavelength domain, the Low Pass filter suppresses features that are smaller than the cut-off wavelength.

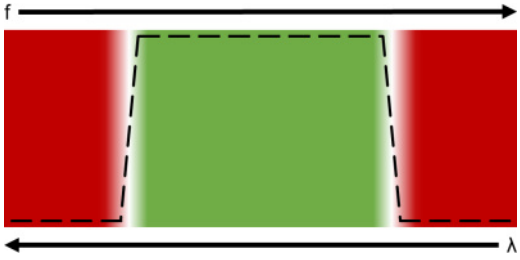
In the Tomography software, the Low Pass filter is specified by:

- **Shortest Wavelength**
  The filter suppresses features that are smaller than the specified value.
- **Shortest Wave Sigma**
  The abruptness (Gaussian width) of the filter's cut-off. A low value corresponds to a sharp cut-off.

  In the illustrations above, the Sigma value corresponds to the steepness of the cut-off, where 0.0 would be a vertical cut-off.

Band Pass Filter

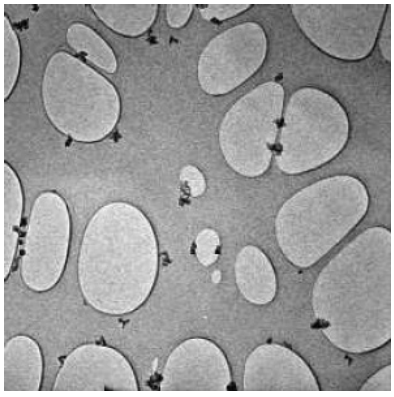
The Band Pass filter multiplies the amplification factors of the High Pass and the Low Pass filters.



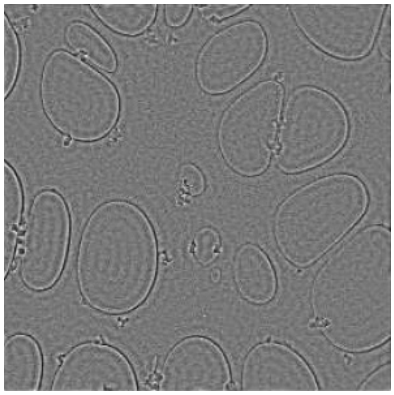
The result is a filter that cuts off the highest and the lowest frequencies, or in the wavelength domain: suppresses the smallest and the largest features.

Example for the application of the Band Pass filter:

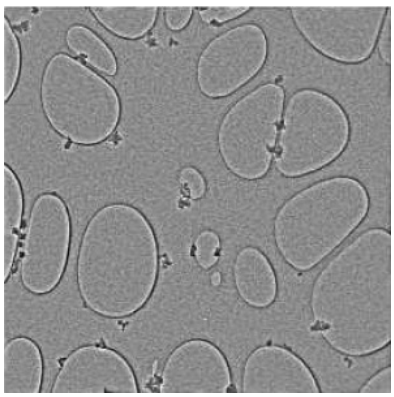
Use the *Shortest Wavelength* and *Shortest Wave Sigma* of the Low Pass filter to suppress noise, features and patterns that are smaller than the markers (gold particles).



Use the *Longest Wavelength* and *Longest Wave Sigma* of the High Pass filter to suppress features and patterns that are larger than the foil holes.



The result is a Band Pass filter that shows the foil hole outlines and the markers.



4.3.1.2 The Hanning Window filter

The first and last pixels of a row or column typically have significantly different counts values. The FFT function processes an image as if it repeats endlessly in horizontal and vertical directions. In the FFT image, the counts difference between pixels on opposing edges of the image will show up as an artefact with a frequency that matches the dimensions of the image and an intensity that matches the counts difference.

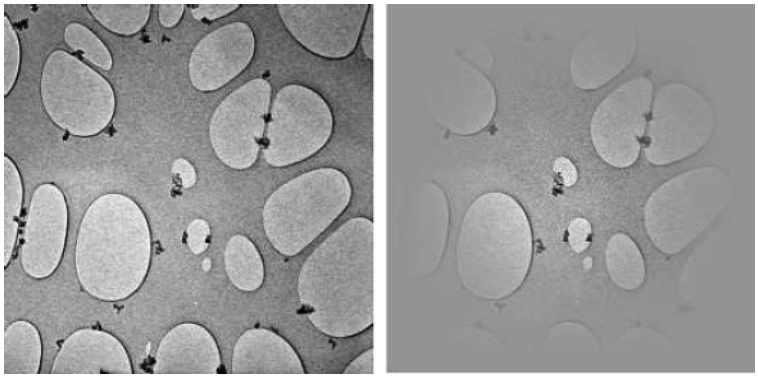
The Hanning Window filter reduces these artefacts by reducing the counts difference between pixels on opposing edges of the image. In Tomography, the Hanning Window filter adjusts the counts value for each pixel as follows:

$C_{Hanning} = C_{avg} + H \times (C_{acq} - C_{avg})$

where:

- $C_{Hanning}$: counts value of the pixel after applying the Hanning factor H.
- C_{avg}: average counts value of all pixels in the image.
- H: the Hanning factor, a *raised cosine* from 1 in the image center to 0 at the image edge.
- C_{acq}: counts value of the pixel before applying the Hanning factor H.

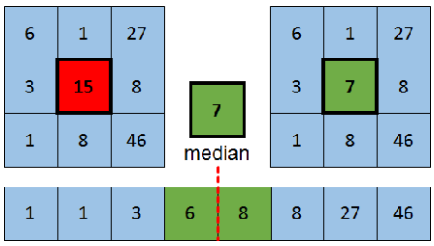
The result is an image in which the contrast is progressively reduced as the distance to the center of the image increases.



A drawback of the Hanning Window is that contrast-rich features that are not near the center of the image become useless for finding cross-correlation peaks.

4.3.1.3 The Median filter

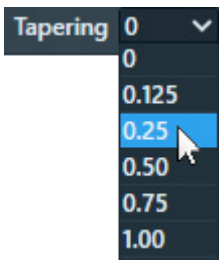
The Median filter reduces noise by replacing the acquired counts value for each individual pixel by the median value of its direct neighbors.



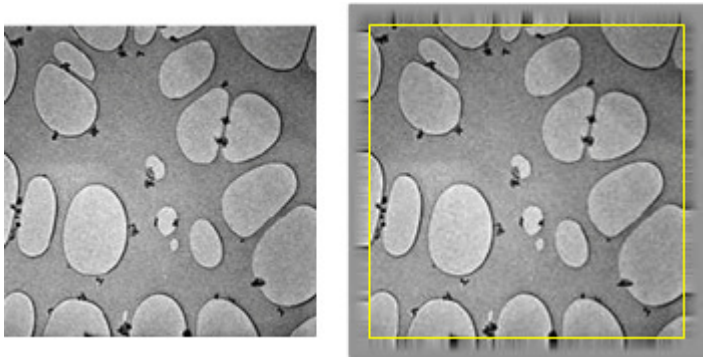
4.3.1.4 The Tapering filter

The FFT function processes an image as if it repeats endlessly in horizontal and vertical directions. The first and last pixels of a row or column typically have significantly different counts values. In the FFT image, the counts difference between pixels on opposing edges of the image will show up as an artefact with a frequency that matches the dimensions of the image and an intensity that matches with the counts difference. The Tapering filter reduces these artefacts, so they do not play a noticeable role anymore.

The Tapering filter first adds a margin around the acquired image. The width of the margin is specified as a fraction of the image size.



The Tapering filter then applies a gradient from the edge of the acquired image toward the outer edge of the added margin. The gradient goes from the counts value at the edge of the acquired image towards the average counts value of the entire image.



Left: acquired image.
Right: acquired image with tapered margin.

4.3.1.5 The Suppress Zero Peak filter

Spurious noise that is present in the reference images is inherently also present in all acquired images. In the cross-correlation of two unfiltered images, the common noise from the reference images results in a peak at zero shift. This peak is typically quite sharp. The *Suppress at Zero* filter identifies the central peak and suppresses it.

4.3.1.6 The Remove X-rays filter

When the electron beam hits the edge of an aperture or filter slit, X-ray photons are spawned. X-ray photons that hit the camera sensor cause isolated pixels with an exceptionally high counts value. The *Remove X-rays* filter identifies these pixels and replaces their counts value by the average value of the surrounding pixels.

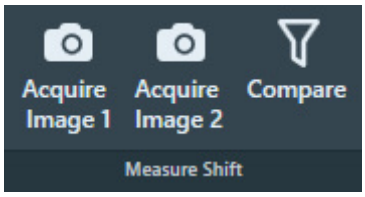
4.3.2 Define the Image Filter Presets

To set the Filter Presets, the steps below must be performed for each magnification range in **Filter Settings Selection** > **Settings**:

For the procedure below, use the following combinations of Acquisition and Optics Preset and Image Filter Preset:

Image Filter Preset	Acquisition and Optics Preset
High LM range	Overview / Positioning
Low SA range	Search / Template
High SA range	Tracking

1. Set the specimen to eucentric height.
2. In the **Acquisition and Optics Settings** task:
   a. Select the appropriate **Preset** for the Image Filter Setting that will be defined.
   b. Select **Optics Settings** > **Set**
3. Select the **Image Filters Settings** task.
4. Select the desired **Preset Selection** > **Presets**
5. Use the **handpanels** and the **live view functions in Velox** to:
   a. Move the **specimen** to an area that can be sacrificed for tuning the Image Filter Settings.
   b. Create an **accurately focused image** with a contrast-rich feature in the center.
6. Select **Measure Shift** > **Acquire Image 1**



7. Change the **stage tilt** by **±5 degrees**, so that the centered feature shifts a small, but noticeable distance from the image center.
8. Select **Acquire Image 2**
9. Select **Compare**
10. Select and/or specify the filter parameters, so that the cross-correlation image shows a single clear bright peak that is not at Zero Shift.
    a. Tune the **Band Pass filter**.
       As a rule of thumb for the initial values, use:
       - Longest Wavelength: $^{1}/_{4}$ of the scale bar in the image.
       - Shortest Wavelength: $^{1}/_{40}$ of the scale bar in the image.
       - Longest Wave Sigma and Shortest Wave Sigma: 0.0

       If the scale bar is 2 µm, then start with a Longest Wavelength of 500 nm, and a Shortest Wavelength of 50 nm.
    b. If the cross correlation image shows undesirable peaks or other artifacts, then select the applicable filters to suppress these peaks.

4.3.3 Suggestions for the application of image filters

- **Low dose imaging of a specimen with markers**
  Specify the **Band Pass filter** to suppress noise and enhance the markers.
  Remove the shorter wavelengths to smoothen the image and the peak at zero shift, while enhancing the signal from the markers.

- **Low dose imaging of a specimen without markers**

  Apply one or more of the following filtering strategies:
  - Specify the **Band Pass filter** to suppress noise and enhance the signal counts of the features of interest.
  - Add the **Suppress Peak at Zero** filter to suppress the correlation between common noise patterns in the two images.
  - Add the **Median** filter to decrease single noise peaks, but retain edges.
  - Add the **Remove X-rays** filter to replace pixels with exceptionally high counts by pixels with average counts.
- **Multiple less suitable features near the feature of interest**
  - Specify the **Band Pass filter** to enhance features of a specific size range.

    If the less suitable features are similar in size as the most suitable feature than it can be difficult to find a robust combination of parameter values.
  - Apply a **Hanning Window** to decrease the contrast of features that are not in the image center.
- **Edge artifacts**

  If the image is dark on one edge and bright on the opposite edge, then a bright line will appear through the center of the cross-correlation image. This is consequence of the Fourier transform. The Fourier transform function assumes that the image can be repeated indefinitely in all directions, so it joins the bright edge to the opposing dark edge and falsely detects a contrast-rich feature with a size that is approximately the same as the acquired image. This artifact can severely decrease the quality of the shift measurement.

  To avoid the bright line artifact:
  - Apply a **Hanning Window**, possibly in combination with a **Tapering** filter to decrease contrast between opposing edges of the image.
  - Specify the **Band Pass filter** to enhance features of a specific size range.

  If multiple images suffer from the same edge artifact, then the Dark and Gain Reference of the camera becomes suspect.
- **High SA magnifications on a system with Gatan OneView**

  Apply a **Hanning Window** filter.

4.4 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.

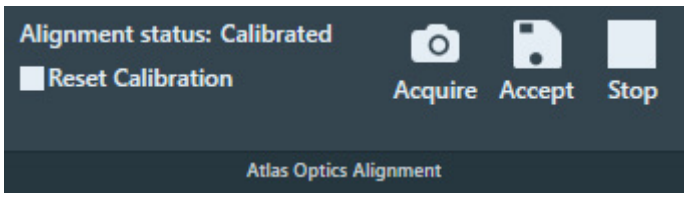
The Atlas Optics Alignment determines the offset of the Cryobox aperture relative to the system's optical axis. It then calculates the amount of Image/Beam Shift that is needed to compensate for the offset, so that the beam passes through the center of the Cryobox aperture. The Image/Beam Shift value 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.

4.4.1 Perform the Atlas Optics Alignment

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

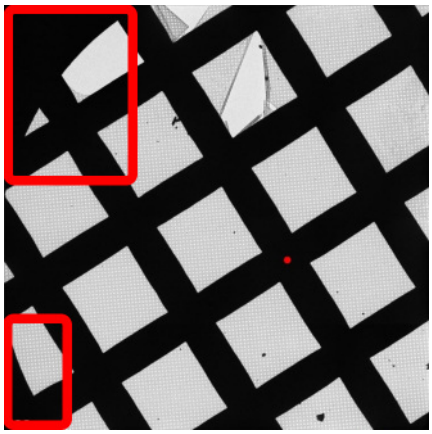
1. Select **Atlas Optics Alignment** > **Acquire**.



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

2. 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.

3. 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.
4. When the image is free from cut-offs, select **Accept**.

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, then the calculated beam shift value may not be accurate anymore, and the calibration might have to be renewed.

To renew the Atlas Optical Alignment calibration:

5. Select **Reset Calibration**.
6. Perform steps 4 - 6 again.

4.5 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.
For example, see: "Using the Volta phase plate with defocus for cryo-EM single particle analysis" (Danev et al, eLife 2017;6:e23006).

The duration of an Automated Acquisition run generally exceeds the lifespan of a Phase Plate area. During an Automated Acquisition run procedure, Tomography 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 Tomography experiment, the Phase Plate should establish a phase shift range of 0.5π ± 0.3π. 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.

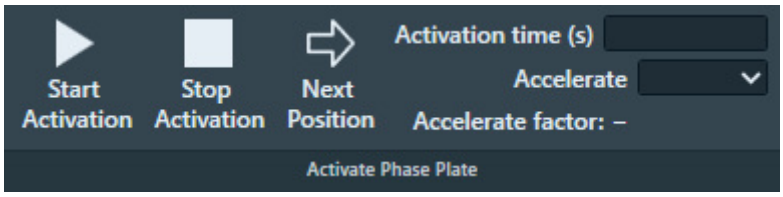
The Accelerate function *only* changes the selected C2 aperture. The Optics settings are not affected.

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.
   For instructions, see: Run the Autostigmate auto-function on page 93.
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.

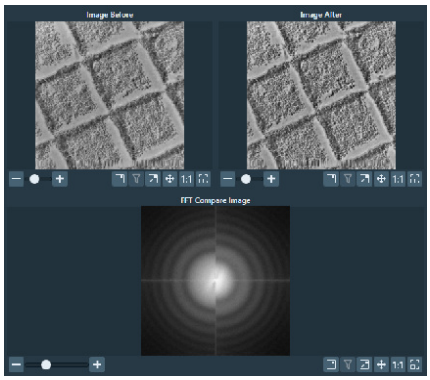
Note After a large move with the aperture mechanism, for example a move to a different Phase Plate, it may take up to 5 minutes for drift to settle.

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

    Tomography 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.



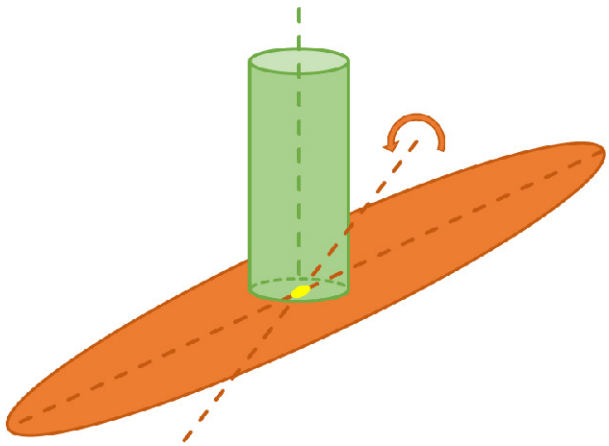
Bottom left half: FFT of an image at the beginning of the activation.

Bottom right half: FFT of an image at end of the activation.

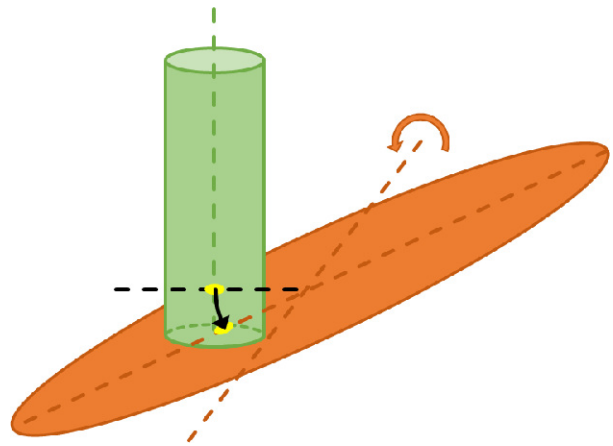
4.6 The Optimized Position task

In an ideal microscope, the optical axis intersects exactly with the tilt axis. In a real microscope however, there can be a small offset between the optical axis of the column and the tilt axis of the stage. This offset has the following undesirable effects:

- The feature of interest goes out of focus.
- At large tilt angles, the feature of interest shifts in Y-direction.
- The result of the Auto Eucentric Height function becomes inaccurate.



Optical axis and tilt axis intersect.
The feature of interest stays in focus and centered.



Optical axis and stage axis have an offset.
The feature of interest goes out of focus
and shifts in Y-direction.

In principle, every magnification could have its own offset. On a well-aligned microscope, the offset between the optical axis and the tilt axis is the same for all magnifications. The Optimized Position calibration measures this offset, so that Tomography can compensate for it with a corresponding Image/Beam Shift. After a successful Optimized Position calibration, the center of the beam intersects with the tilt axis, and their intersection is centered in the field of view of the camera.

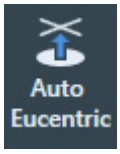
The Optimized Position value is specific for the microscope and its alignment. The Optimized Position calibration must be renewed when the CompuStage is replaced or re-adjusted, or after the optical alignment of the microscope is thoroughly revised.

The alignment of the microscope can be different for TEM and EFTEM. Perform the Optimized Position calibration for both the TEM and the EFTEM mode.

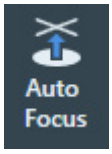
4.6.1 Prepare for the Optimized Position calibration

The Optimized Position calibration automatically selects the Focus Preset. It is recommended to use the same Optics and Camera settings during preparation of the Optimized Position calibration.

1. Make sure that **Auto Functions** > **Calibrations**: **Autofocus** and **Eucentric correction** are completed.
   If not, see Calibration tasks on page 100 for instructions.
2. Load the **Combined Test Specimen (Agar S142)**
   This specimen is a holey carbon foil with gold particles and graphitized carbon. This specimen is delivered with the microscope.
3. In **Preparation** > **Acquisition and Optics Settings**:
   a. Select **Preset Selection** > **Presets**: **Focus**
   b. Select **Optics Settings** > **Set**
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 in the *Focus Preset*.
   b. Select **Binning: 1**
   c. Select **Readout area**: **Full**
   d. Select **Bias/Gain correction**: **Bias/Gain**
   e. Start the *live image view.*
5. Move the **specimen** to an area of thin carbon film, so that:
   - Multiple features, such as markers, are visible in the camera's field of view.
   - A suitable feature is centered in the image.
6. In the **Preparation** > **Optimized Position**:
   a. Select **Auto Eucentric**

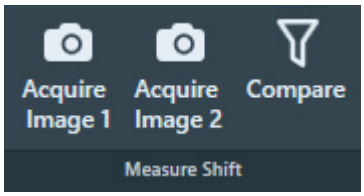
   

   b. Select **Auto Focus**

7. Verify and, if necessary, adjust the *High SA* image filter:
   a. In **Preparation** > **Image Filters Settings**,
   select **Preset Selection** > **Presets**: **High SA (Focus tracking)**

b. Select **Measure Shift** > **Acquire 1**



c. Tilt the **specimen** a few degrees, so that the centered feature shifts a small, but noticeable distance from the image center.

d. Select **Acquire 2**

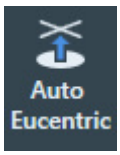
e. Select **Compare**

f. Verify that the **Cross-Correlation image** shows a well-defined peak, away from the zero-shift location.

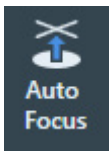
If not, see The Image Filters Settings Task on page 59 for instructions how to tune the Image Filters.

4.6.2 Perform the Optimized Position calibration

1. Select **Preparation** > **Optimized Position**
2. Select **Auto Eucentric**



3. Select **Auto Focus**



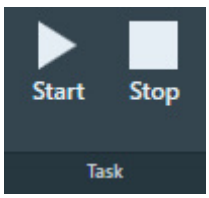
4. If the *CompuStage* or the *FEG* has been serviced, then reset the Optimized Position value:

a. Specify **New Value**: **0.00**

b. Select **Apply**

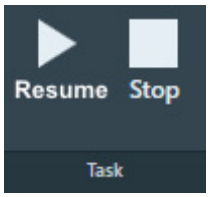
Else, the *Current value* is typically a good starting point for the Optimized Position calibration.

5. Select **Task** > **Start**



6. Monitor the progress and intermediate results in the **Status** side panel.

   The procedure may request to perform manual actions. If so, perform the requested action and select **Resume**

   The Optimized Position calibration procedure starts with the *Current Value*. The *Current Value* is a read-only value.
   - If the result is within 0.05 µm of the *Current Value*, then Tomography updates the *New Value*.
   - If the result is *not* within 0.05 µm of the *Current Value*, then Tomography repeats the calibration once.
     - If the second result is within 0.05 µm of the first result, then Tomography updates the *New Value*.
     - If the second result is *not* within 0.05 µm of the first result, then the *New Value* remains equal to the *Current Value.*
7. Verify that **Optimized Position Calibrations** > **New Value** is not equal to **Current Value**

   This indicates that the calibration is completed successfully. If the calibration is not successful, then New Value is equal to Current Value.
8. If the calibration completed successfully, then select **Apply** to accept the *New Value*.
9. With the **TEM User Interface** and the **Handpanels**:
   a. Verify the **Direct Alignments** control panel > **Tomo Rotation Center** alignment.

      If necessary, adjust the Direct Alignment.
   b. If necessary, correct **astigmatism**
   c. Verify the **Direct Alignments** control panel > **Coma-free** alignments.

      If necessary, adjust the Direct Alignment.
10. Update the Direct Alignments that are stored in the FEG Register:
    a. In the **FEG Registers** control panel, select the **currently loaded FEG Register**
    b. Select **Update** and confirm.

4.6.3 Considerations after a successful Optimized Position calibration

The Optimized Position is a system property. It does not depend on the used specimen, the holder or the software application.

- The Optimized Position calibration result is not shared Tomography for STEM mode.

  If the calibration is successfully completed in either EPU-D or Tomography, then the value is used by both applications.
- The Optimized Position calibration should only be renewed:
  - After the stage is replaced or re-adjusted.
  - After the optical alignments are thoroughly revised.
- To make proper use of the calibrated Optimized Position value:
  - Do *not* use Image Beam Shift to center the feature of interest.
  - If available, use the *Direct Alignments* > *Tomo* versions of the alignments while Tomography is running.

    The regular Direct Alignment versions will reset the calibrated Image Beam Shift value. If a regular Direct Alignment version is performed, then select an Acquisition and Optics *Preset* and select *Set* to re-apply the optics settings. The optics settings include the Image Beam Shift value from the Optimized Position calibration.

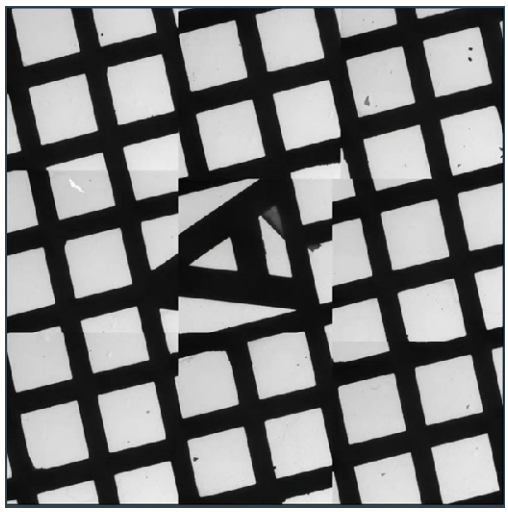
Tomography sets the Optimized Position when the software starts. When the Tomography software is stopped, the Optimized Position is revoked.

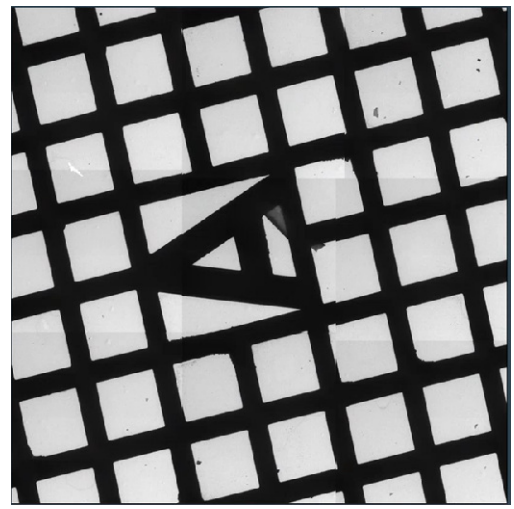
Note While Tomography is running, always use the *Tomo* versions of the Direct Alignments.

The regular *TEM User Interface* > *Direct Alignments* reset the image shift to 0, which cancels the Optimized Position image shift that Tomography has set.

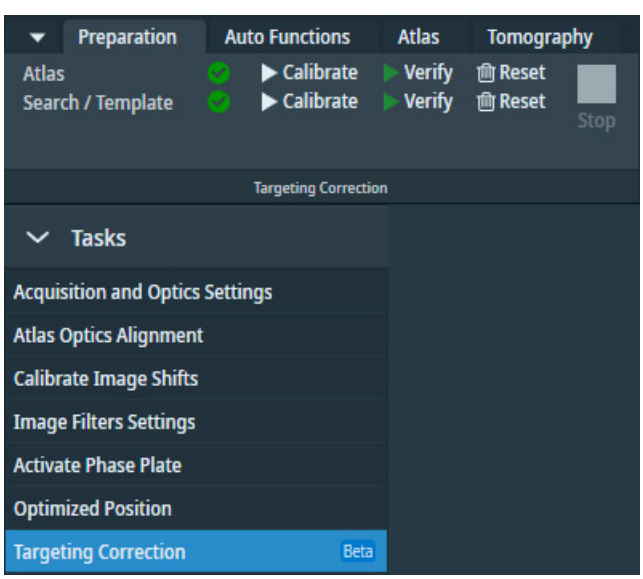
4.7 The Targeting Correction task

Many factors may cause acquired Atlas and Search Maps to have misaligned tiles. This affects Tomography targeting desired areas and features in the specimen. The Targeting Correction task is designed to mitigate targeting errors through software processing for systems that are not well-calibrated. However, there is naturally a limit to the extent of error correction that can be done solely on the software level. Observe the images below showing an Atlas before and after applying the Targeting Correction calibration.

