

## Remote TOMO5 operation guide for eBIC microscopes

Revision:	2.1.3
TOMO version:	5.1.8
Revision date:	05 September 2024
Revision summary:	<ol style="list-style-type: none"><li>1) General updates to images to cover lamella samples</li><li>2) Addition of a 'lamella' option during session set up</li><li>3) Change to 'Batch Position' setup – you can now add more 'Batch Positions' on existing 'Search maps' during data collection</li><li>4) Instructions added for Falcon IVi and Selectris</li></ol>
Comments to:	Lorna Malone
Acknowledgements:	We would like to acknowledge all eBIC staff, both past and present, for their invaluable efforts and contributions towards the writing of this documentation, in particular Dave Farmer, Davide Zabeo, Julika Radecke and Andrew Howe.

## 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 please reach out to the eBIC Local Contact assigned to your session for assistance. Finally, please report any errors in this guide to your Local Contact or [lorna.malone@diamond.ac.uk](mailto:lorna.malone@diamond.ac.uk).

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

## 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 **before 8.30am on the final day of your session** 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

**Note:** If you try to connect via NoMachine and appear to be stuck waiting with a message saying 'waiting for the desktop user to authorise your connection' then contact your LC. For security reasons only one connection can be made to nx-cloud at a time – if the previous user has not properly closed their nx cloud connection then your LC will have to manually close this connection before you can connect.

- ☐ Refer to the '[Remote access and analysis](#)' guide to familiarise yourself with our on-the-fly analysis pipeline

## Contents

<b>Contents .....</b>	<b>3</b>
<b>Quick start guide .....</b>	<b>5</b>
<b>1 Preparation.....</b>	<b>6</b>
1.1 Communication.....	6
1.2 Remote connection .....	6
1.3 Reasonable session timings .....	6
<b>2 User interface summaries.....</b>	<b>7</b>
<b>3 Microscope setup .....</b>	<b>10</b>
3.1 Initial checks and start up .....	10
3.2 Check and adjust your Low Magnification imaging condition presets .....	12
3.2.1 Check and adjust Low magnification Presets (Atlas, Overview/Positioning, Search/Template, Eucentric Height) 12	
3.3 Check and adjust your Exposure Preset .....	14
3.3.1 (Gatan K3 instructions) Checking and adjusting your Exposure preset .....	15
3.3.2 (Falcon IVi instructions) Checking and adjusting your Exposure preset .....	17
3.3.2.1 Checking your Dose Fraction Output on a Falcon IVi .....	17
3.3.2.2 Checking your Dose Rate over sample on a Falcon IVi .....	17
3.4 Other High Magnification Presets.....	19
3.5 Image Shift Calibrations .....	20
3.5.1 Prepare for image shift calibrations .....	20
3.5.2 Find eucentric height .....	20
3.5.3 Autofocus .....	21
3.5.4 Target a feature for Image Shift calibrations.....	21
3.5.5 Calibrate the Image Shifts.....	22
<b>4 Screening.....</b>	<b>25</b>
4.1 Atlas session setup and collection .....	25
4.1.1 Inspect your Atlas.....	26
<b>5 Tomography session setup.....</b>	<b>28</b>
5.1 Session Setup .....	28
5.1.1 Session setup.....	28
5.1.2 Lamella tilt correction (if applicable) .....	29
5.2 Identifying areas of interest using 'Search maps' .....	31
5.2.1 Setting up a 'Search Map' .....	31
5.2.2 Queueing a series of 'Search Map' areas.....	34
5.2.3 Acquiring queued 'Search Maps' .....	34
5.3 Targeting areas for 'Batch Positions' .....	36
5.3.1 (Lamella only) Define Orientation of your Search Maps .....	36
5.3.2 Set up Batch positions .....	37
5.3.3 Define a new Batch position series.....	38
5.3.4 Defining additional 'Batch Position' areas.....	41
5.3.5 Editing the defocus values for 'Batch Position' areas .....	41
5.3.6 (Optional) Eucentric height refinement strategy .....	42
<b>6 Final checks and data collection .....</b>	<b>44</b>
6.1 Auto Functions and alignment.....	44
6.1.1 Alignments via Autofunctions .....	44
6.1.2 Beam centring .....	47
6.1.3 Objective aperture .....	47

6.1.4	Zero-loss peak .....	48
<b>6.2</b>	<b>Start automated acquisition .....</b>	<b>48</b>
6.2.1	Check your tomography data acquisition parameters .....	48
6.2.2	Start tomography acquisition .....	49
6.2.3	Communication with your local contact during your session .....	50
6.2.4	Set up on-the-fly processing .....	50
6.2.5	Plan to request your dewar return in iSPyB .....	50
6.2.6	Monitor your data collection .....	51
6.2.7	Closing Teamviewer and disconnecting NoMachine .....	51
<b>7</b>	<b>Troubleshooting .....</b>	<b>53</b>
<b>7.1</b>	<b>Auto eucentric height failures.....</b>	<b>53</b>
7.1.1	'Auto eucentric by stage tilt' .....	53
7.1.2	Image filter settings .....	53
<b>7.2</b>	<b>Auto coma failures .....</b>	<b>54</b>

## Quick start guide

- 1) Receive handover email from your local contact detailing microscope parameters
- 2) Check your magnification presets and adjust as required (see section 3.2, 3.3 and 3.4)
- 3) Check image shift calibrations (see section 3.5)
- 4) Atlas your grids and screen as required (see section 4)

<b>Naming:</b>	Supervisor_YYYYMMDD_XXXXXX_visit-ID_ATLAS
<b>Image format:</b>	MRC
<b>Output folder:</b>	Z:\[your session ID, i.e., biXXXXXX]/atlas

- 5) Create your TOMO session (see section 5.1)

<b>Naming:</b>	Supervisor_YYYYMMDD_XXXXXX_visit-ID_positionX_TOMO
<b>Sample type:</b>	Slab-like
<b>Options:</b>	Select 'Batch' and 'Low Dose'
<b>Dose Fraction output (K3 only):</b>	Tiff LZW non-gain normalised (Note: on a Falcon IVi the Dose fraction output is set in the 'Data Acquisition' Preset – refer to <b>section 3.3.2.1</b> for full details)
<b>Storage folder:</b>	Z:\[your session ID, i.e., biXXXXXX]

- 6) Targetting areas of interest using 'Search maps' (see section 5.2)
- 7) Setting up areas for data collection using 'Batch Positions' (see section 5.3)

*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.*

- 8) Perform final checks in TOMO (AutoFunctions and check alignments)  
(skip for lamella) (see section 6.1)
- 9) Start data collection (see section 6.2)
- 10) Inform your local contact you have started data collection
- 11) Monitor your session via PaTo (the on-the-fly analysis pipeline)

Visit <https://ebic-pato.diamond.ac.uk> to view your on-the-fly processing. Refer to the '[Remote access and analysis](#)' guide for full guidance

- 12) Return to add more positions as necessary

# 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 ( $\dots \text{e}^-/\text{\AA}^2$ ) ?
- 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

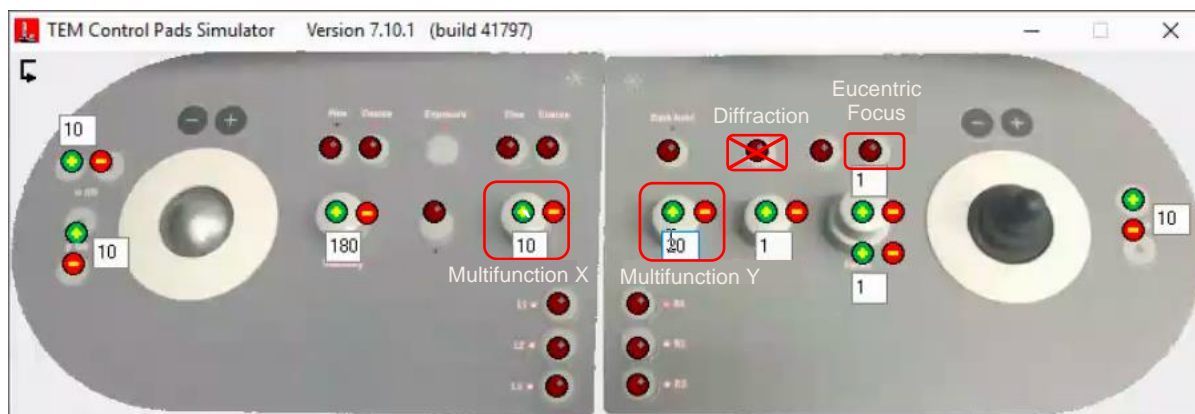
To switch between the virtual monitor hosting the TEM user interface and TOMO, use the Team Viewer Monitor button.



### TEM Control Pads Simulator

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.

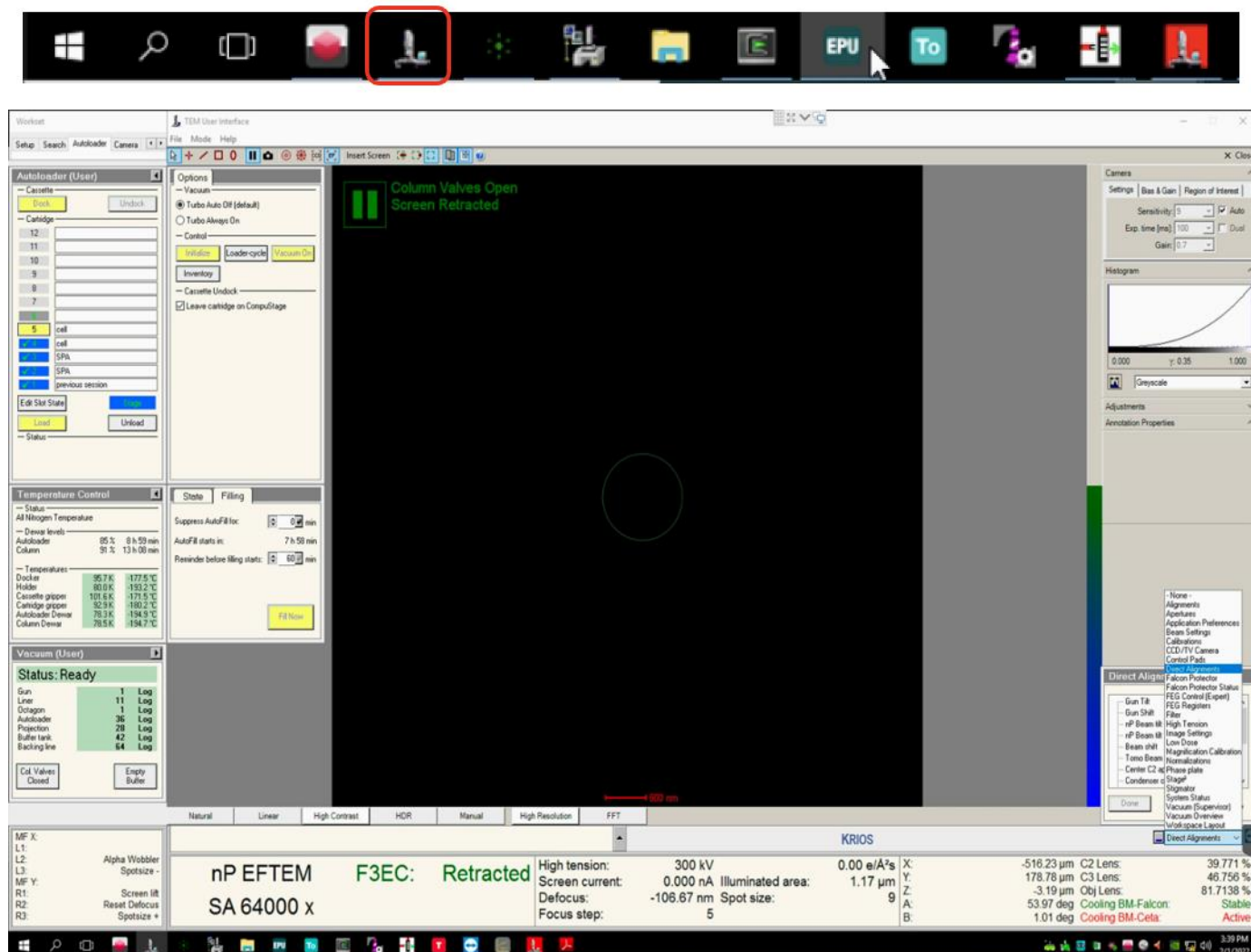
**! WARNING: Take care not to accidentally press the 'Diffraction' button !**





## 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

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.

