Remote TOMO5 operation guide for eBIC microscopes

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TOMO version:	5.1.4
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Revision summary:	1) Added note to ‘Quick start section’
	2) Order of Atlas and Image Shift Calibrations swapped around
	3) Added recommendation note(s) to test your parameters on a single position before setting up **all** of your positions

Comments to: Kyle Morris

Lorna Malone

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Introduction

This document is intended as a guide for trained eBIC users to assist them in setting up their data collection session at eBIC on a Krios, K3 and GIF. The guide is principally aimed at the remote user but is applicable to on-site operation. The level of detail is targeted at a standard eBIC operator. The user is ultimately responsible for their use of eBIC and Diamond systems. If you are in doubt about an action you need to perform or are unable to progress, then contact you eBIC Local Contact. Additionally, please report any errors in this guide to your Local Contact or kyle.morris@diamond.ac.uk.

Please consult the following documentation for guidance on remote microscope access, data analysis and transfer: '**Remote access and analysis**'.

Please note that the workflow we present here is a presentation of the Tomo5 workflow as we approach it on eBIC microscopes. For further information on the Tomo5 workflow in general, please refer to the release notes on the latest version of this software found on Thermo Fisher Scientific instruments.

Information box colour key

Information boxes are presented in green	Optional action instructions are presented in yellow
Terminal commands are presented in violet	Lamella specific instructions are in pink

User and local contact expectations

Please consolidate your grids into the minimum number of autogrid boxes. We standardly load grids from autogrid boxes in the number order they are present and follow the numbering written on the boxes. Your local contact (LC) will load the microscope and take an inventory of the grids in the autoloader before handover.

Ahead of your session, please inform your local contact (LC) of your:

- ☐ Target pixel size and target total dose
- ☐ Grid type (UAu, QF, Cflat or Lacey) and number of grids

Your LC requires this information to complete energy filter tuning, camera gain preparation and grid loading. The gain will be found in your visit processing directory. **Your LC will complete essential alignments (beam shift, pivot points, aperture centring) prior to your session, but the user will complete Image Shift Calibrations and Auto Function alignments prior to starting collection.**
Please consult with your LC to decide on a plan if you wish to recover grids from your session. Grids can be recovered from the cassette, but not the microscope column.

Getting prepared

- ☐ Establish contact with the Local Contact for your session via yours and your LC's preferred choice of communication i.e., Teams, Mail, Notepad, etc.

- ☐ Refer to the '*Remote access and analysis*' guide to access the microscope via NoMachine

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Quick start guide

1) **Receive handover email from your local contact detailing microscope parameters**
2) **Check your magnification presets and adjust as required**
3) **Check image shift calibrations**
4) **Atlas your grids and screen as required**

Naming:	*Supervisor_YYYYMMDD_XXXXXX_visit-ID_ATLAS*
Output folder:	*Z:\[your session ID, i.e., biXXXXXX]/atlas*

5) **Create your TOMO session**

Naming:	*Supervisor_YYYYMMDD_XXXXXX_visit-ID_positionX_TOMO*
Sample type:	*Slab-like*
Options:	*Select 'Batch' and 'Low Dose'*
Dose Fraction output:	*Tiff LZW non-gain normalised*
Storage folder:	*Z:\[your session ID, i.e., biXXXXXX]*

6) **Target area set up (Search maps, Batch positions)**

*If you're unsure of what parameters you should use for your experiment aim to set up one tomogram first to test your parameters before you set up **all** of your positions.*

7) **Target refinement**
8) **Perform AutoFunction alignments in TOMO (skip for lamella)**
9) **Start data collection**

1 Preparation

1.1 Communication

Prior to your session, please reach out to your local contact with the following information about your session and the grids you have shipped.

- How many grids have you sent? (Max. 4 grids per 48 hr session, these should be condensed into as fewer grid boxes as possible)
- What type of grid do you have (e.g., Quantifoil, UltrAuFoil, Cflat, Lacey etc.)
- Are all the grids clipped?
- What pixel size or magnification do you want to collect at? (see Instrument calibrated pixel sizes)
- What is your target total dose per tilt and/or per tilt series (… $e^-/Å^2$) ?
- What is your preferred tilt scheme for this session?
- What is your experience with remote TOMO sessions?
- If you require guidance in use of TOMO, is there someone in your BAG who will be assisting you with your set up or will you require assistance from your Local Contact?

1.2 Remote connection

Prior to your session, please check you can log in on NoMachine as described in the Remote access and analysis guide. You will not be able to access the instrument until your session is scheduled but you should check you can successfully log in to nx-cloud.diamond.ac.uk via NoMachine. From NoMachine you will access your visits instrument via TeamViewer, passwords can be found in the Remote access and analysis guide.

1.3 Reasonable session timings

Day 1

Atlas your grids	by midday
Decide on a grid for collection	after midday
Have search map collection started	by 2 pm
Have targets setup	by 3 pm
Start data collection	by 4pm

*If you are unsure of your parameters for your experiment you should set up one tomogram to test your parameters before setting up **all** of your positions

Day 2

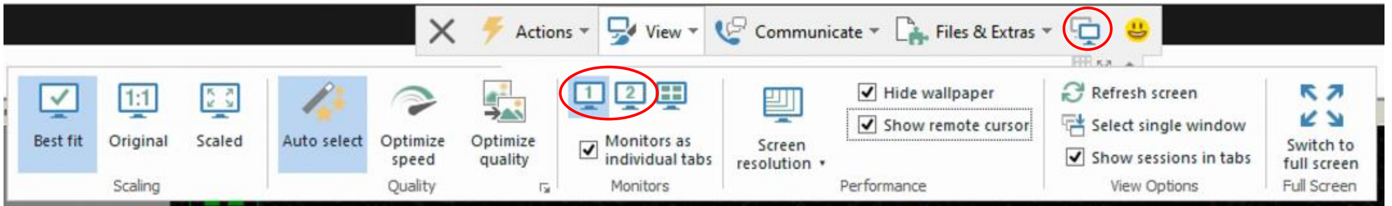
Add further acquisition targets

(as required for session length)

2 User interface summaries

Team Viewer

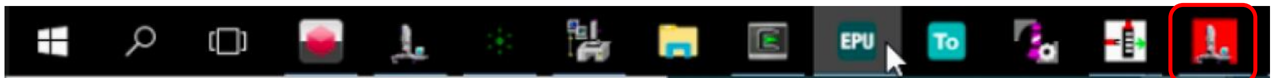
To switch between the virtual monitor hosting the TEM user interface and TFS Tomo, use the Team Viewer Monitor buttons.



TEM Control Pads Simulator – user interface

The Control Pads Simulator provides access to the microscope controls you would find at the instrument itself. It is in general becoming less necessary to use these controls but for some operations later in the guide it is useful to be aware of where to find them.

! WARNING: Take care not to accidentally press the 'Diffraction' button !



TEM User Interface (TUI) – user interface

It is in general becoming less necessary to use TUI but for some operations later in the guide you will need to access this interface.

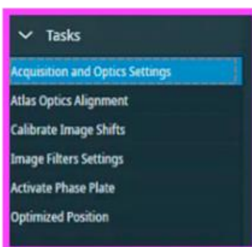
Thermo Fisher Scientific Tomo software – user interface

You can generally understand navigation through the tomography workflow via working left to right through the ‘Workflow tabs’, and top to bottom through the ‘Workflow tasks’ operations. For example, your imaging conditions (Presets) are first configured under the Acquisition and Optics Settings.

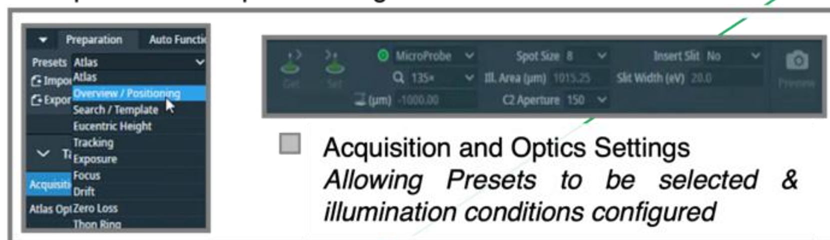
This guide presents a suggested sequence through the tomography workflow.

Workflow tabs: *Preparation, Auto Functions, Atlas, Tomography*

Workflow tasks



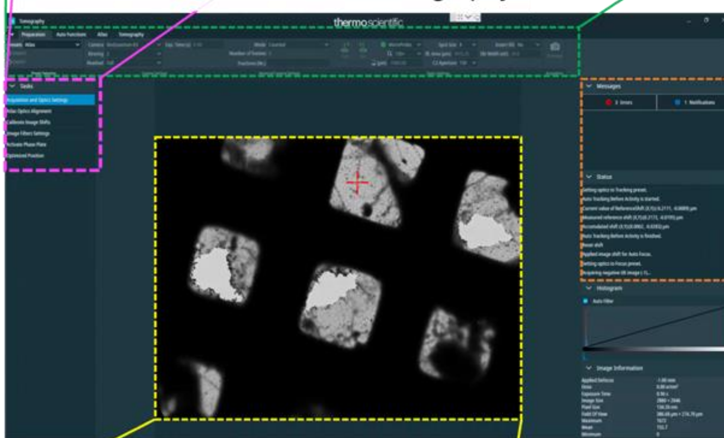
Acquisition and Optics Settings: Presets

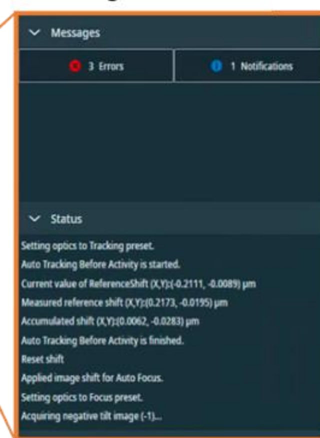


Acquisition and Optics Settings
Allowing Presets to be selected & illumination conditions configured

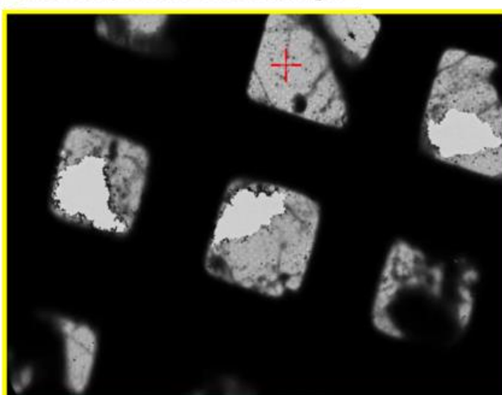
Main user interface for TFS Tomography

Messages & status





Last acquired Preset Image



Workflow tabs
Preparation, Auto Functions, Atlas, Tomography

Workflow tasks
Specific to each workflow section tab

Acquisition and Optics Settings
To configure Preset illumination conditions

Messages and status

Last image acquired under Preparation Preset

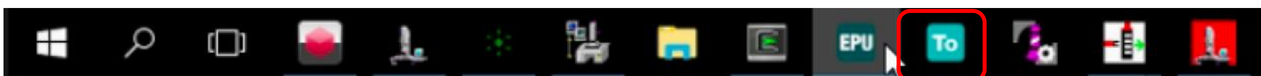
3 Microscope setup and screening

3.1 Initial checks and start up

During setup at the start of your session, your Local Contact (LC) will have loaded and inventoried the autoloader cassette.

Note: *You may find that a grid from the previous session is still on the column or present in 'position 1' of the cassette.*

☐ If a TOMO window is not open, begin by starting this software from the TEM PC



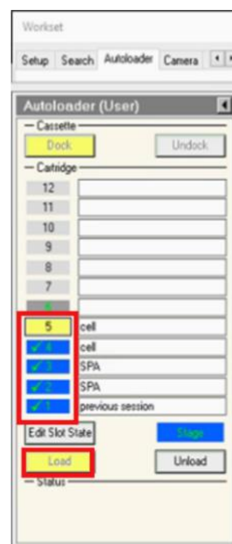
Session set-up can then be controlled by users directly through the Tomo interface. Changes to beam settings should all be controlled directly through the Tomo Preparation presets (TOMO: Preparation > Presets) and navigation around the grid can be done via right-click options over the images in Preparation, Atlas and Tomo image windows.

☐ Check if one of your grids is on the stage

(TUI: Workset > Autoloader > Autoloader (User) > check if a grid is on the stage (blue when in cassette, yellow when on stage)

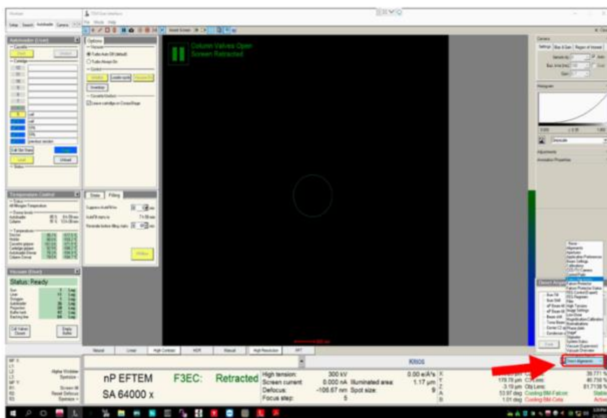
If no grid is loaded then select one of the slots and press 'Load'

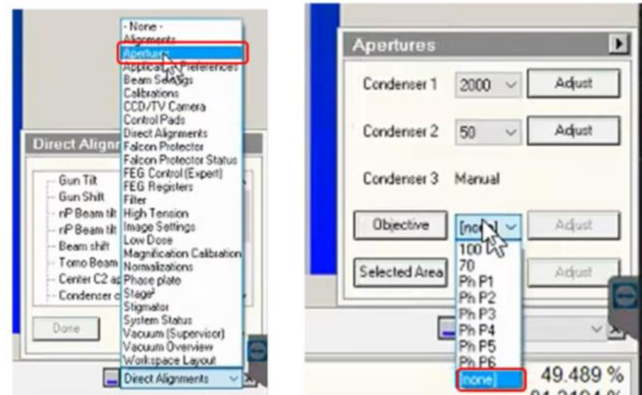
Note: *keep an eye on the 'Status window'*



☐ Make sure the Objective aperture is not inserted

(TUI: drop down menu (bottom right) > Apertures > Objective – check 'Objective' button is grey and 'none' is selected)





3.2 Check and adjusting your imaging condition Presets

Illumination conditions may differ between microscopes. The specimen will also affect how you need to configure your presets. You will need to adjust the magnification of each preset to suit your experiment. Begin with the low magnification presets (Atlas, Overview/Positioning, Search/Template, Eucentric Height) before moving on to you high magnification presets (Tracking, Exposure, Focus, Drift, Zero-Loss, Thon Ring).