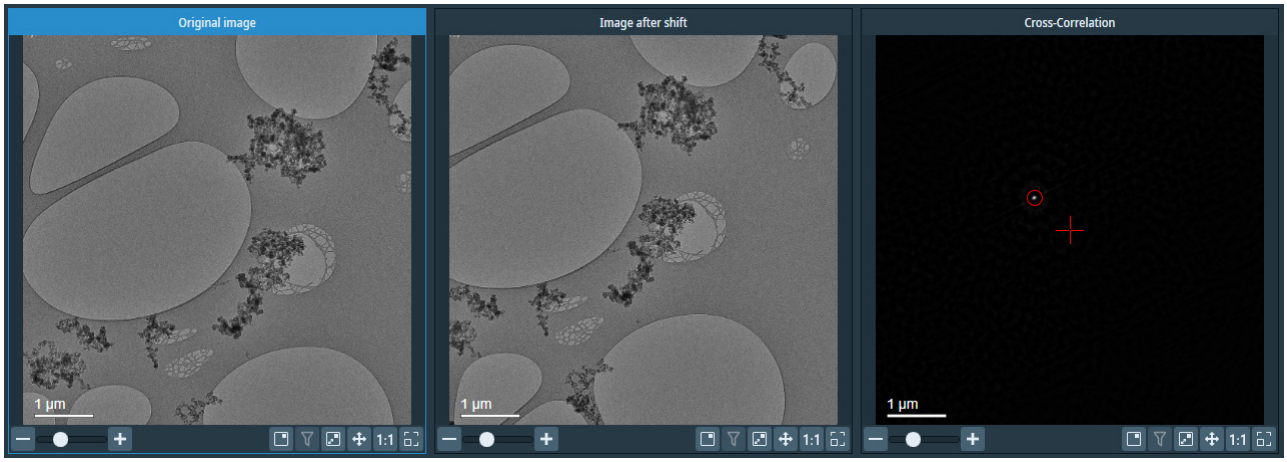




Targeting correction is done on two levels of magnification: the lower magnification is at the level of the Atlas where Stage shift is used to acquire adjacent tiles, whereas the higher magnification is at the level of the Search/Template where Beam/Image shift is used to acquire adjacent tiles. Therefore, there are two different calibration procedures for Targeting correction: Atlas and Search/Template. Both are located in Preparation > Targeting Correction.



Both calibration procedures work essentially as follows. Images are acquired before and after a shift in a certain direction. The distance of the shift is limited within the area that is already observed. This enables comparing the before-shift and after-shift images. The comparison is done in Fourier space (similar to the comparison done in Tracking) and the result is displayed in the Cross-Correlation image display.



The Cross-Correlation image shows a single red-circled dot. If no calibration is needed, the dot appears precisely in the center where the crosshair points. However, if a targeting error is found, the red-circled dot would be off the center by a distance corresponding to the error value. A specific measurement of the error is given in the Status window during the Calibrate procedure.

In summary, the Targeting Correction task allows you to calibrate Stage movements and Beam/Image shifts with greater accuracy when acquiring a feature of interest during automated Data Acquisition (see The Batch Positions task). It also improves the alignment between tiles of Atlas and Search Map images. The calibration for Targeting Correction is available for Atlas and Search presets.

This feature is in Beta. For feedback and questions, please contact tomo5@thermofisher.com.

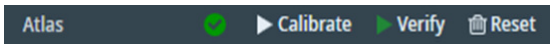
4.7.1 Calibrate the Targeting Correction

To calibrate the Target Correction factor, perform the following actions:

1. Move the specimen to an area in the middle of a grid square, preferably where identifiable features are visible.

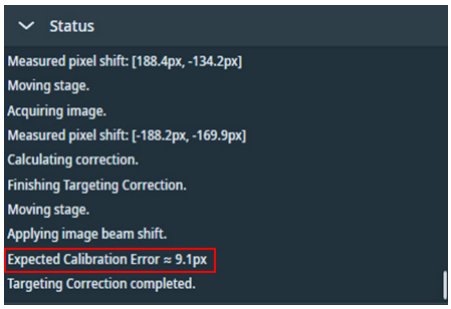
   Note: it is not recommended to perform the calibration on a lamella specimen.
2. Ensure that the specimen is at eucentric height and in focus.

3. In **Calibration > Targeting Correction**, select **Calibrate** in the first row, to calibrate the Targeting Correction factor for the Atlas preset.

   If the Objective Aperture Mechanism is enabled and inserted, then Tomography will automatically retract the aperture before the acquisition starts. After the acquisition is completed, the aperture will be inserted again.

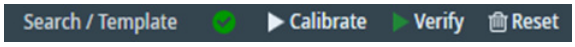
   At the end of the calibration, an estimated margin of error of the calibration, *Expected Calibration Error*, is displayed in the Status area.

4. To verify that the calibration was completed successfully, select **Verify** in the first row. This will re-do the measurement utilizing the new Targeting Correction factor.

   At the end of the verification, a measured calibration error is displayed in the Status area. If the calibration was completed successfully, the measured calibration error is small.

5. Select **Calibrate** in the second row, to calibrate the Targeting Correction factor for the Search preset.

   Although the calibration procedure for the Search preset is different from the Atlas preset one, perform the same actions as described in steps 3 and 4 to verify that the calibration was completed successfully.

4.7.2 When to re-calibrate the Targeting Correction

Once a Targeting Correction calibration is performed for a certain set of parameters, it is stored and re-used when applicable. So, if parameters in the Atlas and Search presets are changed, the calibration is not lost. Reverting the parameter values back restores the calibration that was performed for those values.

Performing a re-calibration is recommended in the following cases:

- When the alignment of the tiles in Atlas and Search Map images becomes worse.
- When the Targeting Correction was performed for the current magnification and camera settings, but several different sets of values were used after that initial calibration.

If the magnification and camera settings are changed, the calibration for that preset is automatically marked invalid. In that case, it is recommended to do the calibration for the new settings.

5 Atlas Tab

An Atlas is an overview of the specimen. To create an Atlas, Tomography acquires a set of large area images at multiple stage positions, and stitches these images together.

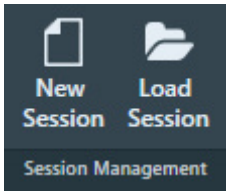
An Atlas can be used to navigate to features of interest during the preparation of an Automated Acquisition run. For Tomography experiments in which only one or a few features at known locations will be processed, the Atlas has little added value and can be omitted.

5.1 Session Setup task

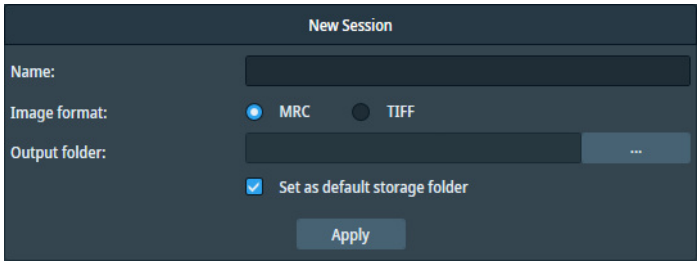
5.1.1 Create a new Atlas Session

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

1. Select the **Atlas** tab > **Session Setup** task.
2. Select **New Session**



3. Enter a **Name** for the session.



4. 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.
     See The MRC2014 Image Format on page 183.
   - **TIFF**: Raster image format: TIFF file format.

5. At *Output folder*, select **[...]** and navigate to the target folder.
   In the specified folder, Tomography creates a sub-folder with the *Name* of the Atlas session.

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

In this folder, Tomography stores the following files:
- The Atlas session file `ScreeningSession.dm`
- For each specimen that is processed in the session: the images and metadata for the Atlas and for all Tiles.
  - On a system with an Autoloader, the Screening task can process multiple specimens.
  - On a system without an Autoloader, the Atlas Acquisition task processes only the specimen that is loaded on the stage.

6. (Optional) 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 subsequent Atlas sessions.
7. Select **Apply**

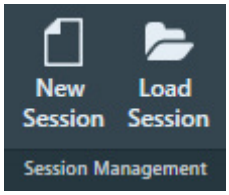
5.1.2 Load an existing Atlas Session

Note If a specimen has already been (partly) processed in an Automated Acquisition run, then do not load an existing Screening Session file for a new Tomography session with that specimen.

The Screening Session file does not contain data about which Grid Squares have been processed already in a preceding Automated Acquisition run. 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 Screening Session, follow the steps below:

1. In the **Atlas** > **Session Setup** task, select **Session Management** > **Load Session**



2. Navigate to the Output folder of the desired Screening Session and select the `ScreeningSession.dm` file.

Note Do not load an `Atlas.dm` file.

5.2 Screening task

The Screening functionality is used to acquire an Atlas. On a system with an Autoloader the Atlas acquisition can be done for multiple specimens from a selection of Slot Positions.

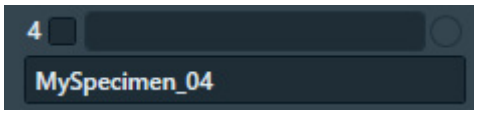
5.2.1 The Screening task for systems with an Autoloader

5.2.1.1 Acquire Atlases for multiple specimens

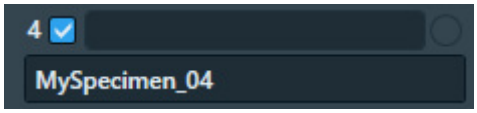
To acquire Atlases for specimens from multiple Slot Positions, follow the steps below:

1. Select the **Atlas** > **Screening** task.
   The Task Selection panel displays all Slot Positions in the Autoloader.
   - Empty slot:

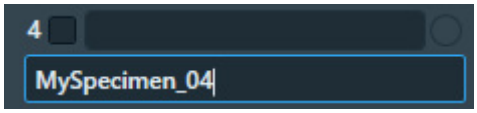
   - Occupied slot:

2. For each occupied slot:
   a. (Optional) Tick the **Slot Position** to schedule it for Atlas acquisition.

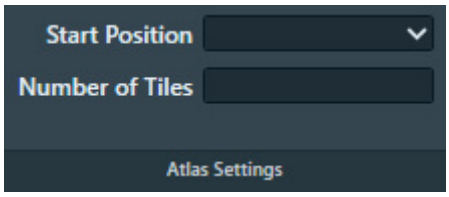
      

      It is not possible to select an empty Slot Position.
   b. (Optional) Edit the default **description**

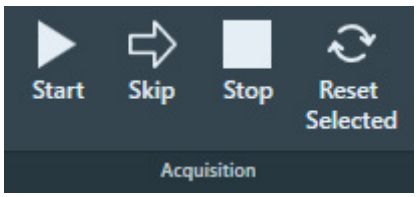
      At the creation of a new Screening session, Tomography copies the slot descriptions that are present the *TEM User Interface* > *Autoloader* control panel. After the Screening session is created, Tomography and the TEM User Interface do not synchronize their slot descriptions.

3. In **Atlas Settings**:



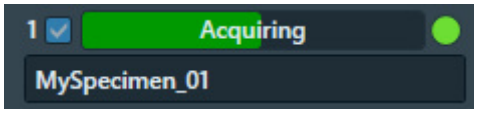
   a. If the currently loaded specimen is included in the set of selected Slot Positions, then select the **Start position**. The selected Start Position only applies to the Slot Position of the currently loaded specimen. For all other Slot Positions, Atlas acquisition starts at *Close to center*. :
      - **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. (Optional) Specify the **Number of tiles** to restrict the area that will be covered in the new Atlas. The specified *Number of tiles* applies to all Slot Positions. It is not possible to specify a different value per Slot Position.

4. Select **Acquisition** > **Start** to begin the screening procedure.

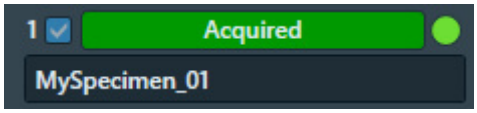


   Tomography prepares the system for Atlas acquisition.
   - If the Objective Aperture Mechanism is enabled and inserted, then Tomography retracts the aperture before acquisition starts. After the acquisition is completed, the aperture is inserted again.
   - If the Autoloader TMP is not running, then Tomography will start it to shorten the exchange time between Slot Positions. After the Screening acquisition is completed, Tomography returns the Autoloader TMP to its previous status.

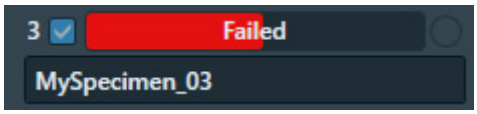
5. Wait until all selected Slot Positions are processed.
   For each included Slot Position, Tomography loads the specimen and acquires an Atlas.
   The progress bar displays the progress or the result of the Atlas acquisition.
   - Acquiring



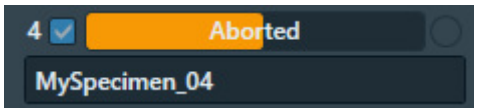
     The **green LED** shows that the specimen from this Slot Position is currently loaded on the CompuStage.
   - Acquired



- Failed



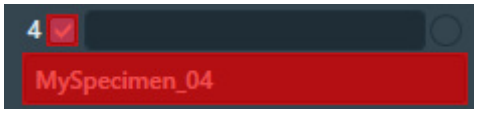
- Aborted



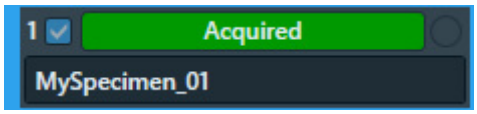
While the screening procedure is running, it is possible to:

- 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, click anywhere in the Slot Position, except for the checkbox and the description.



  The Slot Position is highlighted and the Atlas for the specimen appears in the Image Display.



  Viewing the Atlas for a specimen does not affect an ongoing acquisition for a different specimen.

5.2.1.2 Acquire a single Atlas from an unknown specimen on the stage

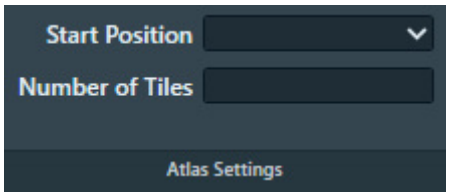
Sometimes the occupation status of the stage is not known. It is unknown if a specimen is currently present on the stage, or it is unknown from which Slot Position the specimen on the stage was loaded. This situation can occur when, for example, the cassette is undocked or when the Autoloader is not initialized.

Under these circumstances, the Single Atlas functionality allows the acquisition of an Atlas from the unknown specimen. If no specimen is present, then the Atlas will be blank.

The Single Atlas functionality is available in the Screening task as an additional (virtual) Slot Position, below the regular Slot Positions. Unlike regular Slot Positions, the Single Atlas slot has its own Acquire function. The Acquire function is only available when the occupation status of the stage is unknown. An Atlas that is acquired with the Single Atlas functionality behaves the same and is treated the same as an Atlas that is acquired for a regular Slot Position in a regular Screening session.

To acquire an Atlas from an unknown specimen on the stage, follow the steps below:

1. (Optional) Verify that a specimen is present on the stage:
   a. In **Preparation** > **Acquisition and Optics Settings**,
      select **Preset Selection** > **Presets**: **Atlas** or another Preset with a large illuminated area to prevent that the specimen is exposed to a converged beam.
   b. Select **Optics Settings** > **Set**
   c. With the TEM User Interface and/or Hand Panels,
      insert the **FluScreen** and open the **Column Valves**.
2. In **Atlas** > **Screening** > **Atlas Settings**:



   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. (Optional) Specify the **Number of tiles** to restrict the area that will be covered in the new Atlas.
   c. At the bottom of the Autoloader Slot Positions, select **Single Atlas** > **Acquire**

      If the Objective Aperture Mechanism is enabled and inserted, then Tomography retracts the aperture before the acquisition starts. After the acquisition is completed, the aperture is inserted again.
   d. Wait until the Single Atlas acquisition is completed.

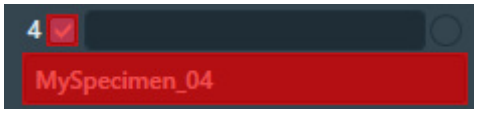
5.2.1.3 Reset the Slot Position status

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:

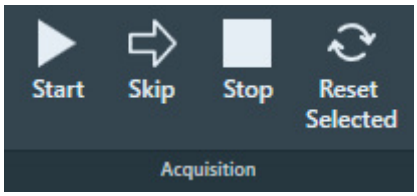
- Slot Position status
- Atlas
- Selected Grid Square categories

To reset the status of a Slot Position:

1. Select the **Slot Position**
   To select a Slot Position, click anywhere except for the checkbox or the description.



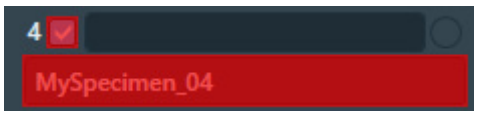
2. Select **Reset Selected**



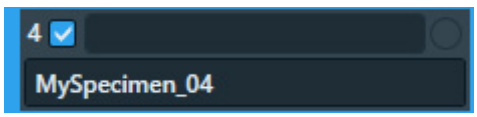
   The status, Atlas, name and selection Grid Square categories are erased, or are returned to their initial values.

5.2.1.4 Load a specimen on the stage

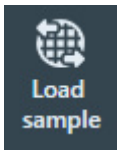
1. Select **Atlas** > **Screening** to display all Slot Positions in the Autoloader.
2. Select the **Slot Position** at which the desired specimen is located.
   To select a Slot Position, click anywhere except for the checkbox or the description.



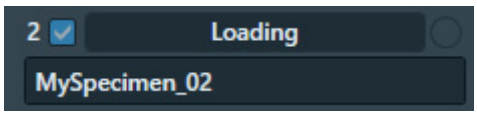
   The Slot Position is highlighted and the Atlas for the specimen appears in the Image Display.



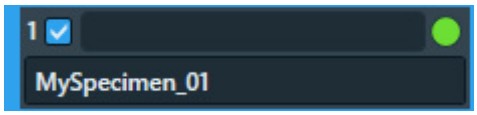
3. Select **Load sample**



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



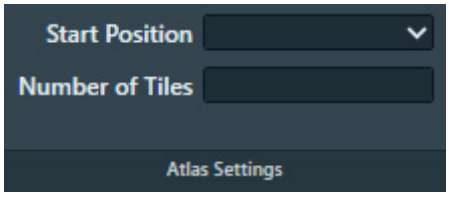
4. Wait until the loading procedure is completed and the **LED** is **green**



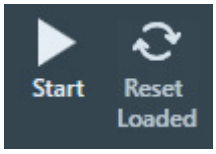
5.2.2 Acquire an Atlas for a single specimen on a system with removable holders (side entry)

To acquire an Atlas on a system with a removable holder (a so-called *side entry* system), follow the instructions below:

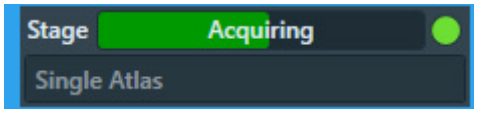
1. Select **Atlas** > **Screening**
2. In the **Atlas settings** section:



   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. (Optional) Specify the **Number of tiles** to restrict the area that will be covered in the new Atlas.
3. Select **Acquisition** > **Start**

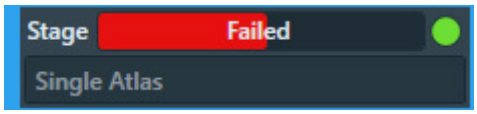


   Tomography prepares the system for Atlas acquisition.
   - If the C2 aperture mechanism is enabled, then Tomography first selects the largest C2 aperture. After the acquisition is completed Tomography returns the aperture mechanism to its initial position.
   - If the Objective aperture mechanism is enabled and inserted, then Tomography first selects the retracted position. After the acquisition is completed Tomography returns the aperture mechanism to its initial position.
4. Wait until Tomography has acquired all images for the Atlas.
   The progress bar displays the progress or the result of the Atlas acquisition.
   - Acquiring

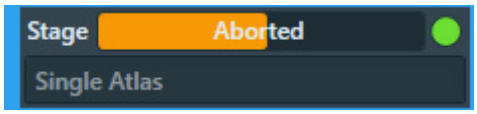


   - Acquired

- Failed



- Aborted



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 **Acquisition and Optics Settings** > **Presets**: **Atlas** and adjust the settings.
c. (For side entry loader) Select **Reset Loaded** to unload the current specimen and prepare for re-acquiring the Atlas.
d. Select **Atlas** > **Screening** > **Start** again to acquire a new Atlas.
   All tiles that were acquired before adjusting the Atlas Preset are discarded.

Note If the Optics Settings parameters of the Atlas Preset are changed, and the system has a Cryobox, then the Atlas Optics Alignment might have to be performed again.

6 Auto Functions Tab

The Auto Functions tab provides various (semi-)automated tasks. These tasks are organized in the following categories:

- *Alignments*: execute an automated alignment.
  Alignments tasks are only available on Tundra systems.
- *Auto-Functions (TEM)*: execute the Auto Functions as stand-alone tasks, outside the context of setting up an Automated Acquisition run.
  For descriptions and instructions, see: Auto-Functions (TEM) tasks on page 89.
- *Calibrations*: calibrate the Auto Functions.
  For descriptions and instructions, see: Calibration tasks on page 100.

The Auto Functions use the Acquisition and Optics Presets. 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.

6.1 Auto-Functions (TEM) tasks

The functions in the **Auto-Functions (TEM)** group of tasks can be executed outside of the workflow context. To set up a properly performing Automated Acquisition run, it is *not absolutely necessary* to run the Auto-Functions (TEM) tasks.

6.1.1 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 have good 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 Tomography workflow the Autofocus function is executed.

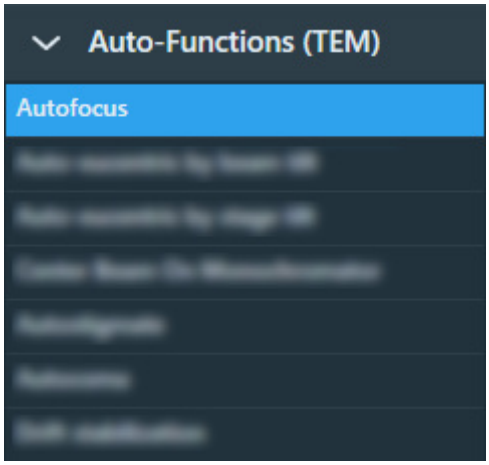
- In the **Auto Functions** tab > **Auto-Functions (TEM)**: **Autofocus** task,
  Tomography uses the **Auto Function Settings** > **Desired Defocus** value.
- At any other time, Tomography uses the defocus value from the active Preset.

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

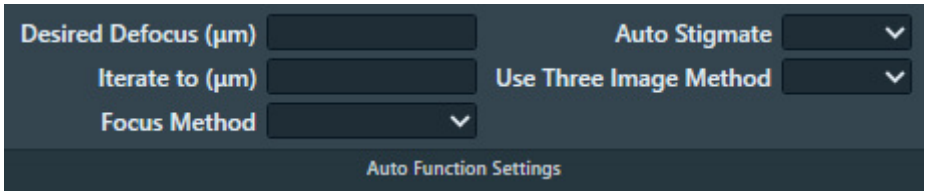
- Adjusts the Objective lens current, similar to using the *Handpanels* > *Focus* knob.
- Adjusts the stage Z-position, similar to the *Auto-eucentric by beam tilt* autofunction.

To run the Autofocus function, follow the procedure below:

1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Autofocus**



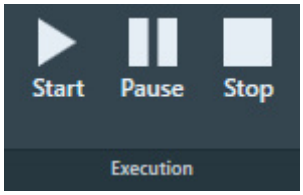
2. Tomography selects the *Focus Preset* automatically. Otherwise, select the **Preset Selection** > **Preset** that should be used for the stand-alone execution of the Autofocus function.
3. In the **Auto Function Settings** section:



   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. (Optional) 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. (Optional) Select **Use Three Image Method**: **Yes** to include drift correction. Tomography will acquire an extra image to measure drift.
4. Select **Execution** section> **Start**



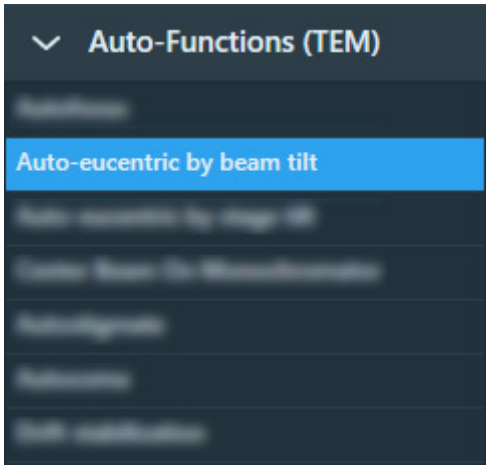
6.1.2 Run the 'Auto-eucentric by beam tilt' auto-function

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. See The Eucentric Correction Calibration task on page 104 for background information and for instructions how to perform the Eucentric correction calibration.

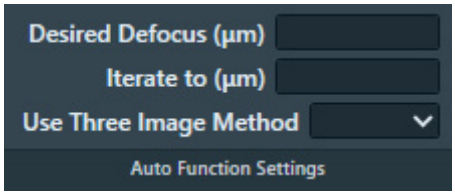
This method is more suited for higher magnifications. At low magnifications the method can be used also, but the accuracy can be less than optimial.

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

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



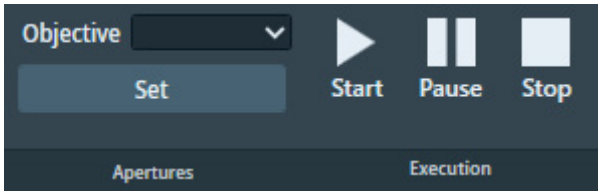
2. Tomography selects the relevant preset automatically, e.g. the *Eucentric Height* preset. Otherwise, select the **Preset Selection** > **Presets** with which the Auto Function will be executed.
3. In the Auto Function Settings section:



For a description of the parameters, see Run the Autofocus auto-function on page 89.

   a. Specify the Desired Defocus
   b. Specify the Iterate to defocus value
   c. (Optional) Select Use Three Image Method: Yes to include drift correction. Tomography will acquire an extra image to measure drift.

4. Select Execution > Start

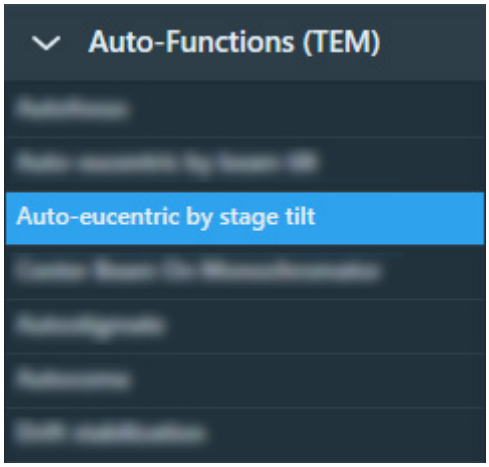


6.1.3 Run the 'Auto-eucentric by stage tilt' auto-function

The *Auto-eucentric by stage tilt* function is used to set the specimen to eucentric height. In Tomography, it is always recommended to use the Stage-tilt method rather than the Beam-tilt one.

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

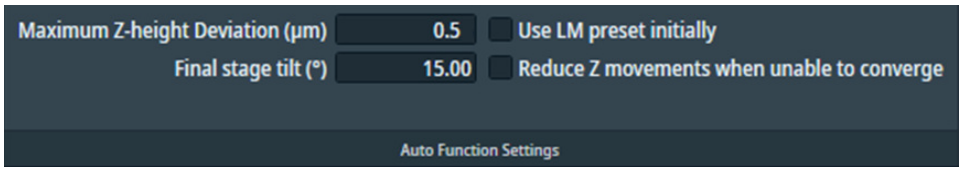
1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Auto-eucentric by stage tilt**



2. Tomography selects the relevant preset automatically, e.g. the *Eucentric Height* preset. Otherwise, select the **Preset Selection** > **Presets** that will be used to execute the auto-function.

   The Auto Functions can be executed with any Preset.

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



   a. Specify the **Maximum Z Deviation**

      The default value, 0.5 µm, is suitable for the majority of all experiments and specimens.

   b. Specify the **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.

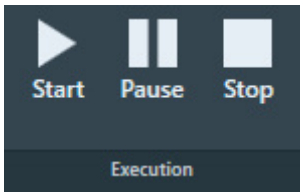
   c. (optional) Tick **Use LM preset initially**. This uses the LM preset for the initial measurement whereas the selected preset is used in the final phase.

      Recommended when Eucentric height is far off 0.

      Recommended when acquiring Search Maps automatically.

   d. (optional) Tick **Reduce Z movements when unable to converge.** When the eucentric height does not converge, select this option to reduce the Z compensation movement by 25%.