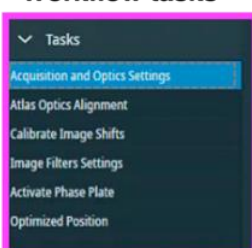
This guide presents a suggested sequence through the TOMO workflow.



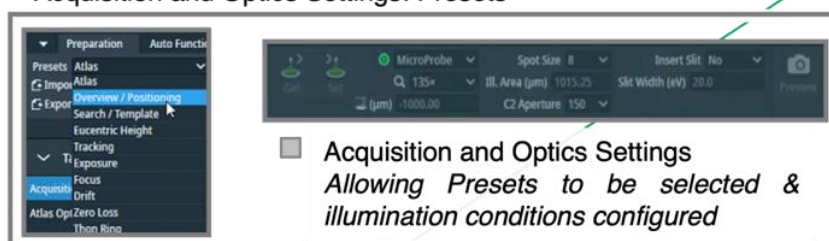
### Workflow tabs: Preparation, Auto Functions, Atlas, Tomography



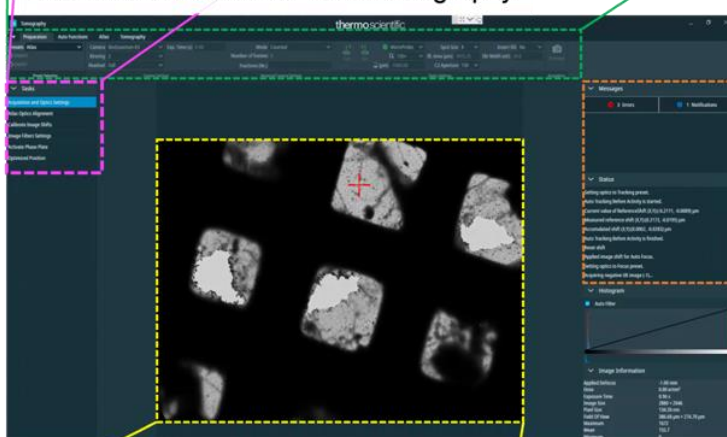
### Workflow tasks



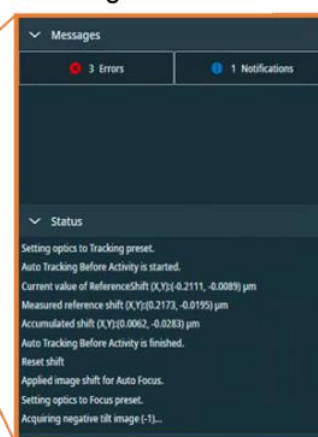
### Acquisition and Optics Settings: Presets



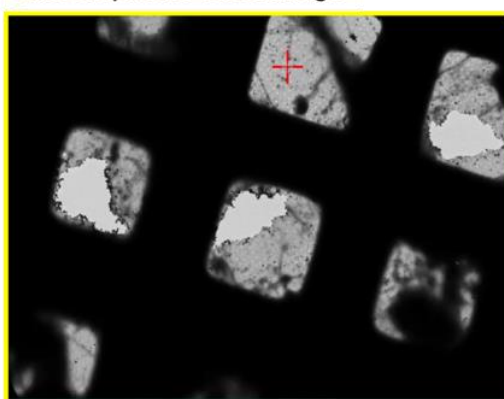
### Main user interface for TFS Tomography



### 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

### 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



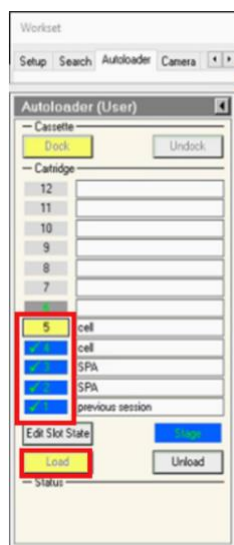
Session set-up can then be controlled by users directly through the Tomo interface. Changes to beam settings should all be controlled directly through the Tomo Preparation presets (TOMO: Preparation > Presets) and navigation around the grid can be done via right-click options over the images in Preparation,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

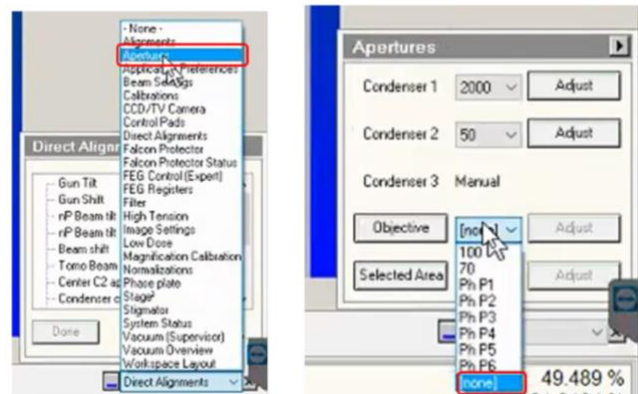
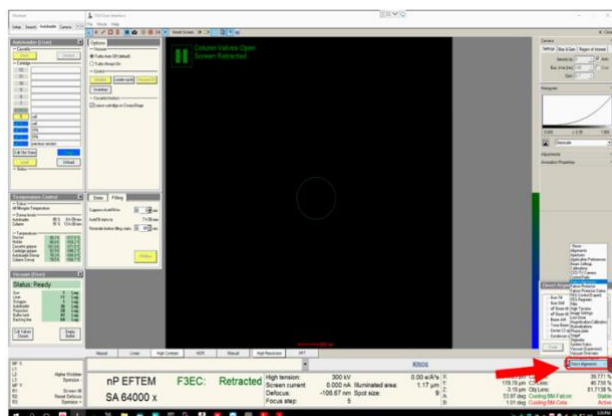
If no grid is loaded then select one of the slots and press 'Load'

**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 adjust your Low Magnification 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 your high magnification presets (Tracking, Exposure, Focus, Drift, Zero-Loss, Thon Ring).

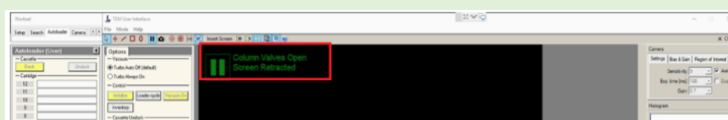
### 3.2.1 Check and adjust Low magnification Presets (Atlas, Overview/Positioning, Search/Template, Eucentric Height)

- ☐ Take an image using the 'Atlas' preset

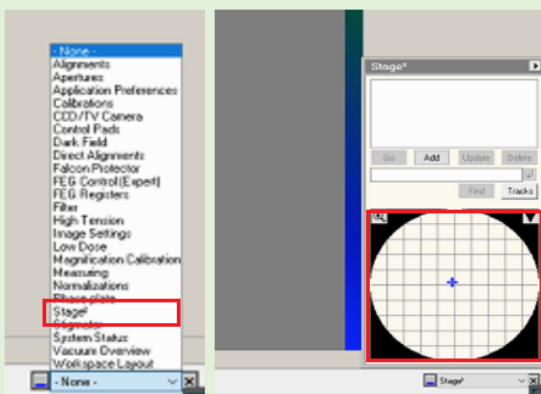
(TOMO: Preparation > Tasks > Acquisition and Optics Settings > Presets > select the 'Atlas' preset > press 'Preview')

#### 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 TOMO 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) Collect a preview image using that preset

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

- 3) Adjust magnification in TOMO 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'*

### 3.3 Check and adjust your Exposure Preset

The **magnification** for the data acquisition preset will have been set by your Local Contact based on communication before your session. Calibrations made by your Local Contact will be dependent on this magnification, **do not** adjust the 'Data Acquisition' **magnification** without discussing with your Local Contact. You should double check that the **exposure time** and other parameters are appropriate for your experiment. The parameters we use here are slightly different depending on your microscope but also on what detector you are using.

For microscopes equipped with a Gatan K3, please refer to **section 3.3.1**

For microscopes equipped with a Falcon IVi, please refer to **section 3.3.2**

Some key differences between a Falcon IVi and a K3:

#### Detector Characteristics

- **Pixel Size and Field of View**  
The Falcon IVi has a 4K x 4K (square) detector, while the K3 has a 6K x 4K (rectangular) detector. This gives the K3 a larger field of view per image, the pixel sizes and magnifications are also different.
- **Frame Rate**  
The Falcon IVi has a higher frame rate than the K3, allowing for more movies/hour to be collected.

#### Data Collection Settings

- **Dose Calculations**  
K3: Manual calculations required for exposure time based on dose rate, pixel size, and total dose  
Falcon IVi: Features a built-in dose calculator
- **Dose Fractionation Output**  
K3: Set in the EPU session setup  
Falcon IVi: Set within the Data Acquisition preset
- **Output File Formats**  
K3: TIFF (recommended) and MRC  
Falcon IVi: MRC, TIFF, and EER (electron event representation) formats. EER or TIFF are recommended.
- **Imaging Modes**  
K3: 'Super-resolution counted mode' with 2x binning in non-CDS mode is recommended  
Falcon IVi: Automatically selects a mode equivalent to 'super-resolution counted mode' with 2x binning in CDS mode. EER mode provides more flexibility with super-resolution.
- **Detective Quantum Efficiency (DQE)**  
Both detectors offer improved DQE compared to previous generations, with specific performance characteristics varying based on operating condition



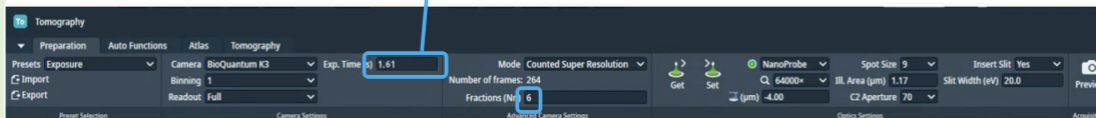
### 3.3.1 (Gatan K3 instructions) Checking and adjusting your Exposure preset

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

There is a '[Dose rate calculation](#)' on our website if required.

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 ( $\text{e}^-/\text{\AA}^2$ ). You may find these [online dose calculators](#) helpful.