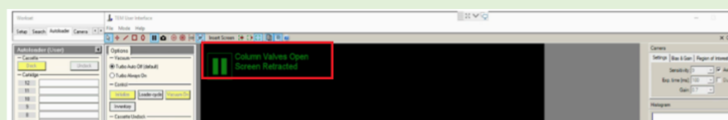
3.2.1 Check and adjust Low magnification Presets (Atlas, Overview/Positioning, Search/Template, Eucentric Height)

☐ Take an image using the 'Atlas' preset

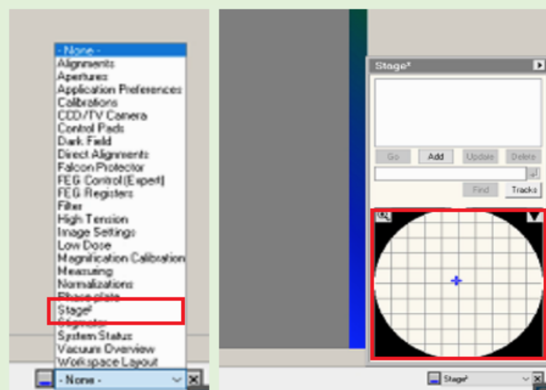
(TOMO: Preparation > Tasks > Acquisition and Optics Settings > Presets > select the 'Atlas' preset > press 'Preview')

If you don't see anything or the image is black

1) Check the screen is retracted and column valves are open



2) You may be over a grid bar or thick ice.
Try moving to the other side of the grid using the 'Stage' in TUI
(TUI: dropdown in bottom right > select 'Stage' > double click on an area of the stage to move to)
Then 'Preview' again in EPU to image the new position



☐ Use the atlas image to navigate to the centre of a grid square (choose one with reasonably thin ice)

(TOMO: right-click on grid square on image > 'Move stage here')

☐ Go to the 'Preparation' tab and check each low magnification preset (Atlas, Overview/Positioning, Search/Template, Eucentric Height) **in turn:**

1) Select the 'preset'

   (TOMO: Preparation > Tasks > Acquisition and Optics Settings > Presets > select 'Atlas' / 'Overview/Positioning' / 'Search/Template' / 'Eucentric Height'

2) 'Set' the Preset to the microscope

   (TOMO: Preparation > Tasks > Acquisition and Optics Settings > Optics Settings > 'Set')

3) Press 'Eucentric Focus' on the control pad
4) Switch to the TUI screen and insert the flu screen *(Control pads: R1)*. Check the size of beam is larger than the sensor green circle on the flu screen
5) Switch back to the TOMO screen and collect a preview image

   (TOMO: Preparation > Tasks > Acquisition and Optics Settings > Acquisition > 'Preview')

6) Adjust imaging parameters for each preset so that:

   Atlas
   Image encompasses the whole screen

   Overview/Positioning
   Image encompasses one grid square or one lamella

   Search/Template
   The magnification is adequate to show your feature of interest and to fit both the Exposure and the Tracking/Focus area in the field of view. For lamella, the 'Search/Template' preset should be adjusted so the targets location is recognisable.

   Eucentric Height
   The 'Eucentric height' and 'Search' magnification preset are usually the same. You may want to set a lower Search preset magnification useful for identifying your target of interest but use a higher magnification for Eucentric height magnification to maintain accuracy in eucentric height determination. When finished setting up 'Search' preset press 'Set' to push the settings to the microscope then select the 'Eucentric Height' preset and click 'Get'

3.2.2 High magnification Presets (Exposure)

Your 'Exposure' **magnification** will have been set by your Local Contact based on prior communication with you and calibrations made by your Local Contact will be dependent on this magnification, **do not** adjust the 'Exposure' magnification **do not** adjust the 'Exposure **magnification** without discussing with your Local Contact. You should double check that the **exposure time** and other parameters are appropriate for your experiment. There is a Dose rate calculation tool on our website if required.

☐ Calculate the exposure time based on the physical pixel dose rate (e^-/px/sec) and pixel size (these values will have been provided to you by your local contact during handover).

Your LC will have told you the **physical pixel dose rate** over vacuum. Follow the example calculation below to calculate and enter your target exposure time based on your desired target dose per tilt or in total (e⁻/Å²). You may find these [online dose calculators]() helpful.

Example exposure time calculation with parallel illumination **targeting optimal dose per tilt**

Physical pixel dose rate: 5.281 e⁻/px/sec
Pixel size (magnified): 1.63 Å/px
Pixel area (magnified): 2.66 Å²/px

$$\frac{e^-/px/sec}{Å^2/px} = e^-/Å^2/sec$$

Dose per tilt (target): 3.2 e⁻/Å²

Dose rate: 1.988 e⁻/Å²/sec

$$\frac{e^-/Å^2}{e^-/Å^2/sec} = sec$$

Exposure time per tilted image (sec): 1.61

Example exposure time calculation with parallel illumination **targeting total dose**

Physical pixel dose rate: 5.281 e⁻/px/sec
Pixel size (magnified): 1.63 Å/px
Pixel area (magnified): 2.66 Å²/px

$$\frac{e^-/px/sec}{Å^2/px} = e^-/Å^2/sec$$

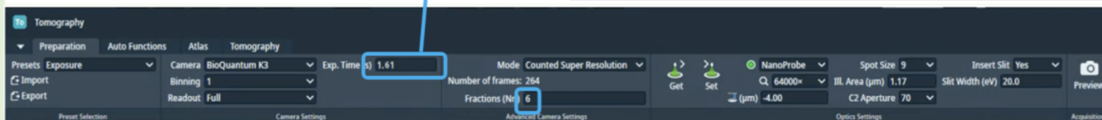
Dose rate: 1.988 e⁻/Å²/sec

Total dose (target): **131 e⁻/Å²**
Tilt range (°): -60 to +60
Tilt step (°): 3
Tilt image number: 41

Dose per tilt = 131 / 41
= 3.2 e⁻/Å²

$$\frac{e^-/Å^2}{e^-/Å^2/sec} = sec$$

Exposure time per tilted image (sec): 1.61



In this example, there are a few considerations to keep in mind:

- The dose per tilted image is 3.20 e⁻/Å² (3.0 - 3.5 is reasonable for sub-tomogram averaging)
- 'Fractions (Nr.)' is set to 6 for a tilt movie with ~ 0.5 e⁻/Å² per fraction, sufficient for motion correction

From your calculation, will each tilt receive sufficient dose for the imaging and tracking to work? If you are unsure, later during set up, you could move to an unused area to acquire data acquisition images at zero and increasing tilts that reflect your collection.

☐ Under the 'Exposure' preset tab enter the calculated exposure time and frame number

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > enter 'Exp. Time' and 'Fractions (Nr.)')

☐ Check that the imaging mode and binning values are suitable for your experiment

'Mode' controls the binning behaviour of TOMO
'Binning' controls the binning behaviour of the camera

Examples for K3:

Example 1 : 'Counted Super Resolution' mode **with '**2' times binning
*The camera will collect in its default super resolution format, the binning of 2 will tell the **camera** to bin the movies 2-fold before transferring standard resolution movies to TOMO*

Example 2: 'Counted Super Resolution' mode **with '**1' times binning
The K3 will collect in its default super resolution format, the Binning of 1 will mean super resolution movies are transferred to TOMO

Example 3: 'Counted' mode **with '**1' times binning
The K3 will collect in its default super resolution format and transfer super resolution movies to TOMO, but 2-fold binning will be performed by TOMO to obtain standard resolution movies.
As in example 1 the outcome is standard resolution movies however binning in TOMO is slower than binning on the camera).

☐ Check all other parameters are correct

3.2.3 Other High Magnification Presets (Tracking, Focus, Zero Loss, Thon Ring, Drift)

The **exposure** magnification settings should have been copied to your other high magnification presets **(Tracking, Focus, Zero Loss, Thon Ring, Drift)** by your local contact but you should double check the settings for these presets are appropriate for your experiment.

☐ Double check exposure times for the high magnification presets, especially for 'Focus and Tracking' to give a reasonable number of counts also considering the thickness at 60 degrees

(TOMO: Preparation > Tasks > Acquisition and Optics settings > Camera Settings > Exp. Time (s) : adjust as required)

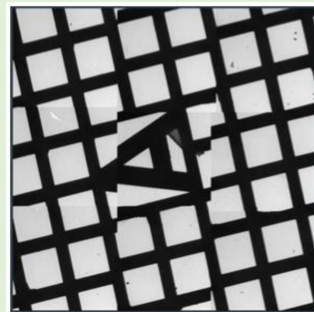
☐ Adjust the ZLP Preset if required to use a brighter spot size, as ZLP needs higher dose

Note: *For the falcon 4 a smaller number is required (i.e., drop 2 spot sizes for K3, 1 for Falcon 4)*

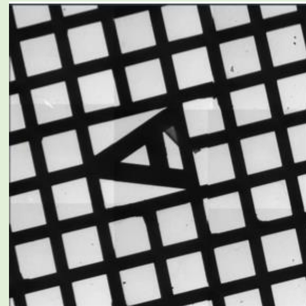
Note: *You may also select binning 2 to help with increasing signal to noise in this Preset*

3.3 Targetting Correction

After you have decided what preset settings to use, it is probably a good time to check whether the 'Targeting Correction' has been calibrated for those presets. Targetting correction calibration improves the alignment and stitching of montage images (i.e., Atlases and Search Maps). This is very helpful for accurately targeting acquisition areas in 'Search Maps'.



Before targeting correction



After targeting correction

3.3.1 Prepare for targeting correction

☐ Use your atlas to find a grid square with recognisable features

Note: *it is not recommended to perform the calibration on a lamella specimen.*

☐ Move to the centre of that square

(TOMO: Atlas > Screening > select Atlas on the left > right-click on grid square on Atlas > 'Move stage here')

3.3.2 Find eucentric height

The stage must be at eucentric height to correctly apply Image Shift Calibrations.

☐ Navigate to centre of your selected grid square

☐ Take an image of the area using the 'Overview/Positioning' magnification preset

(TOMO: Preparation > Acquisition and Optics Settings > select the 'Overview/Positioning' preset > press 'Preview')

☐ Recentre the square as necessary and 'Preview' again.

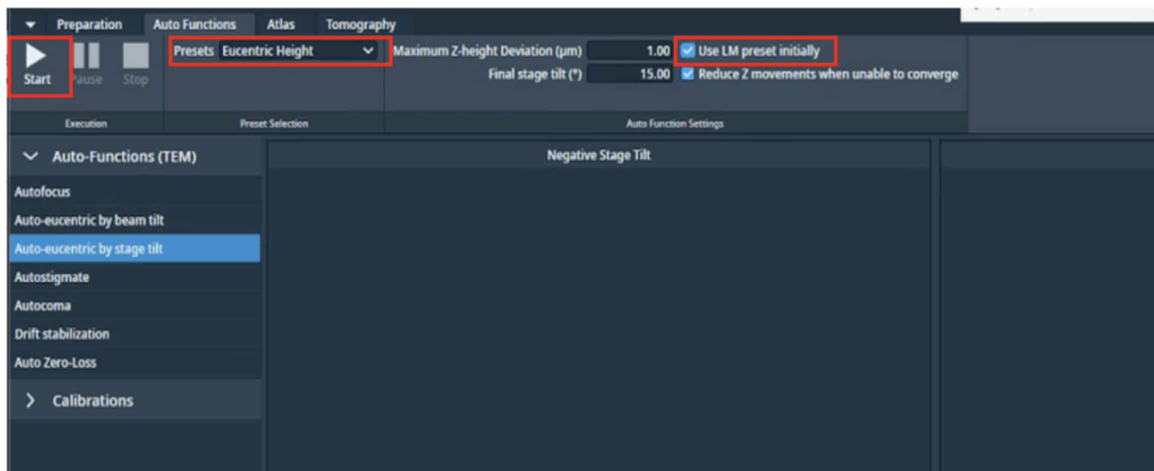
If not done so already, adjust magnification to suit your grid square/lamella size. You may also wish to change the 'Overview/positioning' preset defocus depending on hole contrast

☐ Set the stage to Eucentric height

1) Select Auto Eucentric by stage tilt in the autofuctions tab
   (*TOMO: Autofunctions > Auto Eucentric by stage tilt)*
2) Set to 'Eucentric Height' magnification preset **in autofunctions**
   (*TOMO: Autofunctions > Preset 'Eucentric Height')*

   Note: *we recommend ensuring that the 'Use LM preset initially' option is selected. This will enable a coarse round of 'Autoeucentric by stage tilt' at the 'Overview' magnification before a finer round of 'Autoeucentric by stage tilt' at the 'Eucentric Height' magnification. This is useful to avoid issues when the stage is very far from Eucentric height.*

3) Press 'Start'



Keep an eye on the Status window in TOMO (right hand side) and look for decreasing Z values. On the TOMO view you should see matching images on the left and a sharp 'peak' on the right.

Troubleshooting: Auto eucentric height failures

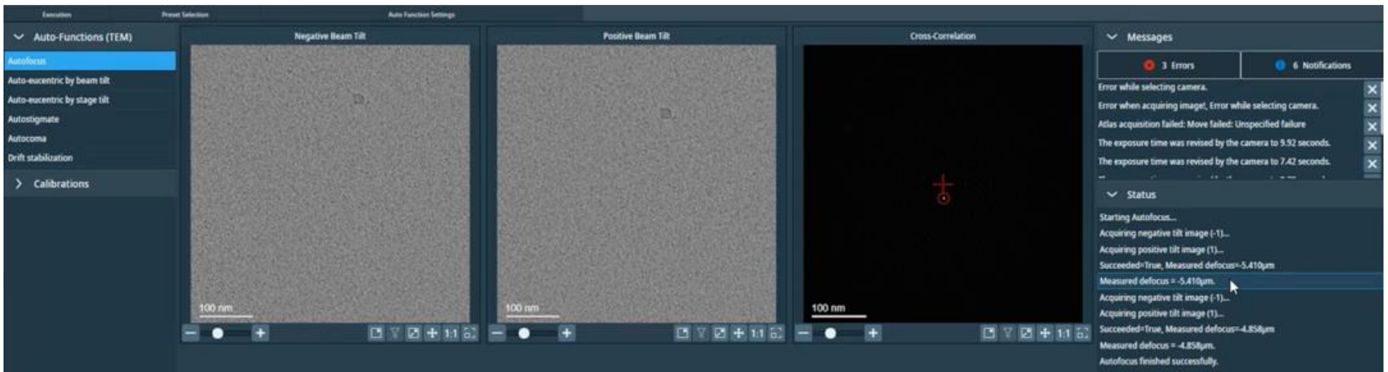
If the 'Auto eucentric by stage tilt' routine fails then refer to section 7.1.1 and 7.1.2

3.3.3 Autofocus

Run the autofocus routine on carbon

1) Select AutoFocus in the autofuctions tab
   (*TOMO: Autofunctions > AutoFocus)*
2) Set to 'AutoFocus' magnification preset **in autofunctions**
   (*TOMO: Autofunctions > Preset 'AutoFocus')*
3) Press 'Start'

Note: *Keep an eye on the Status window in EPU (right hand side) and look for a sharp 'peak' on the right*



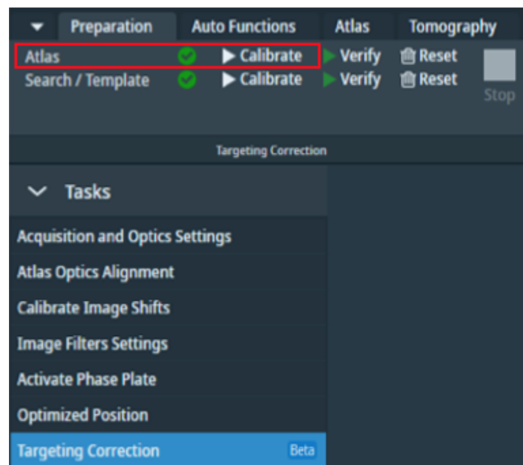
3.3.4 Calibrate the Targetting Correction for the ‘Atlas’ and ‘Search/Template’ magnification presets

Targeting correction is done at 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.