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

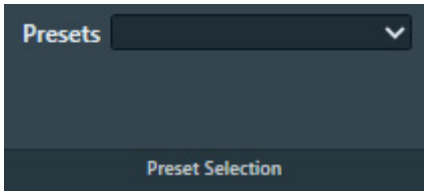


6.1.4 Run the Autostigmate auto-function

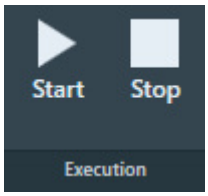
The Autostigmate function uses Thon ring-based aberration corrections. This method corrects astigmatism with higher accuracy than the Auto Stigmate option in the Autofocus function.

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

1. Verify that the *Autofocus* calibration is completed.
   If not, see The Autofocus Calibration task on page 100 for instructions.
2. Move the specimen to an area of continuous amorphous carbon foil that is *not* close to a grid bar.
3. Select **Auto Functions** > **Auto-Functions (TEM)** > **Autostigmate**.
4. Tomography selects the *Thon Ring* preset automatically. Otherwise, select **Preset Selection** > **Presets: Thon Ring**.



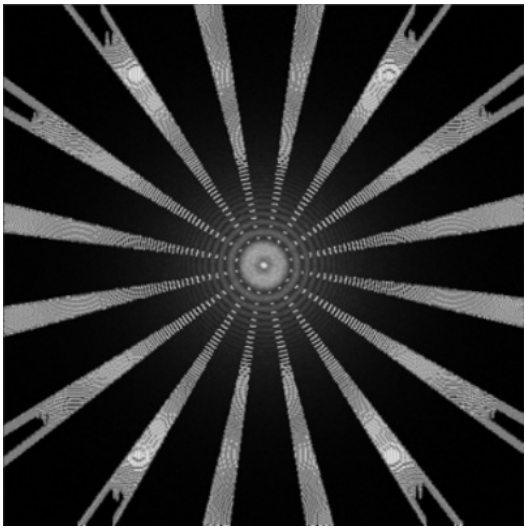
5. Select **Execution** >**Start**.



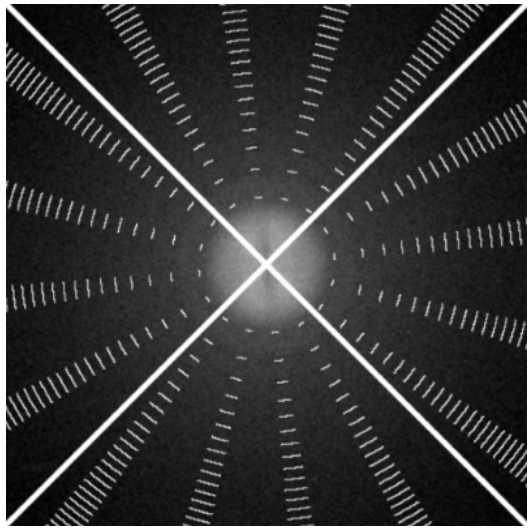
Tomography acquires an image and then attempts to fit Thon Rings.

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.

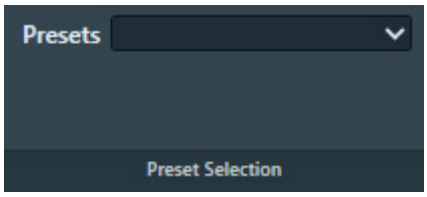
6.1.5 Run the Autocoma auto-function

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.

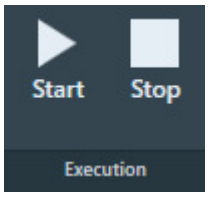
Note For the best result, run the Autostigmate function before *and* after running the Autocoma function.

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 93.
2. Move the specimen to an area of continuous amorphous carbon foil that is *not* close to a grid bar.
3. Verify that the **Objective aperture** is accurately centered.
   If necessary, manually center the **Objective aperture**.
4. Select **Auto Functions** > **Auto-Functions (TEM)** > **Autocoma**.
5. Tomography selects the *Thon Ring* preset automatically. Otherwise, select **Preset Selection** > **Presets: Thon Ring**.



6. Select **Execution** > **Start**.



   Tomography 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 93.

6.1.6 Run the Drift Stabilization auto-function

Each stage move is followed by a certain amount of mechanical and thermal drift.

- 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.

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

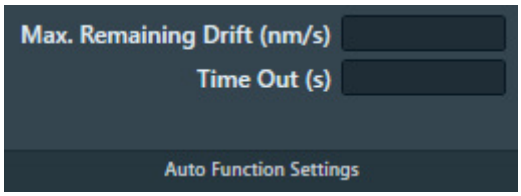
- If the drift speed decreases below a specified threshold value before it times out, then the *Drift stabilization* function reports OK.
- If the drift speed times out before it reaches the specified threshold value, then the *Drift stabilization* function reports a failure.

In Tomography, the *Drift stabilization* function is not used during an Automated Acquisition run. If significant XY drift is suspected before or during an Automated Acquisition run, then it may be useful to run the *Drift stabilization* Auto Function before the run is started or resumed. The Drift stabilization function is not well suited to detect drift on the Z or A-tilt axes.

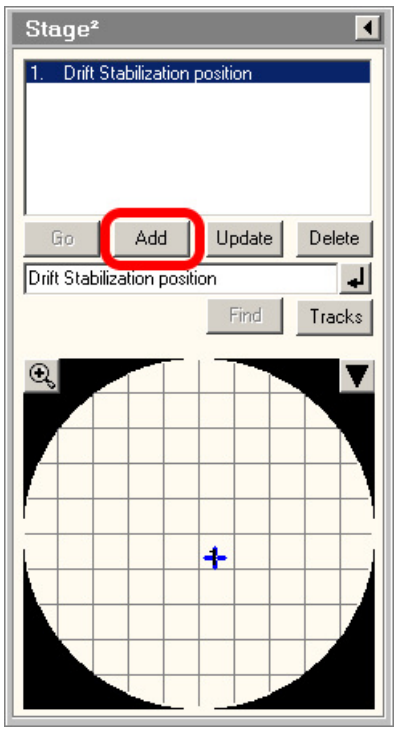
To run the Drift stabilization auto-function, follow the procedure below:

1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Drift stabilization**
2. Select **Preset Selection** > **Presets: Drift**

3. In **Auto Function Settings**:

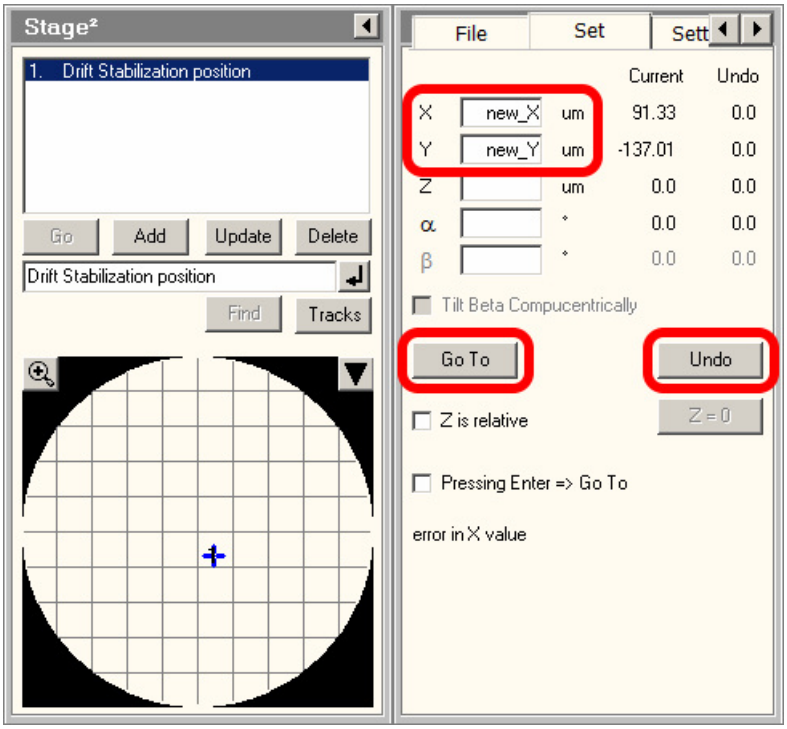


   a. Specify **Max. Remaining Drift (nm/s)**
      This is the threshold value at which the Drift stabilization function reports a successful result.
   b. Specify **Time Out (s)**
      This is the time after which the Drift stabilization function reports a failure.
      During the Automated Acquisition 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 Tracking 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**



   (Optional) enter a name to identify the stage position.

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.

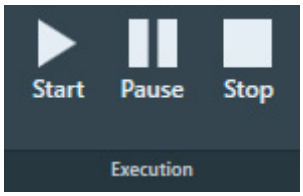


- To mimic a large move, add 160 µm to both X and Y.
  Specimens with a Grid Square width of 80 µm are commonly used. Adding two Grid Square lengths assures there is a decent margin in the measured stabilization time.
- To mimic a short move, add 10 µm to both X and Y.
  On Lacey Carbon, an Acquisition Area spacing of 5 µm is fairly common, but this depends on the beam diameter in the Data Acquisition Preset.

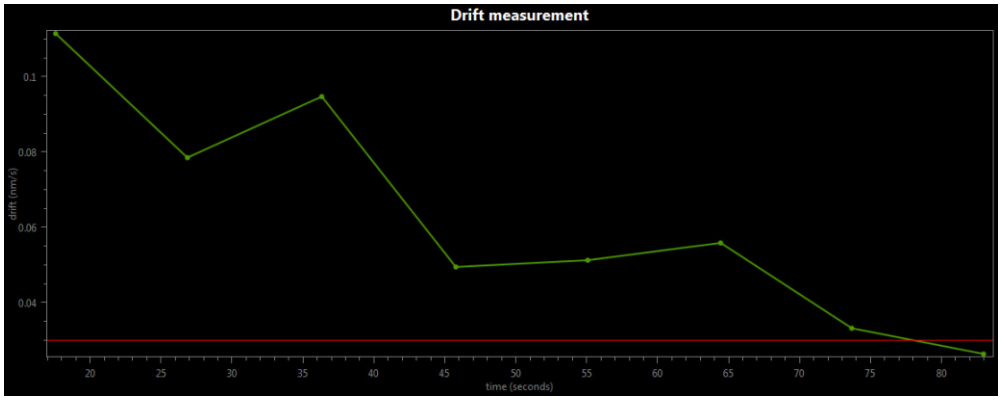
e. Select **Go To** and wait for the CompuStage to complete the move.

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.



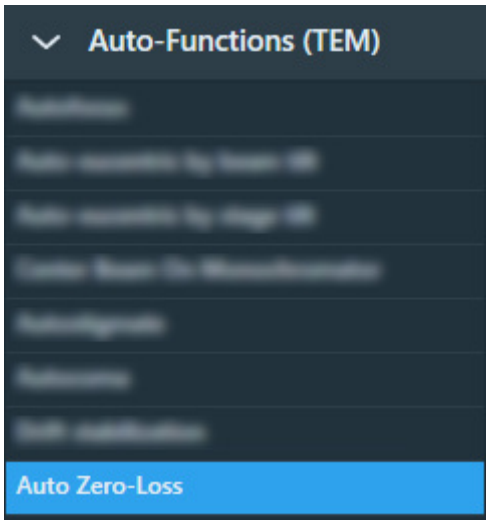
6.1.7 Run the Auto Zero-Loss auto-function

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.

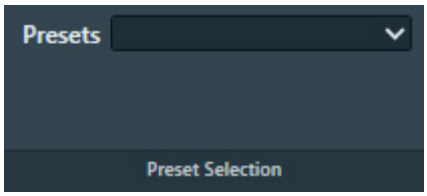
Note The Auto Zero-Loss function is only available when the system is in EFTEM mode when Tomography is started.

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

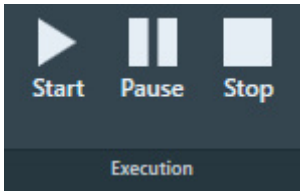
1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Auto Zero-Loss**.



2. Tomography selects the *Zero Loss* preset automatically. Otherwise, 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.

6.2 Calibration tasks

6.2.1 The Autofocus Calibration task

6.2.1.1 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.

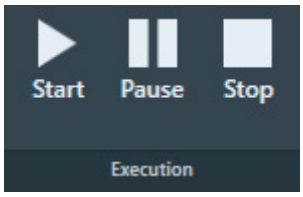
To prepare for the Autofocus calibration, use the Handpanels and the TEM User Interface to perform the procedure below:

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. Adjust **Handpanels** > **Focus** to accurately focus the specimen.

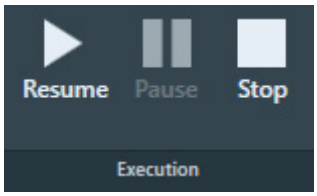
6.2.1.2 Perform the Autofocus Calibration for the Focus Preset

To perform the Autofocus calibration, follow the procedure below:

1. Select the **Auto Functions** > **Calibrations: Autofocus** task.
2. Tomography selects the *Focus* preset automatically. Otherwise, select **Preset Selection** > **Preset**: **Focus**.
   The Autofocus calibration ignores the Defocus value of the selected Preset.
3. Select **Execution** > **Start**



4. Follow the instructions on screen.
   Tomography 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 then calculates their cross-correlation. The encircled bright spot in the Cross-Correlation image corresponds to the shift between the beam tilt images.

5. In the Cross-Correlation image, estimate the length of the shift.
6. 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**: **Focus**.
   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.
7. At the end of the procedure, select **Resume** to accept and store the calibration results.

8. 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.

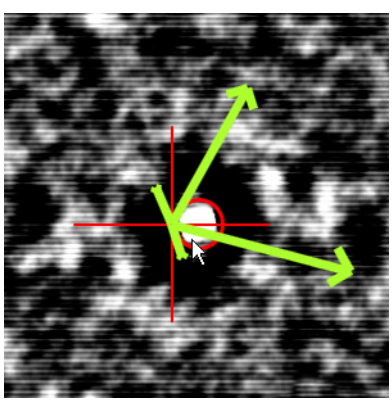
6.2.1.3 Perform the Autofocus Calibration for other Presets

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.

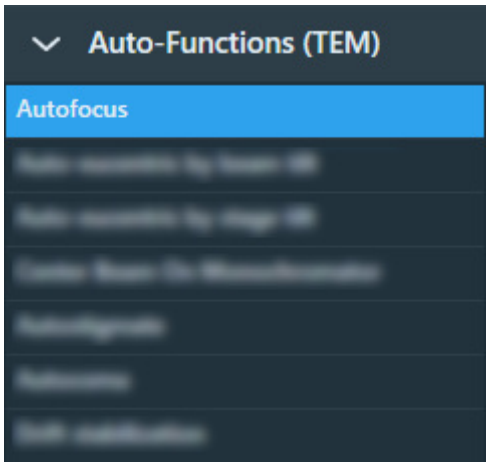
6.2.1.4 How to improve the Autofocus Calibration result

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.

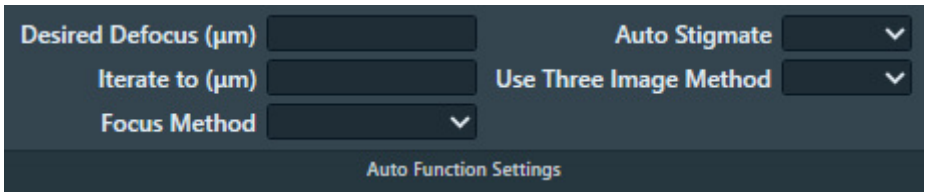


Although the Autofocus function may work well enough for automated data acquisition, it may be worthwhile to improve the stigmation and the accuracy of the calibration.

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

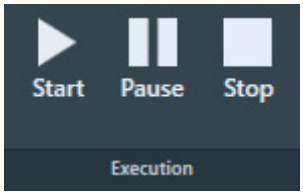


2. In **Auto Function Settings**:



   a. Specify **Desired Defocus**: **0.0**
   b. Select **Auto Stigmate**: **Yes**

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



4. Repeat the calibration procedure.

6.2.2 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:

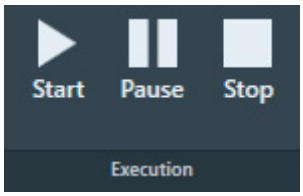
- The beam tilt pivot points must be accurately aligned at eucentric focus height.
- Eucentric focus height and the mechanical eucentric height must be exactly the same.

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.

6.2.2.1 Prepare for the Eucentric Correction Calibration

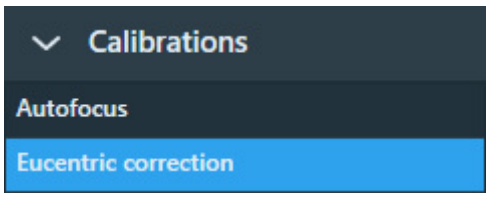
1. Select **Auto Functions** > **Auto-Functions (TEM)** > **Auto-eucentric by stage tilt**
2. Tomography selects the *Eucentric Height* preset automatically. Otherwise, select **Preset Selection** : **Presets**: **Eucentric Height**.
3. Select **Execution** > **Start**



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.

6.2.2.2 Perform the Eucentric Correction Calibration

1. If necessary, select **Auto Functions** > **Calibrations** to expand the Calibrations section.
2. Select **Eucentric correction**.



3. Tomography selects the *Eucentric Height* preset automatically. Otherwise, select **Preset Selection** > **Presets**: **Eucentric Height**.
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.

7 Tomography Tab

The Tomography tab provides tasks and functionalities to setup and execute an Automated Acquisition run. The sequence of actions in the Tomography tab depends on the selected Experiment Settings.

Before starting the tasks in the Tomography tab:

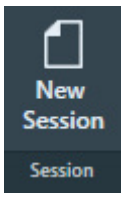
- The tasks in the Preparations tab must be completed.
- The Auto Functions calibrations and alignments must be completed.
- For the Batch option, it is recommended that an Atlas of the specimen is acquired for easy navigation, but this is not a prerequisite.

7.1 Session Setup task

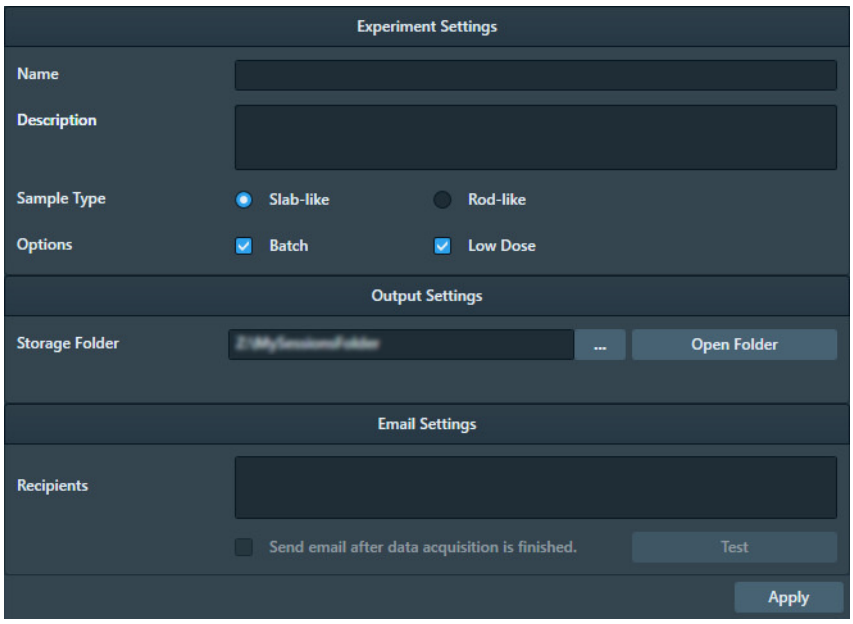
7.1.1 Create a new Tomography Session

To setup a new Automated Acquisition session, follow the procedure below:

1. Select the **Tomography** > **Session Setup** task.
2. Select **Session** > **New Session**



The **New Session** form appears.



3. In the **Experiment Settings** section:
   a. Specify a **Name** for the new Tomography session.
   b. Optionally enter a **Description** for the new Tomography session.

c. Select the **Sample Type**:
   - **Slab-like**
     The specimen is carried by a flat foil. The cross-section of the specimen in the direction of the electron beam increases as the tilt angle increases.
   - **Rod-like**
     The specimen is needle shaped. The cross-section of the specimen in the direction of the electron beam is fairly constant at every tilt angle.

d. (Optional) Tick **Batch**
   Depending on the selection, Tomography activates either:
   - The *Batch Positions* task to acquire data from multiple features of interest.
   - The *Sample Navigation* task to acquire data from a single feature of interest.

e. (Optional) Tick **Low Dose**
   In a Low Dose experiment Tomography does not execute the Autofocus, Tracking and optional Phase Plate Conditioning actions at the same location as Exposure Area where the feature of interest is located. Instead, these action take place at dedicated areas near the feature.
   Use the Low Dose option:
   - To prevent over-exposure of dose sensitive features.
   - To prevent charging of the feature of interest.
   - To prevent failure of the Focus and Tracking functions when the feature of interest has very low contrast.
   - To enable the use of Phase Plates.

   If Low Dose is cleared, then the Autofocus and Tracking actions take place at the same location as the Exposure Area.

4. In the **Output Settings** section:
   a. If the system has a filter with a Gatan K3 camera,
      then select the **Dose fraction output format**:
      - Both output formats use 8-bit unsigned integer pixels.
      - The *TIFF Lzw* format uses a lossless LZW compression.
   b. Specify the **Storage Folder** path,
      or select **Browse** to navigate to the desired file system location.

5. For **Athena Settings**, see The Athena settings on page 108.
6. In the **Email Settings** section:
   a. Specify the **Recipients**
      Enter the email addresses to which a notification is sent when the Automated Acquisition run is completed or otherwise stopped. When entering multiple addresses they need to be separated by commas, semi-colons or spaces.
   b. Tick or clear **Send email...**

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.

   The email settings can not be updated after a run is started.

7. Select **Apply**

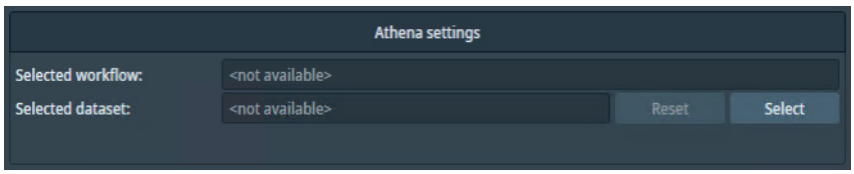
   If an Atlas is available for the currently loaded specimen, then that Atlas is used for the new session.

   Depending on the selected options, Tomography makes the applicable tasks available.

It is possible to change the selected options after the session is created. Depending on the progress and status of the session, some actions may have to be performed again.

7.1.1.1 The Athena settings

If the Microscope PC has a connection to the Athena software on the Data Management Platform (DMP) Server, then Tomography can store the acquired data in an Athena Dataset. Optionally, the Athena Dataset can be related to a Grid, but this is not required for a successful Tomography session. To select the Athena Dataset, the Session Setup > Athena Settings must be defined.



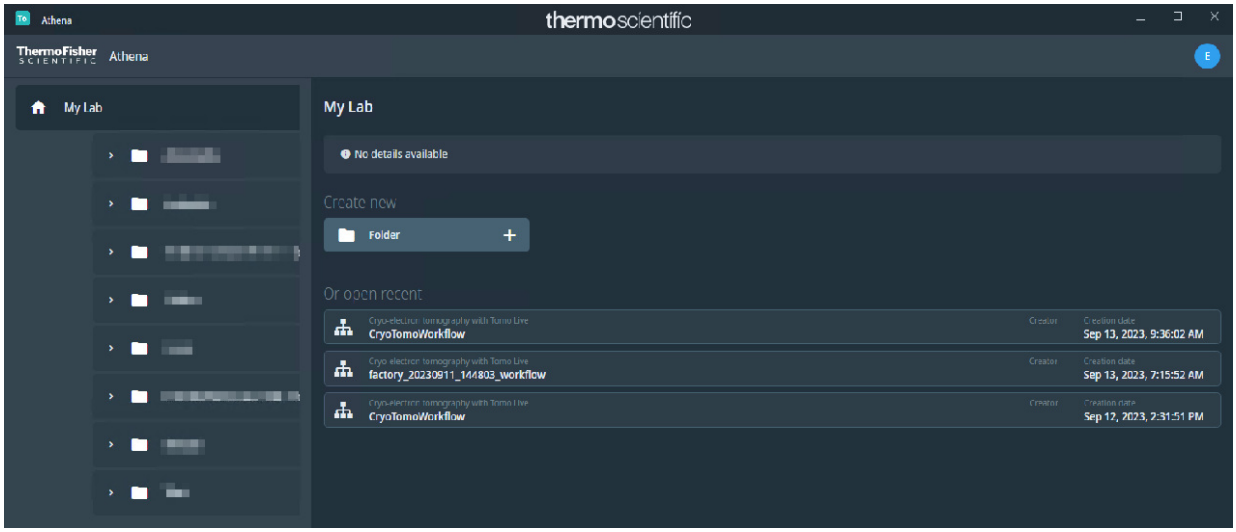
The Athena Settings section is only visible when the Athena client software is installed on the Microscope PC. If the Athena Settings are disabled, then first log in on Thermo Scientific Athena. For instructions, see Log in on Thermo Scientific Athena on page 6. For a successful Automated Acquisition run it is not required to select an Athena Dataset.

Note It is not possible to change the Athena Settings during the Automated Acquisition run.

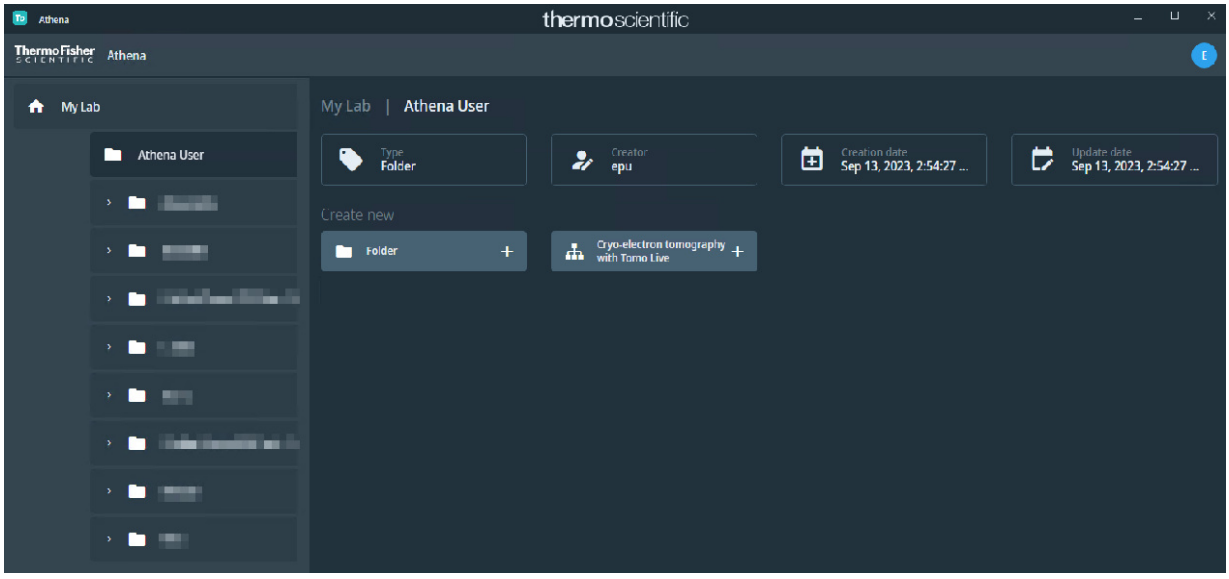
To select an Athena Dataset in the Tomography, perform the actions below in the Session Setup task.

1. In the **Athena settings** section, select **Select**

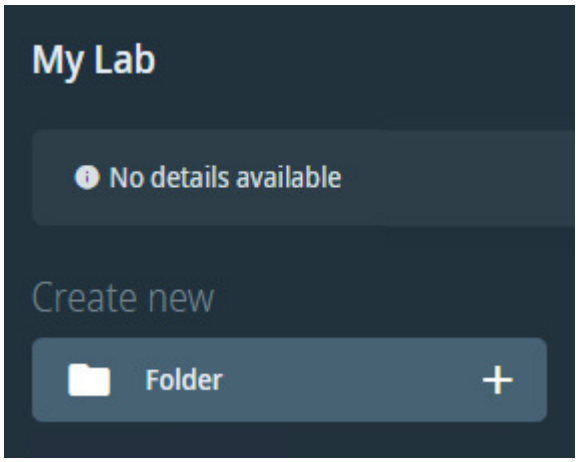
   The *Athena interface* appears.