Example exposure time calculation with parallel illumination <b>targeting optimal dose per tilt</b>	Example exposure time calculation with parallel illumination <b>targeting total dose</b>
<b>Physical pixel dose rate:</b> 5.281 $\text{e}^-/\text{px}/\text{sec}$ <b>Pixel size (magnified):</b> 1.63 $\text{\AA}/\text{px}$ <b>Pixel area (magnified):</b> 2.66 $\text{\AA}^2/\text{px}$ $\frac{\text{e}^-/\text{px}/\text{sec}}{\text{\AA}^2/\text{px}} = \text{e}^-/\text{\AA}^2/\text{sec}$ <b>Dose per tilt (target):</b> 3.2 $\text{e}^-/\text{\AA}^2$ <b>Dose rate:</b> 1.988 $\text{e}^-/\text{\AA}^2/\text{sec}$ $\frac{\text{e}^-/\text{\AA}^2}{\text{e}^-/\text{\AA}^2/\text{sec}} = \text{sec}$ <b>Exposure time per tilted image (sec): 1.61</b>	<b>Physical pixel dose rate:</b> 5.281 $\text{e}^-/\text{px}/\text{sec}$ <b>Pixel size (magnified):</b> 1.63 $\text{\AA}/\text{px}$ <b>Pixel area (magnified):</b> 2.66 $\text{\AA}^2/\text{px}$ $\frac{\text{e}^-/\text{px}/\text{sec}}{\text{\AA}^2/\text{px}} = \text{e}^-/\text{\AA}^2/\text{sec}$ <b>Dose rate:</b> 1.988 $\text{e}^-/\text{\AA}^2/\text{sec}$ <b>Total dose (target):</b> 131 $\text{e}^-/\text{\AA}^2$ <b>Tilt range (°):</b> -60 to +60 <b>Tilt step (°):</b> 3 <b>Tilt image number:</b> 41 <b>Dose per tilt</b> = 131 / 41 = 3.2 $\text{e}^-/\text{\AA}^2$ $\frac{\text{e}^-/\text{\AA}^2}{\text{e}^-/\text{\AA}^2/\text{sec}} = \text{sec}$ <b>Exposure time per tilted image (sec): 1.61</b>



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

- The dose per tilted image is 3.20  $\text{e}^-/\text{\AA}^2$  (3.0 - 3.5 is reasonable for sub-tomogram averaging)
- 'Fractions (Nr.)' is set to 6 for a tilt movie with ~ 0.5  $\text{e}^-/\text{\AA}^2$  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 (we recommend using 'counted super resolution mode' with '2' times binning on the K3)



(TOMO: Preparation > Advanced Camera Settings > Mode > select 'mode')

(TOMO: Preparation > Camera Settings > Binning > select value)

**'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

### 3.3.2 (Falcon IVi instructions) Checking and adjusting your Exposure preset

On a microscope equipped with a **Thermo Fisher Falcon IVi** both the dose fraction output format and dose are found in the Data Acquisition Preset.

#### 3.3.2.1 Checking your Dose Fraction Output on a Falcon IVi

- ☐ Check that the correct dose fraction output value is selected (we recommend using 'manual' on the Falcon IVi unless you want to use EER mode)

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > Exposure Settings > Fractions > select 'Manual' (or 'EER' if you want to use EER mode))

'**Fractions**' controls whether or not to save Dose Fraction images. We recommend using the 'Manual' setting (unless you're using EER format), this will enable you to input a specified number of fractions in the 'Fractions (Nr.)' tab. To use **EER** format, select 'EER'.

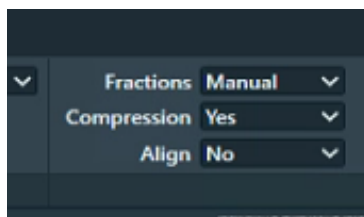
'**EER**': Electron Registration Mode - If you select '**EER**', your dose fraction output will be in EER format. In EER mode, the camera records the coordinates for the individual electrons that hit the camera. This may be useful for some experiments where you want to have fine control over how your processing is dose weighted.

- ☐ Check that the correct compression value is selected (we recommend using compression on the Falcon IVi unless you're using EER mode)

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > Exposure Settings > Compression > select 'Yes')

'**Compression**' controls whether your images are compressed to LZW TIFF format

- If you select just **manual** without compression your dose fraction output will be in **MRC** format (not recommended)
- If you select **manual** with compression your dose fraction output will be in compressed LZW TIFF format (recommended)



#### 3.3.2.2 Checking your Dose Rate over sample on a Falcon IVi

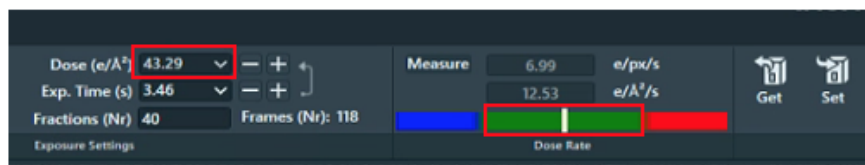
Your Local Contact will have set your Dose parameters for you over vacuum before handing over but you should double check that these values work for your sample.

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.

- ☐ Check that your 'Total Dose' is around your desired total dose and that the 'Dose rate' is within the green range of the 'Dose rate' bar (this is what your local contact has set over vacuum)

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > Exposure Settings > check the value in 'Dose (e/A<sup>2</sup>)')

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > Dose Rate > check that measured dose rate is within the green region of the bar)



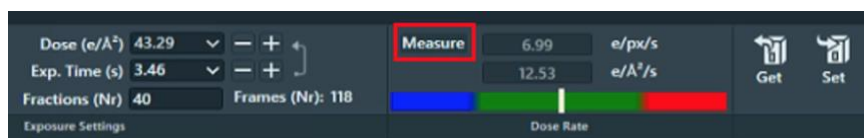
- ☐ Navigate to an area over your sample
- ☐ Ensure that the arrow between 'Dose (e/Å²)' and 'Exp. Time' is pointing **away from** 'Exp. Time', **towards** 'Dose (e/Å²)' (if not, press the arrow to reverse the direction – this ensures that the Exp. Time your local contact has set over vacuum does not change)

The Dose and Exp. Time parameters are interconnected through the measured Dose Rate. Adjusting the Exp. Time automatically calculates the corresponding Total Dose based on the measured Dose Rate, and vice versa depending on which value is selected as the 'leading parameter'. The leading parameter is selected using the arrow on the right of the values, the arrow points away from the leading parameter and towards the parameter to be calculated.



- ☐ Measure the transmitted Dose Rate over your sample

(TOMO: Preparation > Preset Selection > Presets > 'Exposure' > Dose Rate > press 'Measure')



- ☐ Check that the 'Dose (e/Å²)' value is around the same value as you want your total dose, that your exposure time is reasonable and that the 'Dose rate' value displayed is still within the green range of the 'Dose rate' bar
- ☐ Check all other parameters are correct (e.g., defocus, slit width, slit inserted etc.)

### 3.4 Other High Magnification Presets

The other high magnification presets (**tracking, focus, zero loss** and **drift**) should be 'linked' to your **exposure** magnification settings but you should double check the rest of the settings for these presets are appropriate for your experiment.

- ☐ 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 on the K3 to help with increasing signal to noise in this Preset

## 3.5 Image Shift Calibrations

### 3.5.1 Prepare for image shift calibrations

- ☐ Take an image of your inserted grid at 'Atlas' magnification
- ☐ Move to the centre of an intact (not ripped or torn) grid square with identifiable features (e.g., ice crystals)  
(TOMO: right click on image and 'Move stage here')

### 3.5.2 Find eucentric height

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

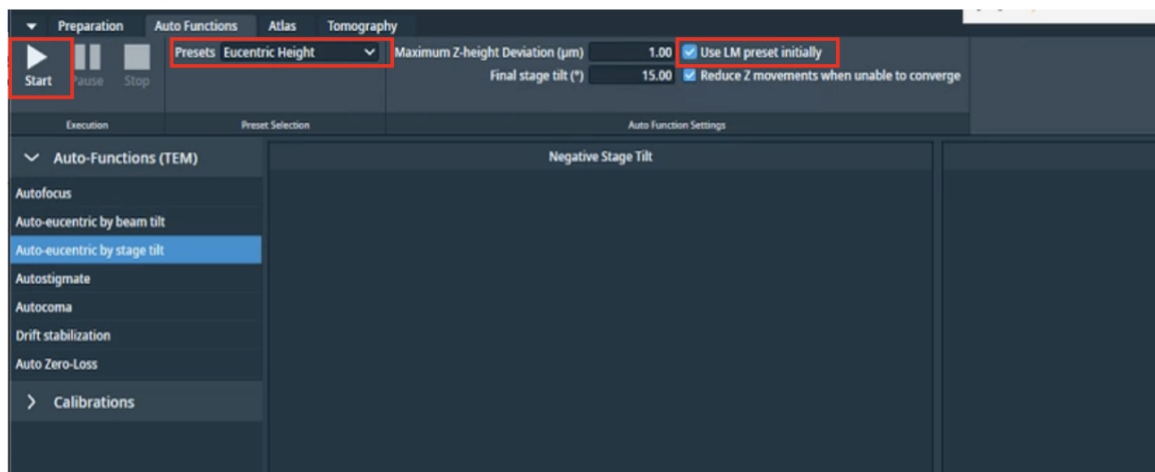
- ☐ Navigate to an area over carbon in the centre of your selected grid square
- ☐ Take an image of the area using the 'Overview/Positioning' magnification preset  
(TOMO: Preparation > Acquisition and Optics Settings > select the 'Overview/Positioning' preset > press 'Preview')
- ☐ Recentre the square over carbon 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 autofunctions 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**

### 3.5.3 Autofocus

Run the autofocus routine on carbon

- 1) Select AutoFocus in the autofunctions 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 TOMO (right hand side) and look for a sharp 'peak' on the right



### 3.5.4 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*)

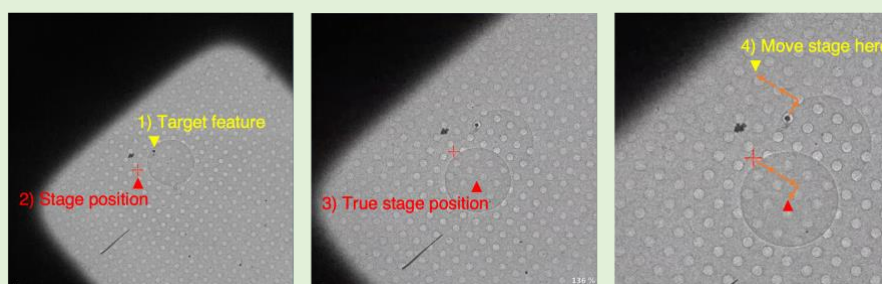
☐ Check the feature remains aligned at higher magnification Presets

- 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.

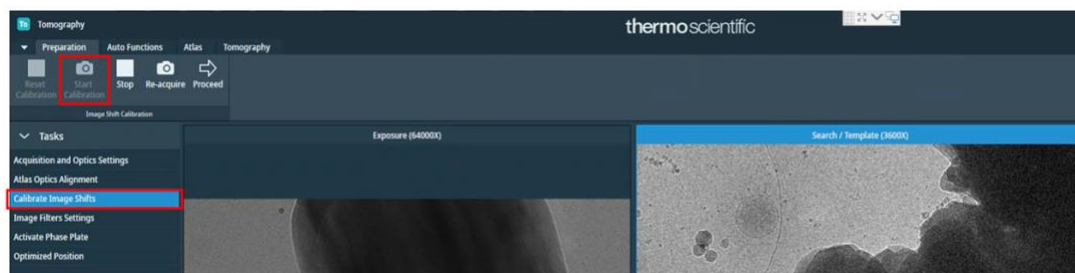


If you found the feature remained centred during targeting, then you may choose to skip image shift calibrations. If you needed to recentre the feature during targeting, you should calibrate the Image Shifts. With the feature now centred at Exposure Preset, proceed to 3.5.5