Atlas magnification:

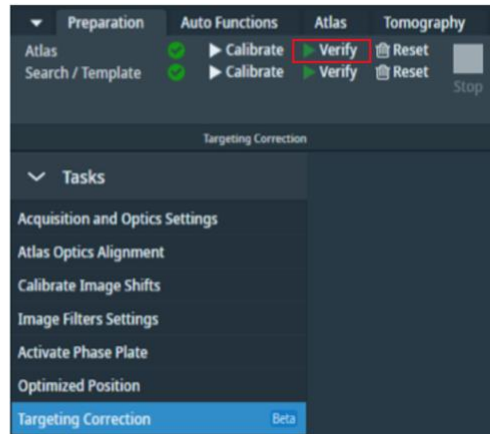
☐ Start targeting correction calibration for the Atlas magnification preset

(TOMO: Preparation > Tasks > Calibrate Image Shifts > press ‘Start Calibration’ next to the Atlas option)



Note: *Keep an eye on the ‘Expected Calibration Error in the ‘Status’ window.*

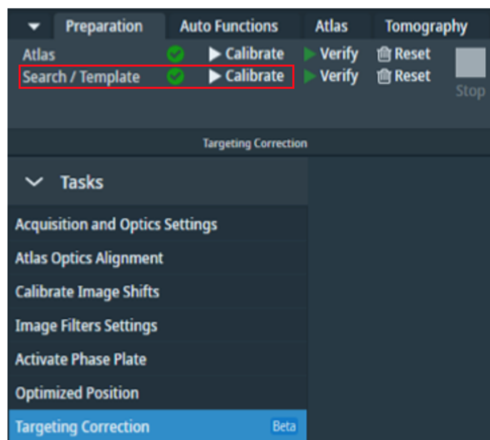
☐ Verify that the targeting correction calibration for the Atlas magnification preset was completed successfully



Search/Template magnification:

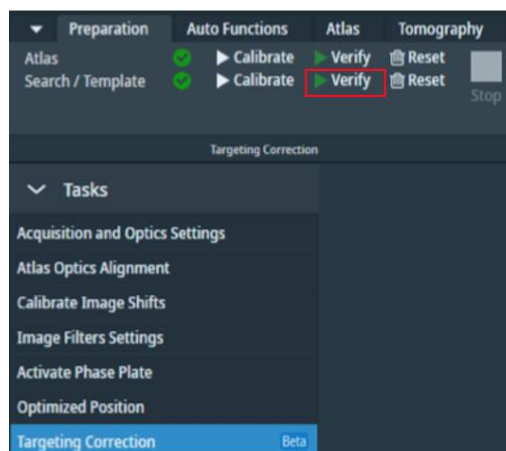
☐ Start targeting correction calibration for the Search/Template magnification preset

(TOMO: Preparation > Tasks > Calibrate Image Shifts > press 'Start Calibration' next to the Atlas option)



***Note:** Keep an eye on the 'Expected Calibration Error in the 'Status' window.*

☐ Verify that the targeting correction calibration for the Search/Template magnification preset was completed successfully



3.4 Image Shift Calibrations

3.4.1 Prepare for image shift calibrations

☐ Take an image of your inserted grid at 'Atlas' magnification and use it to find an identifiable feature which will be recognisable at all magnification presets. Try to choose something which is fairly central on a grid square.

> ***E.g.,** a large non-symmetrical ice crystal*

☐ Move to that square

> *(TOMO: right click on image and 'Move stage here')*

☐ Find Eucentric height as in section 3.3.2

3.4.2 Target a feature for Image Shift calibrations

> Prior to performing Image Shift Calibrations we systematically target a feature at low and up to high magnification. This will ensure that your feature is identifiable at all magnifications, provide indication of whether the Image Shift Calibrations need to be performed and importantly, centre a target feature at high magnification ready for Image Shift Calibrations.

☐ Centre the stage on your feature of choice (i.e., a large asymmetric ice crystal) which will be easily recognisable at both high and low magnifications

1) Collect a 'Preview' using the 'Atlas' Preset

   (TOMO: Preparation > Acquisition and Optics Settings > select the 'Atlas' preset > press 'Preview')

2) Centre on the feature

   (TOMO: Right click on feature > Move stage here)

☐ Check the feature remains aligned at higher magnification Presets

1) Collect a preview using the 'Overview/Positioning' Preset

   (TOMO: Preparation > Acquisition and Optics Settings > select the 'Overview/Positioning'' preset > press 'Preview')

2) Check the feature remains centred, right-click > Move stage here, if necessary
3) Collect a preview using the 'Search' Preset

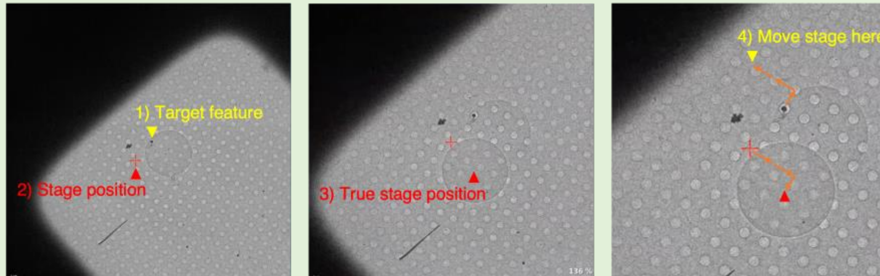
   (TOMO: Preparation > Acquisition and Optics Settings > select the 'Search' preset > press 'Preview')

4) Check the feature remains centred, right-click > Move stage here, if necessary
5) Collect a preview using the 'Exposure' Preset

   (TOMO: Preparation > Acquisition and Optics Settings > select the 'Exposure' preset > press 'Preview')

6) Check the feature remains centred, right-click > Move stage here, if necessary

If you struggle to recentre the feature because it is out of a current field of view, one strategy is to take an image using your current Preset, and then subsequently take a new image using the Overview/Positioning Preset as in (1). The charging mark introduced will be evident in the Overview/Positioning image and indicate what the true stage position is versus where the stage thinks it is. You can then appropriately reposition the stage on the Overview/Positioning image.

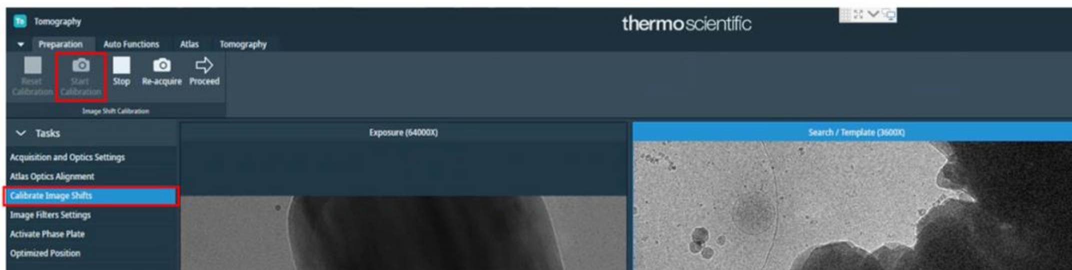


If you found the feature remained centred during targeting, then you may choose to skip image shift calibrations
If you needed to recentre the feature during targeting, you should calibrate the Image Shifts. With the feature now centred at Exposure Preset, proceed to 3.4.3

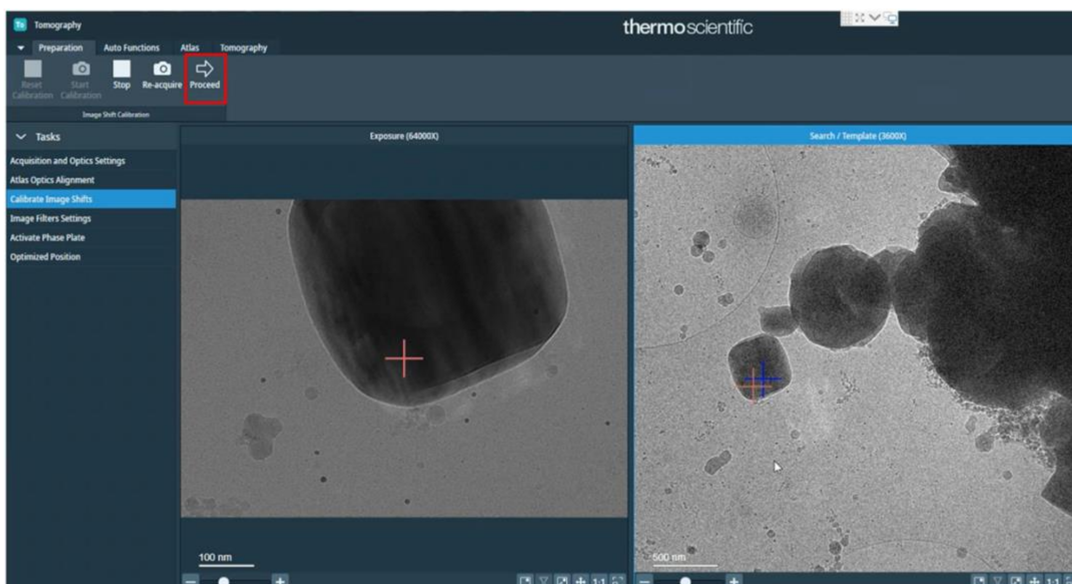
3.4.3 Calibrate the Image Shifts

☐ Start image shift calibration

(TOMO: Preparation > Tasks > Calibrate Image Shifts > press 'Start Calibration')



☐ We strongly suggest not to apply an image shift at the initial 'Exposure' Preset. Click 'Proceed' to begin the calibration



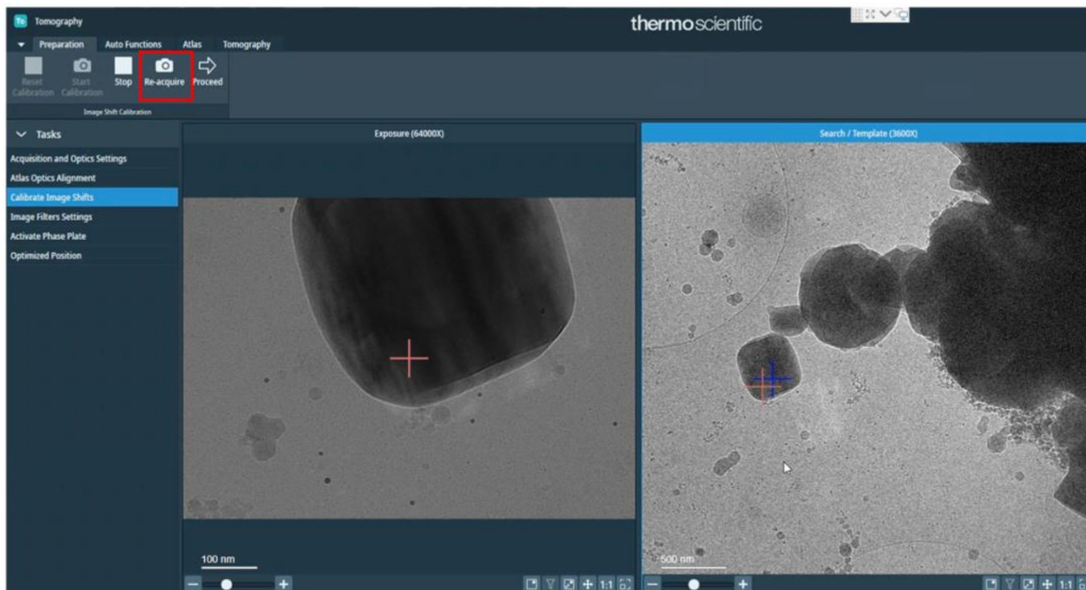
☐ For each magnification in turn:

1) Move the stage (cross) to a particular point on your target feature that you can keep track of (*e.g., the point where an ice crystal meets a hole).*

   (TOMO: Double left click on the right-hand image where you want to move to)

   Note: *The original cross will turn blue and a new red cross will appear at the position you have clicked.*

2) Press 're-acquire' to take a new image



3) Observe whether the position of the stage (red cross) in the right-hand image matches the position in the left hand image.
4) Repeat steps 1 - 3 until satisfied that the position of the stage is well calibrated between the magnifications shown in the left and right images.
5) Press 'Proceed' to move to the next pair of Presets

There is a 180º rotation between the 'Overview/Positioning' and 'Eucentric Height'/'Search/Template' magnification presets. You may find it helpful to take a photo on your phone and manually rotate this to orientate yourself

4 Screening

4.1 Collect your Atlas

Atlas Session setup:

☐ Start a new session for taking an Atlas
(TOMO: Atlas > Session Setup > press 'New session')

☐ Setup the new session

- **Naming convention:** Supervisor_YYYYMMDD_XXXXXX_visit-ID_Atlas

  Note: *DO NOT edit the* ***prefix*** *created by TOMO, this is required by our scripts for data transfer.*

  (i.e., Supervisor_YYYYMMDD_XXXXXX),

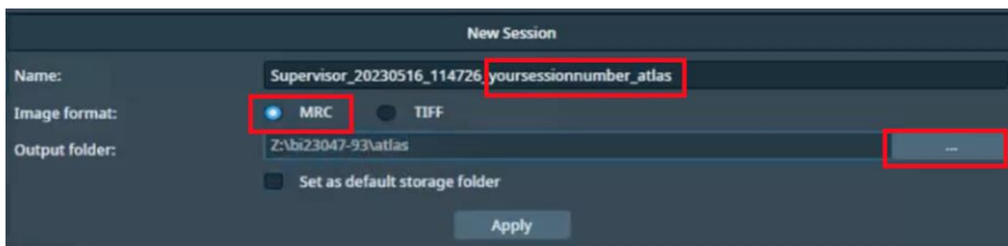
  Note: *We strongly suggest adding a* ***suffix*** *including your visit ID and 'ATLAS'*

  (i.e., Supervisor_YYYYMMDD_XXXXXX_biXXXXXXX_ATLAS)

- **Image format:** MRC
  Note: *These are the Atlas and Atlas tile image formats. Using MRC is recommended to retain the extended MRC header information*

- **Output folder:** Select the SSD storage folder path: Z:\[your session ID, i.e., biXXXXXX]/atlas

  Note: *If you do not see a folder with your session ID and atlas in the Z:\ drive (i.e. :Z\biXXXXX/atlas), ask your LC to initiate 'murfey' before you set up your Atlas session*

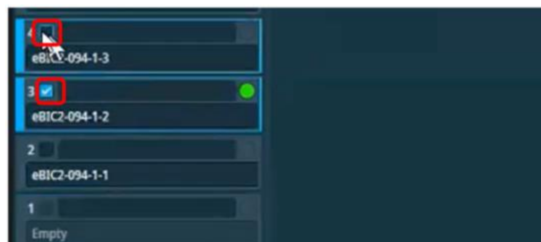


☐ Press 'Apply'

Atlas collection:

☐ Select grids to collect atlases of

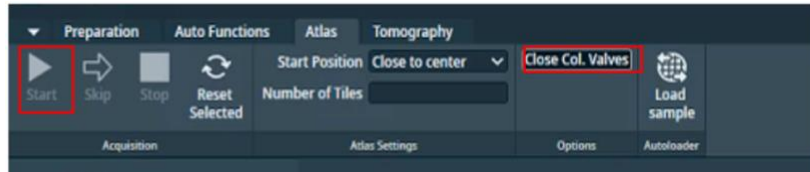
(TOMO: Atlas > Tasks > Screening > Select the checkbox of each grid you want to acquire an Atlas of)



☐ Select 'close the column valves' to ensure TOMO closes column valves when the Atlas is complete
(TOMO: Atlas > Screening > Close Col. Valves)

☐ Click the 'Start' button

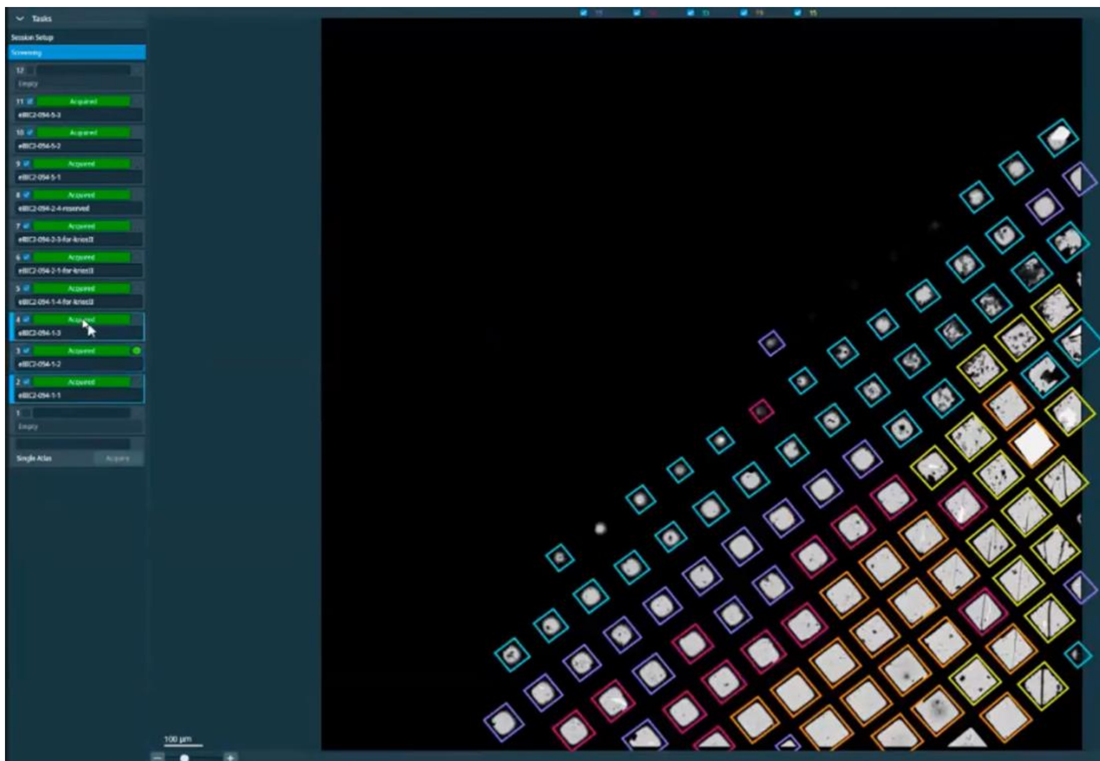
(TOMO: Atlas > Screening > Start)



4.1.1 Inspect your Atlas

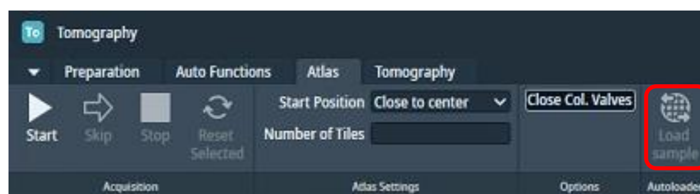
☐ When atlas acquisition is complete, inspect your single (or multiple Atlases) for targets by clicking on the grid in the left panel

Left-click on the central image and drag to move around, middle scroll to zoom in-out



☐ When you have chosen your grid for target setup, select it and click 'Load Sample' from inside Tomo

(TOMO: Atlas > Autoloader > 'Load sample')



IMPORTANT: Once you have chosen a grid for data collection you should **reacquire the Atlas.** This is because shifts and rotation calculations that occur to the grid during loading and unloading are less reliable in TOMO than EPU. You should also ensure that you ONLY load grids through TOMO, not TUI.

5 Tomography setup

IMPORTANT: Once you have chosen a grid for data collection you should **reacquire the Atlas.** This is because shifts and rotation calculations that occur to the grid during loading and unloading are less reliable in TOMO than EPU. You should also ensure that you ONLY load grids through TOMO, not TUI.

5.1 Setup

5.1.1 Session setup

☐ Set up a new TOMO Session

(TOMO: Tomography > New session > Session Setup)

☐ Setup the new session

- **Naming convention:** Supervisor_YYYYMMDD_XXXXXX_visit-ID_autoloader-position_TOMO

  Note: *DO NOT edit the prefix created by TOMO*

  (i.e., Supervisor_YYYYMMDD_XXXXXX)

  We also strongly suggest adding a suffix including your visit ID, autoloader position and TOMO as a suffix

  (i.e. Supervisor_YYYYMMDD_XXXXXX_biXXXXXXX_ positionX_TOMO)
- **Sample Type:** Slab-like
- **Options:** Select 'Batch' and 'Low Dose'
- **Dose fraction output format:** Tiff LZW Non-Gain normalized

  Note: *These are the raw movie stacks from the detector.*
- **Storage folder:** Select the SSD storage folder path: Z:\[your session ID, i.e., biXXXXXX]

  Note: *If you do not see a folder with your session ID and atlas in the Z:\ drive (i.e., :Z\biXXXXX), ask your LC to initiate 'murfey' before you set up your Atlas session*

☐ Click 'Apply'

5.1.2 Lamella tilt correction (if applicable)

If working with lamella, before acquiring Search images to identify targets, the specimen tilt due to milling angle will need to be corrected. The milling angle is known from the FIB-SEM experiment, at eBIC this is typically 10-15°, however the loading orientation of the lamella in the microscope may not be known. The schematic below shows the two possible ‘Loaded orientations #1 & #2’. In the ‘Positive and Negative tilt test’ the lamella long axis will either stay the same or appear narrower, depending on the loaded orientation.
If the lamella long axis narrows in the ‘Positive tilt test’, apply a negative correction. If the lamella long axis narrows in the ‘Negative tilt test’, apply a positive correction. In practise, it can help to apply larger test angles.

- ☐ Tilt the stage to 2X the known milling angle using a positive tilt, by using ‘Set Tilt (°)’ and ‘Set’
- ☐ Acquire an Overview image and note any change in the long axis of the lamella

- ☐ Tilt the stage to 2X the known milling angle using a negative tilt, by using ‘Set Tilt (°)’ and ‘Set’
- ☐ Acquire an Overview image and note any change in the long axis of the lamella
  - If the lamella appearance change is not obvious, try tilting to a higher angle i.e., +/- 40°

- ☐ Apply the appropriate Tilt Correction, by using ‘Set Tilt (°)’ and ‘Set’
- ☐ Acquire a new Overview with the Tilt Correction applied

5.2 Identifying areas of interest using ‘Search maps’

The ‘Search maps’ feature in TOMO allows you to set up areas in which to montage a set of images at the ‘Search’ magnification. This is useful to identify areas to target for data collection. You can add multiple Search Maps on the specimen then queue them for automatic acquisition. During the acquisition of multiple Search Maps, Tomography ensures that the specimen is at Eucentric Height before acquiring each individual Search Map.

5.2.1 Setting up a ‘Search Map’

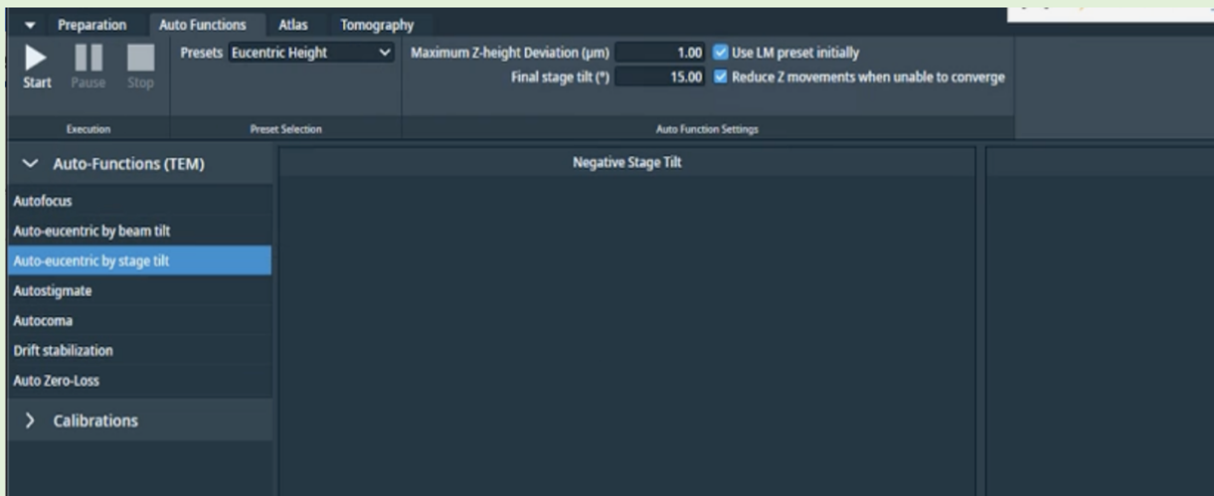
☐ If an Atlas is available for the currently loaded specimen, then that Atlas is used for the new session.

IMPORTANT: Once you have chosen a grid for data collection you should **reacquire the Atlas.** This is because shifts and rotation calculations that occur to the grid during loading and unloading are less reliable in TOMO than EPU. You should also ensure that you ONLY load grids through TOMO, not TUI.

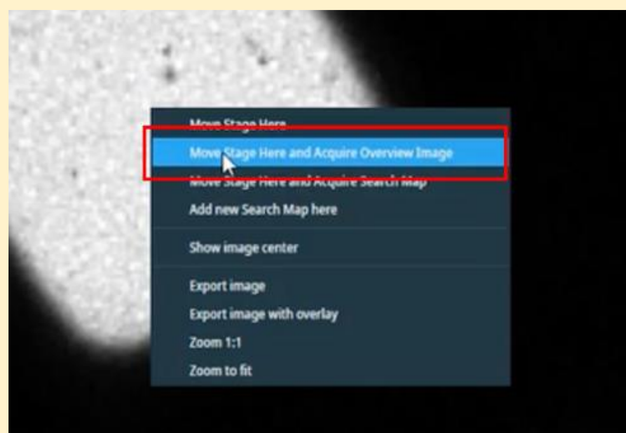
☐ Use your Atlas to navigate to an area of interest

A note on the autoeucentric routine:
Before setting up Search maps, we recommend ensuring that the 'Use LM preset initially' option is selected for the 'Autoeucentric by beam tilt' autofunction routine. This will enable a coarse round of 'Autoeucentric by stage tilt' at the 'Overview' magnification before a finer round of 'Autoeucentric by stage tilt' at the 'Eucentric Height' magnification. This is useful to avoid issues when the stage is very far from Eucentric height.

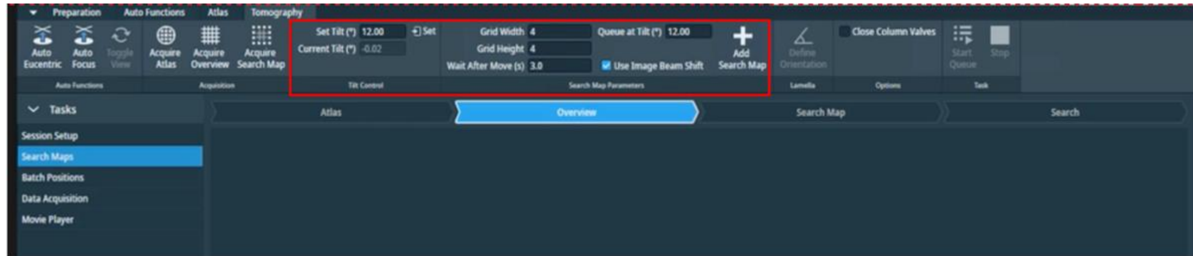
(TOMO: Autofunctions > Auto-Functions (TEM) > Auto-eucentric by stage tilt > Auto Function Settings > select 'Use LM preset initially'



(Optional) Acquire a more detailed image of the area at the 'Overview' magnification
(TOMO: Tomography > Search Maps > Atlas > Right-click on area and press 'Move Stage here and acquire Overview Image')

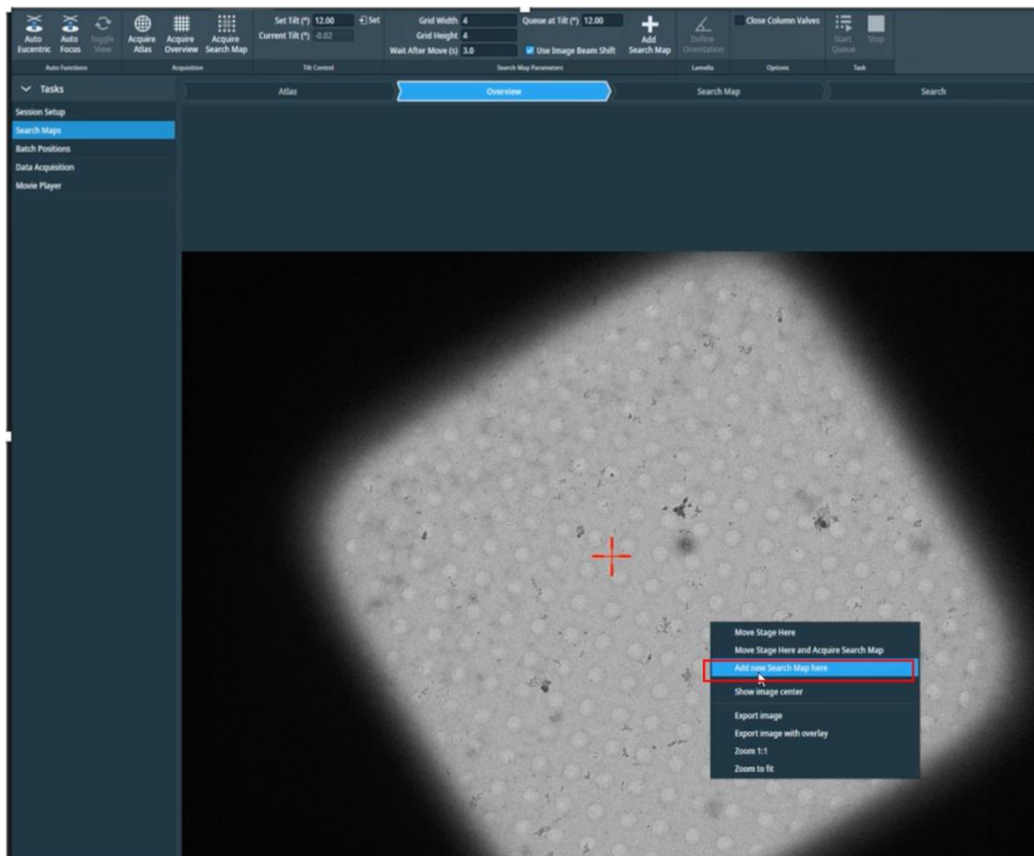


☐ Adjust your search map parameters as required to suit your experiment
(TOMO: Tomography > Search Maps > Overview > adjust parameters as required)
i.e., *Grid Width (X direction), Grid Height (Y direction), Wait After Move (s), Queue at Tilt (˚) etc.*
(Optional) *Deselect Use Image Beam Shift to go to the next Search Map tile location with Stage Shift instead of Beam/Image Shift.*