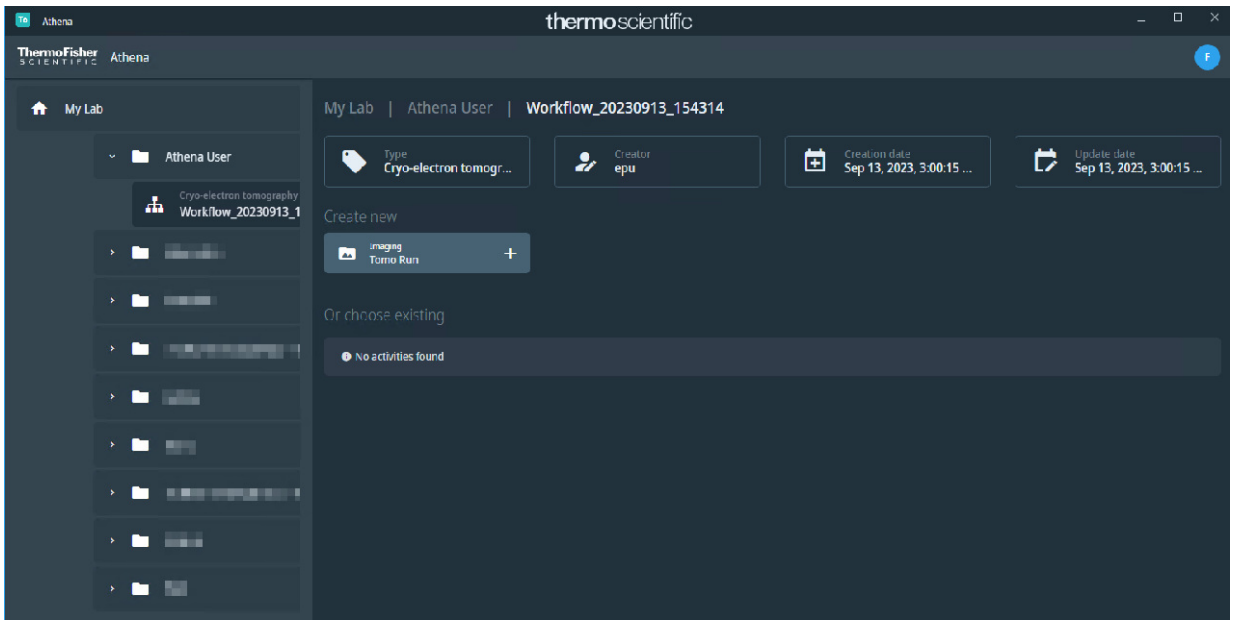
2. Select the **Folder** for which the Tomography session is executed, in the left panel.



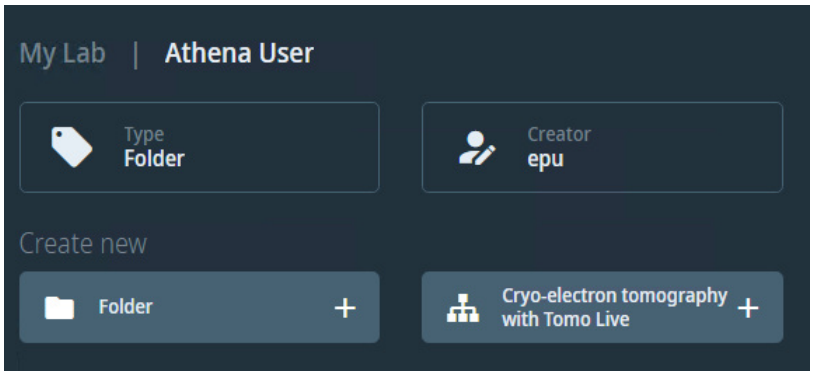
If not yet available, create a new folder by using the **Folder+** button. Adjust name and description and click **OK**.



3. Select the **Workflow** for which the Tomography session is executed, in the left panel.



If not yet available, create a new workflow by using the **Cryo-electron tomography with Tomo Live+** button. Adjust name and description and click **OK.**



4. Create a new dataset by using the **Tomo Run+** button. Adjust name and description and click **OK**.

   It is not possible to change the Dataset selection for data that is already acquired. If the Athena Settings are changed after data has already been acquired, then the new Athena Settings apply only to the data that is acquired from that moment onwards

7.1.2 Load an existing Tomography Session

You can load a Tomography session file (`.dm`) which contains the following data:

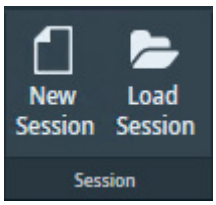
- Experiment Settings
- Athena Settings
- Output Settings
- Email Settings.

In addition, loading a Tomography session automatically loads the data saved in the corresponding Storage Folder. This allows you to review acquired data and utilize Tomography features, such as batch annotations.

Note To ensure proper functionality, manually load the corresponding Atlas session before loading the Tomography session. This ensures that the Atlas session is correctly displayed within the Tomography session, if applicable.

To load an existing Tomography session, follow the steps:

1. Go to the **Tomography > Session Setup task**.
2. Select **Session Management > Load Session**.



Navigate to the Output folder of the desired Tomography session and select the session's `.dm` file.

7.2 The Search Maps task

To identify each individual feature, Tomography uses *Search Maps*. A Search Map is matrix of Search images that is assembled like an Atlas.

In the Search Maps task, multiple Search Maps on the specimen can be added and acquired automatically. During the acquisition of multiple Search Maps, Tomography ensures that the specimen is at Eucentric Height before acquiring each individual Search Map.

For easy navigation and accurate target selection, Tomography provides a process flow that uses a stepped sequence of views on the specimen at increasing magnifications. This flow helps accurately identify multiple features of interest and helps prepare a Tilt Series for each individual feature.

Tomography also provides a way to select an Atlas that does not have to be currently loaded on the stage, allowing you to define Search Maps for multiple samples without needing to load each sample individually. However, acquiring images (including Overview images) and navigating on them with the stage is not allowed until the corresponding sample is loaded.

To speed up defining Batch Positions (see The Batch Positions task on page 131), multiple Search Maps can be acquired separately without user interaction.

To acquire multiple Search Maps for areas of interest, perform the following actions:

1. (Optional) Acquire an Overview of the area of interest.

   The Overview has a wide field of view. On a slab-like specimen, this is typically a single GridSquare. In the Overview view it is possible to:

   - Acquire a Search Map immediately.
   - Add a Search Map to the list of Search Maps to be acquired.
2. Add a Search Map of the area of interest to the list of Search Maps to be acquired.

   A Search Map has a smaller field of view at a higher accuracy than the Overview image. The tiles of a Search Map are marked in the Atlas and in the Overview. The currently active Search Map has green tile outlines, all acquired Search Maps have yellow tile outlines, and all Search Maps yet to be acquired have orange tile outlines.
3. (Optional) Edit, refine, re-order, and delete the defined Search Maps as needed.

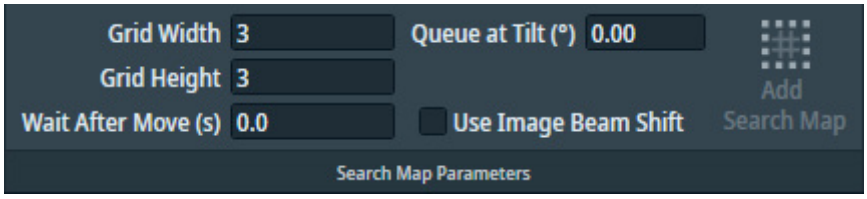
7.2.1 Add a Search Map

Search Maps can be added on the Atlas image, or on an Overview image. For information on how to acquire an overview image, refer to Acquire an Overview image on page 122, Acquire an Overview image on page 132.

It is also possible to acquire a single Search Map directly using the Acquire Search Map button. However, using this button does not run the Auto-eucentric function automatically. See Acquire a Search Map on page 125, Acquire a Search Map on page 134.

To define and add a Search Map:

1. In the **Search Map Parameters**:

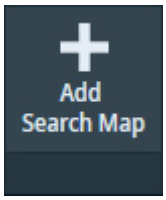


   a. Specify the **Grid Width** (X direction) and **Grid Height** (Y direction).
   b. Specify **Wait After Move (s)**: the time between the stage move and the acquisition of a search map image.
   c. Specify **Queue at Tilt (˚)**: the Alpha tilt used specifically for defining queued Search Map acquisitions.

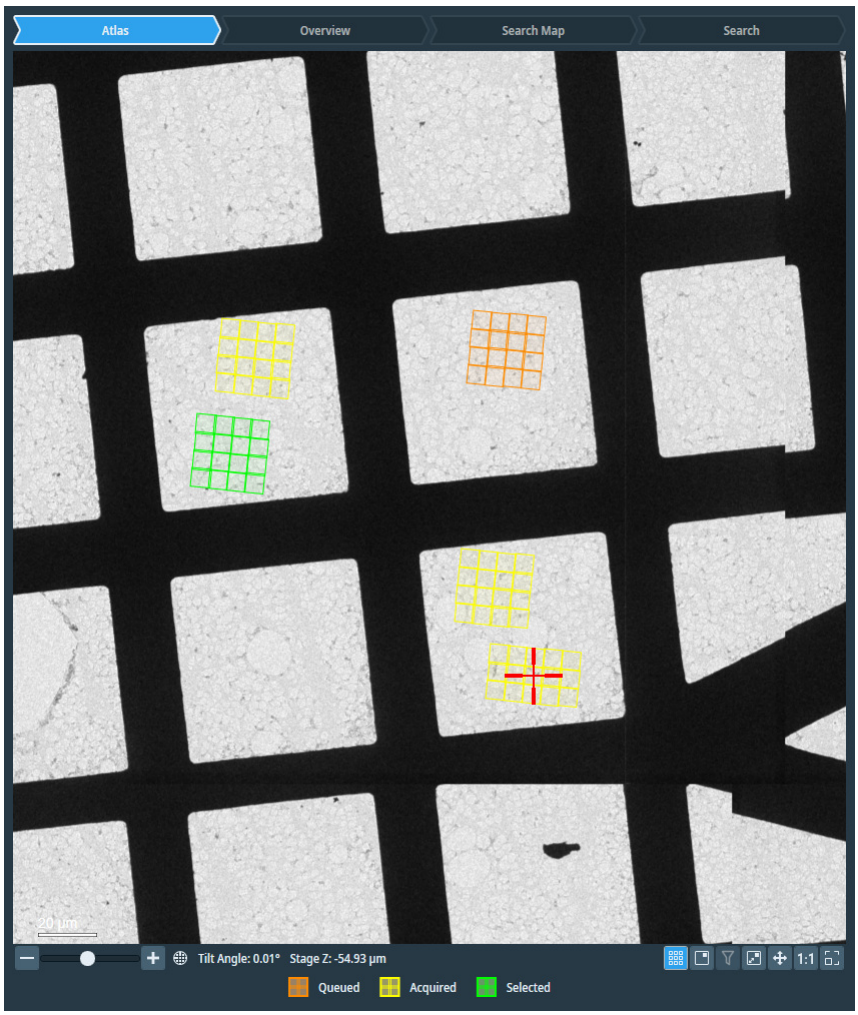
Note *Queue at Tilt* is used within the context of adding Search Maps for acquisition and is distinct from the *Set Tilt* function.

   d. (Optional) Tick **Use Image Beam Shift**: if active, image beam shift is used, rather than stage shift, for Search Map acquisition.
2. In the Atlas image or in the Overview image, right-click on the center of the **area of interest** and select **Add Search Map here**.
3. (Optional) Alternatively, in the Atlas image or the Overview image, right-click on the center of the **area of interest** and select **Move Stage here**.

4. Click **Add Search Map**.



The outlines of the Search Map are visible in the Atlas, and are displayed in orange for Search Maps that are not yet acquired. If an Overview is available that contains the Search Map area, then the outlines of the Search Map tiles are also visible in that Overview.

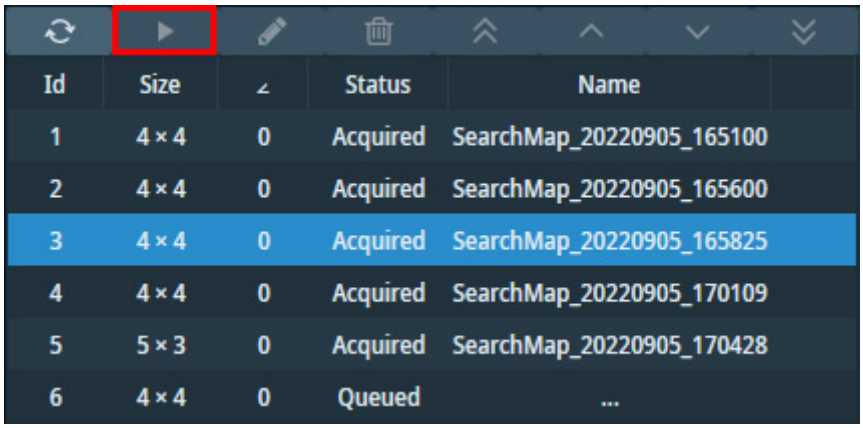


7.2.2 Manage Search Maps

7.2.2.1 Acquire a single Search Map

To acquire a single Search Map from the Search Maps List:

1. In the Image Display or in the Search Maps List, select the Search Map that must be acquired.
2. Select the Acquire button above the Search Maps List column header.



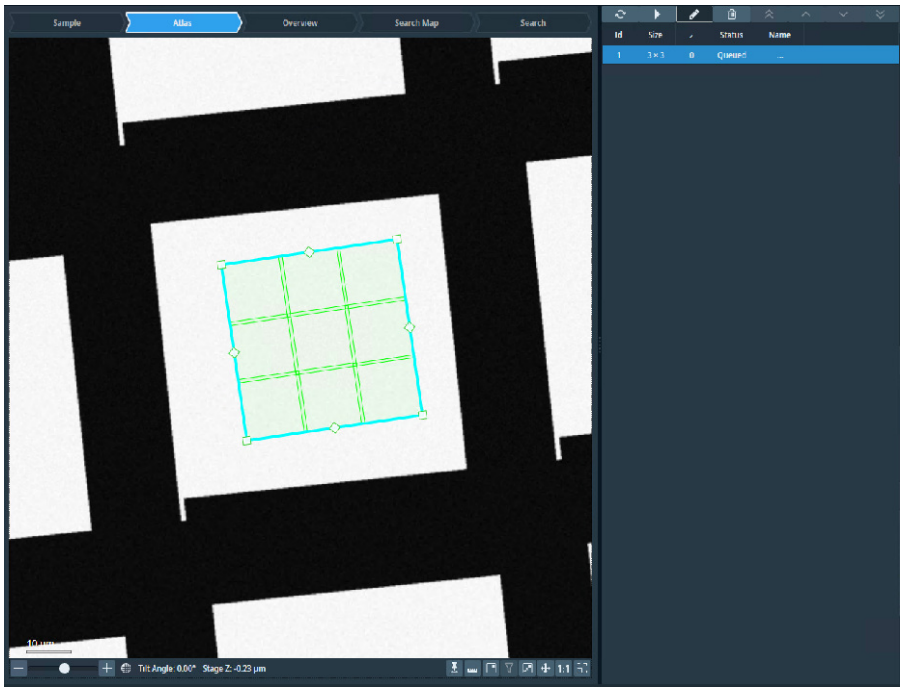
Id	Size	∠	Status	Name
1	4 × 4	0	Acquired	SearchMap_20220905_165100
2	4 × 4	0	Acquired	SearchMap_20220905_165600
3	4 × 4	0	Acquired	SearchMap_20220905_165825
4	4 × 4	0	Acquired	SearchMap_20220905_170109
5	5 × 3	0	Acquired	SearchMap_20220905_170428
6	4 × 4	0	Queued	...

7.2.2.2 Edit a single Search Map

To change a Search Map:

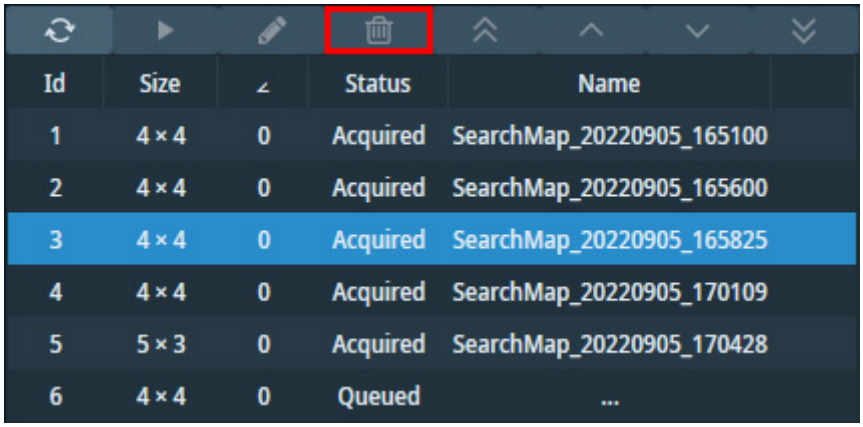
- Select the Search Map (either in the list of Search Maps, or by clicking on the Search Map annotation).
- Click, hold and move the center of a Search Map to pan the position of Search Map.
- Click, hold and move the squares on edges of the Search Map to change size of Search Map.
- To enter/exit the edit mode of a search map, either:
  - Double click on a search map annotation or
  - Click the edit button in the search maps list

Note You cannot edit the Acquired Search Maps.



7.2.2.3 Delete a single Search Map

1. In the Image Display or in the Search Maps List, select the Search Map that must be deleted.
2. Either:
   - Right-click and select Delete
   - Select the Delete button above the Search Maps List column header.



Id	Size	∠	Status	Name
1	4 × 4	0	Acquired	SearchMap_20220905_165100
2	4 × 4	0	Acquired	SearchMap_20220905_165600
3	4 × 4	0	Acquired	SearchMap_20220905_165825
4	4 × 4	0	Acquired	SearchMap_20220905_170109
5	5 × 3	0	Acquired	SearchMap_20220905_170428
6	4 × 4	0	Queued	...

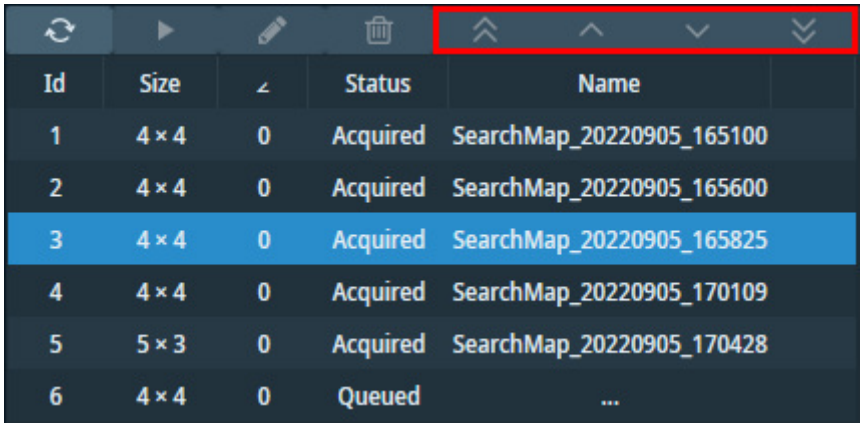
Note There is no Undo function. It is not possible to retrieve deleted Search Maps.

7.2.2.4 Change the processing order of the Search Maps

During the Automated Acquisition run, the Search Maps in the list are processed in top-to-bottom order. By default, this is the order in which the Search Maps have been added to the list.

To change the processing order, either:

- Select a Search Map, then select a re-ordering action from the toolbar above the Search Maps List.
- Right-click on a Search Map and select a re-ordering action from the context menu.



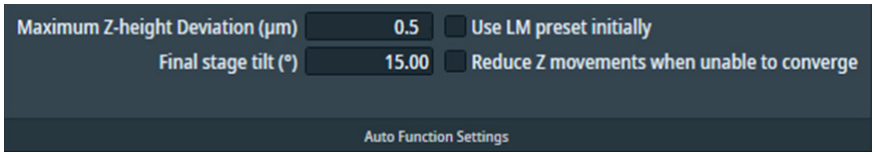
Id	Size	∠	Status	Name
1	4 × 4	0	Acquired	SearchMap_20220905_165100
2	4 × 4	0	Acquired	SearchMap_20220905_165600
3	4 × 4	0	Acquired	SearchMap_20220905_165825
4	4 × 4	0	Acquired	SearchMap_20220905_170109
5	5 × 3	0	Acquired	SearchMap_20220905_170428
6	4 × 4	0	Queued	...

Note Note: The Acquired Search Maps will always be displayed at the top of the list.

7.2.3 Acquire all Search Maps in the list

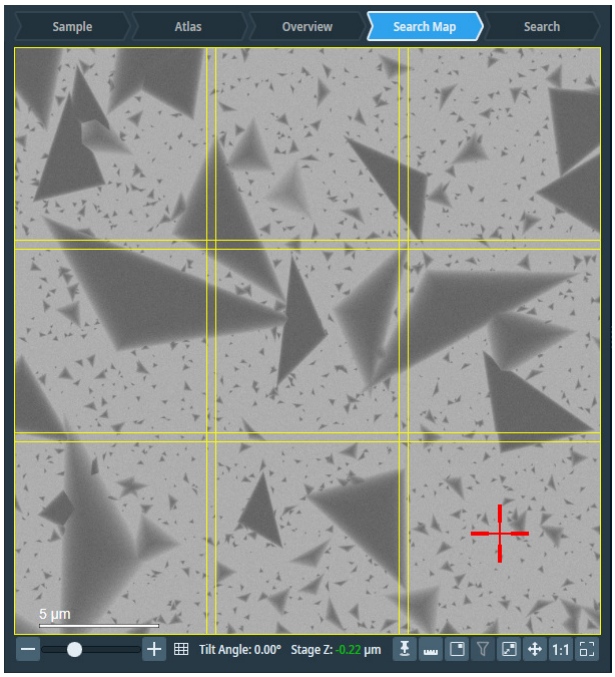
During automatic acquisition of all Search Maps, the Auto-eucentric function runs at the center of each area of interest before acquiring the corresponding Search Map. After the Search Map is acquired, an Overview image is acquired at the center of the Search Map and saved. This image can then be used to gain information about the surroundings of the area of interest.

1. In **Auto Functions > Auto-eucentric by stage tilt > Auto Function Settings**:
   a. Select **Use LM preset initially**.
   b. Select **Reduce Z movements when unable to converge**.



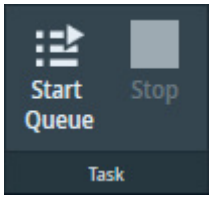
Using these two options is recommended because they can improve the reliability of Auto-eucentric, especially when far from the eucentric height.

Search Maps acquired from the queue have a highlighted Z height, indicating that they have been acquired at Eucentric height.



Note When Search Maps are defined on an Atlas that is not on the stage, the automatic acquisition of Search Maps firstly loads the correct sample, re-aligns the Atlas to correct for any shift and rotation during reload, then continues with eucentric height, and finally, the Search Map is acquired.

2. Select **Start Queue** to acquire the Search Maps in the list.

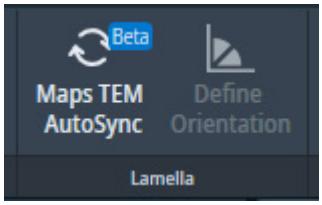


7.2.4 Synchronize Data with Maps TEM Automatically

For detailed information about Importing Presets and synchronizing Batch Positions from Tomography to Maps TEM, see *section Tomography, Maps User Guide*.

Note For the synchronisation to work, you must have both Maps and the Tomography 5 Software installed. Also, Maps Data Exchange Service must be installed.

Click **Maps TEM AutoSync** button in **Search Maps** task -> **Lamella** section to activate automatic synchronization of data with Maps TEM, and enable sending annotations in Maps.



These annotations can be sent from Maps into Tomography:

- Sites of interest
- Areas of interest

- Lamella sites

If available, Milling angle of selected Lamella site is automatically shown. If the absolute value of Current Tilt and Queue at Tilt values differ from the absolute value of Milling angle, a warning is shown.

This feature is in Beta. For feedback and questions, please contact tomo5@thermofisher.com.

7.2.5 Define the Lamella orientation

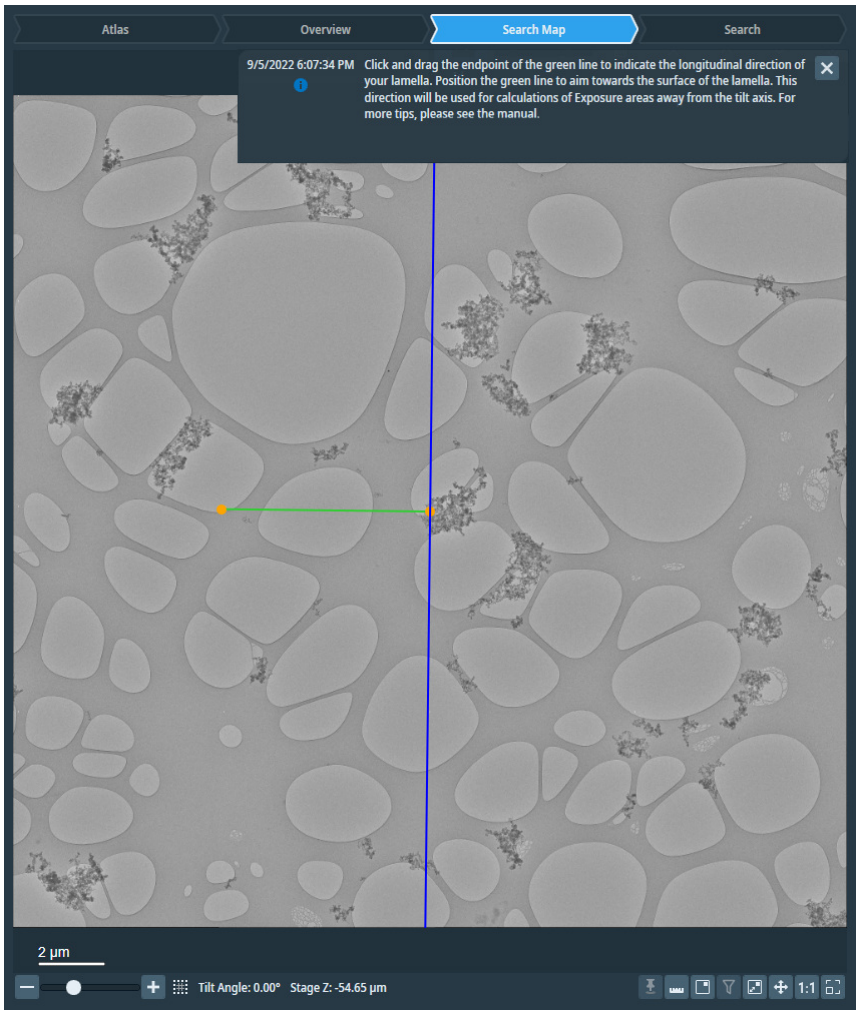
When loading specimens containing lamellae, it is recommended to align a lamella with the tilt axis. If the specimen is not oriented correctly with respect to the tilt axis, features of interest at Exposure Areas that are not located on the tilt axis might not be tracked and acquired accurately. Tomography allows you to correct for small misalignment in the orientation of a lamella with respect to the tilt axis in order to acquire exposure areas relatively far from the tilt axis more accurately.

The orientation of a lamella needs to specified manually once per specimen.

Follow these steps to define the orientation of a lamella:

1. Open a previously-acquired Search Map.
2. Select **Define Orientation**.

A green line with two orange endpoints appears in the image display. One end is fixed to the center of the tilt axis while the other end is free to drag around.