### 3.5.5 Calibrate the Image Shifts

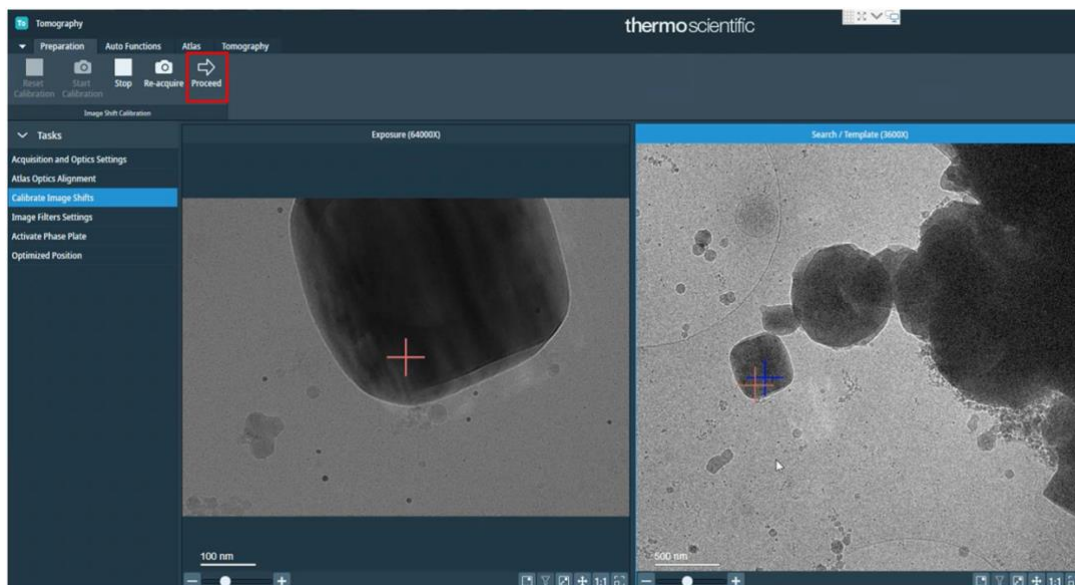
- ☐ Start image shift calibration

(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





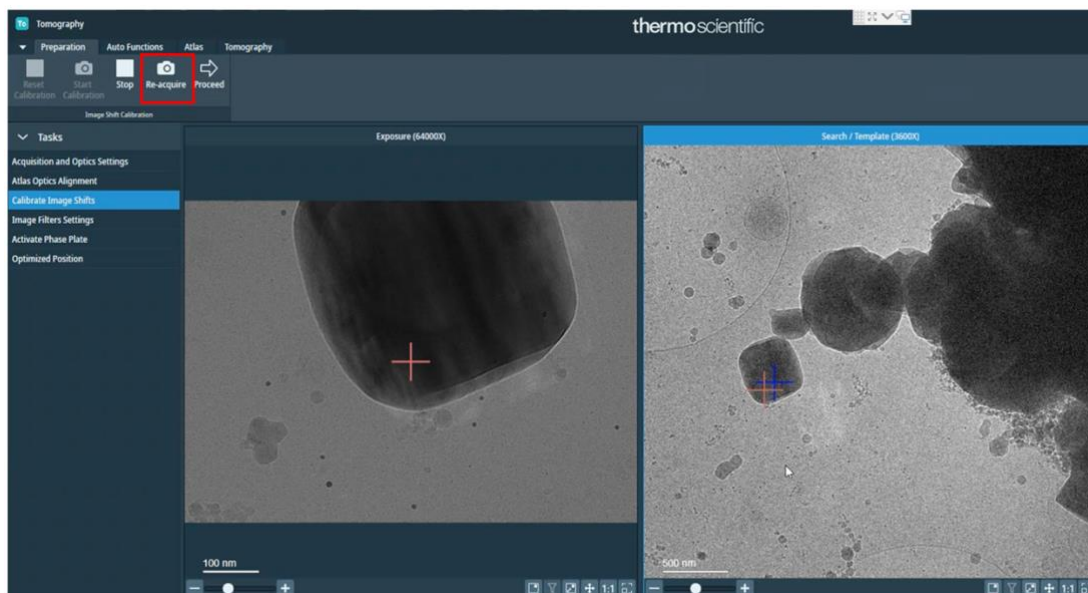
☐ For each magnification in turn:

- 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 180° rotation between the 'Overview/Positioning' and 'Eucentric Height'/'Search/Template' magnification presets on some microscopes with a K3. You may find it helpful to take a photo on your phone and manually rotate this to orientate yourself

## 4 Screening

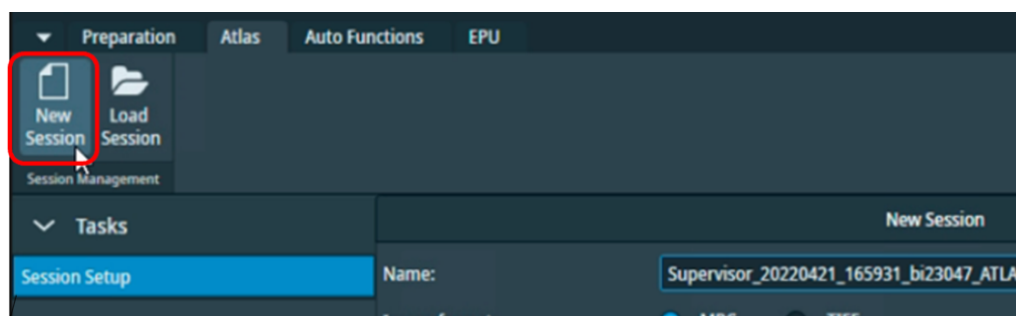
We will not go into the details of thoroughly screening grids here since it is expected that eBIC users will be working on pre-screened grids during their sessions. However, if you need to inspect and document areas to confirm ice characteristics etc., now is an appropriate time to perform some quick screening.

### 4.1 Atlas session setup and collection

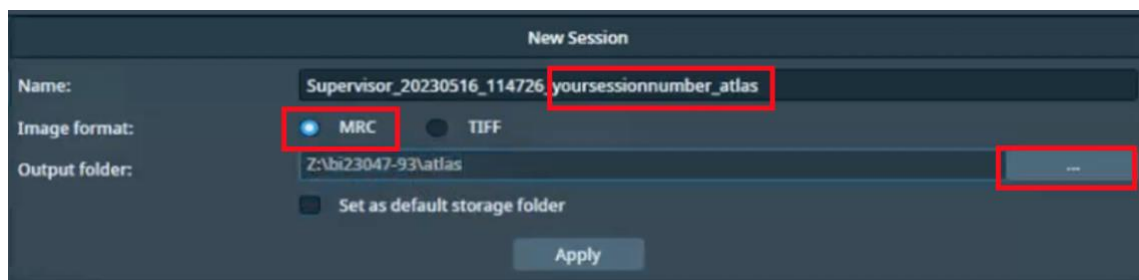
#### Atlas Session setup:

- ☐ Start a new session for taking an Atlas

(TOMO: Atlas > Session Setup > press 'New session')



- ☐ Setup the new session parameters



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

**Naming:** Supervisor\_YYYYMMDD\_XXXXXX\_visit-ID\_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:** Z:\[your session ID, i.e., biXXXXXX]/atlas

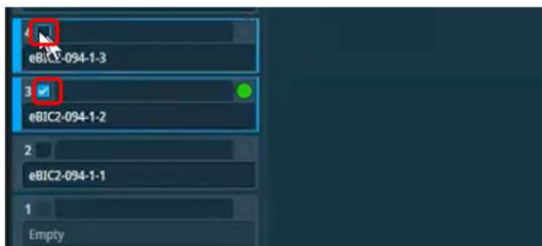
**Note:** If you do not see a folder with your session ID and atlas in the Z:\ drive (i.e. :Z\biXXXXXX/atlas) ask your LC to initiate 'murfey' before you set up your Atlas session

- ☐ Press 'Apply'

#### Atlas collection:

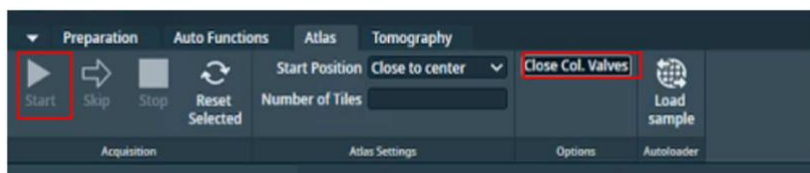
- ☐ Select grids to collect atlases of

(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)

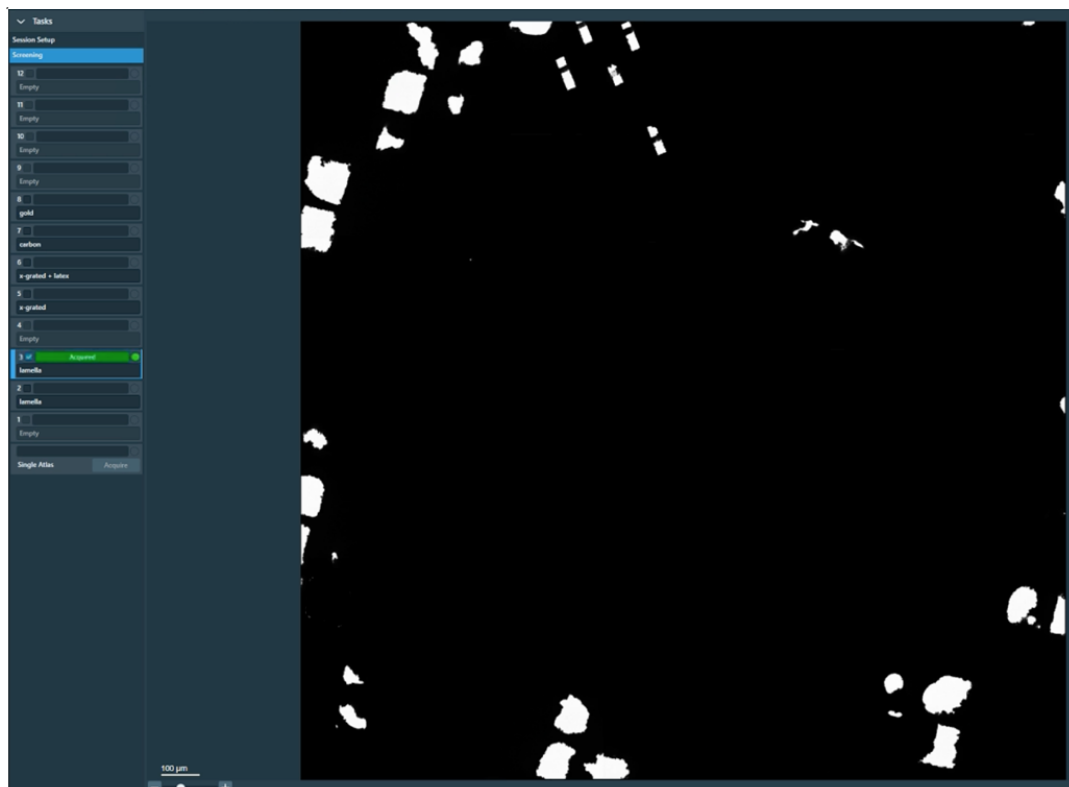
- ☐ Click the 'Start' button to start acquiring atlases of your selected grids  
(TOMO: Atlas > Screening > Start)



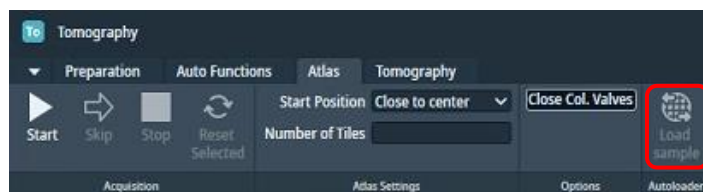
#### 4.1.1 Inspect your Atlas

- ☐ 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:** If you acquired multiple atlases, once you have chosen a grid for data collection you should reacquire the Atlas of that grid. 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.

**A note on atlas montage 'stitching':** On each Atlas, TOMO will have collected a montage of images taken at Atlas magnification and stitched them together to provide a view of the entire grid. Sometimes this 'stitching' is a bit off but this should not affect navigation or data collection. You can ask your LC to perform correction if required.