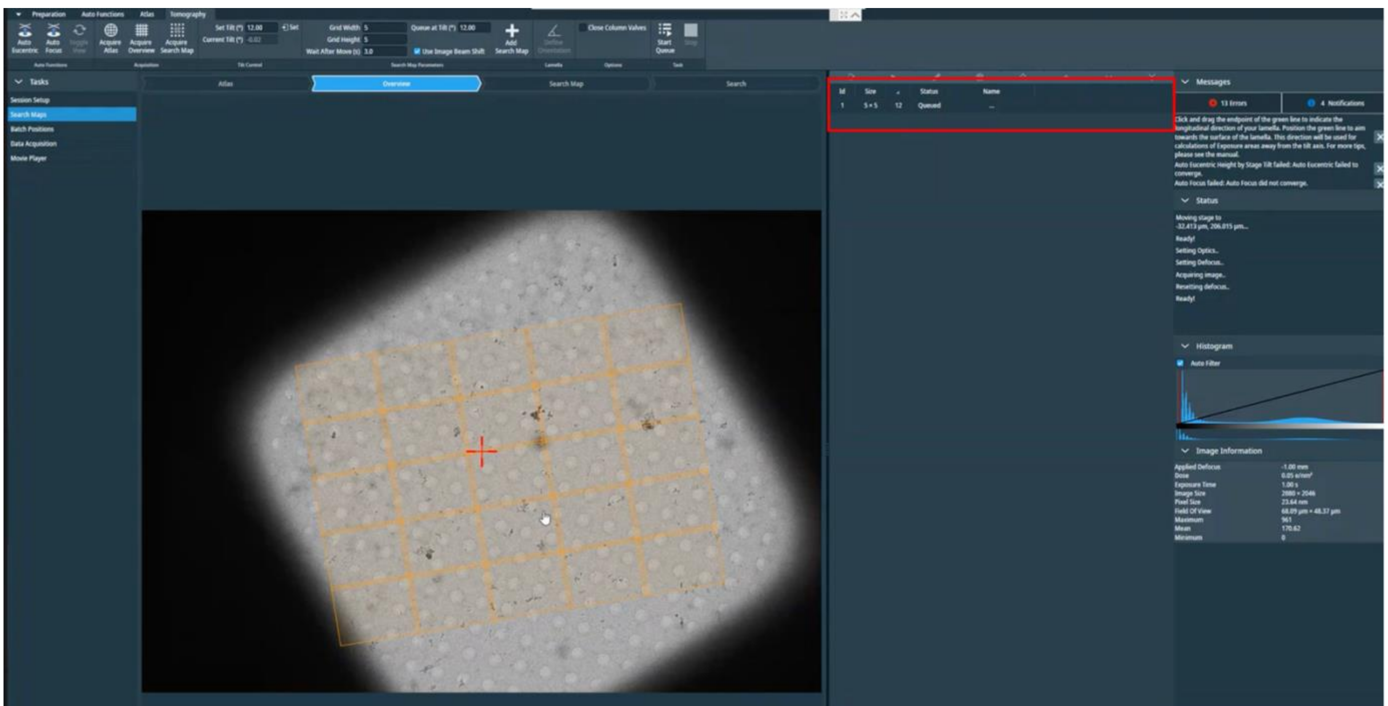


A note on the 'Queue at Tilt' field
Ensure this field is **filled in correctly**, especially for lamella tomography where the **initial tilt angle is not 0°.** This value is not automatically updated with the current stage tilt, so you will need to manually set the desired angle before queuing the Search Maps for collection (this should be done at the correct start tilt angle). If the wrong angle is specified, you will have to re-acquire all the Search Maps again and re-do the entire setup, as the targeting will be inaccurate during collection.

☐ In the Atlas image or the Overview image, select an area of the image to set up a Search Map
(TOMO: Tomography > Search Maps > Atlas or Overview > Right-click on area and press 'Add new Search Map here')



☐ Your search map area will appear as an orange grid on the image. It will also appear in a list on the right hand side of the screen



OPTIONAL BUT ADVISED:
If you're unsure of what parameters you should use for your experiment, we'd advise setting up one tomogram first to test your parameters before you set up **all** of your positions.

5.2.2 Queueing a series of 'Search Map' areas

☐ Continue adding 'search maps' as described in section 5.2.1. Each 'Search map' will be added to queue of search maps on the right-hand side of the screen.

> ***Note:*** *You may alter the parameters used for each search map (i.e., Grid width/height, tilt etc.) as required before adding the search map to an area*
>
> ***Note:*** *Search maps will initially appear orange unless selected from the queue. List on the right-hand side of the screen (selected search map turns green)*

Acquiring a single Search map

To acquire a single Search Map from the Search Maps queue, select the Search Map to acquire from the list and press the 'Acquire' button

Deleting Search maps

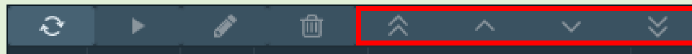
To delete a single Search Map from the Search Maps queue, select the Search Map to delete from the list and press the 'Delete' button

Editing Search maps

If you need to change search map parameters, add a new Search Map with the updated parameters and Delete the incorrect one

Change the processing order of the Search Maps

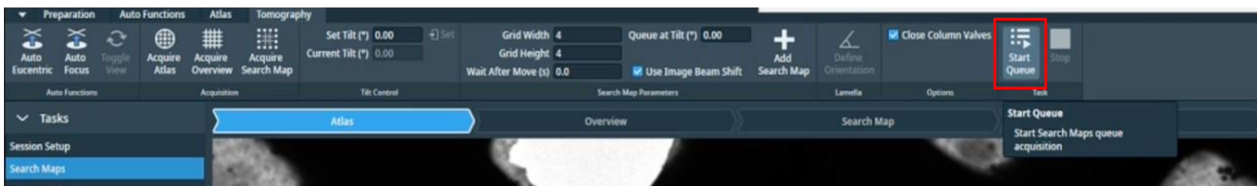
During the Automated Acquisition run, the Search Maps in the queue are processed in top-to-bottom order. By default, this is the order in which the Search Maps have been added to the list. To change the processing order, select the Search Map to reposition from the list, then select a re-ordering action from the toolbar above the Search Maps List.

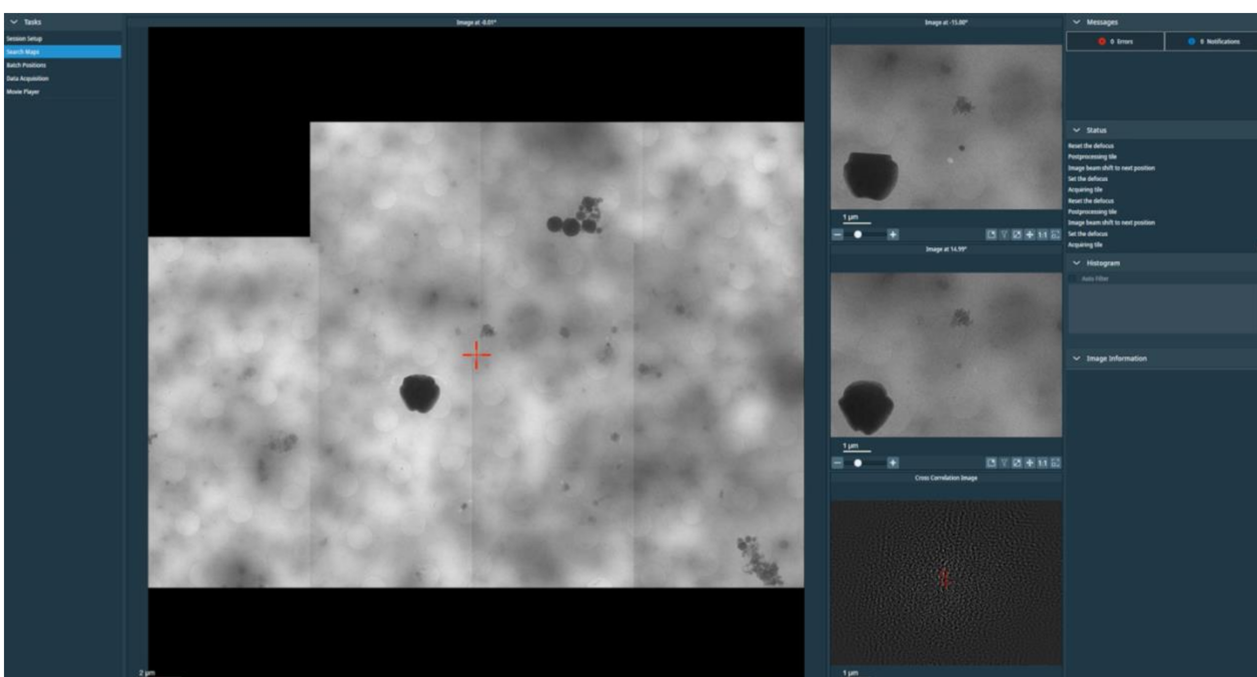


5.2.3 Acquiring queued 'Search Maps'

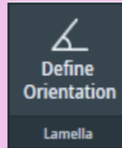
When acquiring a queue of Search maps, TOMO will go to each 'Search Map' position, determine the eucentric height for that position, then acquire and montage a set of images at the 'Search' magnification as specified in the 'Search Map' parameters.

☐ Acquire 'Search maps' of all queued areas
(TOMO: Tomography > Tasks > Search Maps > Task > 'Start Queue')

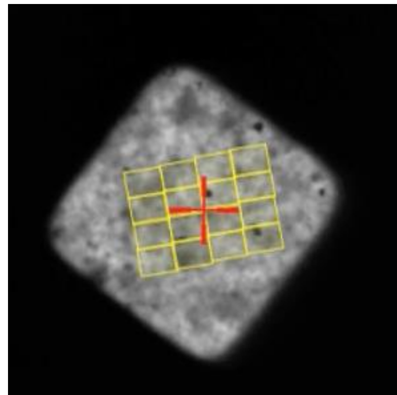




Note for lamella:
After collecting a Search Map, the ‘Define Orientation’ button becomes available. If the lamella wasn’t loaded perfectly (which it almost never is) and isn’t exactly perpendicular to the tilt axis, you should use this feature to define the orientation of the lamella with respect to the tilt axis. This helps with more accurate targeting of off-axis acquisition areas.



☐ Once ‘Search map’ areas have been acquired they will turn from orange to yellow and appear as ‘Acquired’ in the queue list summary on the right-hand side of the screen



Id	Size	∠	Status	Name
1	4×4	0	Acquired	SearchMap_20230117_135307
2	6×6	0	Queued	...
3	6×6	0	Queued	...
4	4×4	0	Queued	...

5.3 Targeting areas for ‘Batch Positions’

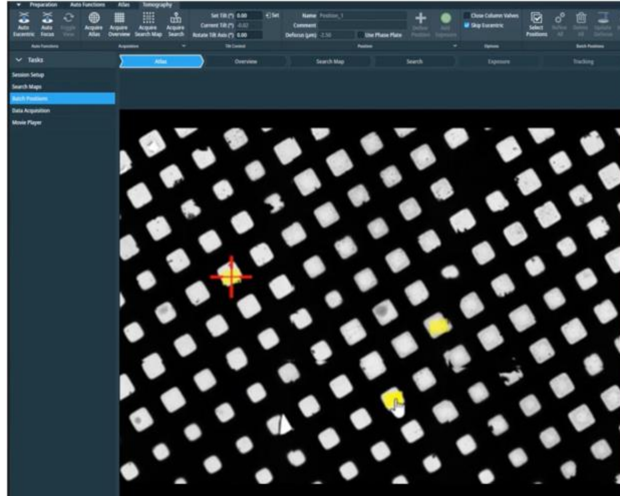
Using ‘Batch Positions’ enables a user to set up multiple positions for data collection. During automated Data Acquisition, TOMO will acquire a Tilt Series for each Batch Position.

IMPORTANT NOTE:

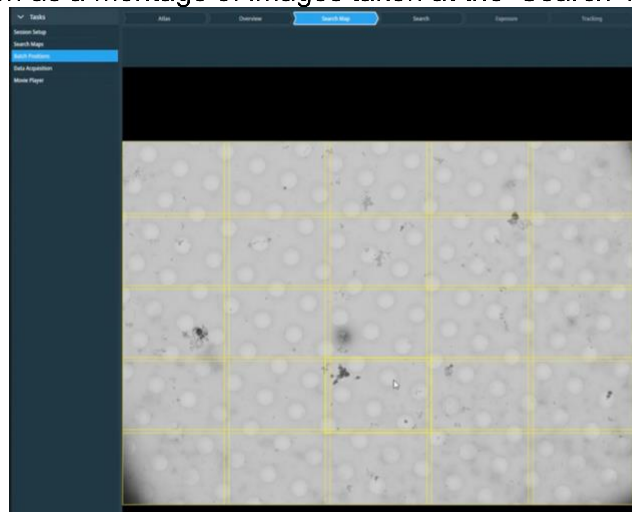
As you are adding batch positions, **be aware:**

- Of **nearby features** that will become exposed as the stage tilts.
- Look for **features** that may **enter the field of view** and **interfere** with tracking and/or acquisition, including grid bars.