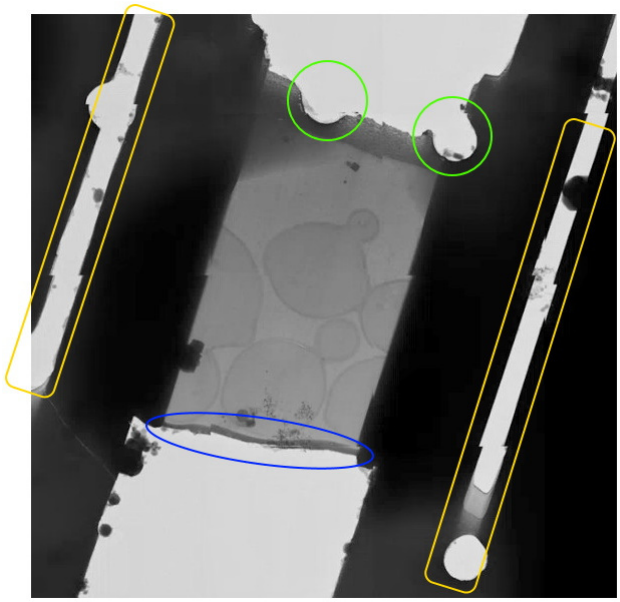


Click and drag the free end to adjust the alignment and the direction of the line according to the following.

a. Alignment: The green line needs to be longitudinal to the lamella, i.e., parallel to the orientation of the milling.

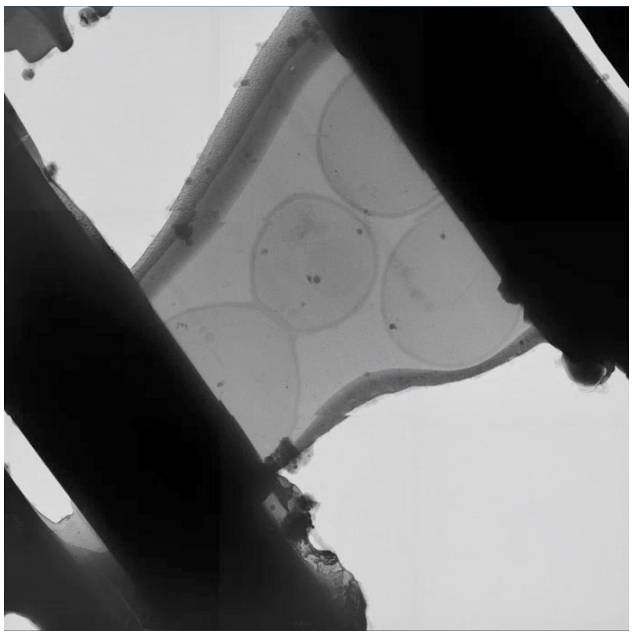
b. Direction: The green line needs to be aimed towards the surface or the higher part of the lamella. That is the the point from which the milling started, i.e., the source point of the ion beam.

To help you identify the orientation of any particular lamella, we present the following illustrated example.

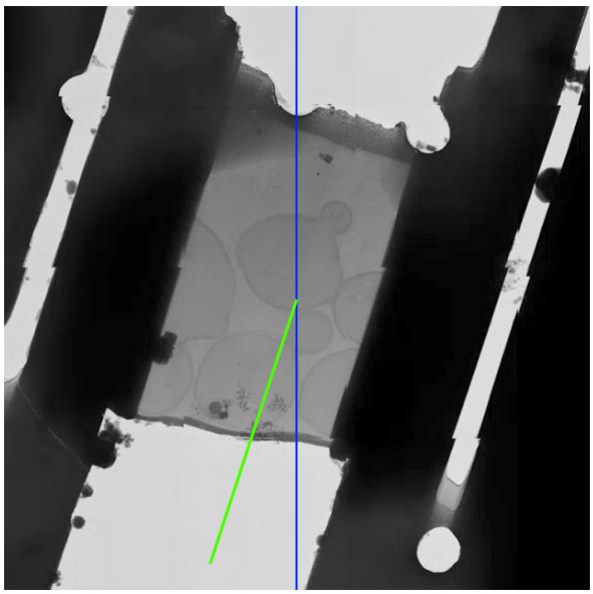


- (Marked yellow) The tension-relief cuts indicate the longitudinal axis of the lamella. Therefore, we look for the higher and lower parts of the lamella on the two ends of that longitudinal axis. In addition, you should notice which of the two sides across the length of a tension-relief cut has an abrupt start indicating the position where the cut started. The starting positions of the two tension-relief cuts are usually on the side where the milling started, i.e., on the higher side of the lamella.
- (Marked blue) The sputter-deposited platinum indicates the source of the ion beam.
- (Marked green) The round holes are foil holes which indicate the lower side of the lamella.

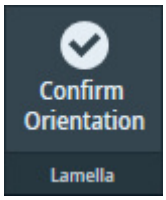
Tip regarding similar-looking sides: In some instances, the lower side of the lamella has no visible holes, and looks similar to the higher side. Observe the image below where the southeast side is the higher side of the lamella. While the lower side of the lamella (northwest in the image) has no visible holes, we can see a double layer indicating that this is not where the milling started; so we exclude this side from being the higher side. Another clue from this image is the tension-relief cut appearing on the left side; the starting position of this cut is towards the southeast where the milling started from.



Once you have determined the correct orientation, adjust the green line to the correct alignment and direction as shown below.



3. Select **Confirm Orientation**.



7.3 The Sample Navigation task

In the Sample Navigation task, a single position on the specimen is prepared for the acquisition of Tilt Series during the Data Acquisition run.

To prepare a Tilt Series for a feature of interest, perform the following actions.

1. Acquire an *Overview* of the area of interest.

   The Overview has a wide field of view. On a slab-like specimen, this is typically a single Grid Square. In the Overview view it is possible to:

   - Acquire a *Search Map* for easier and more accurate navigation and target selection.
   - Acquire a *Search* image at the location of a single feature of interest.
2. Acquire a *Search Map* of the area of interest; or use a previously-acquired Search Map.

   A Search Map has a smaller field of view at a higher accuracy than the Overview image. It is a matrix of Search images that is assembled like an Atlas. The tiles of a Search Map are marked in the Atlas and in the Overview. The currently active Search Map has green tile outlines, all other Search Maps have yellow tile outlines.
3. Acquire a *Search* image with the feature of interest in the center.

   The Search view is used to define the locations of the Template Areas and the Tilt Series settings.

   For single positions, this step is mandatory.

   For batch positions, this step is recommended, not mandatory.
4. Define the Template Area locations and the Tilt Series settings.

The sections below provide detailed information and instructions for the actions above.

7.3.1 Acquire an Overview image

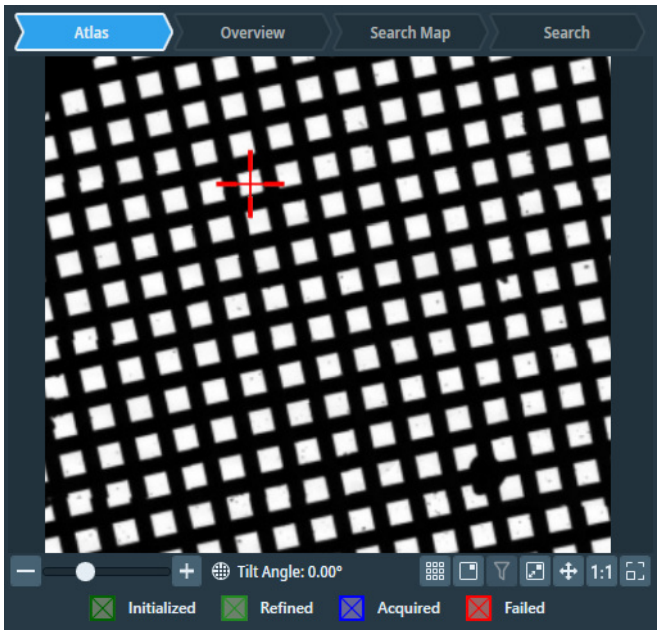
An Overview image can be acquired in the context of an Atlas of the specimen. How to do this is described in the steps below.

It is also possible to acquire an Overview image without an Atlas of the specimen. How to do this is also described below.

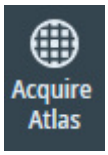
7.3.1.1 Acquire an Overview with using an Atlas

To acquire an Overview image *with* the use of an Atlas:

1. Select the **Atlas** view.

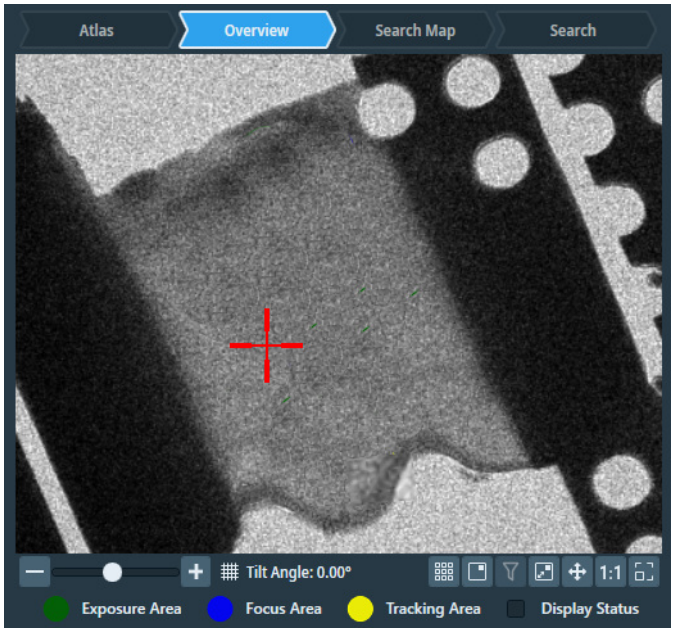


- If no Atlas is available yet, then select **Acquire Atlas**

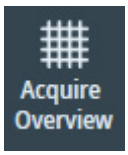


  Tomography switches to the *Atlas* > *Screening* task for the acquisition of a new Atlas. For instructions how to acquire an Atlas for the currently loaded specimen, see: Atlas Tab on page 80.
- If an Atlas is available for the currently loaded specimen, then Tomography displays the Atlas. If there already are Batch Positions in the list, then their locations are marked in the Atlas view.

2. Right-click in **the center of the area of interest**,
   then select **Move Stage Here and Acquire Overview Image**
   Tomography moves the specimen to the selected position, and then acquires and displays an Overview image.



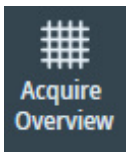
3. If the Overview image does not show the entire area of interest, then:
   a. In the current Overview image, right-click on the **center of the area of interest** and select **Move stage here**
   b. Select **Acquire Overview**



7.3.1.2 Acquire an Overview without using an Atlas

To acquire an Overview image *without* using an Atlas:

1. In **Velox**, start a *live image view*.
2. Use the **handpanels** to move the **area of interest** to the center of the live image.
3. Select **Acquire Overview**

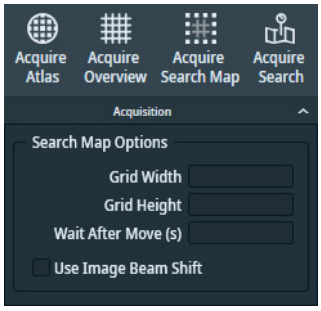


4. If the Overview image does not show the entire area of interest, then:
   a. In the current Overview image, right-click on the **center of the area of interest** and select **Move stage here**
   b. Select **Acquire Overview** again.

7.3.2 Acquire a Search Map

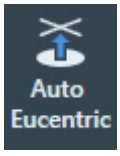
To acquire a Search Map:

1. In the **Acquisition** > **Search Map Options**:

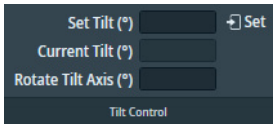


   a. Specify the **Grid Width** (X direction) and **Grid Height** (Y direction).
   b. Specify **Wait After Move (s)**: the time between the stage move and the acquisition of a search map image.
   c. Specify **Queue at Tilt (˚)**: the Alpha tilt used for Search Map acquisition.
   d. (Optional) Tick **Use Image Beam Shift** to go to the next Search Map tile location with Image Beam Shift instead of stage moves.

2. If this is the first Search Map that will be acquired in this field of view, then select **Auto Eucentric**



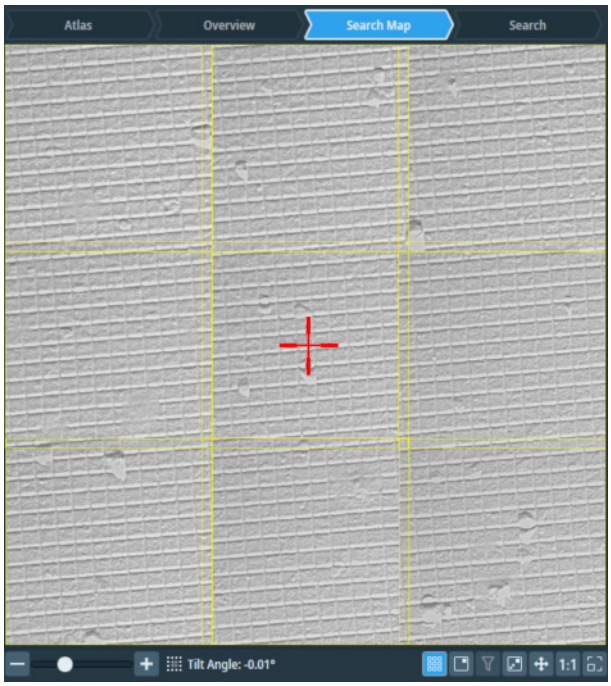
3. (Optional) Specify the *Tilt Control* settings:



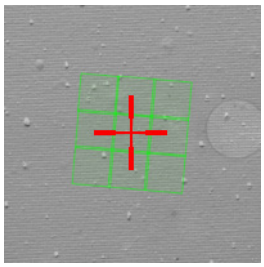
   - Specify **Set Tilt**, then select **Set** to move the stage to a different tilt angle.
     On narrow screens, the Tilt Control ribbon must be expanded to make the settings visible.
   - The **Rotate Tilt Axis** value is a system property. It specifies the offset of the tilt axis in the Search Map image relative to the physical tilt axis of the stage. The Rotate Tilt Axis value can be calculated with 3D reconstruction software, based on the acquired data from preceding Tilt Series.

4. In the Overview image, right-click on the center of the **area of interest** and either:
   - Select **Move stage here**, then select **Acquisition** > **Acquire Search Map**
   - Select **Move stage here and acquire Search Map**

     Tomography moves the specimen to the selected position, and then acquires and displays a Search Map.

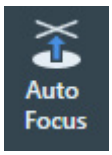


The outlines of the Search Map tiles are visible in the Atlas. If an Overview is available that contains the Search Map area, then the outlines of the Search Map tiles are also visible in that Overview.

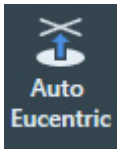


7.3.3 Acquire a Search image

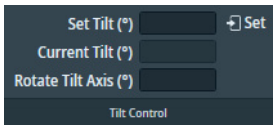
1. In the Overview image or Search Map view, right-click on the **feature of interest** and select **Move Stage Here and Acquire Search Image**
2. (Optional) Select **Auto Focus**



3. If this is the first feature of interest in the current area of interest, then select **Auto Eucentric**

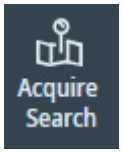


4. Accurately center the feature of interest:
   a. (Optional) Specify the *Tilt Control* settings:



   - Specify **Set Tilt**, then select **Set** to move the stage to a different tilt angle.
     On narrow screens, the Tilt Control ribbon must be expanded to make the settings visible.
   - The **Rotate Tilt Axis** value is a system property. It specifies the offset of the tilt axis in the Search image relative to the physical tilt axis of the stage. The Rotate Tilt Axis value can be calculated with 3D reconstruction software, based on the acquired data from preceding Tilt Series.

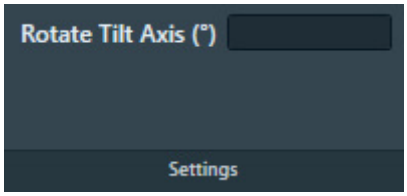
   b. Select **Acquire Search**



   c. If the feature of interest is not accurately centered in the Search image, then right-click on the **feature of interest** and select **Move Stage Here and Acquire Search Image**

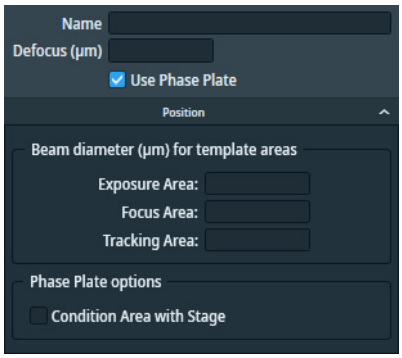
7.3.4 Define the Tilt Series position

1. (Optional) Specify **Rotate Tilt Axis**



The Rotate Tilt Axis value specifies the offset of the tilt axis in the Search image relative to the physical tilt axis of the stage. The Rotate Tilt Axis value can be calculated with 3D reconstruction software, based on the acquired data from preceding Tilt Series.

2. Specify the **Name** and **Defocus** value for the Tilt Series position.



3. If *Session Setup > Low Dose* is ticked, then:
   a. If the selected Objective aperture when Tomography is started is a Phase Plate, then Phase Plate settings become available:
      - (Optional) Tick **Use Phase Plate**

        The *Condition Area* becomes available in the Search image.

        Use Phase Plate is only available when the selected Objective aperture when Tomography is started is a Phase Plate.
      - (Optional) Tick **Condition Area with Stage** to move the specimen with the stage so that the Condition Area is illuminated.

        If *Condition Area with Stage* is cleared, then Tomography always uses beam shift, unless the distance exceeds the maximum achievable beam shift.

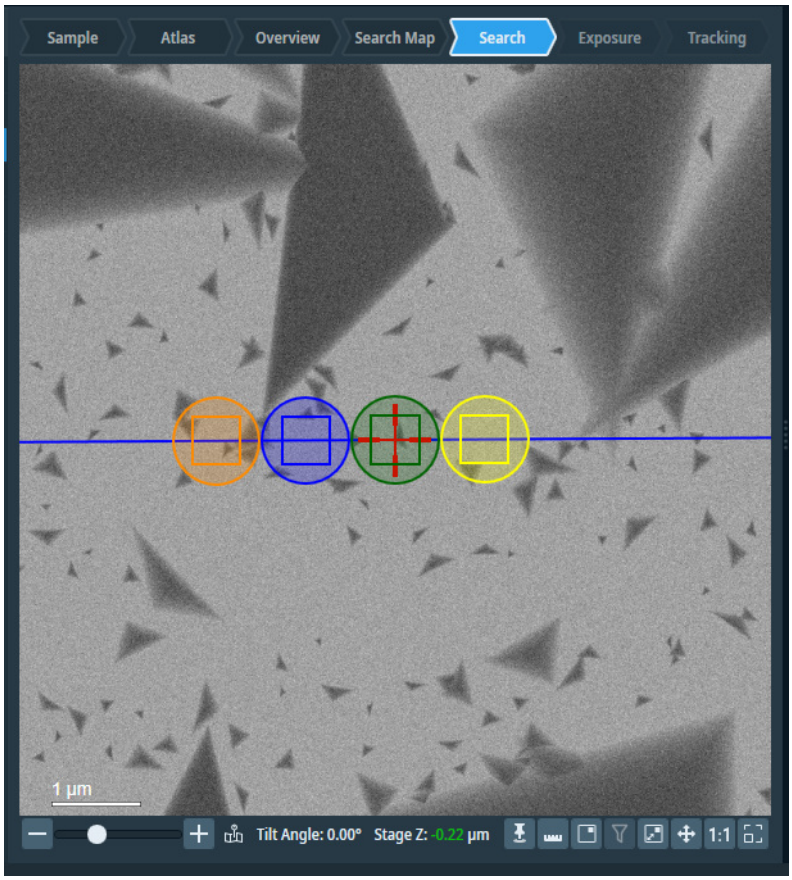
        Condition Area with Stage applies to all Batch Positions. It is *not* possible tick or clear *Condition Area with Stage* after the first Batch Position is added to the Batch Positions list.
   b. (Optional) *On a system with two condenser lenses with TEM 7.12 or lower* (typically systems that run on Talos software), specify the **beam diameter for the template areas**.

      The specified diameters are used only for display of the template areas in the Image Display. They have no impact on the actual beam diameter. To change the actual beam diameter of a template area, adjust the *Intensity* for the related Preset in the *Preparation > Acquisition and Optics Settings*.

      This step is not required on other systems. Specifically, on systems with two condenser lenses with TEM 7.13 or higher, and on systems with three condenser lenses (typically systems that run on Titan software), the size of each Template Area is determined by the *Intensity* or the *Illuminated Area* that is specified in the corresponding *Acquisition and Optics Preset*.
   c. (Optional) Right-click in the Search image and select **Lock Focus and Tracking Area**

      When locked, the Focus Area and the Tracking Area stay on top of each other. In most experiments, these areas can be on the exact same position to save time without sacrificing accuracy.

d. Drag the **Template Areas** to their desired locations on the tilt axis.



- The square outline represents the field of view of the camera sensor.
- The circular area represents the illuminated area.
  In the example above, the illuminated areas (circles) are much larger than necessary to illuminate the field of view (squares).

It is not possible to assign a location away from the tilt axis.

Note Do not change *Rotate Tilt Axis* for the purpose of dragging Template Areas to positions away from the current tilt axis.

The purpose of the Rotate Tilt Axis parameter is to compensate for a difference between the tilt axis orientation in Tomography and the physical tilt axis of the stage. If the tilt axis in Tomography is not aligned with the physical tilt axis, then the Z-height of the Focus Area will vary with the tilt angle, which may result in unfocused images of the Exposure Area.

7.3.5 Guidelines for the locations of the Template Areas

The Tracking, Focus and Condition Areas can usually be located at the same position, preferably on an area of amorphous carbon foil. For an optimal location of the Template Areas, keep the following guidelines in mind:

- The Tracking, Focus and Condition Areas must not overlap with the Exposure Area.
  The accumulated dose of multiple illuminations can cause local damage to the feature of interest.

- It is common practice to put the Focus Area, Tracking Area and optional Condition Area at the same position. This saves time and has a negligible impact on the result of the focus, tracking and conditioning functions. Use the *Lock Focus and Tracking Area* option to assign the exact same position for the Focus Area and Tracking Area. The Condition Area must always be positioned separately.
- In a Low Dose Tomography session, the Tracking function and Focus function are performed on an area near the feature of interest, so that the feature itself is not exposed. If the feature of interest moves more than half of the field of view after a tilt step, then move the Tracking Area and Focus Area a bit further away from the Exposure Area, so that the feature of interest is not exposed.
- There must be no large structures nearby any of the Template Areas that could cast a shadow on the Template Area when the specimen is tilted.

  For example, avoid positions near:
  - Grid bars.
  - Ice crystals.
  - The edge of a FIB lamella.
- The Z-height of the specimen at the *Focus Area* must be as close as possible to the Z-height of the feature of interest. This is typically the case close to the Exposure Area.

  Avoid positions near damaged or unstable carbon foil.
- The field of view of the *Tracking Area* must contain features with sufficient contrast, so that the Cross-Correlation image shows a well-defined peak.

  See The Image Filters Settings Task on page 59 for suggestions how to tune the filter settings to achieve a well-defined peak in the Cross-Correlation image.

7.4 The Batch Positions task

In the Batch Positions task, multiple positions on the specimen can be prepared for a Data Acquisition run. During the automated Data Acquisition run, Tomography will acquire a Tilt Series at each individual Batch Position.

For easy navigation and accurate target selection, Tomography offers a process flow that uses a stepped sequence of views on the specimen at increasing magnifications. This flow helps to accurately identify multiple features of interest, and to prepare a Tilt Series for each individual feature. In Batch-mode, a prepared Tilt Series for a feature of interest is called a *Batch Position*.

Note You can view the Atlas you have selected and loaded, but you cannot change this selection here. If you need to change the selected Atlas, you must do so in the Search Maps task.

To prepare a Tilt Series for a feature of interest, perform the following actions.

1. Acquire an *Overview* of the area of interest.

   The Overview has a wide field of view. On a slab-like specimen, this is typically a single Grid Square. In the Overview view it is possible to:
   - Acquire a *Search Map* for easier and more accurate navigation and target selection.
   - Acquire a *Search* image at the location of a single feature of interest.
2. Acquire a *Search Map* of the area of interest; or use a previously-acquired Search Map.

   A Search Map has a smaller field of view at a higher accuracy than the Overview image. It is a matrix of Search images that is assembled like an Atlas. The tiles of a Search Map are marked in the Atlas and in the Overview. The currently active Search Map has green tile outlines, all other Search Maps have yellow tile outlines.
3. Acquire a *Search* image with the feature of interest in the center.

   The Search view is used to define the locations of the Template Areas and the Tilt Series settings.

   For single positions, this step is mandatory.

   For batch positions, this step is recommended, not mandatory.
4. Define the Template Area locations and the Tilt Series settings.
5. Add the new Batch Position to the Batch Positions list.
6. (Optional) Edit, refine, re-order and/or delete the defined Batch Positions.

The sections below provide detailed information and instructions for the actions above.

7.4.1 Add a Batch Position

To add a Batch Position, the following workflows are possible:

- Acquire an *Overview* image
  > Acquire a *Search Map* image
  > Acquire a *Search* image
  > Define and add a *Batch Position*.
- Acquire an *Overview* image
  > Acquire a *Search* image
  > Define and add a *Batch Position*.
- **Recommended workflow**:
  Acquire an *Overview* image
  > Acquire a *Search Map* image
  > Define and add a *Batch Position*.

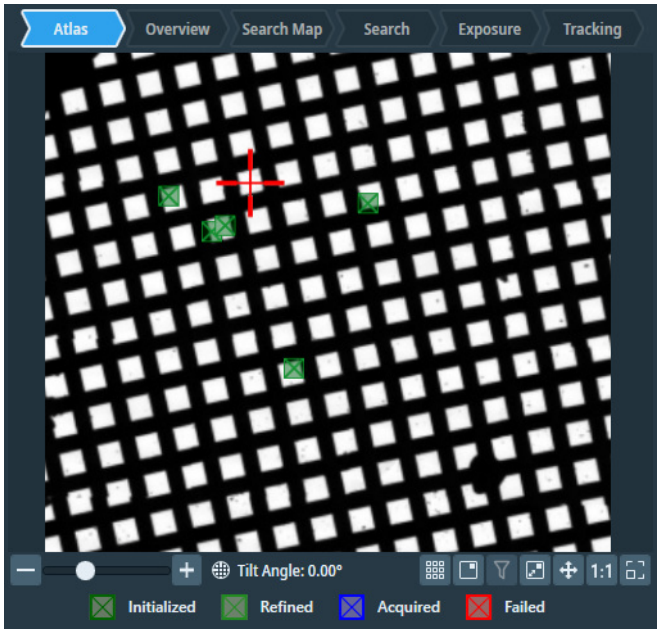
7.4.1.1 Acquire an Overview image

An Overview image can be acquired in the context of an Atlas of the specimen. How to do this is described in the steps below.
It is also possible to acquire an Overview image without an Atlas of the specimen. How to do this is also described below.

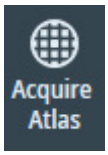
7.4.1.1.1 Acquire an Overview with using an Atlas

To acquire an Overview image *with* the use of an Atlas:

1. Select the **Atlas** view.



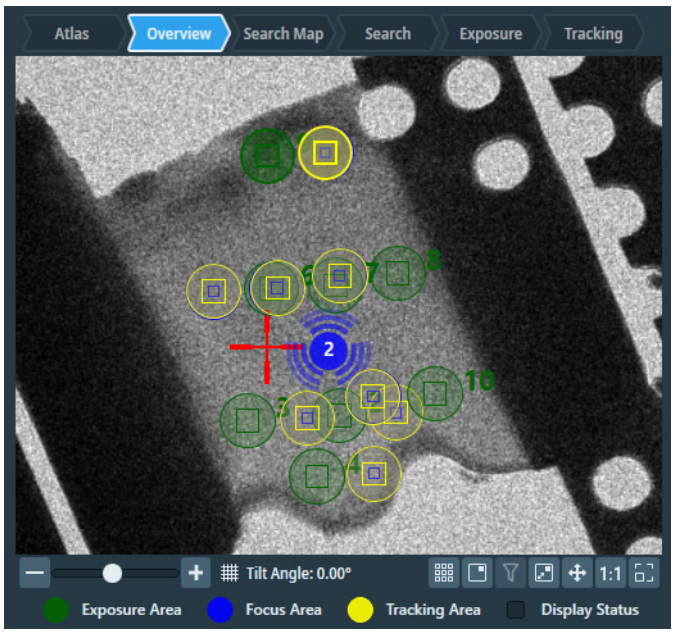
- If no Atlas is available yet, then select **Acquire Atlas**



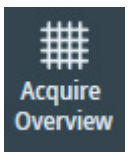
Tomography switches to the *Atlas* > *Screening* task for the acquisition of a new Atlas. For instructions how to acquire an Atlas for the currently loaded specimen, see: Atlas Tab on page 80.