## 5 Tomography session setup

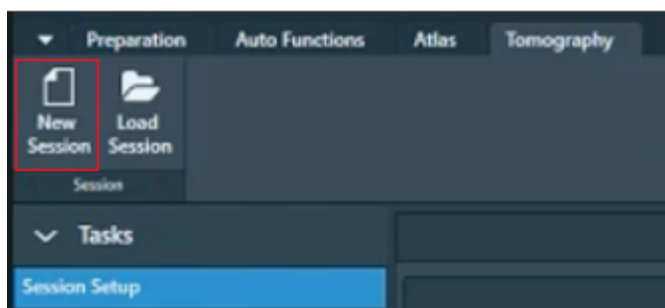
**IMPORTANT:** Once you have chosen a grid for data collection you should **reacquire the Atlas**. This is because shifts and rotation calculations that occur to the grid during loading and unloading are less reliable in TOMO than EPU. You should also ensure that you **ONLY** load grids through TOMO,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

### 5.1 Session Setup

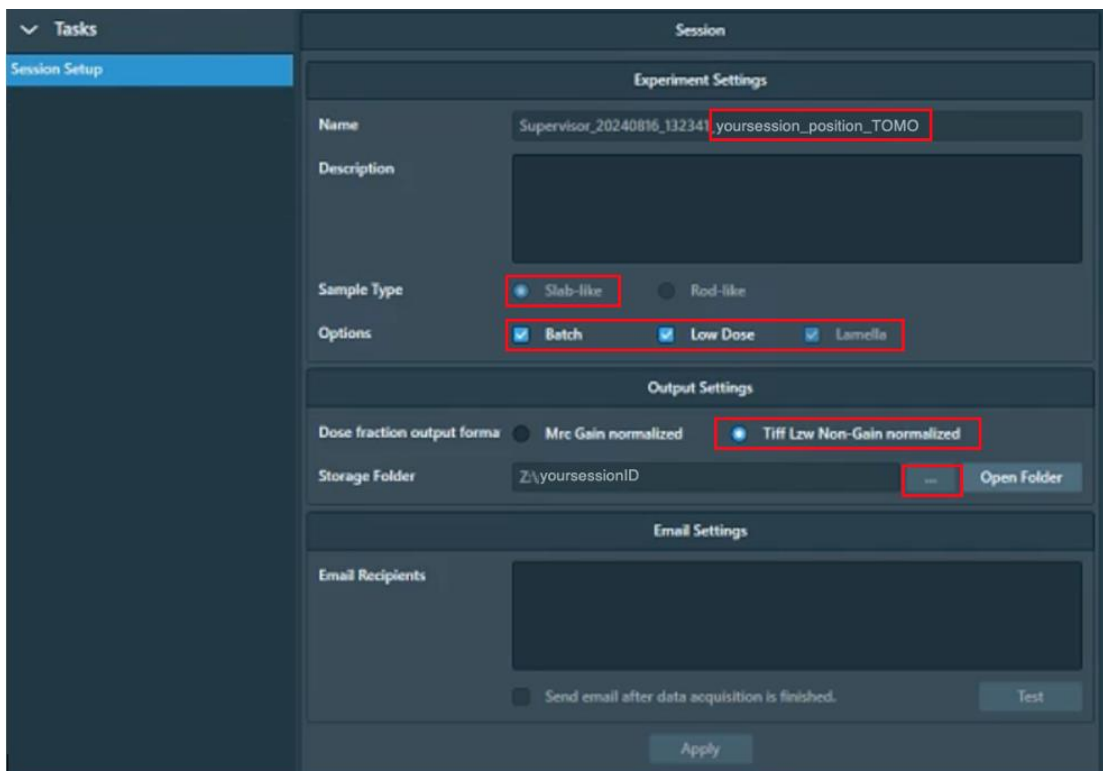
#### 5.1.1 Session setup

- ☐ Set up a new TOMO Session

(TOMO: Tomography > New session > Session Setup)



- ☐ Setup the new session parameters



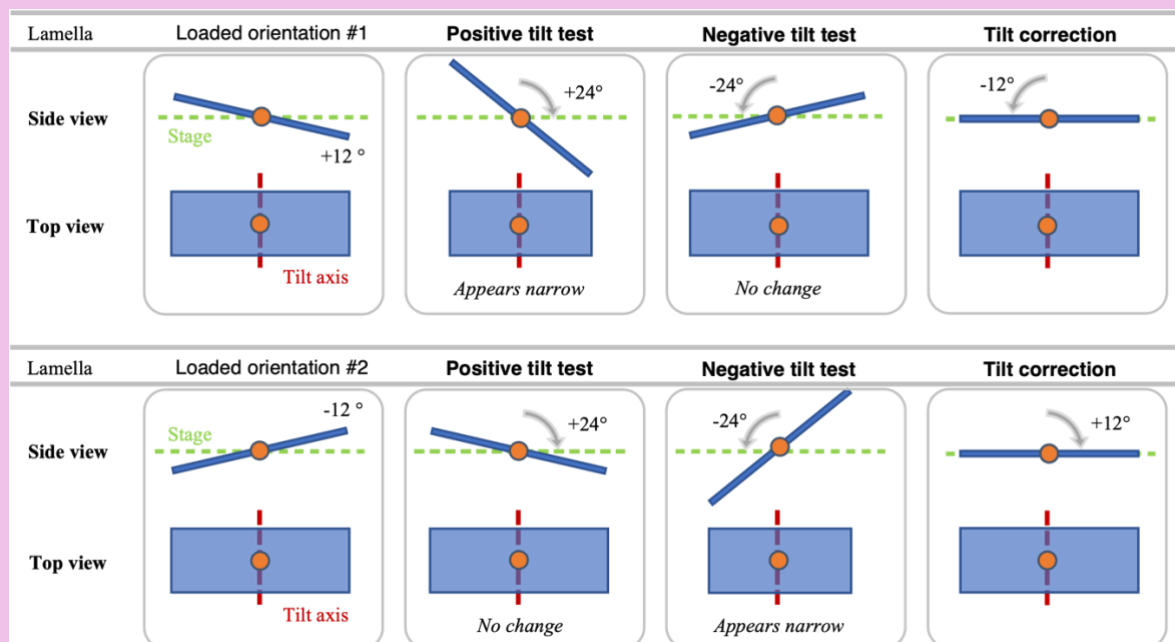
<b>Naming:</b>	Supervisor_YYYYMMDD_XXXXXX_visit-ID_positionX_TOMO <b>Note:</b> DO NOT edit the prefix created by TOMO
<b>Sample type:</b>	Slab-like
<b>Options:</b>	Batch / Low Dose / Lamella (select all that apply)
<b>Image format:</b>	MRC <b>(Note:</b> These are the Atlas, overview/positioning etc. images stored in the TOMO folder. Using MRC is recommended to retain the extended MRC header information)
<b>Dose Fraction output (K3 only):</b>	Tiff LZW non-gain normalised <b>(Note:</b> These are the raw movie stacks from the detector. Using Tiff LZW non-gain normalized is required by eBIC. Please note that if TOMO crashes this will revert back to .mrc format so <b>always double check</b> 'Tiff LZW Non-Gain normalized' is selected) <b>(Note:</b> on a Falcon IVi the Dose fraction output is set in the 'Data Acquisition' Preset – refer to <b>section 3.3.2.1</b> for full details)
<b>Output folder:</b>	Z:\[your session ID, i.e., biXXXXXX] <b>(Note:</b> Press the ... to navigate to your session ID folder. If you do not see a folder with your session ID and atlas in the Z:\drive 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.

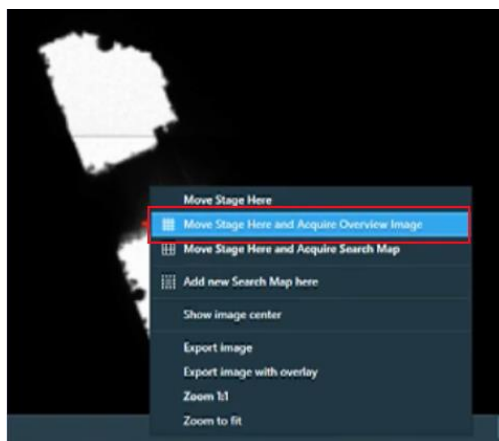


☐ Use your atlas to find a lamella

☐ Acquire an Overview image of the lamella at 0° tilt

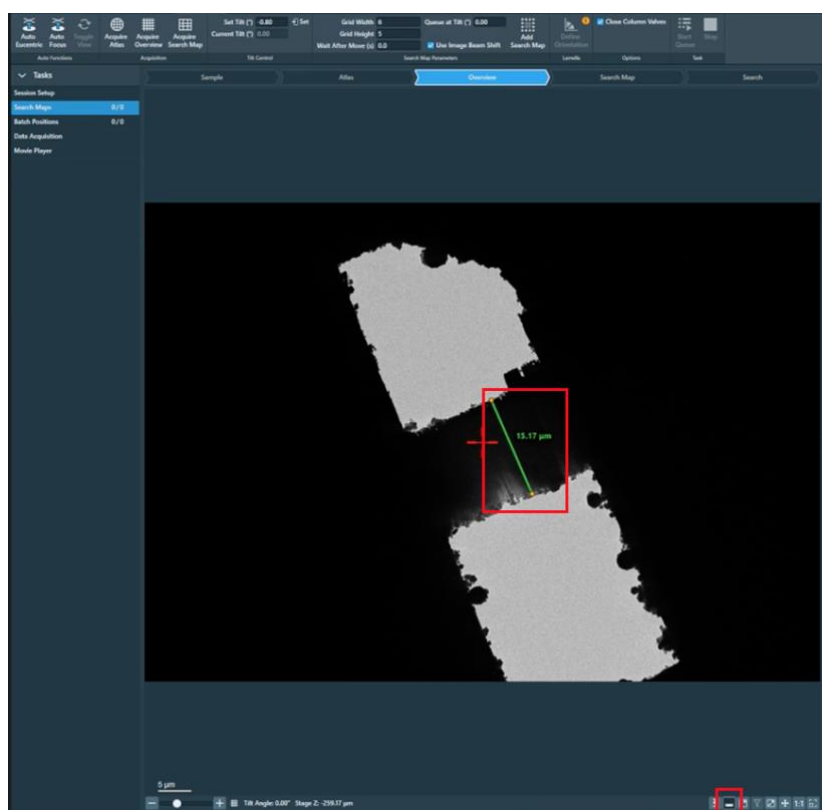
(TOMO: Right click > Move stage here and Acquire Overview Image)





- ☐ Measure the width of your lamella using the measuring tool

*Select the measuring tool (bottom right of image panel) then Left Click and Drag to measure*



- ☐ Tilt the stage to 2X the known milling angle using a positive tilt, acquire an overview and take a note of the change in width of the lamella. Repeat in the negative direction.

*(TOMO: Tomography > Search Maps > Tilt Control > Set Tilt (°) > press 'Set')*



If the lamella appearance change is not obvious, try tilting to a higher angle i.e., +/- 40°

- ☐ Once you have determined which direction the tilt correction needs to be applied, apply the appropriate Tilt Correction

*(TOMO: Tomography > Search Maps > Tilt Control > Set Tilt (°) > press 'Set')*

## 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.

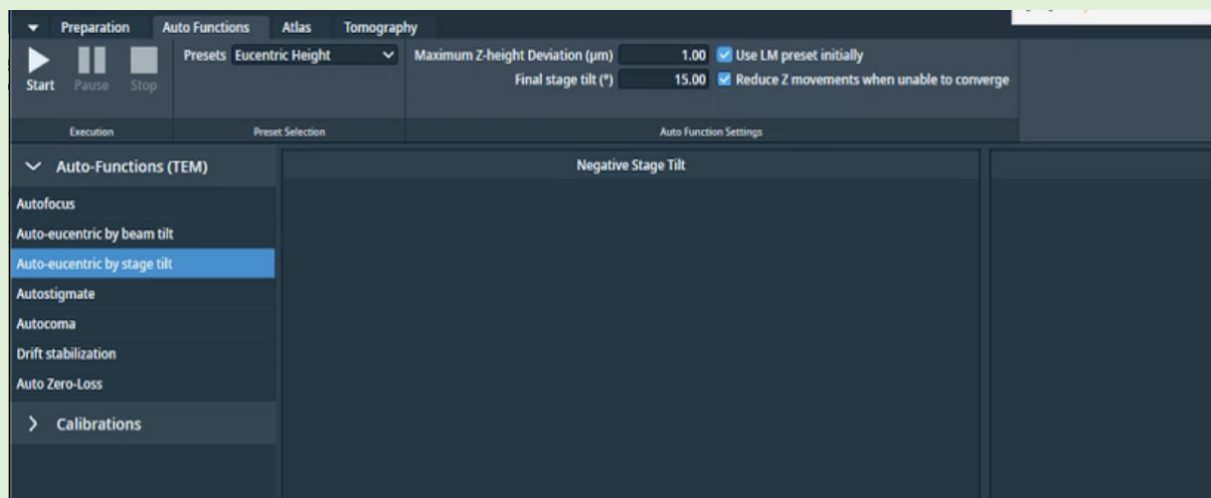
**IMPORTANT:** Once you have chosen a grid for data collection you should **reacquire the Atlas**. This is because shifts and rotation calculations that occur to the grid during loading and unloading are less reliable in TOMO than EPU. You should also ensure that you **ONLY** load grids through TOMO,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