☐ Go to the ‘Batch positions’ tab
(TOMO: Tomography > Tasks > Batch position)
☐ From the Batch positions Atlas overview, select a ‘Search map’ to view
(TOMO: Tomography > Tasks > Batch position > Atlas > left click on a yellow acquired ‘Search map’)

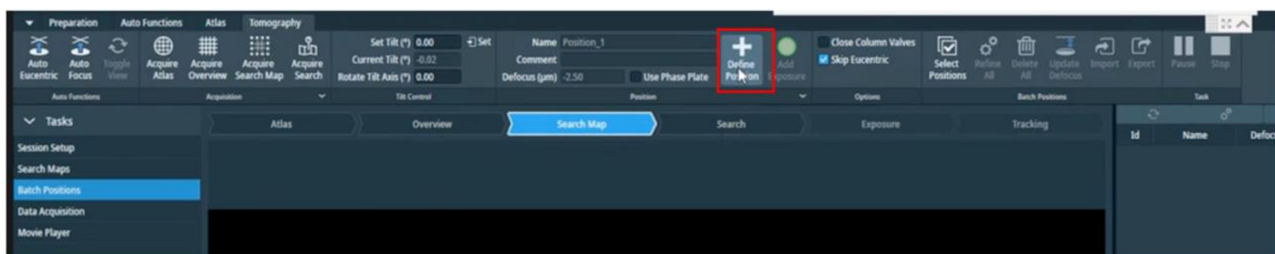


☐ The search map will open as a montage of images taken at the 'Search' magnification



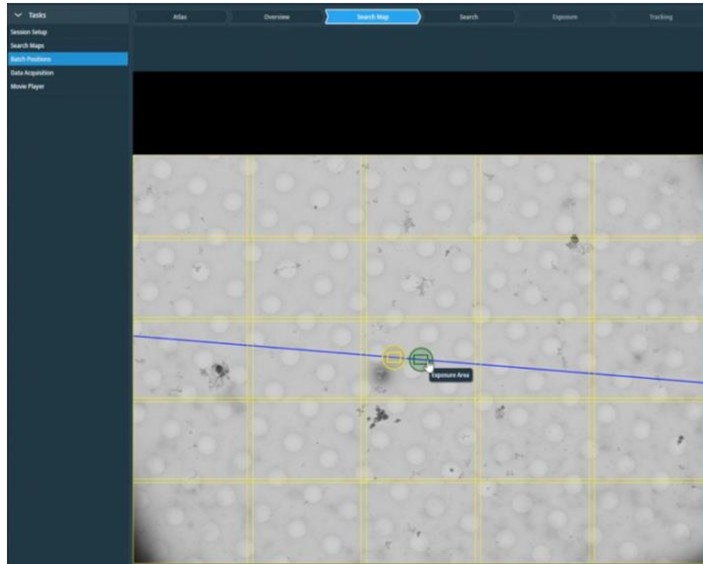
5.3.1 Define a new Batch position series

☐ Add an acquisition area to the Search map
(TOMO: Tomography > Tasks > Batch position > Search Map > Define position)

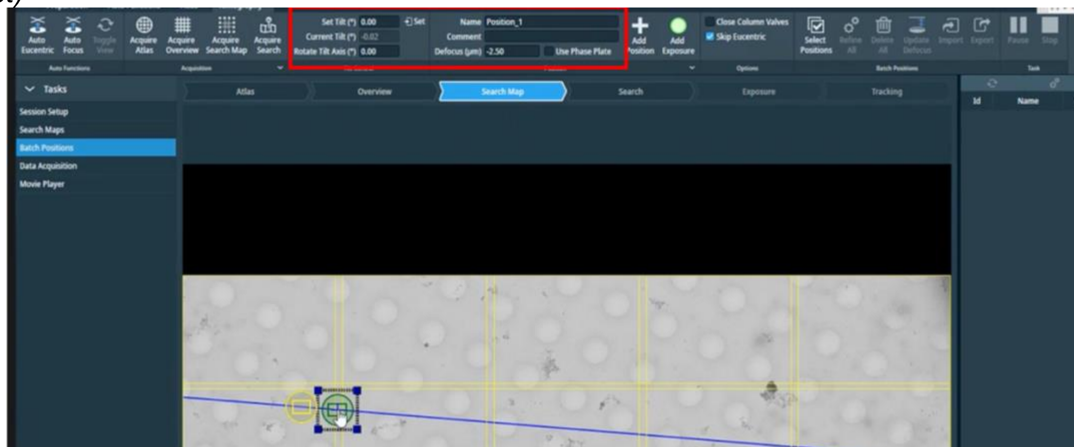


A blue line will appear along the tilt axis, this will be accompanied by a green circle (exposure area), a yellow circle (tracking area) and a blue circle (autofocus area).

Note: *the yellow and blue circles will likely be locked together and appear as one overlapping circle.*



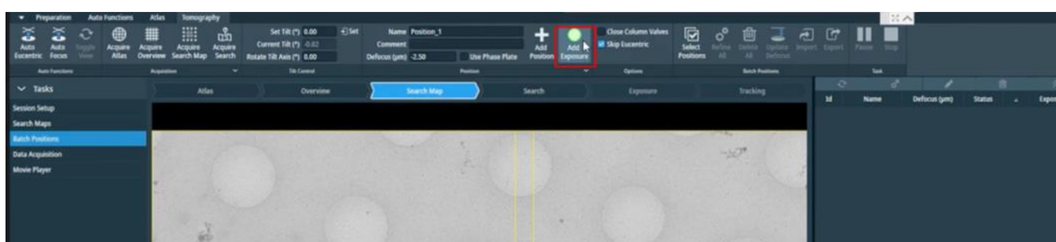
☐ Reposition the green ‘Exposure area’ to an area of interest
(TOMO: Tomography > Tasks > Batch position > Search Map > left-hand click on the green circle and drag to reposition)
Note: *when you reposition the green ‘Exposure area’ the blue tilt axis line and yellow/blue tracking/focus areas should automatically move with it*
☐ Adjust parameters of the ‘Exposure area’ to suit your experiment
(TOMO: Tomography > Tasks > Batch position > Search Map > left-hand click on the green circle to select)

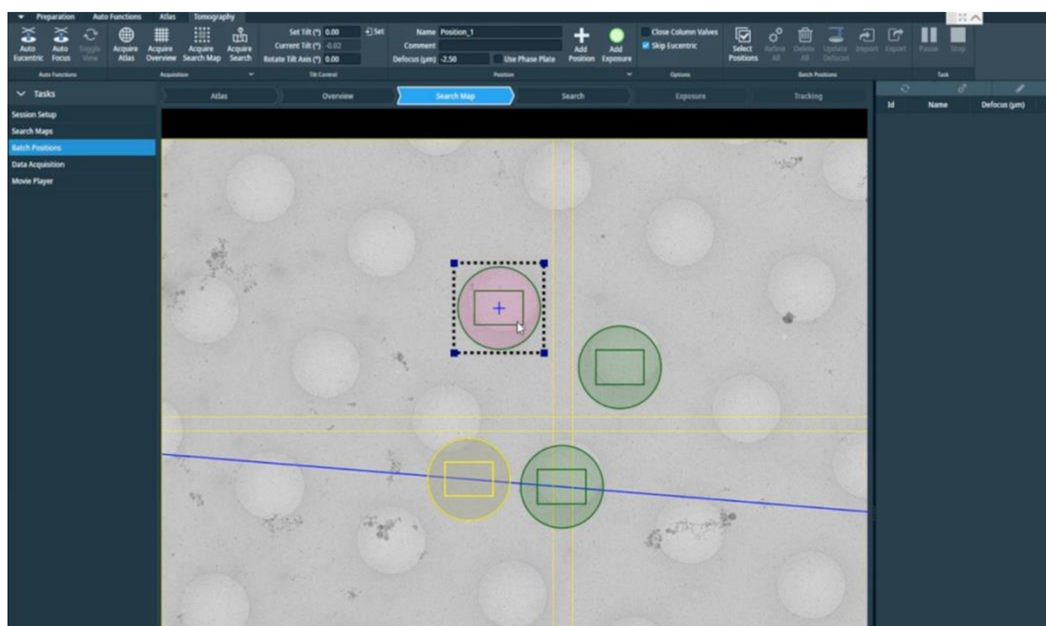


☐ Adjust the position of the ‘Focus’ and ‘Tracking’ areas so they are on the tilt axis positioned over carbon and there are features that are trackable. Avoid cracks, ice crystals, thick regions. Move both ‘Focus’ and ‘Tracking’ areas to the same position.
☐ Adjust parameters of the ‘Focus’ and ‘Tracking’ areas to suit your experiment.
(TOMO: Tomography > Tasks > Batch position > Search Map > left-hand click on the yellow or blue circle to select)

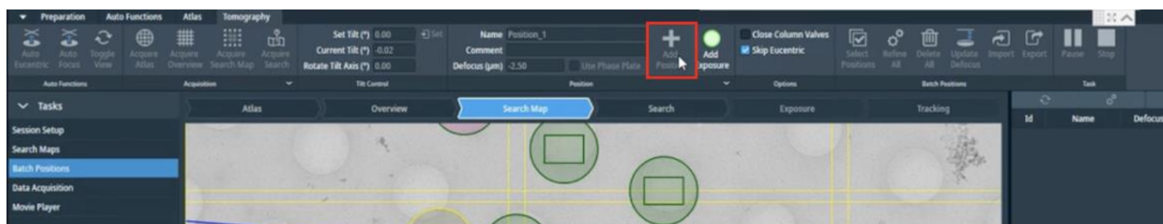
☐ Add additional exposure areas around the batch position area and reposition them as required
(TOMO: Tomography > Tasks > Batch position > Search Map > ‘Add Exposure’)

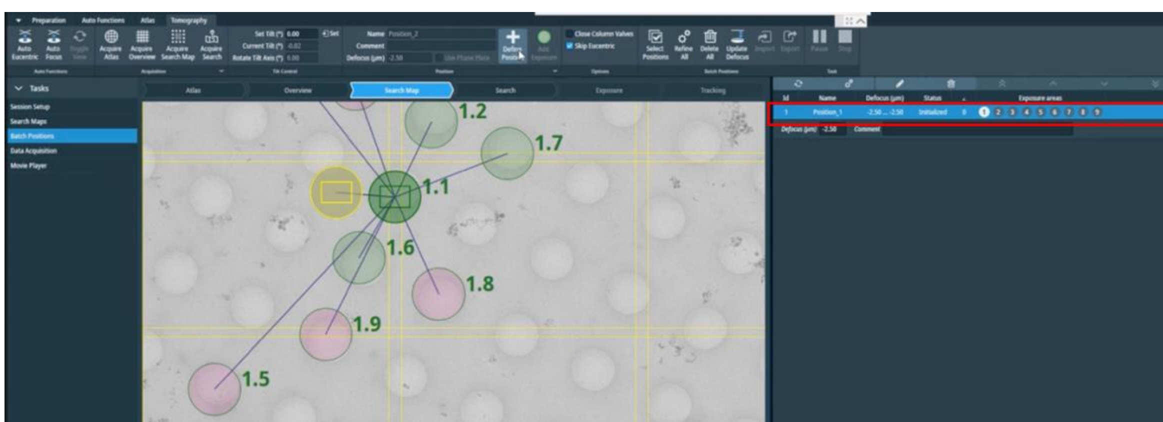
Note: *You should try to keep your exposure areas as close as possible to the primary exposure area and the tilt axis . If you go too far away the exposure area will turn from green to red.*
Note: *Remember that as the stage tilts during tilt-series acquisition, the circular electron beam will expose an oval area perpendicular to the tilt axis. Adjacent positions lined up perpendicularly to the tilt axis should have enough spacing between them to prevent overlap and overexposure (one exposure area diameter in between is a reasonable distance).*





☐ Press ‘Add Position’ to add the set of positions to the ‘Batch positions’ queue





IMPORTANT INFO ON BUG IN TOMO 12.14:

If you are adding many areas (e.g. > 15) and notice at some point that your tracking is no longer on the tilt axis, you should reset the template (Right click > 'Reset template')

5.3.2 Adding additional 'Batch Position' areas

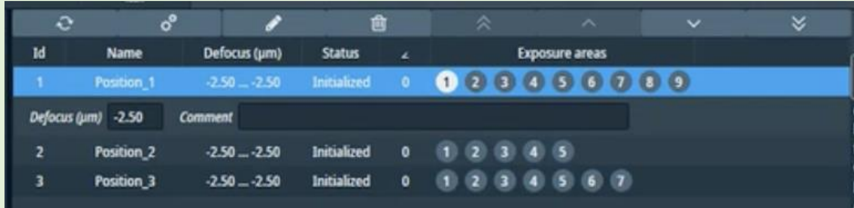
☐ Continue adding 'Batch Position' areas to the search map as described in **section 5.3.1**

Note: *when you press 'Define position' TOMO will copy and paste all of the positions from the last batch position set. You can either keep these and reposition as required or right click 'reset template areas'*

☐ When you have finished adding 'Batch Positions' to your selected Search map you can repeat steps in **section 5.3** to add 'Batch positions' to additional Search maps

Editing Individual Batch Positions

1) Select the 'Batch Position' set you want to edit from the queue list on the right-hand side of the screen
2) Expand the Batch position row to reveal the 'Defocus' and 'Comment' areas



3) Adjust as required and select Enter

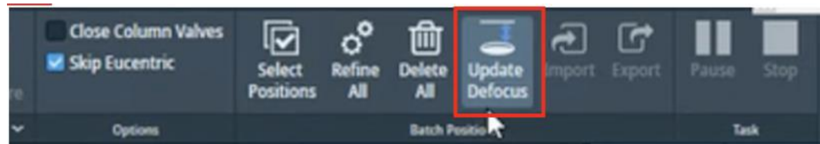
Note: *Remember the starting tilt angle for a Batch Position is only defined as the current stage angle when the Batch Position is added, thus this cannot be edited here.*

5.3.3 Editing the defocus values for 'Batch Position' areas

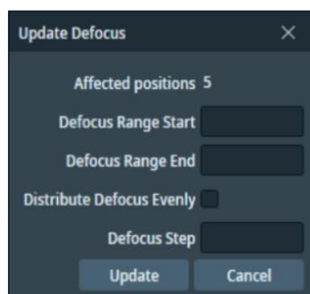
Using the 'update defocus' button allows you to assign a defocus value (or a range of defocus values) to all selected Batch Positions in the queue list.

☐ Select 'Select Positions' then tick all desired Batch positions in the queue

☐ Press 'Update focus'



☐ Adjust the defocus parameters to suit your experiment then press 'update':



5.3.4 (Optional) Eucentric height refinement strategy

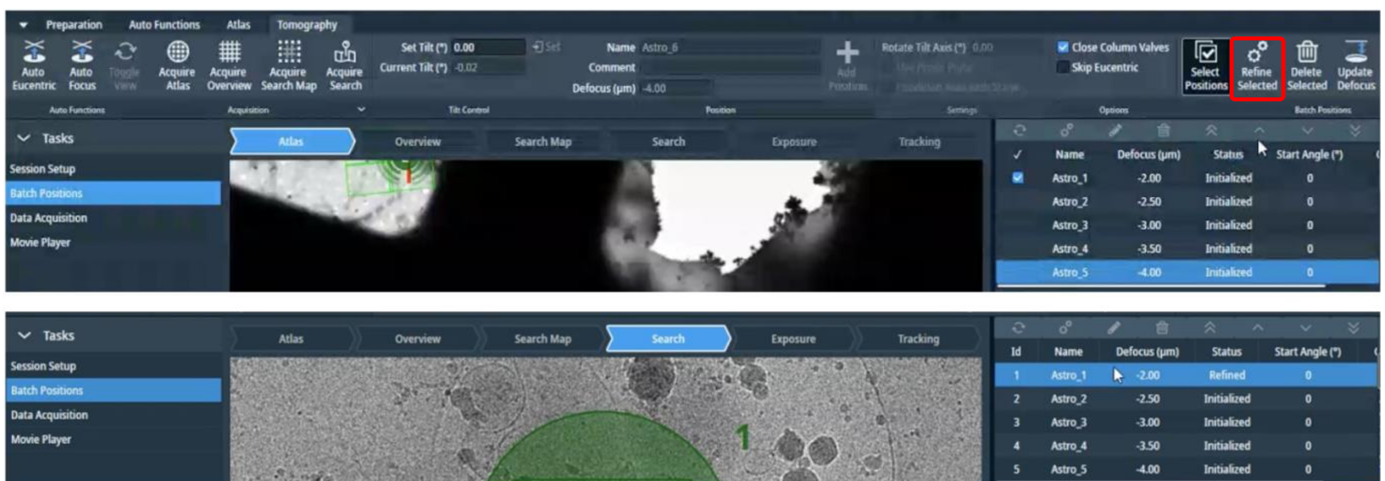
This is an optional action but some experiments may require it. To check the eucentric height is properly calibrated for your batch positions, there are a number of available strategies. You may wish to choose one based on the type of tomography session you are performing (single particle like, cellular, lamella). For single particle like specimens your grid height is most likely to be the same across the positions on a square, so finding eucentric height for each batch position is not likely required. For cellular or lamella samples, it is more likely each position will have a different z-height, in which case eucentric height should be refined for each batch position. Eucentric height can be refined during batch position setup or during data acquisition.