- If an Atlas is available for the currently loaded specimen, then Tomography displays the Atlas. If there already are Batch Positions in the list, then their locations are marked in the Atlas view.

2. Right-click in **the center of the area of interest**,
   then select **Move Stage Here and Acquire Overview Image**
   Tomography moves the specimen to the selected position, and then acquires and displays an Overview image.



3. If the Overview image does not show the entire area of interest, then:
   a. In the current Overview image, right-click on the **center of the area of interest** and select **Move stage here**
   b. Select **Acquire Overview**

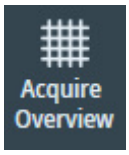


7.4.1.1.2 Acquire an Overview without using an Atlas

To acquire an Overview image *without* using an Atlas:

1. In **Velox**, start a *live image view*.
2. Use the **handpanels** to move the **area of interest** to the center of the live image.

3. Select **Acquire Overview**



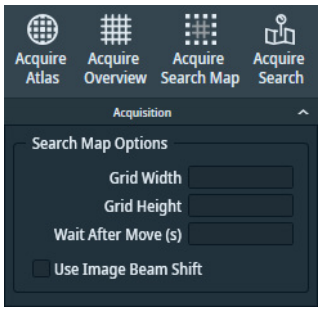
4. If the Overview image does not show the entire area of interest, then:
   a. In the current Overview image, right-click on the **center of the area of interest** and select **Move stage here**
   b. Select **Acquire Overview** again.

7.4.1.2 Acquire a Search Map and/or a Search image

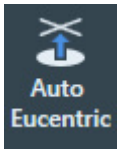
7.4.1.2.1 Acquire a Search Map

To acquire a Search Map:

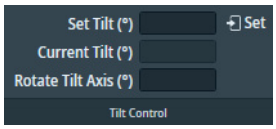
1. In the **Acquisition** > **Search Map Options**:



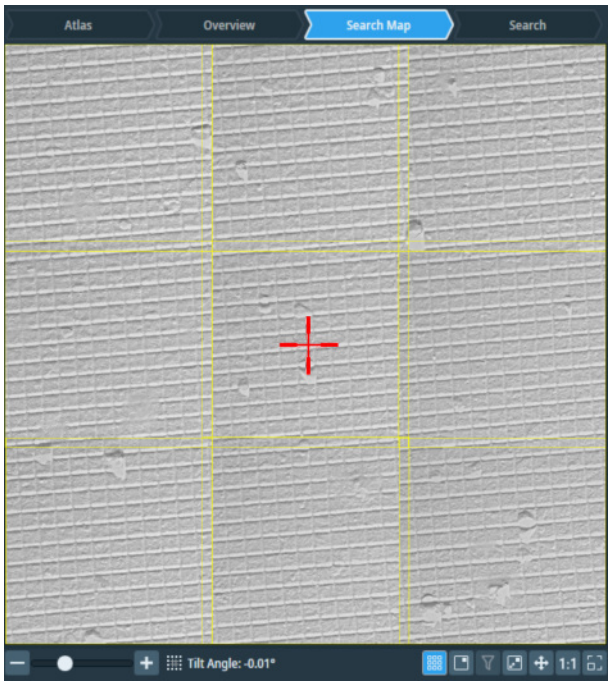
   a. Specify the **Grid Width** (X direction) and **Grid Height** (Y direction).
   b. Specify **Wait After Move (s)**: the time between the stage move and the acquisition of a search map image.
   c. Specify **Queue at Tilt (˚)**: the Alpha tilt used for Search Map acquisition.
   d. (Optional) Tick **Use Image Beam Shift** to go to the next Search Map tile location with Image Beam Shift instead of stage moves.
2. If this is the first Search Map that will be acquired in this field of view, then select **Auto Eucentric**



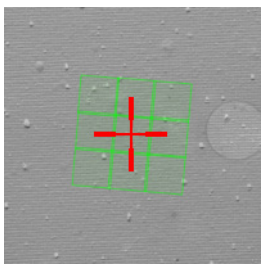
3. (Optional) Specify the *Tilt Control* settings:



   - Specify **Set Tilt**, then select **Set** to move the stage to a different tilt angle.
     On narrow screens, the Tilt Control ribbon must be expanded to make the settings visible.
   - The **Rotate Tilt Axis** value is a system property. It specifies the offset of the tilt axis in the Search Map image relative to the physical tilt axis of the stage. The Rotate Tilt Axis value can be calculated with 3D reconstruction software, based on the acquired data from preceding Tilt Series.
4. In the Overview image, right-click on the center of the **area of interest** and either:
   - Select **Move stage here**, then select **Acquisition** > **Acquire Search Map**
   - Select **Move stage here and acquire Search Map**
     Tomography moves the specimen to the selected position, and then acquires and displays a Search Map.



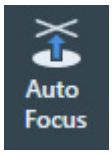
The outlines of the Search Map tiles are visible in the Atlas. If an Overview is available that contains the Search Map area, then the outlines of the Search Map tiles are also visible in that Overview.



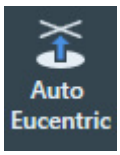
7.4.1.2.2 Acquire a Search image

1. In the Overview image or Search Map view, right-click on the **feature of interest** and select **Move Stage Here and Acquire Search Image**

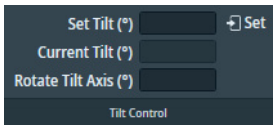
2. (Optional) Select **Auto Focus**



3. If this is the first feature of interest in the current area of interest, then select **Auto Eucentric**

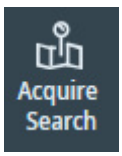


4. Accurately center the feature of interest:
   a. (Optional) Specify the *Tilt Control* settings:



   - Specify **Set Tilt**, then select **Set** to move the stage to a different tilt angle.
     On narrow screens, the Tilt Control ribbon must be expanded to make the settings visible.
   - The **Rotate Tilt Axis** value is a system property. It specifies the offset of the tilt axis in the Search image relative to the physical tilt axis of the stage. The Rotate Tilt Axis value can be calculated with 3D reconstruction software, based on the acquired data from preceding Tilt Series.

   b. Select **Acquire Search**

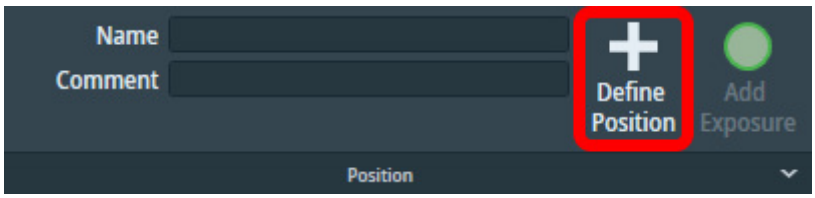


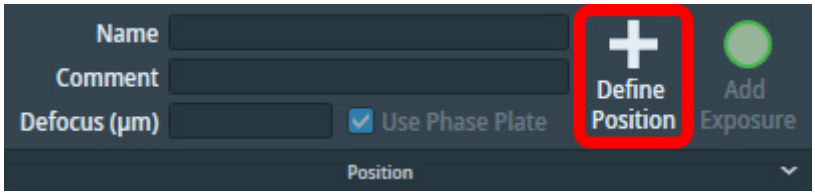
   c. If the feature of interest is not accurately centered in the Search image, then right-click on the **feature of interest** and select **Move Stage Here and Acquire Search Image**

7.4.1.3 Define and add the Batch Position

A new Batch Position can be defined in a *Search Map* image or in a *Search* image.

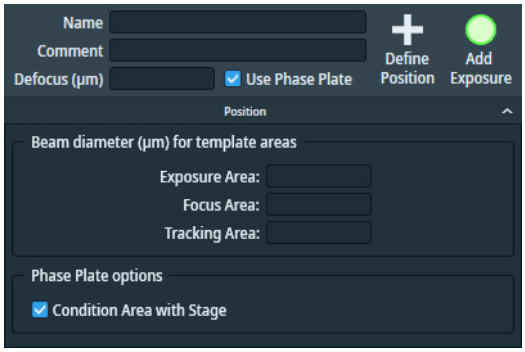
1. Determine the location of the new Batch Position:
   - In a *Search* image, select **Define Position**





- In a *Search Map* image, right-click at the **exact location in the Search Map** where the new Batch Position must be created, and either:
  - (*Recommended*) Select **Add position to batch** from the context menu.
  - Select **Move stage here** from the context menu,
    then select **Position** > **Define Position** in the ribbon bar.

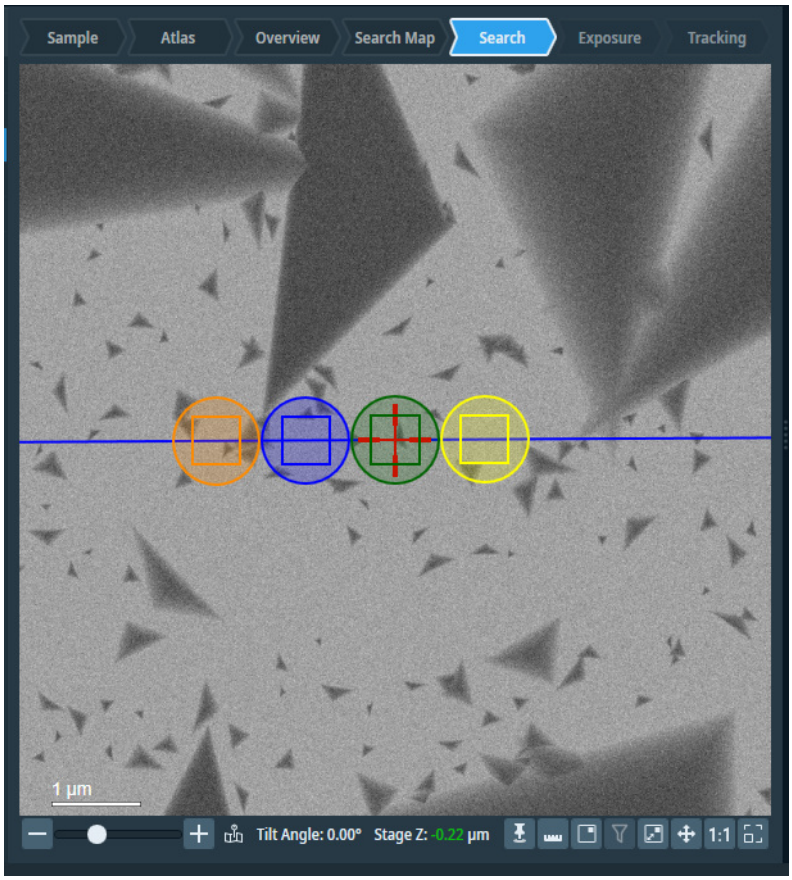
2. Specify the **Name**, (optional) **Comment** and **Defocus** value for the Batch Position.
   It is also possible to specify the Defocus values after all Batch Positions have been defined, see: Edit the Defocus value for multiple Batch Positions on page 143.
3. If *Session Setup* > *Low Dose* is ticked, then:



   a. If the selected Objective aperture when Tomography is started is a Phase Plate,
      then Phase Plate settings become available:
      - (Optional) Tick **Use Phase Plate**
        The *Condition Area* becomes available in the Search image.
        Use Phase Plate applies to all Batch Positions. It is *not* possible tick or clear *Use Phase Plate* after the first Batch Position is added to the Batch Positions list.
      - (Optional) Tick **Condition Area with Stage** to use the stage to move the Condition Area into the center of the beam.
        If *Condition Area with Stage* is cleared, then Tomography always uses beam shift, unless the distance exceeds the maximum achievable beam shift.
        Condition Area with Stage applies to all Batch Positions. It is *not* possible tick or clear *Condition Area with Stage* after the first Batch Position is added to the Batch Positions list.

   b. (Optional) Right-click in the Search image and select **Lock Focus and Tracking Area**
      When locked, the Focus Area and the Tracking Area stay on top of each other. In most experiments, these areas can be on the exact same position to save time without sacrificing accuracy.

c. Drag the **template areas** to their desired locations on the tilt axis.
The default template is copied from the most recently defined Batch Position.
For guidelines, see: Guidelines for the locations of the Template Areas on page 129.



- The square outline represents the field of view of the camera sensor.
- The circular area represents the illuminated area.
  In the example above, the illuminated areas (circles) are much larger than necessary to illuminate the field of view (squares).

It is not possible to assign a location away from the tilt axis.

Note

Do not change *Rotate Tilt Axis* for the purpose of dragging Template Areas to positions away from the current tilt axis.

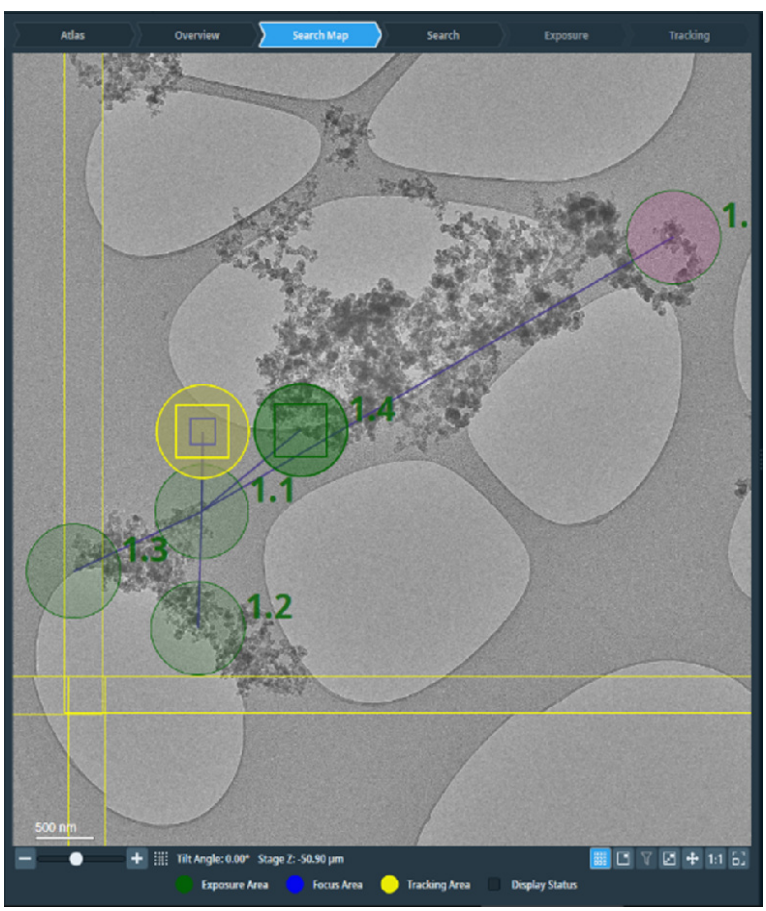
The purpose of the Rotate Tilt Axis parameter is to compensate for a difference between the tilt axis orientation in Tomography and the physical tilt axis of the stage. If the tilt axis in Tomography is not aligned with the physical tilt axis, then the Z-height of the Focus Area will vary with the tilt angle, which may result in unfocused images of the Exposure Area.

4. (Optional) Select **Add Exposure** to add an additional Exposure Area to the template.
   - By default, the additional Exposure Area uses the same Defocus value as the primary Exposure Area.
   - Because additional Exposure Areas are part of the Batch Position template, the next new Batch Position will also have more than one Exposure Area.
   - During acquisition, each Exposure Area is tracked using the *Tracking After Acquisition* function.

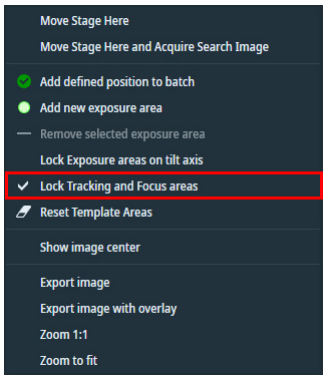
   It is also possible to right-click in the image and then select **Add new exposure area**
5. (Optional) To remove an additional Exposure Area,
   right-click on the Exposure Area and select **Remove Exposure Area**
   It is not possible to remove the primary Exposure Area.
6. Select **Add Position**
   Tomography adds the new Batch Position to the Batch Positions List.
   The Start Angle depends on how the Batch Position is created:
   - In a Search Map: the Start Angle is the Tilt Angle that is displayed at the bottom of the Search Map image.
   - In a Search image: the Start Angle is the current A-tilt position.

Best practice

The recommended best practice is to place exposure areas within close proximity to the primary exposure area. If an area is placed too far away, it is displayed in red as in the following image.

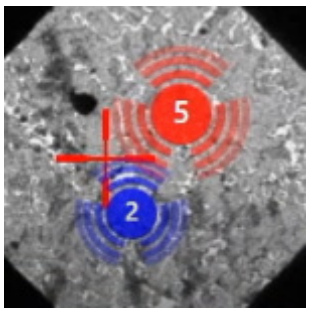
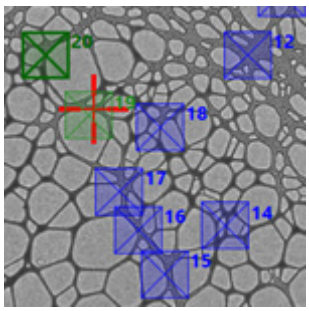
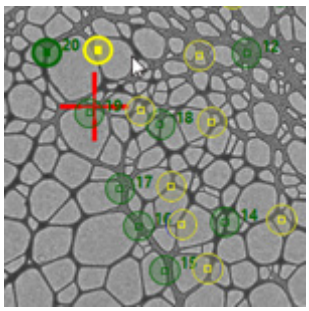


To restrict exposure areas to the tilt axis, use the Lock exposure areas on tilt axis setting from the context menu.



7.4.2 Manage the Batch Positions

7.4.2.1 Description of the position markers in the Image Display

Marker type	Description
	*Only used in the Batch Positions task*. Location of multiple Batch Positions. The color of the marker indicates the status of the Batch Positions. Hover on the Group marker with the mouse to display a tooltip with the Batch Position IDs and statuses of the grouped Batch Positions.
	Location and status of a Tilt Series. In Batch-mode, the label shows the Batch Position ID.
	Location and field of view (square shapes) of the Template Areas. In TEM and EFTEM mode, also the beam diameter (circular shapes) is displayed. In Batch-mode, the label shows the Batch Position ID.

The displayed marker type can change depending on the view and the zoom level. The meaning of the color of the marker is described in the legend at the bottom of the Image Display.

Depending on the availability of acquired images, the highlighted Batch Position in the Image Display and in the Batch Positions list is the same:

- When a marker is selected in the Image Display, then the corresponding row in the Batch Positions list is also highlighted.
- When a row is selected in the Batch Positions list, then the Image Display highlights the corresponding marker. If the location of a Batch Position is outside the currently displayed field of view, then the Image Display switches to a same-type view in which the selected Batch Position is visible. The Image Display will not switch to a different type of view.

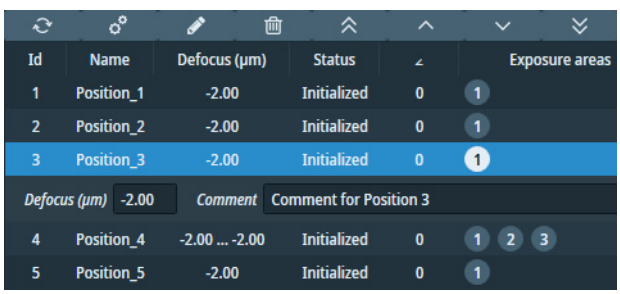
For example: in the Image Display, the currently selected Batch Position is highlighted in a Search Map view. In the Batch Positions list, a different Batch Position is selected that is not visible in the currently displayed Search Map. If a different Search Map is available that contains the selected Batch Position, then that Search Map will be displayed. If such a Search Map is not available, then the Image Display will not follow the selection in the Batch Positions list.

7.4.2.2 Edit a single Batch Position

1. In the Image Display or in the Batch Positions List, select the **Batch Position**.

   If the Batch Position has multiple Exposure Areas, then it is also possible to directly select a particular Exposure Area.

   In the Batch Positions List, the selected Batch Position row expands, and the *Defocus* value and *Comment* become visible.



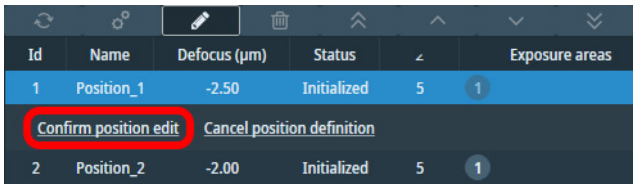
2. (Optional) Adjust the **Defocus** value and select **Enter**
3. (Optional) Edit the **Comment** and select **Enter**
4. (Optional) Select **Edit** to update the template.

   The following updates and adjustments are now available:

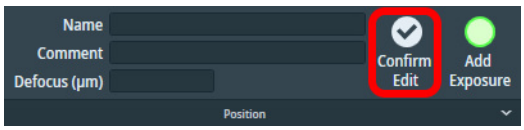
   - Add or remove an Exposure Area.
   - Adjust the locations of the template areas.

To save the changes, either:

- Select **Confirm position edit**



- Select **Confirm Edit**



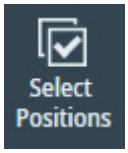
To cancel the template adjustments and revert to the stored template area positions, either:

- Select **Cancel position definition**
- Select **Edit** again.

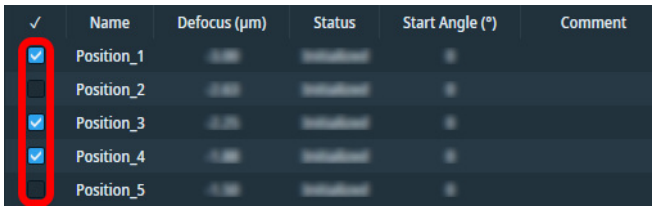
7.4.2.3 Edit the Defocus value for multiple Batch Positions

The Update Defocus function assigns a Defocus value to all (selected) Batch Positions from which no data has been acquired yet. To update the Defocus values for all (selected) Batch Positions:

1. (Optional) Select a subset of Batch Positions:
   a. Select **Select Positions**

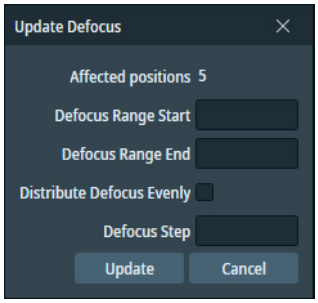


   b. In the Batch Positions list, tick the desired **Batch Positions**



2. Select **Update Defocus**

3. In the *Update Defocus* dialog:



   a. Specify the **Defocus Range Start** and **Defocus Range End** values.
   b. (Optional) Tick **Distribute Defocus Evenly** to let Tomography calculate the *Defocus Step*, based on the specified range and the number of affected positions.
   c. If *Distribute Defocus Evenly* is cleared, then specify the **Defocus Step**
   d. Select **Update** to assign new Defocus values to all Batch Positions.

7.4.2.4 Refine a single Batch Position

The Refine function:

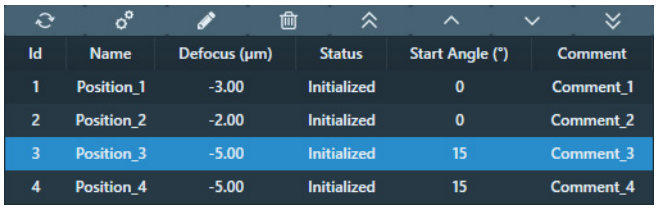
- Adjusts the Batch Position to compensate for any mechanical play or hysteresis on the X, Y and Alpha axis of the stage.
- Runs Auto-eucentric to bring the specimen to the correct Z-height if it has not been done yet.

- Acquires focused reference images for increased centering accuracy during the Data Acquisition run.

If the Refine action is not performed before the Data Acquisition run is started, then Tomography refines the Batch Positions during the Data Acquisition run.

To refine a single Batch Position:

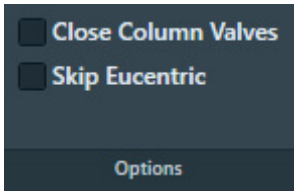
1. Select the *Batch Position* that will be refined.
   Either:
   - If the desired Batch Position is visible in the Image Display,
     then select the desired **Image Display** > **Batch Position**
     Tomography automatically highlights the selected Batch Position in the Batch Positions list.
   - In the **Batch Positions** List, select the desired **Batch Position**



Id	Name	Defocus (µm)	Status	Start Angle (°)	Comment
1	Position_1	-3.00	Initialized	0	Comment_1
2	Position_2	-2.00	Initialized	0	Comment_2
3	Position_3	-5.00	Initialized	15	Comment_3
4	Position_4	-5.00	Initialized	15	Comment_4

   In the Image Display, the marker for the selected Batch Position is highlighted. If necessary, Tomography first switches to a different Overview or Search image to display the Batch Position.

2. If *Auto Function* > *Auto Eucentric* has already been executed for this Grid Square,
   then tick **Skip Eucentric**

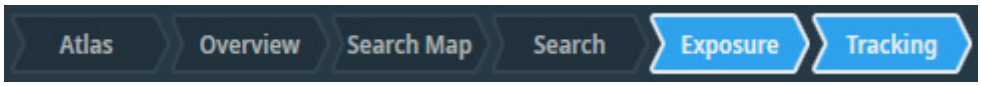


   By default, the Refine Batch Positions procedure executes the *Auto-eucentric height by stage tilt* function for each Batch Position. Within the same Grid Square, the Z-height of the features of interest does not change significantly. To speed up refinement, re-adjusting the eucentric height can be skipped.

3. Either:
   - Right-click and select **Refine**
   - Select the **Refine** button above the Batch Positions List.

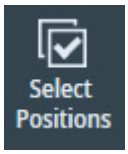
   After the Refine action has completed successfully, the status of the Batch Position changes from *Initialized* to *Refined*.

4. (Optional) Above the Image Display area, select **Exposure** and/or select **Tracking** to inspect the acquired Exposure and Tracking images for the refined Batch Position.

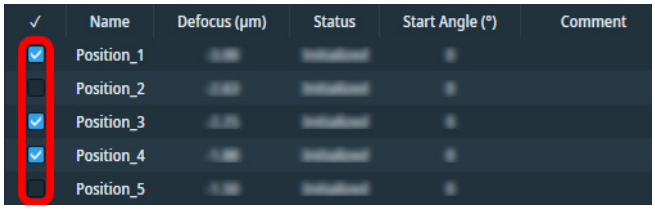


7.4.2.5 Refine multiple Batch Positions

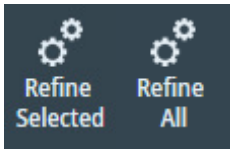
1. (Optional) Select a subset of Batch Positions:
   a. Select **Select Positions**



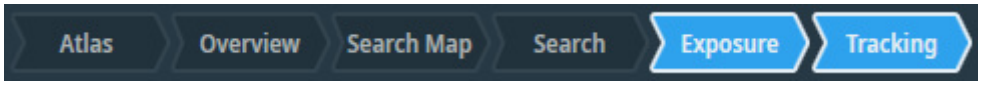
   b. In the Batch Positions list, tick the desired **Batch Positions**



2. Select **Refine Selected** / **Refine All**



3. (Optional) Inspect the Exposure and Tracking images for each refined Batch Position.
   a. In the **Batch Positions** list, select the desired **Batch Position**
   Tomography loads the image view for the selected Batch Position.
   b. Above the Image Display area, select **Exposure** and/or select **Tracking** to inspect the acquired Exposure and Tracking images for the refined Batch Position.



7.4.2.6 Delete a single Batch Position

It is not possible to delete a Batch Position for which a Tilt Series has been acquired.

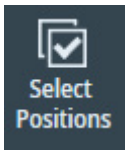
To delete a single Batch Position:

1. In the Image Display or in the Batch Positions List, select the **Batch Position** that must be deleted.
2. Either:
   - Right-click and select **Delete**
   - Select the **Delete** button above the Batch Positions List column header.

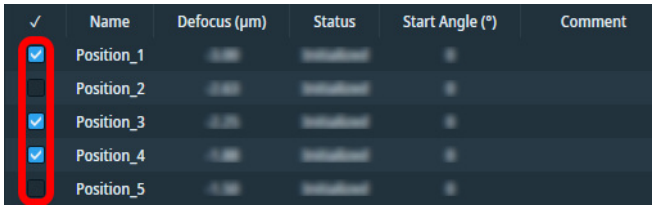
Note There is no Undo function. It is not possible to retrieve deleted Batch Positions.