- ☐ Use your Atlas to navigate to an area of interest

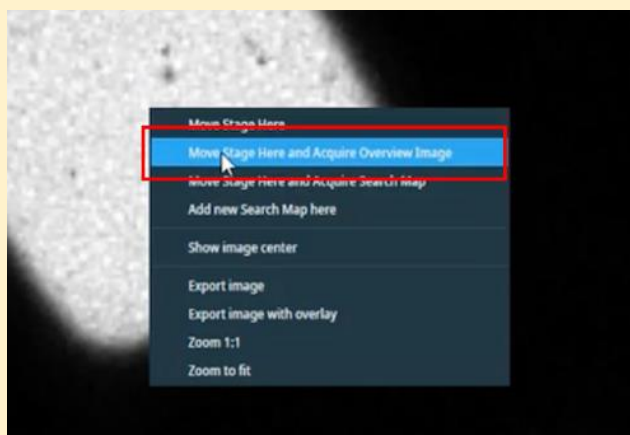
#### **A note on the autoeucentric routine:**

*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')

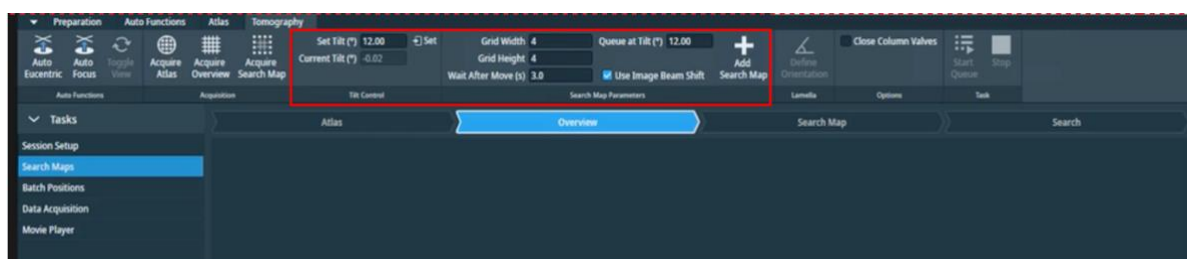


☐ Adjust your search map parameters as required to suit your experiment

(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.

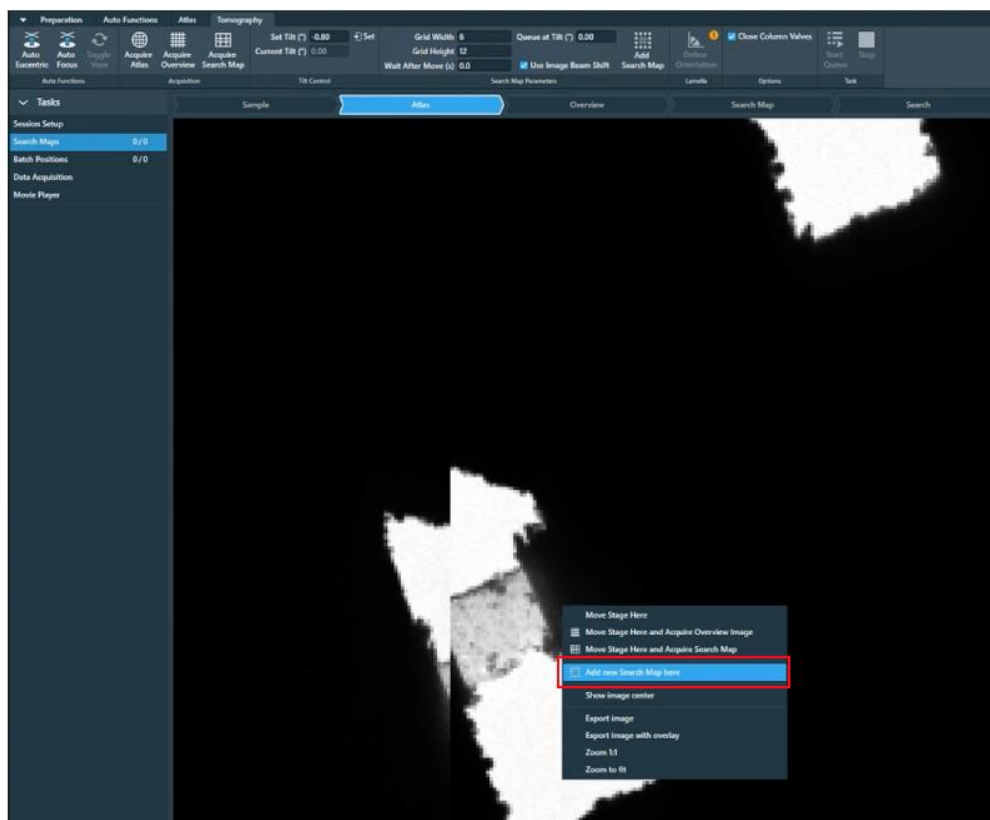


#### A note on the 'Queue at Tilt' field

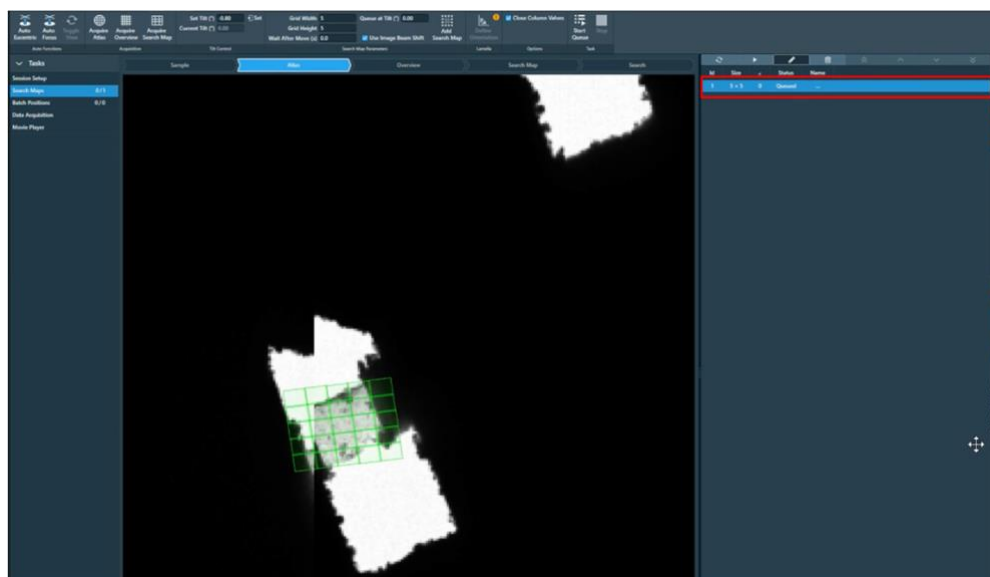
Ensure this field is **filled in correctly**, especially for lamella tomography where the **initial tilt angle is not 0°**. This value is not automatically updated with the current stage tilt, so you will need to manually set the desired angle before queuing the Search Maps for collection (this should 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 a green 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. As you add search maps, you will notice the currently selected search map will appear green while the other search maps appear orange.

**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

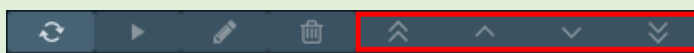


### Editing Search maps

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.



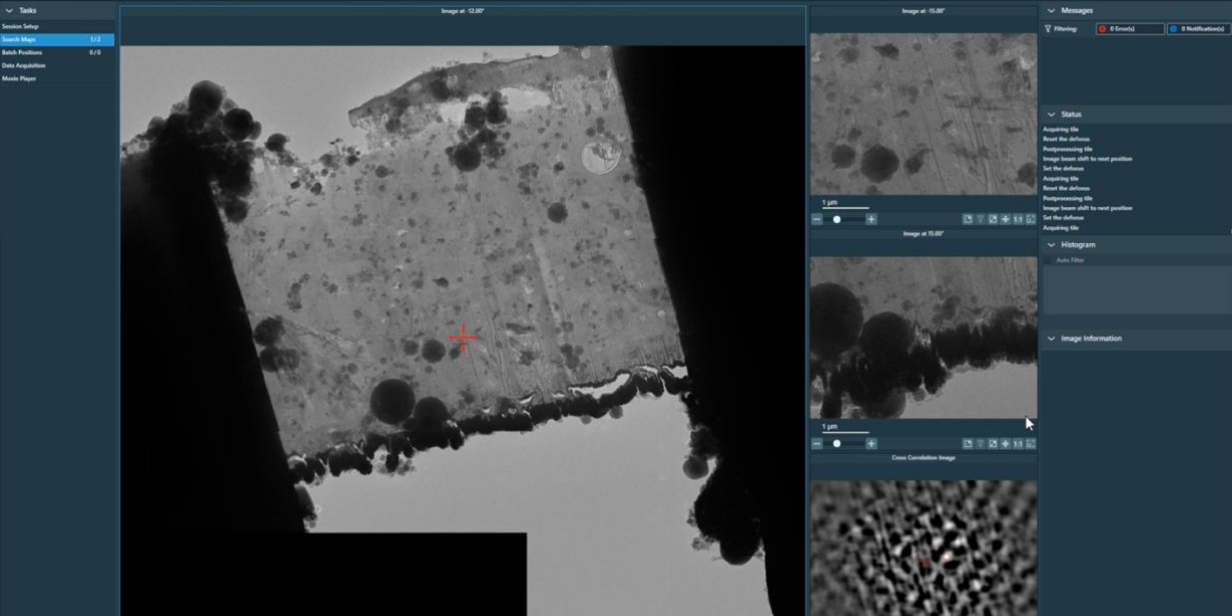
## 5.2.3 Acquiring queued 'Search Maps'

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.

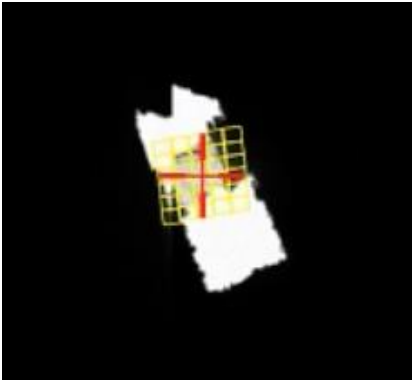
☐ Acquire 'Search maps' of all queued areas

(TOMO: Tomography > Tasks > Search Maps > Task > 'Start Queue')





□ 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:

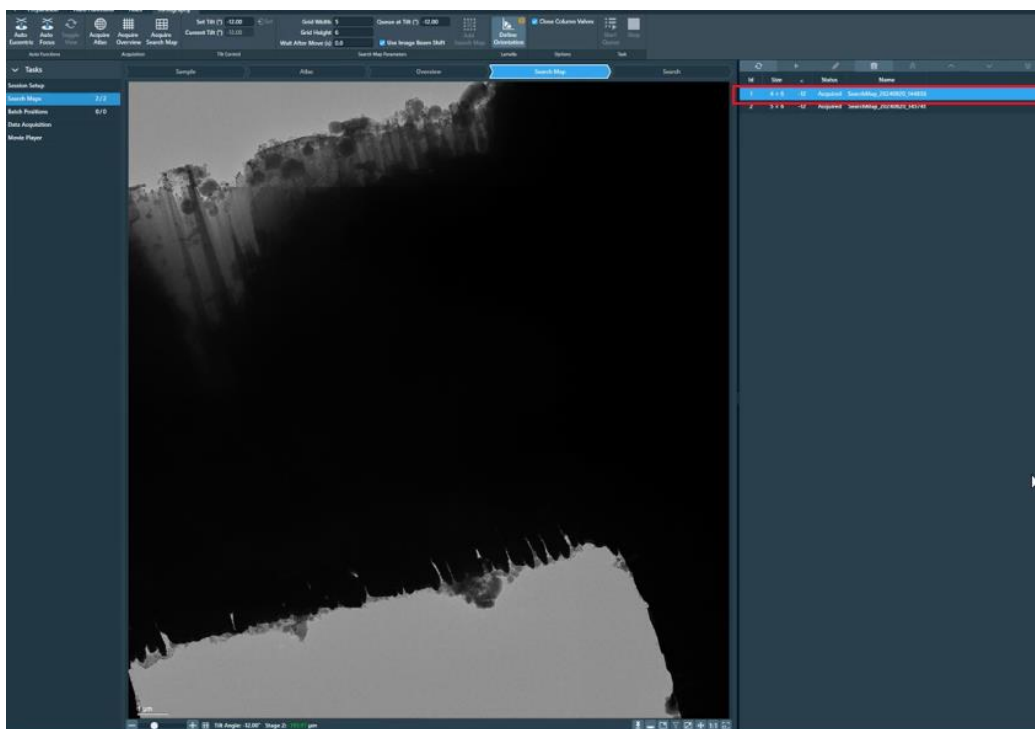
As you are adding batch positions, **be aware:**

- 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.