For single particle like tomography, your eucentric height will have been calibrated and stored when following the procedures in this workflow. Thus, proceed to Final checks and data collection in section 6.

For cellular or lamella samples, it is more likely that each batch position is at a different z-height and in this case you should proceed to 5.3.4.1.

5.3.4.1 *Refine target positions*

☐ Refine the selected square positions
- Tomography > Batch Positions > Refine all
- Tomography > Batch Positions > Select Positions > Refine selected



☐ Check and revisit squares that have failed refinement.
- If eucentric height fails, you could find eucentric by stage-tilt (as in section 3.3.2), acquire a new search image to update the z-height and add the position again. Delete the failed/initialized position.

☐ Tick the 'Skip Eucentric' option as eucentric height is already found with 'Refine all'
Note: *If you do not 'Refine all' then Skip Eucentric should not be checked which will instruct Tomo to run eucentric refinement before each tomogram is acquired during the data acquisition*

6 Final checks and data collection

6.1 Auto Functions and alignment

For lamella grids autofunctions and alignments will already have been completed by your Local Contact before you set up. You should not repeat these on lamella grids.

Proceed directly to **section 6.2.** *Do not pass Go. Do not collect £200…*

☐ For alignments, use your Atlas to navigate to an area of carbon

(TOMO: Tomography > Search Maps > Atlas or Overview > Right-click and 'Move Stage here')

6.1.1 Alignments via Autofunctions

☐ Perform Autofunctions in the following order:

Note: *Ensure that you select the corresponding Preset for each Auto Function alignment*

Step 1: Auto Eucentric

1) Select 'AutoEucentric by stage tilt' in the autofuctions tab

   (*TOMO: Autofunctions > AutoEucentric by stage tilt)*
2) Set to 'Eucentric Height' magnification preset **in autofunctions**

   (*TOMO: Autofunctions > Preset 'Eucentric Height')*
3) Press 'Start'

Step 2: AutoFocus

1) Select 'AutoFocus' in the autofuctions tab

   (*TOMO: Autofunctions > AutoFocus)*
2) Set to 'AutoFocus' magnification preset **in autofunctions**

   (*TOMO: Autofunctions > Preset 'AutoFocus)*
3) Press 'Start'

Step 3: Autostigmate

1) Select 'Autostigmate' in the autofuctions tab

   (*TOMO: Autofunctions > Autostigmate)*
2) Set to 'Thon Ring' magnification preset **in autofunctions**

   (*TOMO: Autofunctions > Preset 'Thon ring')*
3) Press 'Start'

Note: *The astigmatism should ideally be less than 5 nm*

Step 4: Autocoma

1) Select 'Autocoma' in the autofuctions tab

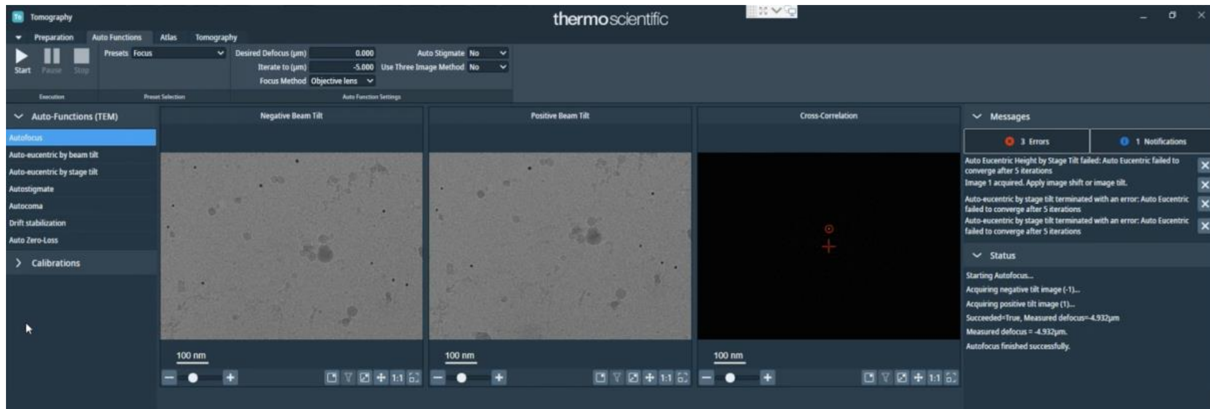
(*TOMO: Autofunctions > Autocoma)*

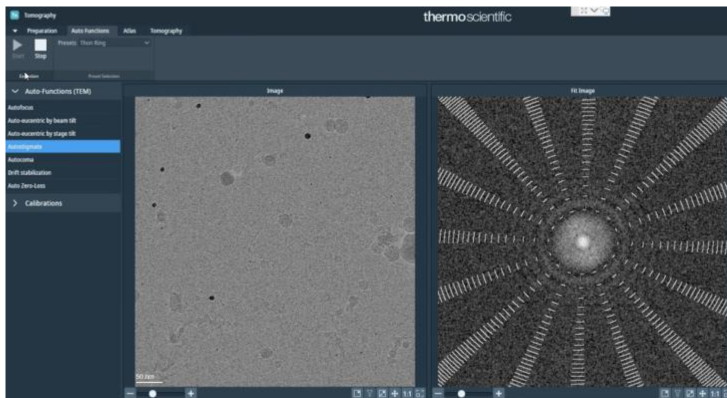
2) Set to 'Thon Ring' magnification preset **in autofunctions**

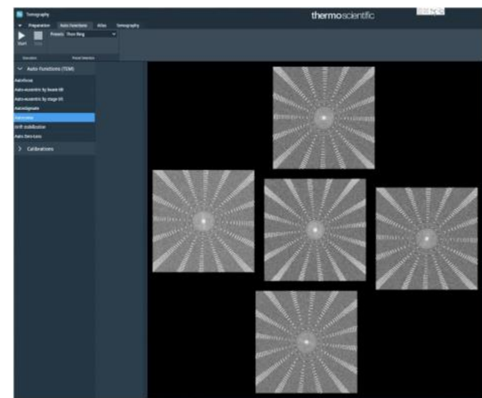
   (*TOMO: Autofunctions > Preset 'Thon ring')*

3) Press 'Start'

Note: *The coma would typically be be less than 200 nm on eBIC Krioses*







6.1.2 Beam centring

Your Local Contact will have aligned and centred your beam for you but you may wish to double check. You can recentre the beam using the 'TEM Control Pads Simulator' if required.

If you are doing this immediately after the Autofunctions in **section 6.1.1** then you should already be at eucentric height and focussed – if not perform autoeucentric and autofocus before proceeding to centre your beam.

☐ Switch to the TEM user interface (TUI)

☐ Insert the flu screen

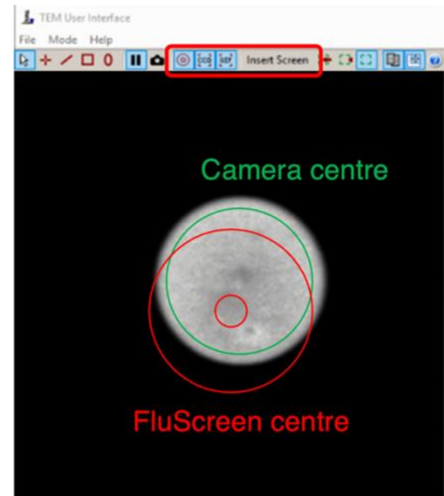
(TUI: Insert Screen)

☐ Load the TEM Control Pads Simulator

☐ Inspect if the beam is centred

☐ Recentre if necessary

1) Select **'Tomo Beam Shift'** from the Direct Alignments
   (TUI: Direct alignments (bottom right drop drown) > Tomo Beam Shift)
2) Use the **multifunction X/Y** on the simulator pads to centre the beam
3) Click 'Done' on the Direct Alignment panel



6.1.3 Objective aperture

Your Local Contact will have aligned and centred your objective aperture for you.

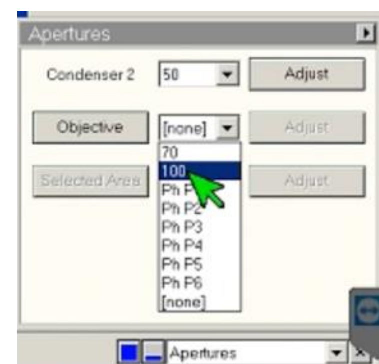
☐ Insert the 100 µm objective aperture

(TUI: drop down menu (bottom right) > Apertures > Objective – yellow when in, grey when out

☐ Inspect if the beam is centred

☐ Recentre if necessary

1) Select 'Tomo Beam Shift' from the Direct Alignments
   (TUI: Direct alignments (bottom right drop drown) > Tomo Beam Shift)
2) Use the **multifunction X/Y** on the simulator pads to centre the beam



Click 'Done' on the Direct Alignment panel

☐ Repeat the Autostigmate Auto Function on a carbon area

Autostigmate

1) Select 'Autostigmate' in the autofuctions tab

   (*TOMO: Autofunctions > Autostigmate)*
2) Set to 'Thon Ring' magnification preset **in autofunctions**

   (*TOMO: Autofunctions > Preset 'Thon ring')*
3) Press 'Start'

Note: *The astigmatism should ideally be less than 5 nm*

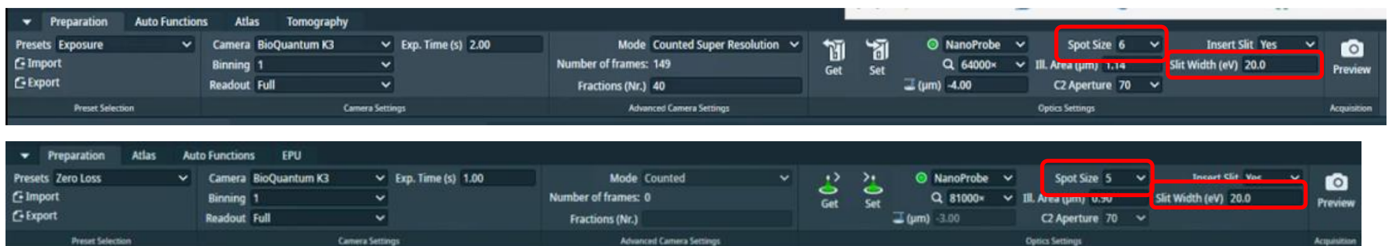
6.1.4 Zero-loss peak

The zero-loss peak centring routine can also be checked using the option in the Auto Functions tab. You may wish to run and check this routine before starting the data collection if you are using the energy selecting slit. This routine periodically runs during data collection using the Zero Loss preset and on the same area that you set up your autofocus area. Hence run the ZLP Auto Function on a carbon area representative of what would be encountered during data collection.

☐ Check your ZLP parameters

e.g., *Check the slit width is set to your desired width and that the width is the same for both the 'Zero Loss' preset and 'Exposure' preset)*

Note whether the ZLP preset uses a larger spot size (lower number) than Exposure preset or the ZLP may fail.



☐ Perform Auto Zero-Loss Auto Function

Auto Zero-Loss

1) Select 'Auto Zero-Loss' in the autofuctions tab

   (*TOMO: Autofunctions > Auto Zero-Loss)*

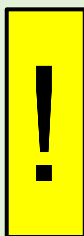
2) Set to 'Zero Loss' magnification preset **<u>in autofunctions</u>**

   (*EPU: Autofunctions > Preset 'Zero Loss')*

Press 'Start'

6.2 Start automated acquisition

6.2.1 Check your tomography data acquisition parameters



You should refer to your dose calibration in **section 3.2.2** to double check those collection parameters for your Exposure Preset are reasonable.

From your calculation, will each tilt receive sufficient dose for the imaging and tracking to work?

If you are unsure, you could move to an unused area and acquire test data acquisition images at zero and increasing tilts that reflect your collection.

☐ Go to Exposure preset and check your exposure time is consistent with your dose calculation (refer to **section 3.2.2**)

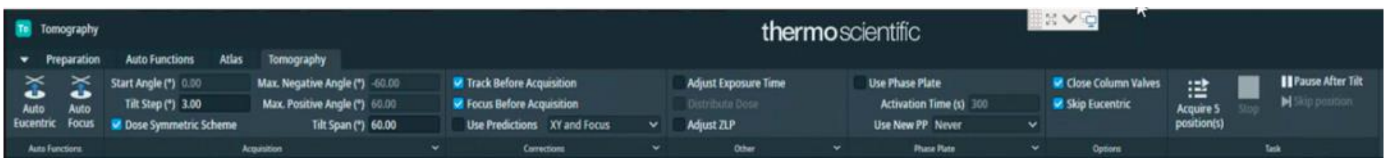
(TOMO: Preparation > Preset Selection > Presets > 'Exposure')

☐ Go to your Tomography Data Acquisition settings and double check your parameters

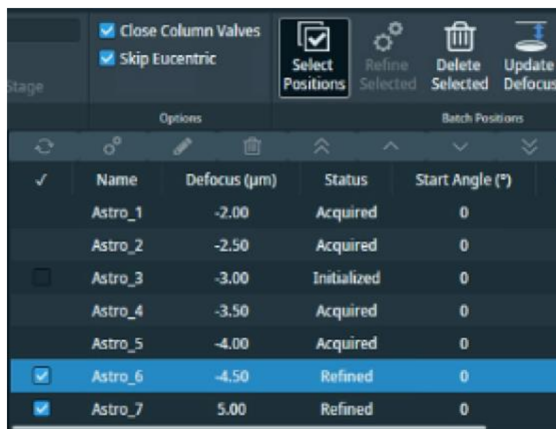
(TOMO: Tomography > Tasks > Data Acquisition)

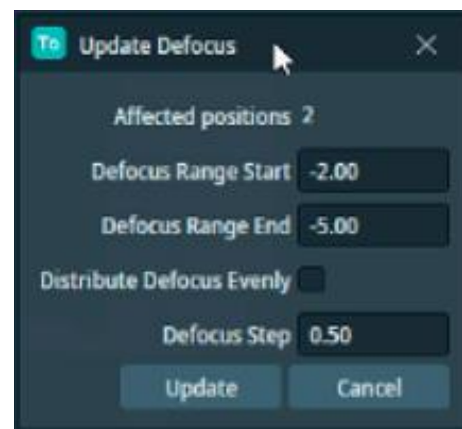
Check:

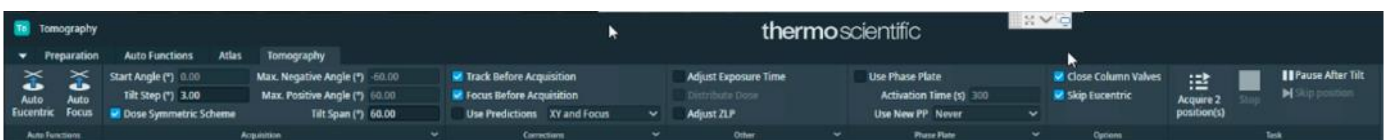
- **Tilt step, max positive and negative angle, tracking scheme (**Standard settings are shown in the image below)
- Unselect 'Adjust ZLP' (this might fail and has just been calibrated using the AutoFunctions)
- Select 'Close Column Valves'
- You may wish to select 'Skip Eucentric' if you have refined positions or your grid is flat
- You may wish to select 'Adjust Exposure Time' if you want to increase your dose at higher tilts



- You may also select specific positions for collection
- You may also 'Update Defocus' to batch modify the defocus series of a set of batch positions





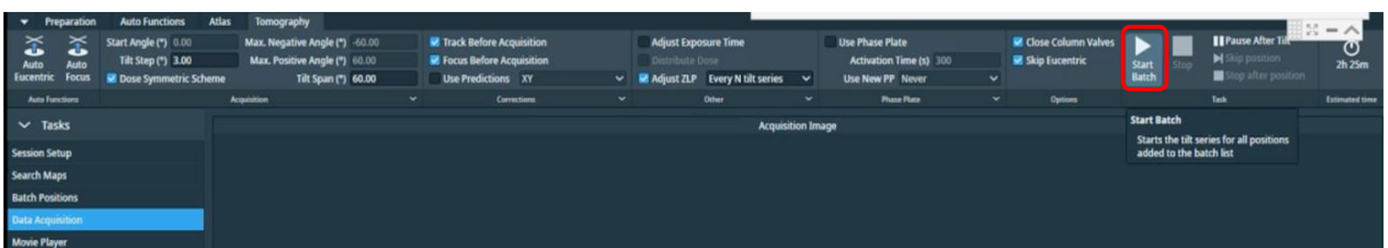


6.2.2 Start tomography acquisition

☐ Double check data collection parameters agree with dose calculation and desired tilt scheme

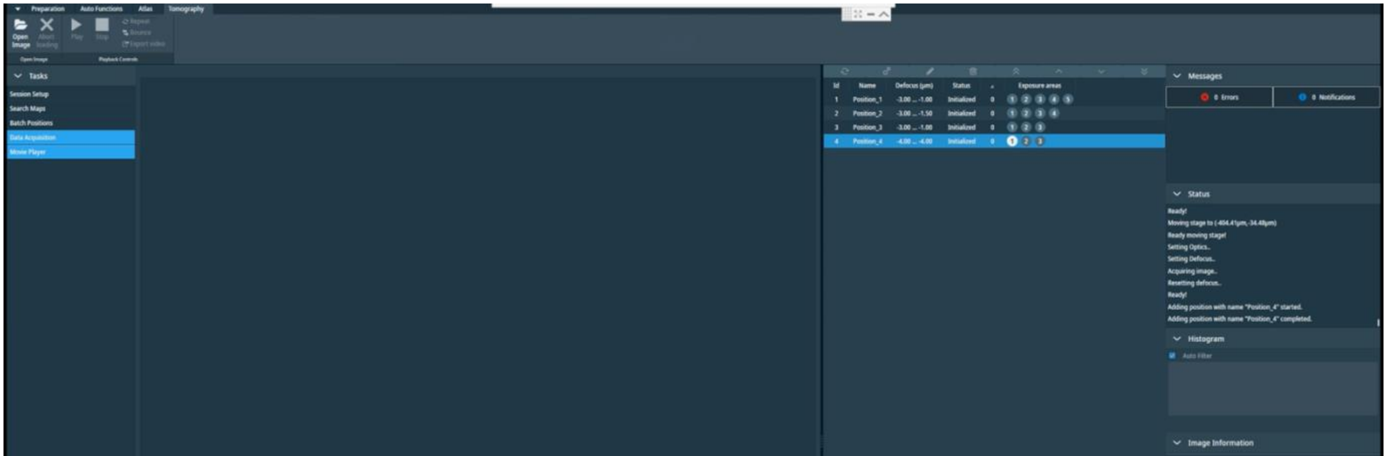
☐ Start the automated acquisition

(TOMO: Tomography > Data Acquisition > Task > press 'Start Batch')



☐ Monitor the beginning of data acquisition and frequently during your session

- [ ] **Inform your local contact that you have started your data acquisition**
- [ ] As batch positions have their tomograms collected, their status will update



6.2.3 Communication with your local contact during your session

Once collection is underway, you need to inform your Local Contact, who will commence the data transfer to your project directory.

If you wish to set up multiple data collections from different grids during your session, this is possible, provided that the same collection parameters are being used.

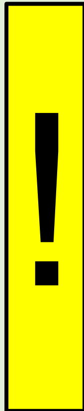
You will need to inform your local contact in advance if you are planning to set up multiple collections so they can pause the automatic data monitoring system. Please inform your local contact once you have set up a collection on a new grid, as they will have to run new data transfer scripts for each session that is run.

6.2.4 Plan to request your dewar return in iSPyB

You should inform you local contact of your plans regarding the return of your dewar.

*Please refer to the **'Shipping your dewar home'** section on our website for further instructions.*

6.2.5 On-the-fly processing and monitoring your data collection



It is the user's responsibility to monitor their data collection and ensure that the quality is as expected. Please refer to the ***'Remote access, analysis and data download'*** for how to access and monitor your data.

If at any point you leave the microscope unattended, please ensure you close the NoMachine connection in case your LC requires access (only one NoMachine connection is permitted at a time for security reasons).

For any issues:

- During working hours (Mon-Fri, 9am-5pm) contact your **Local Contact** directly
- Outside working hours contact the **Diamond Experimental Hall Coordinators**
  phone: 01235 77 8787
  email: experimentalhall@diamond.ac.uk
  Tell them who your Local Contact is and the m0X number of the microscope you are on:

Microscope	m0X number
Krios I	m02
Krios II	m03
Krios III	m06
Krios IV	m07

Anticipated pauses in data collection:
Please note that EPU will pause collecting images when LN_2 is being refilled, or when GIF ZLP is being adjusted (for K3). These brief pauses in data collection should not last longer that 15-20 minutes. Additional time might be required for EPU to move to a new target and acquire an image.

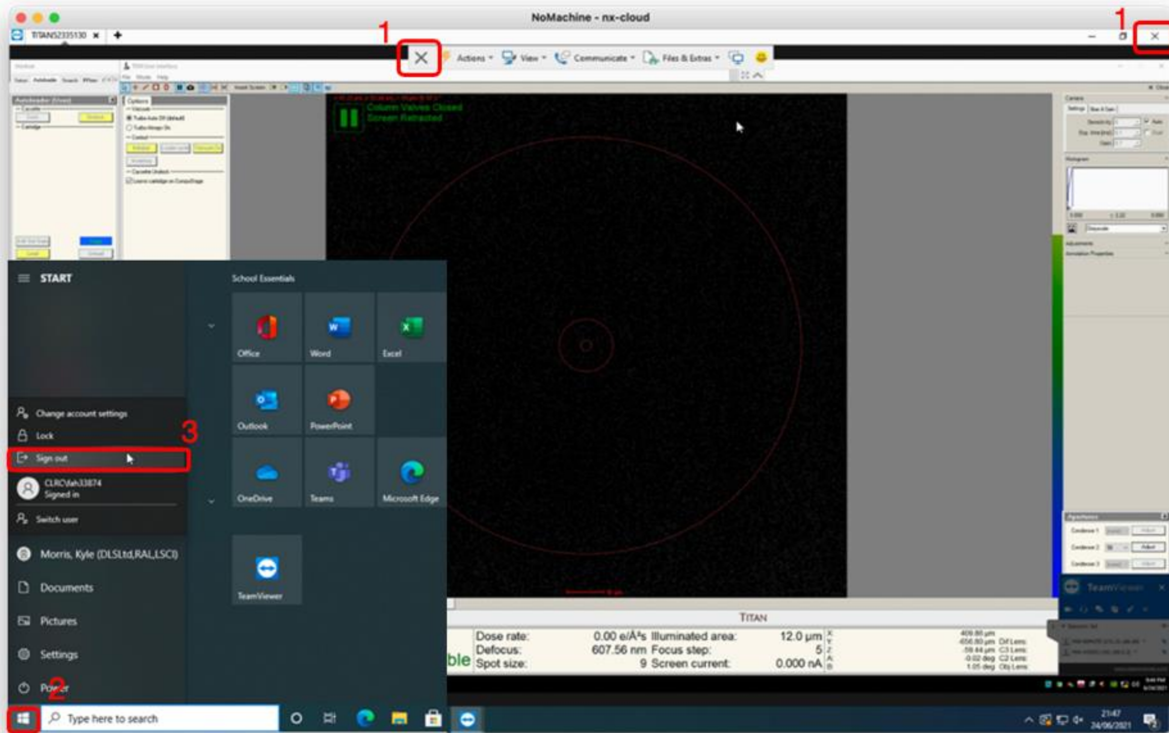
If, after 30 minutes, no new files appear as described in the ***'Remote access, analysis and data download'***, please contact your Local Contact (LC) directly (during working hours) or via the Diamond Experimental Hall Coordinators (outside normal working hours) as above.

6.2.6 Final steps

If at any point you leave the microscope unattended, please ensure you close the NoMachine connection in case your LC requires access (only one NoMachine connection is permitted at a time for security reasons).

☐ Click on (1) the highlighted cross to close TeamViewer
- **(Take care that you do not accidentally close the 'TEM User Interface')**

☐ Click on (2 and 3) to sign out of the remote support PC
- **(Take care that you do not accidentally log out the Microscope operating system)**



- ☐ Press Ctrl+Alt+0 (win) or Ctrl+Option+0 (apple) on your keyboard to access NX options
- ☐ Click on (4) 'Connection'
- ☐ Click on (5) 'Disconnect' to disconnect from the NoMachine nx-cloud session

7 Troubleshooting

7.1 Auto eucentric height failures

7.1.1 'Auto eucentric by stage tilt'

If the 'Auto eucentric by stage tilt' routine fails then try the following options:

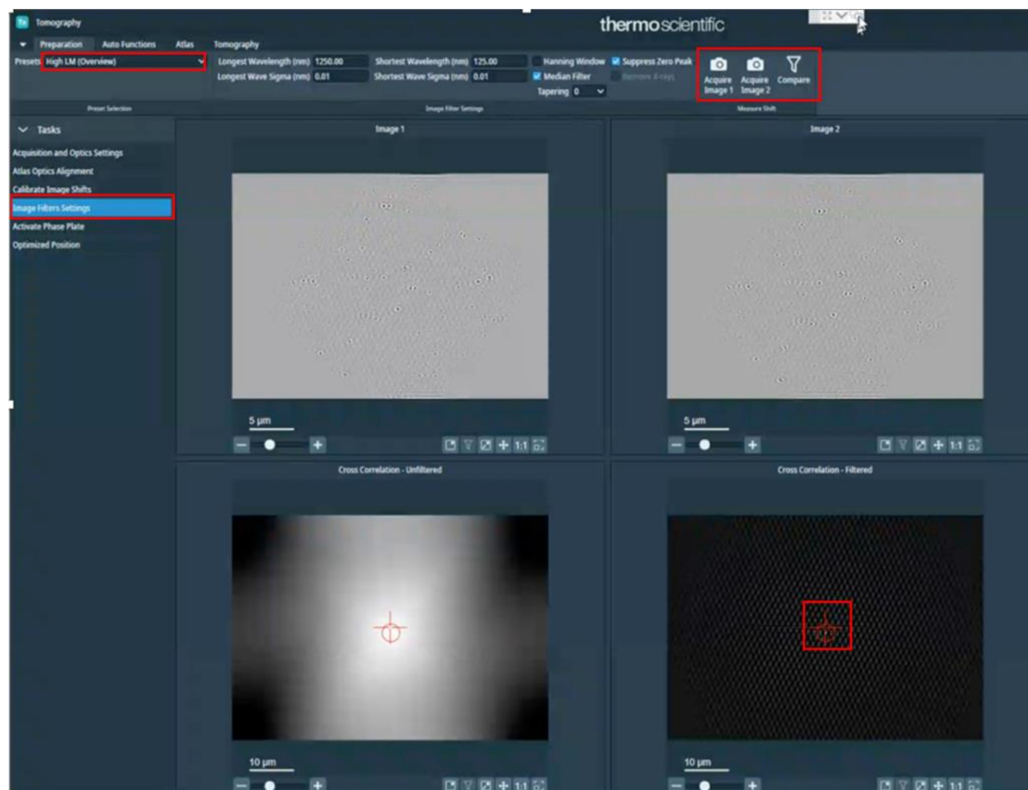
1) Re-run 'Auto-eucentric by stage tilt' with 'Presets: Overview' for courser eucentric optimisation. If this succeeds, subsequently re-run 'Auto-eucentric by stage tilt' with 'Presets: Eucentric Height' to increase precision.
2) Try again with a smaller stage tilt of 5°, then if this succeeds try again with 15°
3) Check to see that a strong cross-correlation peak is found. If not, then proceed to troubleshoot the Image Filter Settings (see next page).
4) **If using quantifoil grids:** the repeating pattern of holes may be preventing detection of a single cross-correlation peak. Try changing the 'Eucentric Height' Preset to a smaller defocus offset (- 25 to - 50 µm) and/or a shorter exposure time to decrease cross-correlation from hole patterns
5) **If using lacey grids or lamella:** there may not be enough signal for a strong cross-correlation peak. Try changing the Eucentric Height Preset to a larger defocus offset (- 50 to - 75 µm) and/or a longer exposure time to improve the cross-correlation peak.

If TOMO **still** fails to find eucentric height automatically, speak to your LC.

7.1.2 Image filter settings

Tomography grids vary more than SPA grids (regular hole pattern versus cells on a grid), so there is no general image filter that can be applied when calculating a cross-correlation. If there is not a good cross-correlation peak when finding eucentric height, then the Image Filter may need to be adjusted for your grid.

1) Open the 'Image Filter Settings'
   (TOMO: Preparation > Tasks > Image Filter Settings)
2) Select the Preset you want to optimise the Image Filter Settings for:
   (TOMO: Preparation > Tasks > Image Filter Settings > Preset Selection > select preset)
   i.e., Low SA (Eucentric Height / Overview)
   High SA (Focus, Tracking)
3) Set stage to 0° and Acquire Image 1
4) Set stage to 5° and Acquire Image 2
5) Click compare
6) Look for reliably found cross-correlation peak



7) In the case of not finding a cross-correlation peak, try:
   - Longest wavelength (nm) – ¼ of the scale bar
   - Shortest wavelength (nm) – 1/40 of the scale bar
   - Systematically try using different filters
   - Try adjusting the Tapering value

7.2 Auto coma failures

At lower magnifications autocoma may fail. In these cases you should try unbinning the thon ring preset. If this does not work speak to your Local Contact.