7.4.2.7 Delete multiple Batch Positions

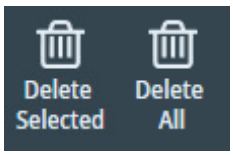
1. (Optional) Select a subset of Batch Positions:
   a. Select **Select Positions**



   b. In the Batch Positions list, tick the desired **Batch Positions**



2. Select **Delete Selected** / **Delete All**



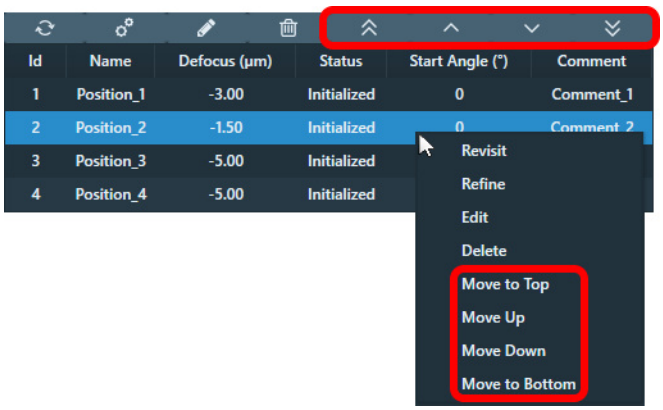
Note There is no Undo function. It is not possible to retrieve deleted Batch Positions.

7.4.2.8 Change the processing order of the Batch Positions

During the Automated Acquisition run, the Batch Positions in the list are processed in top-to-bottom order. By default, this is the order in which the Batch Positons have been added to the list. If a Template Area overlaps with the Exposure Area of a nearby Batch Position that is lower on the list, then it is advised to adjust the processing order so that the Exposure Area is not illuminated before data is acquired from it.

To change the processing order, either:

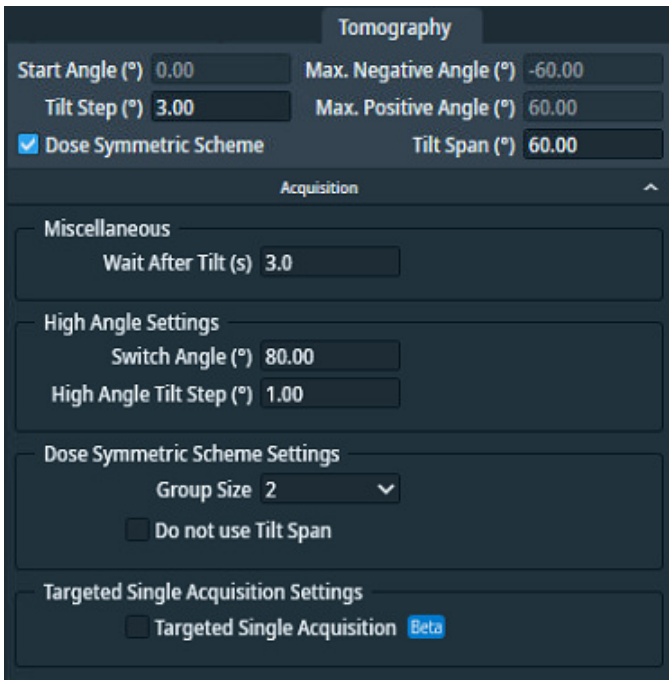
- Select a **Batch Position**, then select a **re-ordering action** from the toolbar above the Batch Positions List.
- Right-click on a Batch Position and select a **re-ordering action** from the context menu.



7.5 The Data Acquisition task

7.5.1 Description of the Acquisition Settings

The Acquisition settings define the tilt scheme that is executed during a Tilt Series acquisition. The basic settings are available in the main ribbon bar. Select the ribbon bar title to display the advanced settings.



Start Angle

The tilt axis position at which the first image of the Tilt Series will be acquired.

Tilt Step

The tilt axis rotation between consecutive Tilt Series images.

Dose Symmetric Scheme

A Dose Symmetric Scheme is a sequence of angles that alternate between the positive and negative side in increasing tilt angles. This way, the accumulated dose at low Tilt Angles is minimized.

Targeted Single Acquisition

When activated, automatic acquisition is executed only on the specified angle. This type of acquisition is useful on samples with sparse distribution of particles. This type of acquisition is available only in Batch mode.

This feature is in Beta. For feedback and questions, please contact tomo5@thermofisher.com.

Max. Negative Angle, **Max. Positive Angle**

The outer most tilt axis positions at which a Tilt Series image will be acquired.

On a system with TEM Server 7.10 or later, the *Max. Negative Angle* and *Max. Positive Angle* are validated against the actual maximum tilt angle of the stage.

Tilt Span

Only for Dose Symmetric Scheme

The maximum deviation from the Start Angle. Tomography automatically calculates the Max. Negative Angle and Max. Positive Angle, based on the Start Angle and the Tilt Span.

Group Size

Only for Dose Symmetric Scheme

Select the number of tilts desired in a single sweep by setting the Group Size to 2, 3, or 4.

File Name

Filename of the MRC image to which the acquired Tilt Series images are recorded. This MRC file is saved in the **Session Setup** > **Storage Folder**

Wait After Tilt

After each Tilt Step, Tomography waits the specified amount of time to let any mechanical tension or drift stabilize before executing the Template or acquiring an image.

Do not use Tilt Span

Only for Dose Symmetric Scheme

Disables *Tilt Span* and the automatic calculation of the Max. Negative Angle and Max. Positive Angle. For a Dose Symmetric Scheme, the Max. Negative Angle and Max. Positive Angle must be symmetric relative to the *Start Angle*.

Switch Angle and **High Angle Tilt Step**

When the tilt axis rotation exceeds the Switch Angle, then the rotation increment for the next Tilt Series image changes from the Tilt Step value to the High Angle Tilt Step.

- For experiments with a *slab-like specimen*, the Switch Angle value is typically set at around 40º.
- For experiments with a *rod-like* specimen, the Switch Angle can be specified equal to largest value of *Max. Positive Angle* and *Max. Negative Angle*.

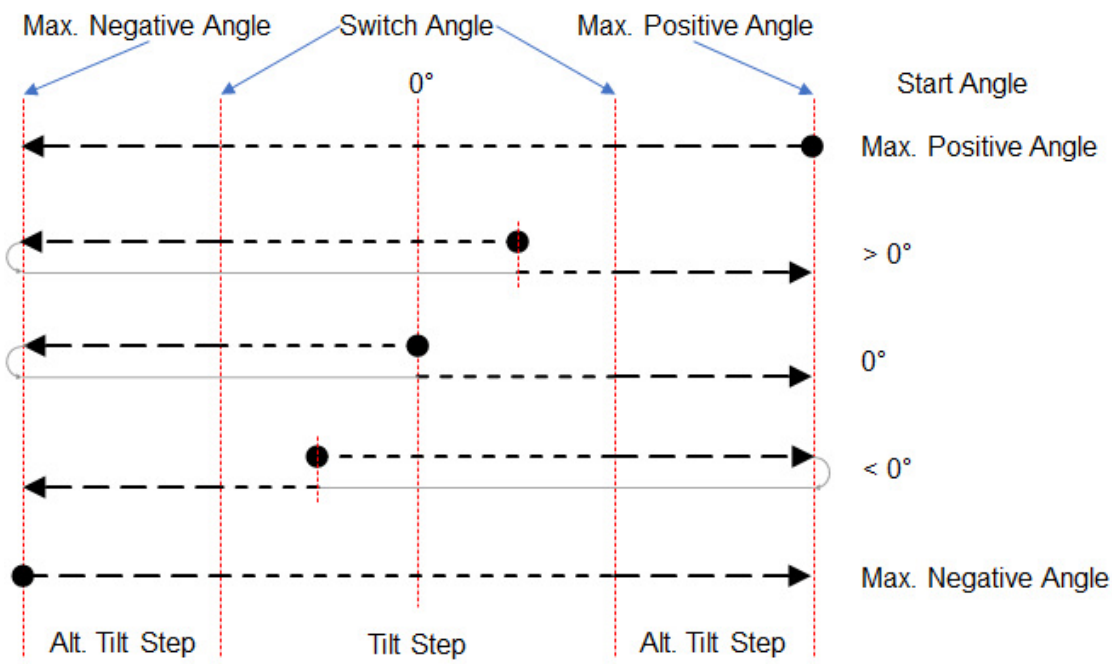
7.5.1.1 Tilt sequences when Dose Symmetric Scheme is not selected

If Dose Symmetric Scheme is cleared, then a Tilt Series is typically acquired in two branches:

- The first branch always passes through 0° tilt.

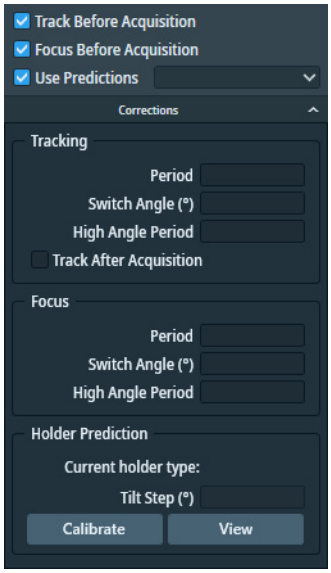
  After the first branch is acquired, the specimen returns to the Start Angle to acquire the second branch.

  If the Start Angle is 0°, then the first branch starts in the negative direction.
- If the Start Angle is equal to the Max. Positive Angle or Max. Negative Angle, then there is no second branch. All tilt angles are covered by the first branch. This is also referred to as a *Continuous Scheme*.


Max. Negative Angle
Switch Angle
Max. Positive Angle
0°
Start Angle
Max. Positive Angle
> 0°
0°
< 0°
Max. Negative Angle
Alt. Tilt Step
Tilt Step
Alt. Tilt Step

7.5.2 Description of the Corrections Settings

The basic correction settings are available in the main ribbon bar. Select the ribbon bar title to display the detailed settings.



Track Before Acquisition

The Tracking Before Acquisition function executes the Tracking function before a Tilt Series image is acquired. The Tracking > Period value determines how often the Tracking function is executed.

In a Low Dose experiment the Tracking function is performed on the Tracking Area.

Focus Before Acquisition

The Focus Before Acquisition function executes the Autofocus function before a Tilt Series image is acquired. The *Focus* > *Period* value determines how often the Autofocus function is executed.

In a Low Dose experiment the AutoFocus function is performed on the Focus Area.

Use Predictions

If the *Holder Prediction Calibration* has been performed, then Tomography applies a calibrated XY and/or Focus correction before executing the Tracking and/or Autofocus functions.

- The XY correction is performed by applying an image shift.
- The Focus correction is applied by adjusting the Objective lens value.

Predictions improves the accuracy and reliability of the Focus and Tracking action, and does not add a time penalty.

Period

The number of tilt steps between consecutive Autofocus or Tracking function executions.

Switch Angle

The tilt angle (positive and negative) at which the Autofocus or Tracking execution interval toggles to from *Period* to *High Angle Period* or vice versa.

High Angle Period

The number of tilt steps between consecutive Autofocus or Tracking function executions, when the tilt axis position is beyond the *Switch Angle*.

Track After Acquisition

After an image acquisition, Tomography measures the tracking error in the acquired image and calculates a corresponding correction for the next image acquisition in the Tilt Series.

The Tracking After Acquisition function is enabled by default. It does not require an additional image acquisition and does not take noticeable extra time. In Low Dose experiments, Tracking After Acquisition is a necessity. The specimens are typically not flat, so the movement of the Tracking Area may not accurately represent the movement of the feature of interest.

Holder Prediction:

- **Current Holder Type**
  The type of specimen holder that is currently inserted in the stage.
  Systems with an Autoloader have a fixed holder.
- **Tilt Step**
  The interval at which images are acquired during the Holder Prediction calibration.
- **Calibrate**
  Start the calibration procedure. See Perform the Holder Calibration on page 154 for instructions.
- **View**
  View the most recent calibration result for the current tilt scheme. It is also possible to manually select a calibration result file from a different tilt scheme.

7.5.2.1 Working principles of the Track Before Acquisition and the Track After Acquisition functions

The *Track Before Acquisition* function acquires a dedicated image after the specimen is tilted, before the Tilt Series image is acquired. The function then measures the tracking error and applies a corresponding corrective image shift before the Tilt Series image is acquired.

In a Low Dose experiment, the tracking image is acquired at a dedicated Tracking Area. The Tracking Area is located on the virtual tilt axis, at an offset from the Exposure Area.

The *Track After Acquisition* function uses the acquired Tilt Series image to measure the tracking error in the X and Y directions. The result is used:

- To cross-check the correction that is applied by the *Track Before Acquisition* function.
- To feed-forward an initial correction to the next tilt angle, so that the overall tracking correction performance becomes more accurate and reliable.

The use of the acquired Tilt Series image for calculation of the tracking correction has these advantages:

- The correction is based on a measurement at the Exposure Area itself instead of the nearby Tracking Area.
  - Although the Tracking Area is nearby, the appearance and condition of the specimen at the Tracking Area may be slightly different from the Exposure Area. This may cause small but noticeable inaccuracies.
  - The virtual tilt axis in the Search image can have a small offset relative to the physical tilt axis. If the *Rotate Tilt Axis* value is not accurate, then the measured shift of the Tracking Area may be slightly different than the actual shift of the Exposure Area. These differences typically become larger as the tilt angle increases.
- There is no tracking image acquisition prior to the Tilt Series image acquisition, so:
  - The specimen is not exposed to an additional dose.
  - No additional acquisition time is spent.

The most prominent drawback of the *Track After Acquisition* method is that the specimen is tilted to the next tilt angle *after* the correction has been calculated. If *Tracking Before Acquisition* is not enabled, then any incidental shift that is caused by the tilt step cannot be corrected.

7.5.2.2 Recommendations for the Tracking, Focus and Holder Prediction Corrections

The *Tracking Before Acquisition* function measures the tracking error (TE_{before}) at the Tracking Area, before the Tilt Series image is acquired.
The *Tracking After Acquisition* function measures the tracking error (TE_{after}) at the Exposure Area, after the Tilt Series image has been acquired.

These tracking functions complement each other as follows:

1. After a Tilt Series image is acquired, the *Tracking After Acquisition* function measures TE_{after}.
   If a TE_{before} correction has already been applied, then TE_{after} is the difference between the shift of the feature of interest and the Tracking Area.
2. The specimen moves to the next tilt angle.
3. The *Tracking Before Acquisition* function measures TE_{before} on the Tracking Area.
4. Tomography applies a tracking correction for TE_{before} and adds a correction that corresponds to TE_{after} at the preceding tilt angle.

For the highest tracking accuracy:

- Tick **Tracking Before Acquisition** and **Tracking After Acquisition**
  Specify **Period**: **1**
- Tick **Focus Before Acquisition**
  Specify **Period**: **1**
- If the *Holder Prediction* calibration is completed, then tick **Predictions** and select the **XY and Focus** mode.
  See Perform the Holder Calibration on page 154 for instructions.

For higher throughput rates:

- Increase the **Period** values for *Tracking* and/or *Focus*
- If the *Holder Prediction* calibration is completed, then tick **Predictions** and select the **XY and Focus** mode.
  See Perform the Holder Calibration on page 154 for instructions.

7.5.2.3 (Optional) Limit the tilt range for the Track Before Acquisition function

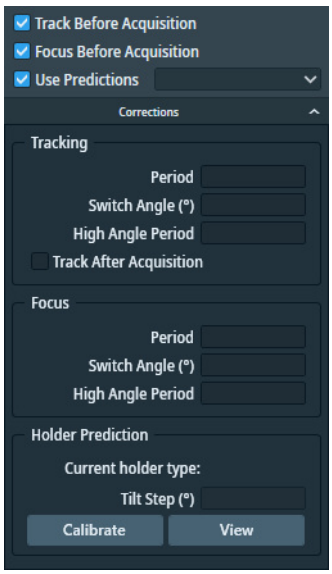
For Slab-like specimens, the time that is required to complete a Tilt Series can be shortened by limiting the tilt angle range in which *Track Before Acquisition* is active

- For small tilt angles, *Track Before Acquisition* is necessary to accurately correct for specimen shift. At small angles, specimen shift directly affects the achievable reconstructed volume.
  - If *Session Setup* > *Low Dose* is cleared, then Tomography automatically skips the *Track After Acquisition* function in the tilt range where *Track Before Acquisition* is active.

    In experiments that are *not* marked as Low Dose, there is no need to place a dedicated Tracking Area at an offset from the Exposure Area in the Template. The *Track Before Acquisition* function can acquire its tracking image on the Exposure Area. Using the acquired Tilt Series image from the same Exposure Area to cross-check the applied correction does not add significant value.
  - If *Low Dose* is ticked, then both tracking correction methods are active.
- For large tilt angles, the *Track After Acquisition* is sufficient. The effective field of view is already quite large due to foreshortening, so a small residual shift has a limited impact on the reconstructed volume. Spending time on *Track Before Acquisition* would not result in a significantly better reconstructed volume.

Follow the steps below to limit the tilt angle range in which *Track Before Acquisition* is active. It is not possible to limit the tilt angle range in which the *Tracking After Acquisition* function is active.

1. Tick **Corrections** > **Track Before Acquisition**
2. Select the **Corrections ribbon bar title** to display the detailed settings.



3. In the **Tracking** section:
   a. Specify the **Period**
      This is typically **1**
   b. Specify the **Switch Angle**
   c. Specify a very large value for **High Angle Period**, for example **100**
      By specifying a value that is larger than the number of tilt steps between the **Switch Angle** and the **Max. Negative Angle** or **Max. Positive Angle** values, the **Track Before Acquisition** function will not be triggered.
   d. Verify that **Track After Acquisition** is ticked.
      If the tilt range for the *Track Before Acquisition* function is limited and *Track After Acquisition* is not ticked, then there will be no tracking correction at all outside the *High Tilt Angle* range. Execution of the *Track After Acquisition* function is independent of the *Switch Angle* and *High Angle Period* values.

7.5.2.4 Perform the Holder Calibration

The Holder Calibration is specific for the system mode. The results from a Holder calibration in TEM mode is not valid for STEM mode, and vice versa. If Tomography is used for TEM and for STEM experiments, then the Holder Calibrations must be performed for TEM mode and for STEM mode.

Within a system mode, Holder Calibrations are specific for:

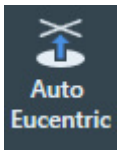
- All non-symmetric tilt schemes: the Start Angle and the holder type.
- Dose Symmetric Scheme: the Start Angle, the Tilt Step and the holder type.

The Holder Calibration is independent of the selected Prediction mode *XY*, *Focus* or *XY and Focus*, it always includes the *XY* as well as the *Focus* values.

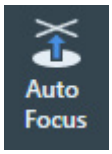
The Holder Calibration is not available when *Session Setup* > *Batch* is ticked.

To perform the Holder Calibration, follow the steps below:

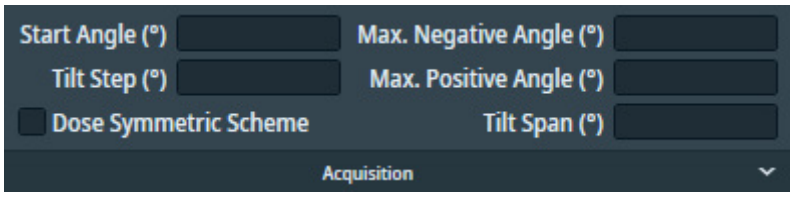
1. Load the **Combined Test Specimen (Agar S142)**
   This specimen is a lacey carbon foil with gold particles and graphitized carbon. This specimen is delivered with the microscope.
2. Move the **specimen** to an area that has:
   - Flat, stable carbon foil.
   - Gold particles or other contrast-rich features that are easily recognizable at the Tracking Preset magnification.
3. Select **Auto Eucentric**



4. Select **Auto Focus**

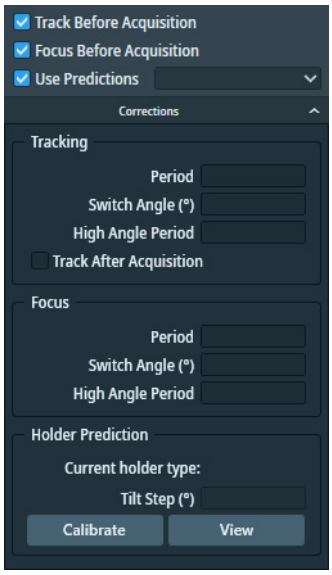


5. Set the **Max. Negative Angle** and **Max. Positive Angle** to the widest usable range.



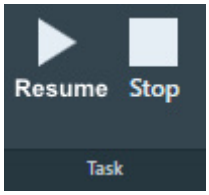
6. In **Corrections** > **Holder Prediction**, specify **Tilt Step**: **5.00**

   If *Acquisition* > *Dose Symmetric Scheme* is selected, then Holder Calibration uses the same Tilt Step as specified in the Acquisition settings.



7. Select **Calibrate**
8. Monitor the progress and intermediate results in the **Status** side panel.

   The procedure may request to perform manual actions. If so, perform the requested action and select **Resume**

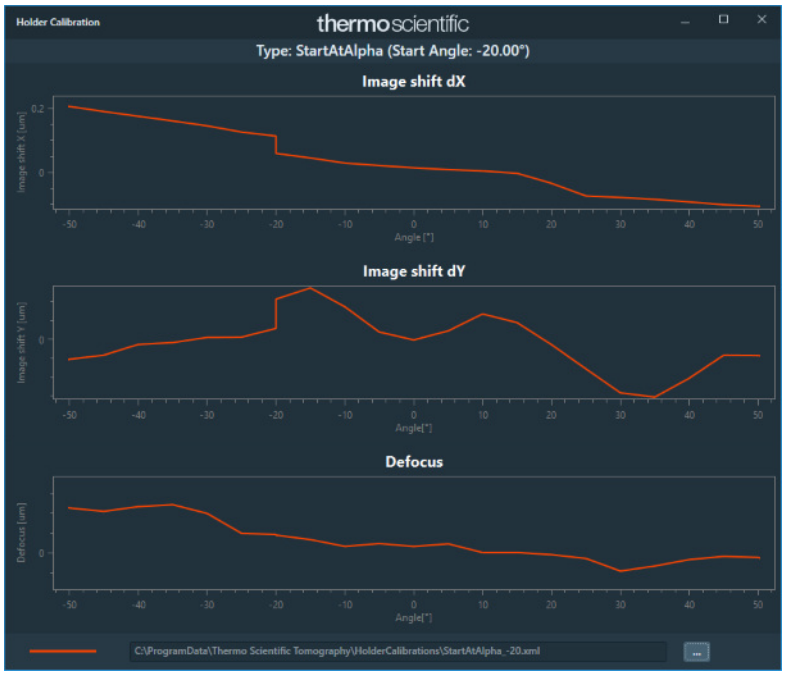


After the calibration is completed, Tomography automatically stores the results in `C:\ProgramData\Thermo Scientific Tomography\ HolderCalibrations` as a TXT file, a CSV file, and an XML file. If desired, use a spreadsheet application to visualize the calibration result in a graph.

7.5.2.5 View the Holder Calibration results

1. Select the **Correction ribbon title** to show the detailed Correction settings.
2. In the **Holder Prediction** section, select **View**

   The *Holder Calibration* viewer appears. By default, the Holder Calibration viewer opens the most recent calibration results that match with the current tilt scheme.



   If there are no matching calibration results, then the graphs are empty.

3. (Optional) View the Holder Calibration results for a different tilt scheme:
   a. Select the file picker

   b. Select the **XML file** for the desired Holder Calibration, then select **Open**

For a regular user or supervisor, Tomography shows only one Holder Calibration file.

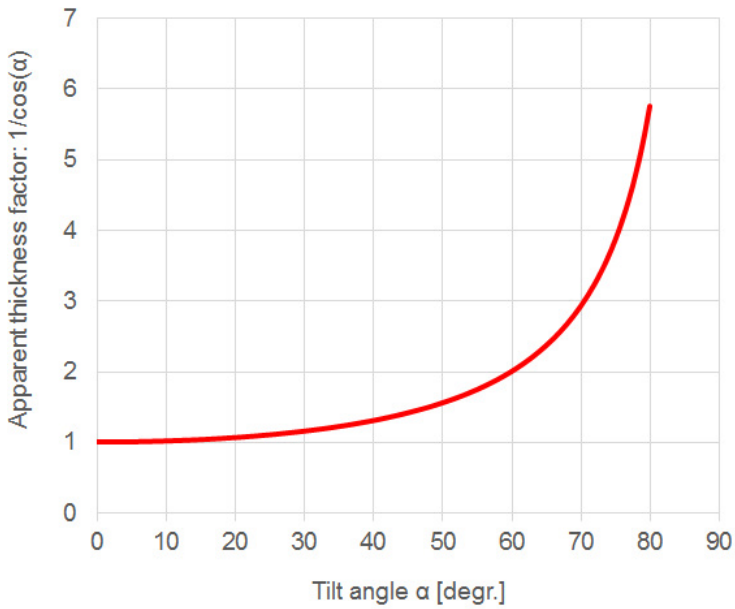
For Thermo Fisher Scientific engineers, the Holder Calibration viewer can open up to three Holder Calibration files, so the results can be compared.

7.5.3 Description of the Other settings

The available functions are available in the main ribbon bar. To specify their settings, select the ribbon bar title to expand the flap-out.

Adjust Exp. Time, **Ratio I0/I60** and **Measure**

When a slab-like specimen tilts towards higher angles, the apparent thickness of the specimen along the electron beam's path increases.



Due to increased electron scattering, the signal that is recorded by the sensor becomes weaker. The purpose of the *Adjust Exp. Time* function is to maintain the same recorded signal strength at all tilt angles. To do so, it multiplies the default exposure time by a factor that depends on the current tilt angle. The basis for the multiplication factor is the value of *Ratio I0/I60*. This is the ratio between the recorded intensity at 0° tilt and the recorded intensity at 60° tilt.

Follow the steps below to determine the value of *Ratio I0/I60*:

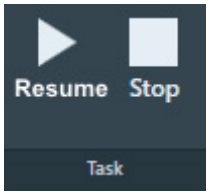
1. Move the **specimen** to an area that is similar to a feature of interest.
2. Select **Measure**

   Tomography starts an automated sequence of image acquisitions and A-tilt moves.
3. Monitor the **Status** side panel for progress, intermediate results and possibly instructions for manual actions.

   If a manual action is required:

   a. Perform the requested actions.

   b. Select **Resume**



Distribute Dose

When *Adjust Exp. Time* is active, the exposure increases with the tilt angle. As a result, the total dose that is received by the feature of interest during the Tilt Series becomes considerably larger. For dose sensitive features of interest, this could cause so much damage that the acquired images become unusable for accurate and reliable 3D reconstruction.

The *Distribute Dose* function prevents overexposure of the feature of interest due to increased exposure times at high tilt angles. To do so, the *Distribute Dose* function:

- Calculates the total theoretical dose for the entire Tilt Series as if all acquisitions were using the default exposure time.

  $D_{default} = D_0 \times [\#tilt\ angles]$
- Calculates the total theoretical dose for the entire Tilt Series with the *Adjust Exp. Time* option.

  $D_{AET} = \Sigma_{tilt\ angles}(D_0 \times [Adj.Exp.Time\ factor])$
- Scales down the exposure time for each tilt angle, so that D_{AET} does not exceed the $D_{default}$

Adjust ZLP and **Period**

This option is only available when the microscope is in EFTEM mode when Tomography is started. When *Adjust ZLP* is ticked, Tomography regularly adjusts the position of the filter's energy slit, so that it is centered at the Zero Loss Peak (ZLP).

- The ZLP adjustment is executed at the start of each Tilt Series or at the start of each Nth Tilt Series.
- If desired, the ZLP adjustment is repeated at a specified interval during the Tilt Series.

  Specify *Period* as the number of tilt steps between consecutive adjustments. If the specified number of steps exceeds the number of tilt angles in the Tilt Series, then the ZLP adjustment is executed only at the start of each Tilt Series.

7.5.4 Description of the Phase Plate settings

The basic functions are available in the main ribbon bar. Select the ribbon bar title to expand the flap-out for advanced functions and detailed settings.