### 5.3.1 (Lamella only) Define Orientation of your Search Maps

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. If you selected the 'lamella' option during session set up, you will need to 'define your orientation' of at least one search map before you can proceed to batch positions.

☐ Go to one of your Search Maps



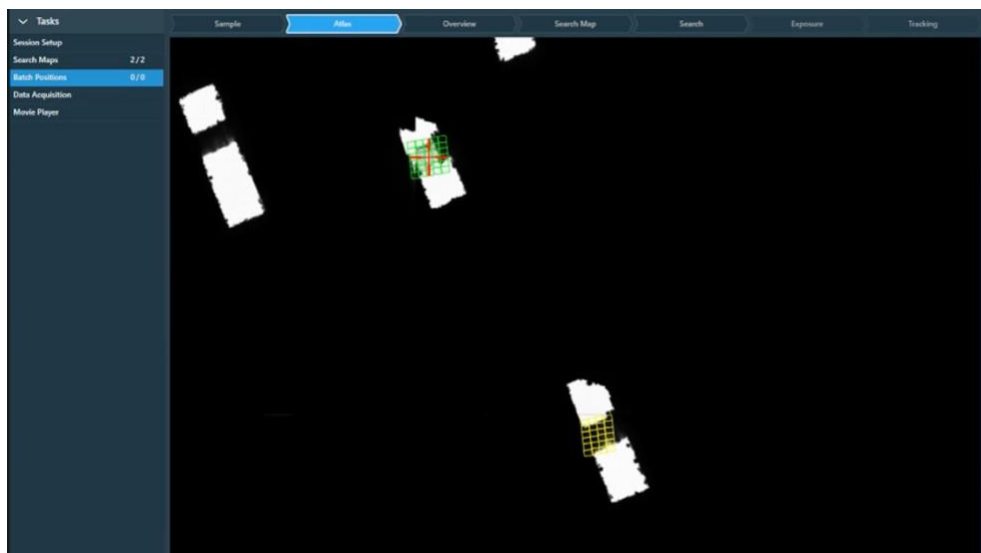
☐ Select 'Define Orientation'



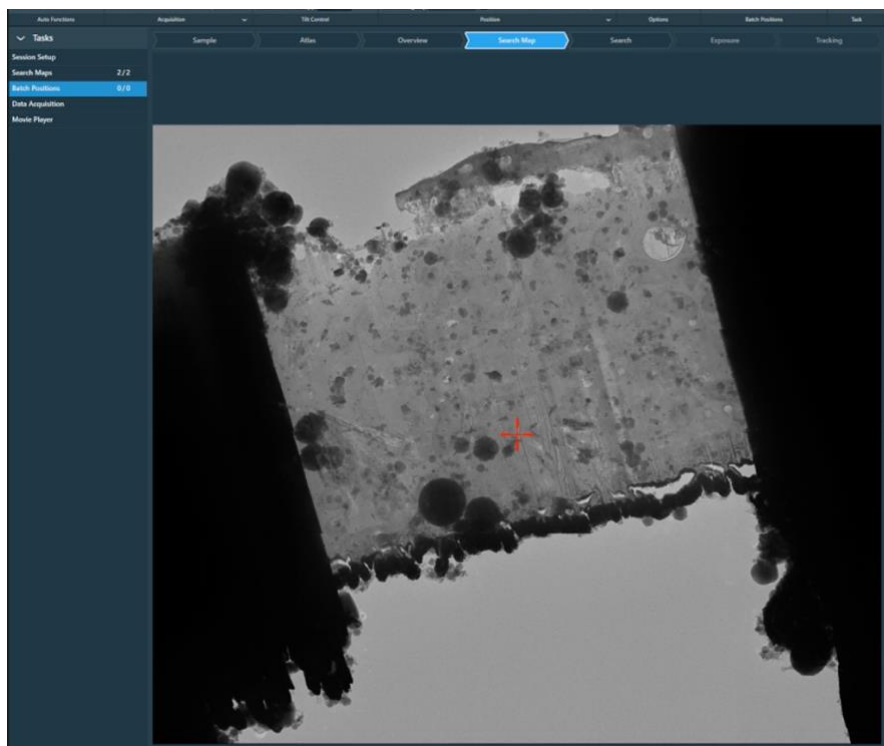


- 

(TOMO: Tomography > Tasks > Batch position > Atlas > left click on a yellow acquired 'Search map')



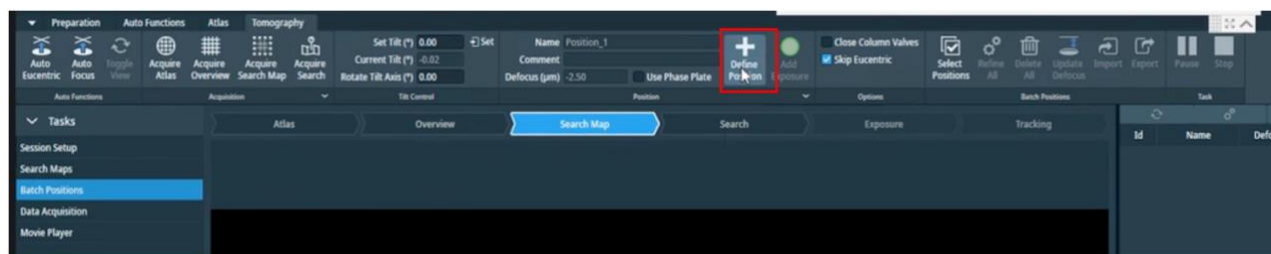
- Page 37 of 54



### 5.3.3 Define a new Batch position series

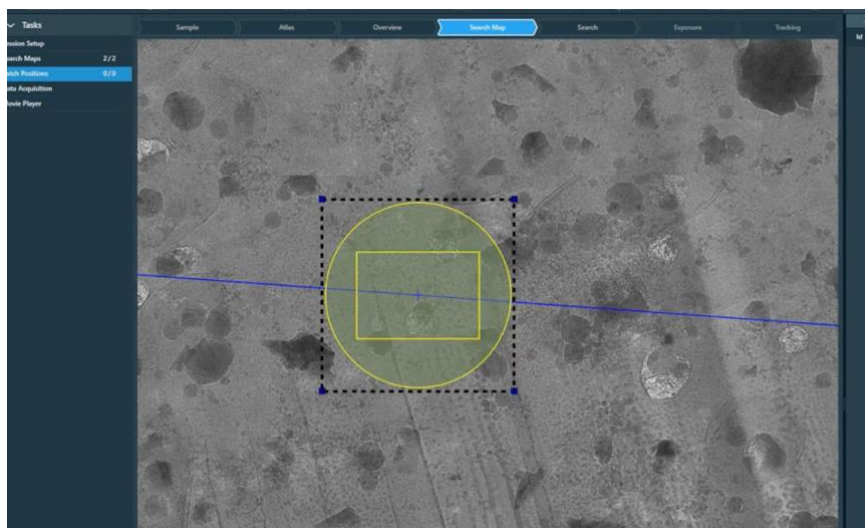
- ☐ Add an acquisition area to the Search map

(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).

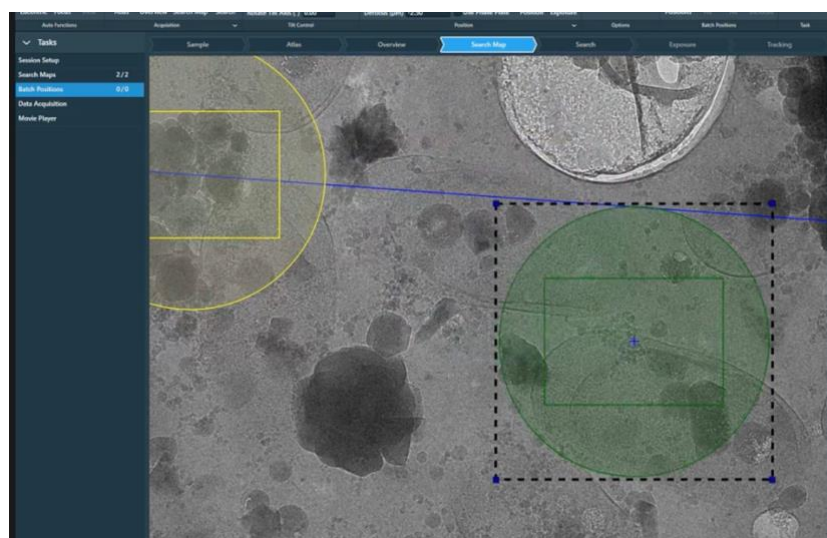
**Note:** the yellow and blue circles will likely be locked together and appear as one overlapping circle. The green circle might be underneath the Yellow and Blue circles.



- ☐ Reposition the green 'Exposure area' to an area of interest

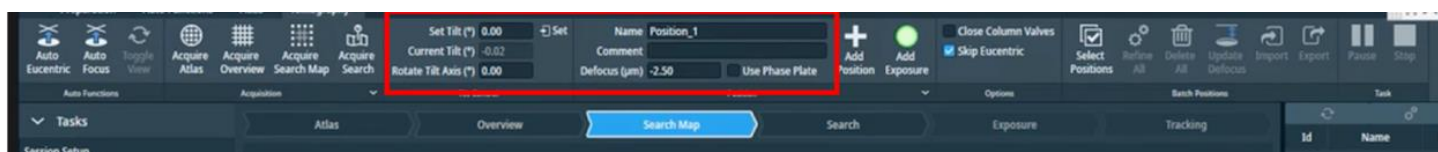
(TOMO: Tomography > Tasks > Batch position > Search Map > left-hand click on the green circle and drag to reposition)

**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)*

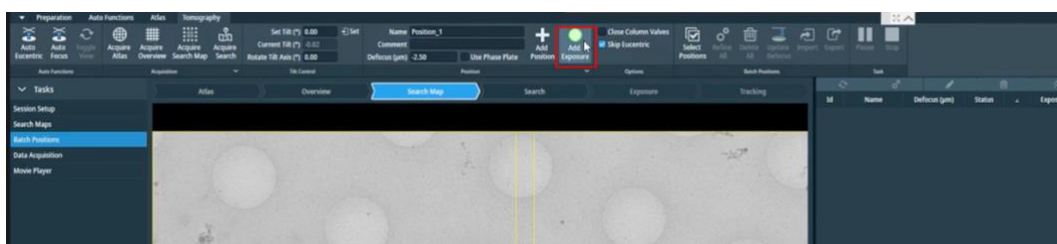
☐ Add additional exposure areas around the batch position area and reposition them as required

*(TOMO: Tomography > Tasks > Batch position > Search Map > 'Add Exposure')*

#### A note on positioning your exposure areas:

*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. At eBIC we have tested acquisition away from the tilt axis to a limit of ~ 16 m*

*Also, 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



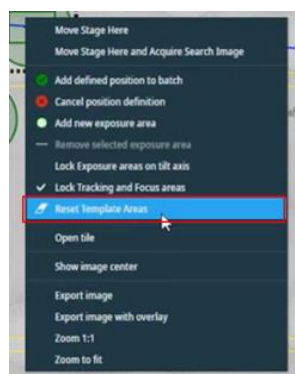




### 5.3.4 Defining additional 'Batch Position' areas

- ☐ Continue adding 'Batch Position' areas to the search map as described in **section 5.3.3**

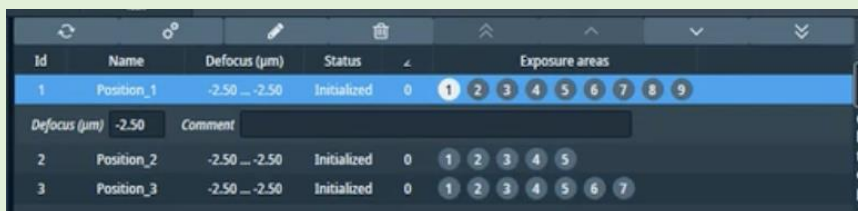
**Note:** when you press 'Define position' TOMO will copy and paste all of the positions from the last batch position set. You can either keep these and reposition as required or right click 'reset template areas'



- ☐ 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.