Use Phase Plate

Tick *Use Phase Plate* to use Phase Plates during the Automated Acquisition run.

Although Tomography can move to the next index position within a Phase Plate, it cannot select a different aperture position. Before starting the Automated Acquisition run, move the Objective Aperture Mechanism to a Phase Plate position.

Activation Time

The illumination time that is needed to create a new Phase Plate area that meets the desired phase shift.

See Determine the Phase Plate Activation Time on page 70 to determine the Activation Time value.

Use new PP

It is possible to activate a new Phase Plate before starting the Automated Acquisition with the *Preparation > Activate Phase Plate* task. During the manual and automated actions between activation and the first Tilt Series image acquisition, the illumination conditions could change. The beam could shift or tilt away from the activated area, or the Phase Plate may even be destroyed.

To ensure that the activated Phase Plate area remains valid for image acquisition, it is also possible to include Phase Plate activation in the Automated Acquisition procedure.

Use new PP offers the following options:

- **Never**:
  Do not activate a new Phase Plate during the Automated Acquisition run.
- **Once**:
  Activate a new Phase Plate after the feature of interest is centered and the Exposure Preset values are set to the microscope. In a Batch run, Tomography activates a new Phase Plate for each Batch Position.
- **Before each branch**:
  Select *Before each branch* to activate a new Phase Plate before starting the second branch of the Tilt Series. This could increase quality of the acquired Tilt Series images, but activation time can be significant, especially under Low Dose conditions.
  If the Start Angle is not equal to the *Max. Negative Angle* or the *Max. Positive Angle*, then the Tilt Series is acquired in two branches (see Description of the Acquisition Settings on page 147). After the first branch is completed, the stage returns to the Start Angle and centers the feature of interest again. During this action there is a chance that the Phase Plate is not always properly illuminated, because Tomography uses various Presets to do so. This may deteriorate the Phase Plate.
  Besides the centering action at the start of the second branch there may also be a build-up of small electromagnetic fields due to charging. Although this is a generic effect that is not related to the centering action, the impact of charging is reduced or even even eliminated by the activation of a new Phase Plate at the start of the second branch.
- **Every N tilt series**:
  Similar to *Before each branch*, select *Every N tilt series* to activate a new Phase Plate before starting every N^{th} Tilt Series.

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.

Conditioning Time

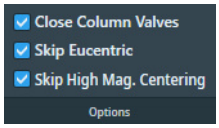
The illumination time that is needed to maintain the phase shift performance of an activated Phase Plate.

Period

The number of tilt steps after which the active Phase Plate is conditioned again.

Period (N)
The number of tilt series after which the active Phase Plate is conditioned again.

7.5.5 Description of the Options



The Options section of the Ribbon Bar provides the following functionalities.

Close Col. Valves
Closes the Column Valves after the Automated Acquisition run is completed.

Skip Eucentric
Only available when Session Setup > Batch is ticked.
When ticked, Tomography skips the Auto Eucentric function during Batch Position refinement.

Skip High Mag. Centering
Only visible when Session Setup > Batch and Low Dose are cleared.

The automated centering procedure at the start of a new Tilt Series is as follows:

1. Center with the stage at 0 degrees at Search magnification.
2. Center with Beam Shift at the Start Angle at Search magnification.
3. Center with Beam Shift at the Start Angle at Tracking magnification.
   When *Skip High Mag. Centering* is ticked, this action is skipped. The availability and default value of the *Skip High Mag. Centering* option depend on the configured Tilt Scheme.

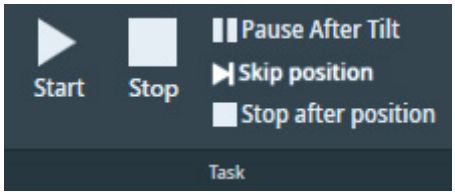
Stop / Reduce Emission
Only available on systems with a thermionic filament.
Not displayed in the screen image above.

Switches off the emission, or brings the electron source to a safe standby state after the Automated Acquisition run is completed.

7.5.6 Setup and start the automated Data Acquisition run

1. Select or specify the parameters for the Data Acquisition run.
   See:
   - Description of the Acquisition Settings on page 147
   - Description of the Corrections Settings on page 150
   - Description of the Other settings on page 157
   - Description of the Phase Plate settings on page 158
   - Description of the Options on page 160

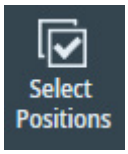
2. (Optional) Select **Pause After Tilt** to inspect the image after each tilt step and, if necessary, to make adjustments before the next Tilt Series image is acquired.



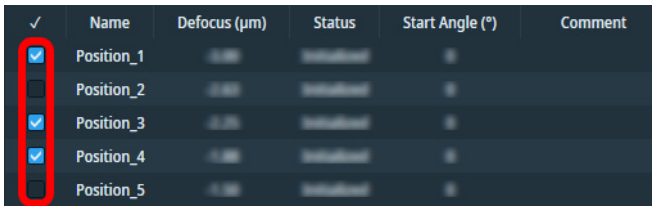
Depending on the session settings, the Start button can have the following functions:

- *Start* to acquire data for a single Tilt Series.
- *Start Batch* to acquire data for all Batch Positions.
- *Acquire [N] Position(s)* to acquire data for a selected subset of the Batch Positions.

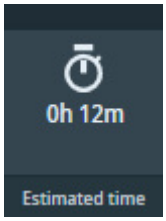
3. (Optional) If *Session Setup* > *Batch* is ticked and if *not all* Batch Positions must be processed, then select a subset of the Batch Positions:
   a. Select **Select Positions**



   b. In the Batch Positions list, tick the desired **Batch Positions**



4. Select **Start**, **Start Batch** or **Acquire [N] Position(s)**
   - Tomography shows the estimated remaining duration of the Automated Acquisition run.



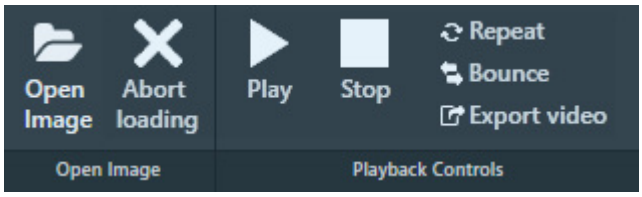
     - The estimated time is updated during acquisition.
     - The tooltip shows the estimated end date and time.
   - Depending on the used camera and the *Session Setup* > *Output Settings*, the acquired data is stored as MRC files or TIFF files. Additionally, Tomography stores the metadata as MDOC files.
   - If the system has a CFEG, then the tip is flashed automatically at convenient times during the Automated Acquisition run to ensure optimal illumination.
   - If a Gatan filter with Gatan K3 camera is used to acquire the Tilt Series, then Tomography automatically refreshes the Dark Reference at the start of each Tilt Series.

5. Wait until the Data Acquisition run is completed.
   At any time during the Data Acquisition run, it is possible to:
   - Select **Pause** to make adjustments.
     After the adjustments are made, select **Resume** to continue the Data Acquisition run.
   - Select **Stop** to abort the Data Acquisition run.
   - Select **Pause After Tilt** to activate or de-activate *Pause After Tilt* mode.
   - If the Data Acquisition run acquires data for multiple Batch Positions,
     select **Skip position** to abort data acquisition for the current Batch Position.
   - Select **Stop after position** to abort the Data Acquisition run after Tilt Series at the current Batch Position is completed.

7.6 The Movie Player task

The Movie Player tasks offers functionalities to view the images of a Tilt Series as a movie. It is possible to view a Tilt Series that has just been acquired in an automated Data Acquisition run, or to load the MRC file from a previously acquired Tilt Series.

7.6.1 Description of the Movie Player functionalities

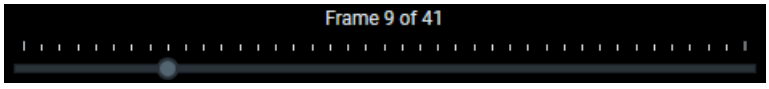


To open an image, image series or frame series:

- **Open Image**
  Open an MRC file.
- **Abort loading**
  Stop loading the selected MRC file.

If the opened MRC file contains an image series or frame series, then the following playback functions become available:

- **Play**
  Start the playback of a frame series.
- **Stop**
  Stop the ongoing playback of a frame series.
- **Repeat**
  Continuously repeat the playback of a frame series until Stop is selected.
  The Repeat function can be toggled before and during the playback of a frame series.
- **Bounce**
  Reverse the playback direction when the last frame is reached.
  The *Bounce* function can be toggled before and during the playback of a frame series.
- **Export video**
  Export the frame series as a video file.
  See Export a frame or image series to a video file on page 164.
- **Frame Selection Slider**
  The Frame Selection slider is available at the bottom of the Image Display.

  Drag the slider with the mouse to display the desired frame number.

7.6.2 View the Tilt Series images for a single feature of interest

Use the **Slider** or the functions in the **Playback Controls** ribbon to inspect the most recently acquired Tilt Series images.

7.6.3 View the Tilt Series images in a Batch Mode Session

If Tilt Series have been acquired for multiple Batch Positions, then follow the steps below to inspect the acquired images:

1. In the **Positions** list, select the desired **Batch Position**

Id	Name	Defocus (µm)	Status	Start Angle (°)	Comment
1	Position_1	-3.00	Acquired	0	Comment_1
2	Position_2	-1.50	Acquired	0	Comment_2
3	Position_3	-5.00	Acquired	15	Comment_3
4	Position_4	-5.00	Acquired	15	Comment_4

2. The Movie Player will automatically show a scaled down version, a *thumbnail*, of your tilt series movie if it was acquired in Batch mode. The thumbnail file is saved in a Thumbnails folder found in the corresponding sessions's folder.
3. Use the **Slider** or the functions in the **Playback Controls** to inspect the Tilt Series frames.

7.6.4 View the Tilt Series images from a previous Tomography session

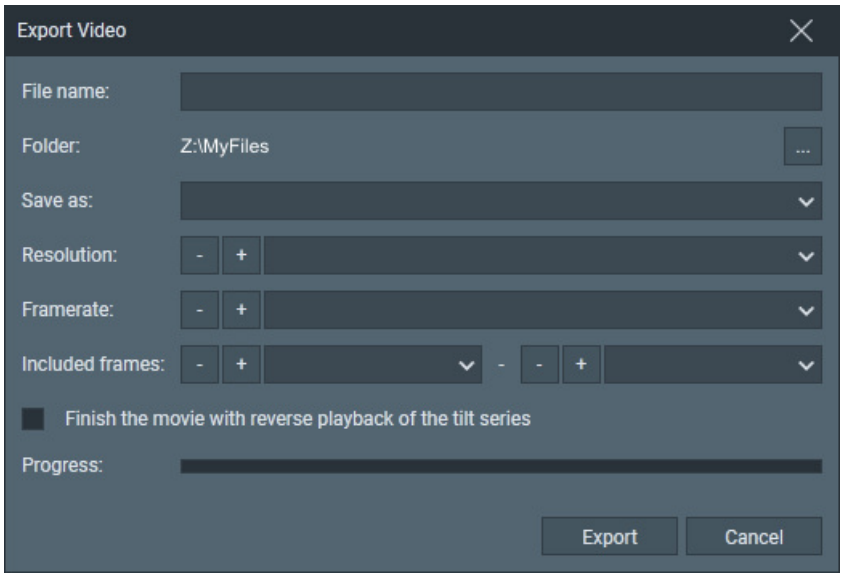
1. Select **Open Image**, then navigate to the desired **MRC file** and select it.
2. Use the **Slider** or the functions in the **Playback Controls** to inspect the Tilt Series frames.

7.6.5 Export a frame or image series to a video file

An acquired frame series can be exported as an H.264 (MPEG 4 part 10) video file. To do so, follow the steps below:

1. Either:
   - Right-click in the Image Display and select **Export video**
   - From the Image Display video controls, select **Export video**

2. In **Export Video**:



a. Specify the **File name**
b. Select the **Folder** where the video will be saved.
c. In **Save as**, select the video format.
   Currently, **H.264 (*.mp4)** is the only option.
d. Select the **Resolution**
e. Select the **Framerate**
f. In **Included frames**, select the first frame and the last frame to be included in the exported video file.
g. (Optional) Tick **Finish the movie with reverse playback of the tilt series**
   When ticked, the exported video will show the frame series from the first to the last selected frame and back again. If the exported video is played in a loop, then the frame series will bounce back and forth between the first and last included frames.
h. Select **Export**

8 Inspect the Acquired Images

8.1 View and post-process MRC images with Thermo Scientific Velox software

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.

For detailed descriptions and instructions of the viewing and processing functionalities in Velox, see the Velox User Manual.
The Velox software does not provide 3D reconstruction functionalities.

8.2 Install the Thermo Scientific Imaging Codec Pack to add native support for common microscopy formats to Windows

The Thermo Scientific Imaging Codec Pack adds commonly used microscopy image formats to the natively supported image formats in Windows. After installation, the supported microscopy image formats can be opened in the Windows Photo Viewer, and the thumbnails and previews in Windows Explorer show the actual image, instead of a generic icon.

For an overview of the supported image formats, see the Imaging Codec Pack User Guide.

9 Detailed Preconditions for Successful Tomography Usage

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.

9.1 Preconditions for the specimen and specimen holder

Side entry holders must be cleaned and pumped down before insertion into the microscope.

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 Tomography.

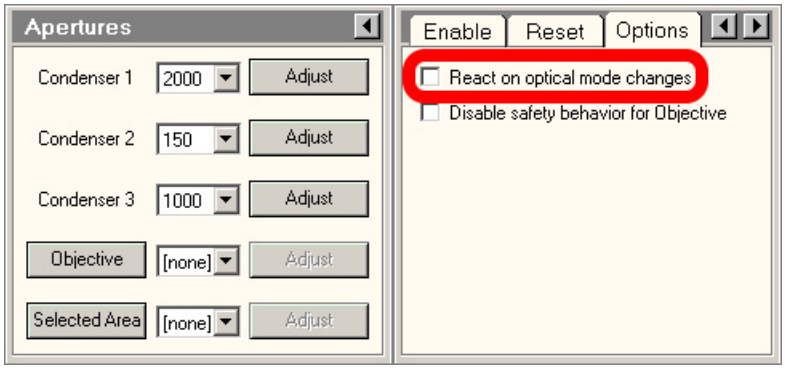
Note To work in both Nanoprobe and Microprobe modes, the focus calibration must be done separately for both modes.

- Direct alignments should be checked and, if necessary, adjusted in the modes that are used for Tomography.

  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 cooled and at a stable temperature.
- After any actions that may have introduced exceptionally strong drift, such as inserting a holder, enough time should be allowed for settling.

9.2.1 Clear the Apertures > Options > React on Optical Mode Changes option

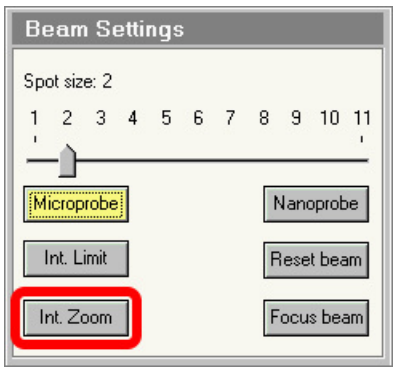
Note Do not use the *React on optical mode changes* function to change the apertures automatically.



Among others, the *React on optical mode changes* function automatically returns to 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*.

9.2.2 Disable Beam Settings > Intensity Zoom

On systems with two condenser lenses, make sure that *TEM User Interface* > *Beam Settings* > *Int. Zoom* is off when using magnifications below 10.000X.



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.

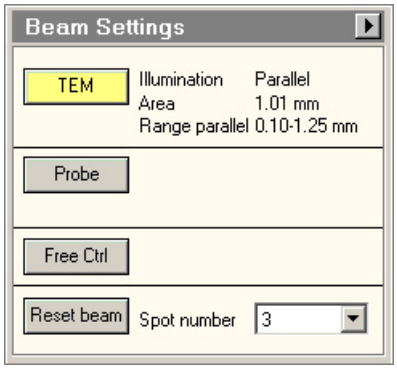
   If the verifications in steps 5c and 5d fail, perform the **Align HM-TEM** > **All SA Magnifications** alignment.
6. In the **TEM User Interface** > **Beam Settings** control panel:
   - Titan: disable **Auto Zoom**
   - Talos:disable **Intensity Zoom**

9.3.1.3 Test the Parallel Beam range on Titan Systems

Perform this test in the following optical modes:

- TEM nP
- TEM uP

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 Flucam Viewer:
   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 Flucam Viewer:
    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 Flucam Viewer:
    a. Place a **marker** on the recognizable feature.
    b. Place a **circle** around the beam.
17. Select the **lowest HM magnification**

18. In the Flucam Viewer:
    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 Flucam Viewer:
    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 Flucam Viewer:
    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 Flucam Viewer:
    a. Place a **marker** on the recognizable feature.
    b. Place a **circle** around the beam.
17. Select the **lowest HM magnification**
18. In the Flucam Viewer:
    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.

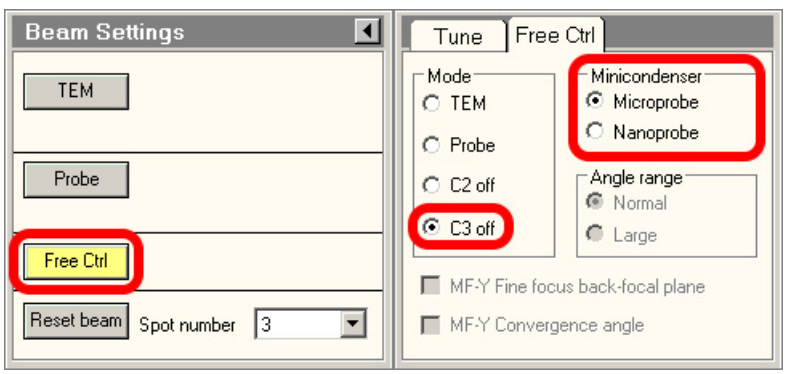
9.4 Aperture Alignments

9.4.1 Check the C2 Apertures center positions on systems with a C3 lens

Three Lens Condenser systems include all Titan systems.

Not all C2 apertures are used for the acquisition of the Atlas or 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 (C3 off), 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 the C2 Apertures center positions on a system without a C3 lens

Two Lens Condenser systems include all Talos and Tecnai systems.

Not all C2 apertures are used for the acquisition of the Atlas or 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.

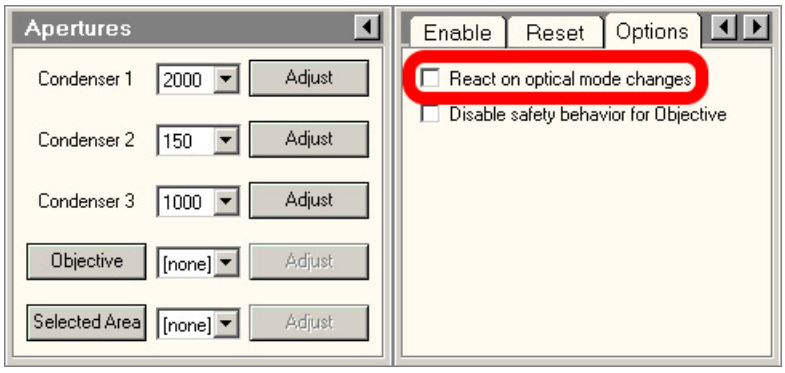
   If the beam does not expand symmetrically or does not stay centered, **adjust** the C2 aperture position.
5. Repeat step 4 for **every C2 aperture**.
6. Select **TEM User Interface** > **Beam Settings** > **Nanoprobe**.
7. Repeat steps 4 and 5.

9.4.3 Check the Objective Apertures center positions

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 returns to 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*.

9.4.4 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 Selected Area apertures are accurately centered around the beam.
3. Switch to **LM mode**
4. Select a **lower LM magnification**.
5. Verify that all Selected Area apertures are accurately centered around the beam.
6. Select a **lower SA magnification**.
7. Verify that all Selected Area apertures are accurately centered around 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.
- **(Tomo) Beam Shift**
  Center the beam when no user beam shift is applied. The *(Tomo) Beam Shift* alignment does not reset any image shift that may have been set due to the *Optimized Position calibration*.
  Perform this Direct Alignment at the highest magnification that will be used during the Automated Acquisition run.

- **(Tomo) Rotation Center**

  When focusing, the central image features should stay on the optical axis and should not move. The *(Tomo) Rotation Center* alignment does not reset any image shift that may have been set due to the *Optimized Position calibration*.

  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 **Tomography** > **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 100.

9.6.3 Eucentric Correction Calibration (Optional)

The Eucentric Correction Calibration is executed in the **Tomography** > **Auto Functions** tab > **Calibrations**: **Eucentric Correction** task. Follow the instructions in The Eucentric Correction Calibration task on page 104.

10 Troubleshooting: Symptoms and Solutions

At the moment there is only a troubleshooting chapter for the Atlas tasks.

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 Flucam Viewer.	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.

A The MRC2014 Image Format

A.1 The Main Header and Extended Headers in an MRC file

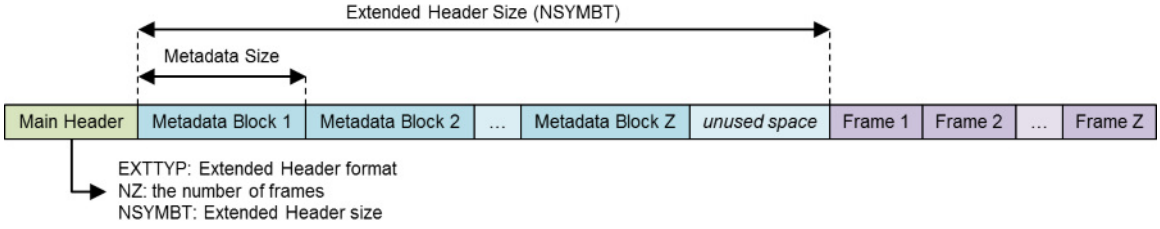
MRC files have a generic Main Header and an optional Extended Header.

- The Main Header contains generic image information, such as the image dimensions and the pixel format. For the specification of the main header,
  see MRC/CCP4 File Format for Images and Volumes.
- The Extended Header contains 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 and many other relevant parameters.
  - Image acquisition information, such as binning and exposure time.

Among others, the Main Header contains the following fields:

- `NZ`: the number of frames in the MRC file.
- `NSYMBT`: the reserved size for the Extended Header.
- `EXTTYP`: the format of the Extended Header: `FEI1` or `FEI2`.
  The FEI2 format is an extended version of the FEI1 format.

For every frame in the file, the Extended Header contains one Metadata Block. The first element of each block contains the Metadata Block size. All Metadata Blocks in the Extended Header have the same size and contain the same fields. The sum of the Metadata Block sizes fits within the reserved size for the Extended Header.



A.2 The Extended Header specification

The FEI1 and FEI2 Extended Header formats allow for the addition of new fields without breaking compatibility. When a new field is added, the `Metadata Size` and `Metadata Version` fields are updated. Image reading and processing software can use the `Metadata Size` value from the first Metadata Block to index the blocks for the other frames in the MRC file.

With the introduction of the FEI2 format, the format of the FEI1 Extended Header is frozen. For MRC files with an FEI1 Extended Header, image reading and processing software can assume the values of the `Metadata Size` and `Metadata Version` fields are 768 bytes and version number 0.

The tables below specify the content of the FEI1 and FEI2 Extended Headers for the 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.
  - Int64: Signed integer of 8 bytes (only used in FEI2 Extended Header).
  - UInt32: Unsigned integer of 4 bytes.
  - Float64: Floating point number of 8 bytes.
- **IsPresent**:
  UInt32 value that is used as a 32-bit / little-endian bitmask. If a metadata field is set, then the value of the *IsPresent* bit in the bitmask is 1.

A.2.1 FEI1 Extended Header specification

Image, System and Application

Name	Offset (dec)	Offset (hex)	Format	IsPresent	Description
Metadata size	0	0x0000	Int32	NA	Metadata size [bytes] All Metadata Blocks in the file have the same size. • FEI1: 768 bytes • FEI2: updated for each version.
Metadata version	4	0x0004	Int32	NA	Version ID of the metadata format. All Metadata Blocks in the file have the same format. • FEI1: 0 • FEI2: initial value: 2 The value is updated for each new version.
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
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 Detector

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	

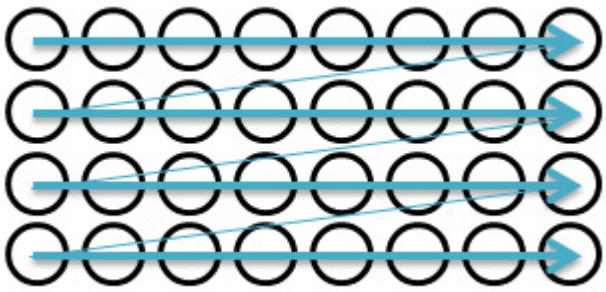
A.2.2 FEI2 Version 2 Extension to the Extended Header specification

Name	Offset (dec)	Offset (hex)	Format	IsPresent	Description
Scan rotation	768	0x0300	Float64	Bitmask 4 – #2	Rotation of the scan pattern in STEM mode [radians]
Diffraction pattern rotation	776	0x0308	Float64	Bitmask 4 – #3	Rotation of the diffraction pattern in diffraction mode [radians]
Image rotation	784	0x0310	Float64	Bitmask 4 – #4	Rotation of the image in imaging mode [radians]
Scan mode enumeration	792	0x0318	Int32	Bitmask 4 – #5	• 0: Other • 1: Raster • 2: Serpentine raster
Acquisition time stamp	796	0x031C	Int64	Bitmask 4 – #6	Microseconds since 1970-01-01T00:00:00Z at which the image was acquired
Detector commercial name	804	0x0324	16 chars	Bitmask 4 – #7	Commercial name of the detector or camera
Start tilt angle	820	0x0334	Float64	Bitmask 4 – #8	Start tilt angle of a tomography series [degr.]
End tilt angle	828	0x033C	Float64	Bitmask 4 – #9	End tilt angle of a tomography series [degr.]
Tilt per image	836	0x0344	Float64	Bitmask 4 – #10	Tilt increment per image in a tomography series [degr.]
Tilt speed	844	0x034C	Float64	Bitmask 4 – #11	Tilt speed in a tomography series [degr./sec]

Beam center X pixel	852	0x0354	Int32	Bitmask 4 – #12	Beam center X on image [pixels]
Beam center Y pixel	856	0x0358	Int32	Bitmask 4 – #13	Beam center Y on image [pixels]
CFEG flash timestamp	860	0x035C	Int64	Bitmask 4 – #14	Microseconds since 1970-01-01T00:00:00Z of the most recent CFEG flashing
Phase plate position index	868	0x0364	Int32	Bitmask 4 – #15	Position index of the phase plate aperture
Objective aperture name	872	0x0368	16 chars	Bitmask 4 – #16	Name of the inserted objective aperture

A.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.

A.3.1 The MRC image pixel data encoding for Thermo Scientific Ceta cameras

If the image is acquired with a Ceta camera, then the MRC image pixel data encoding depends on the presence of the Ceta Speed Enhancement (Ceta-2).

Camera	MRC Pixel Data
Ceta without Speed Enhancement	32-bit floating point
Ceta with Speed Enhancement	16-bit signed integer

11 Copyright, Limited Rights and Revision History

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Revision Table

Revision	Date	Description of Changes
5.0	OCT-2019	Initial Release
5.1	FEB-2020	Update for Tomography 5.1
5.2	APR-2020	Update for Tomography 5.2
5.3	JUL-2020	Update for Tomography 5.3
5.4	OCT-2020	Update for Tomography 5.4
5.5	JAN-2021	Update for Tomography 5.5
5.6	APR-2021	Update for Tomography 5.6
5.7	JUL-2021	Update for Tomography 5.7
5.8	OCT-2021	Update for Tomography 5.8
5.9	JAN-2021	Update for Tomography 5.9
5.10	APR-2022	Update for Tomography 5.10
5.10.A	APR-2022	Secondary update for Tomography 5.10
5.11	OCT-2022	Update for Tomography 5.11

Revision	Date	Description of Changes
5.12	OCT-2022	Update for Tomography 5.12
5.13	JAN-2023	Update for Tomography 5.13
5.14	APR-2023	Update for Tomography 5.14
5.15	JUL-2023	Update for Tomography 5.15
5.16	OCT-2023	Update for Tomography 5.16
5.17	JAN-2024	Update for Tomography 5.17

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