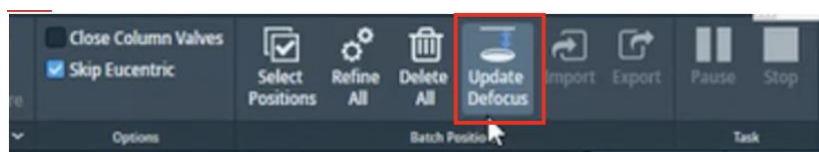
### 5.3.5 Editing the defocus values for 'Batch Position' areas

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.

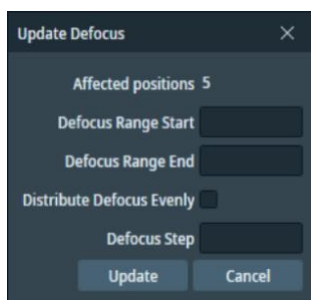
- ☐ Select 'Select Positions' then tick all desired Batch positions in the queue



- ☐ Press 'Update focus'



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



### 5.3.6 (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**.

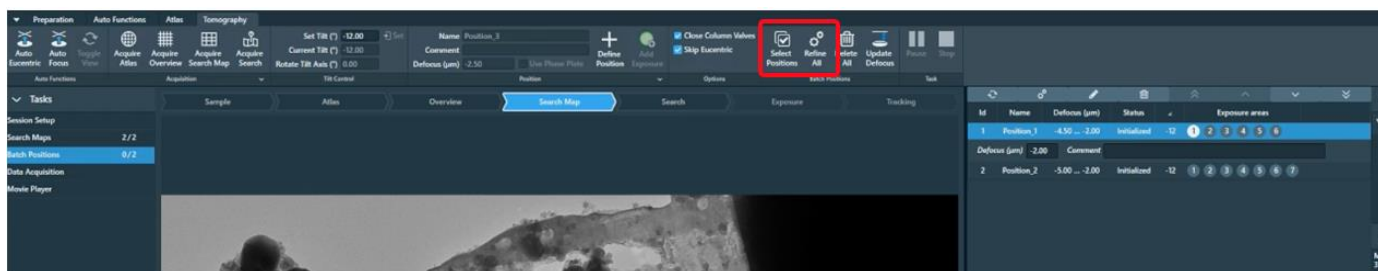
For cellular or lamella samples, it is more likely that each batch position is at a different z-height and in this case you should carry out the eucentric height re

- ☐ Select positions to refine or press 'refine all' to refine all positions

(TOMO: Tomography > Batch Positions > Search Map > Batch positions > Refine all)

OR

(TOMO: Tomography > Batch Positions > Search Map > Batch positions > Select positions)



☐ Check and revisit squares that have failed refinement.

- If eucentric height fails, you could find eucentric by stage-tilt (as in **section 3.5.2**), acquire a new search image to update the z-height and add the position again. Delete the failed/initialised 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

#### 6.1.1 Alignments via Autofunctions

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.

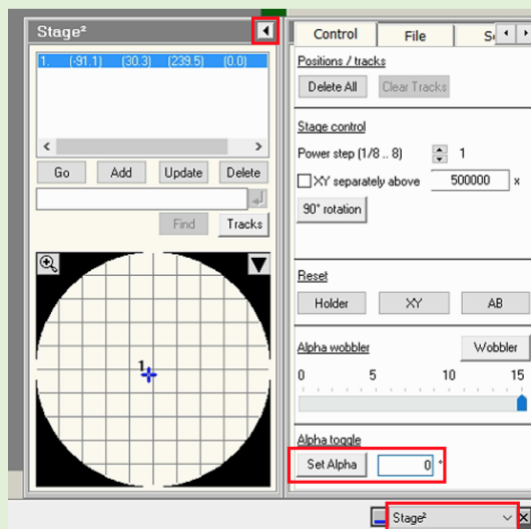
Proceed directly to **section 6.2** *Do not pass Go. Do not collect £200...*

Alignments must be performed on an untilted grid, over carbon and at eucentric focus which is achieved by bringing the sample to eucentric height and adjusting the objective lens to be at focus. The objective aperture should also initially be removed.

**A note on UltraAuFoil and HexAuFoil grids:** Please inform your local contact before your session if you are using grids with a gold film, certain autofunctions (autocoma and autostigmat) must be performed over carbon so your local contact will do these before handing over to you.

**A note on tilted grids:** The following autofunction tasks/checks should be performed at 0 degrees tilt. If you are collecting data at a tilt, you should adjust the grid manually to 0 degrees in TUI:

(TUI: dropdown in bottom right > select 'Stage' > press the tab out arrow > 'Input a value of '0' then 'Press 'Set Alpha'')



- ☐ Navigate to an area of carbon
- ☐ Remove the objective aperture if inserted
- ☐ Perform Autofunctions in the following order:

#### Step 1: Auto Eucentric

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

## Step 3: Autostigmatize

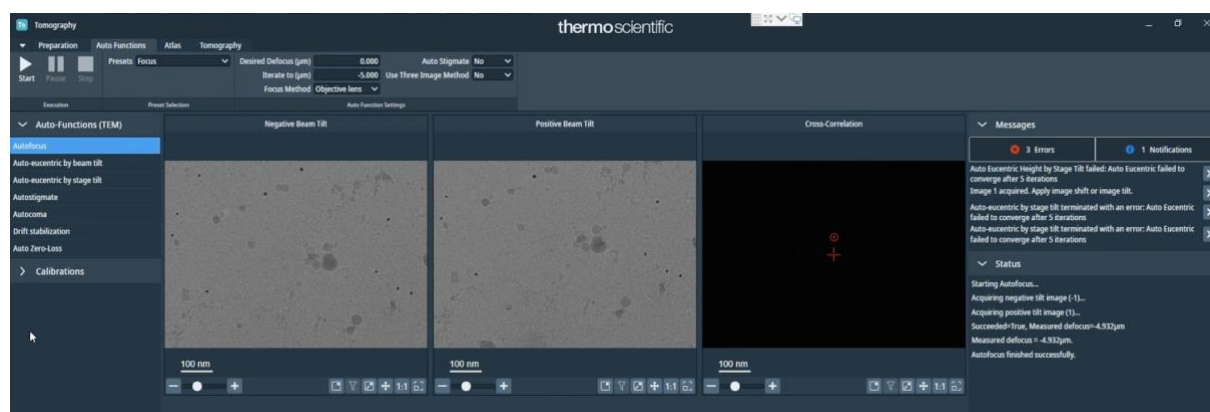
- 1) Select 'Autostigmatize' in the autofunctions tab  
(TOMO: Autofunctions > Autostigmatize)
- 2) Set to 'Thon Ring' magnification preset **in autofunctions**  
(TOMO: Autofunctions > Preset 'Thon ring')
- 3) Press 'Start'

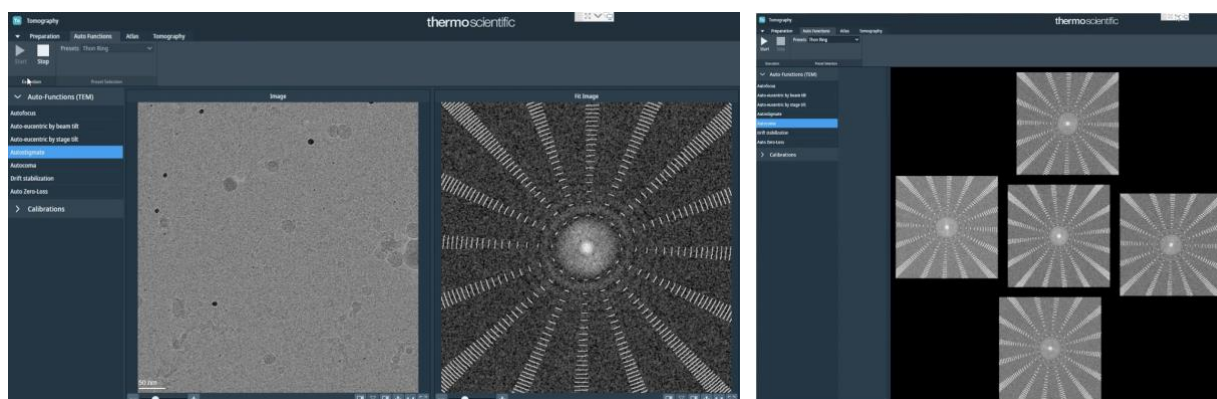
**Note:** The astigmatism should ideally be less than 5 nm

## Step 4: Autocoma

- 1) Select 'Autocoma' in the autofunctions 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 less than 200 nm on eBIC Krioses



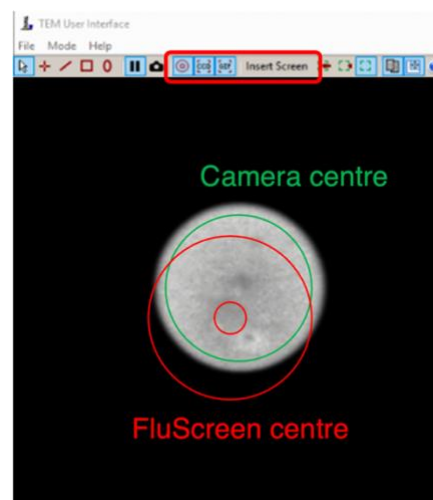


### 6.1.2 Beam centring

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.

This assumes the objective lens is at eucentric focus from the previous alignments. If you are in doubt you may repeat Step 1 & 2 from 6.1.1.

- ☐ 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 down) > 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  $\mu\text{m}$  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 down) > Tomo Beam Shift)
  - 2) Use the **multifunction X/Y** on the simulator pads to centre the beam

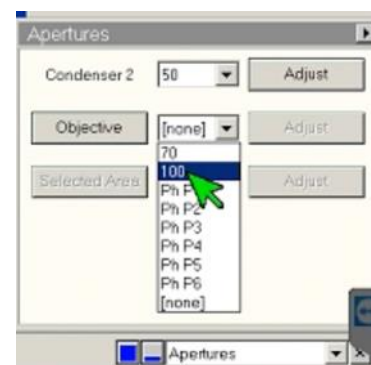
Click 'Done' on the Direct Alignment panel

- ☐ Repeat the Autostigmat Auto Function on a carbon area

#### Autostigmat

- 1) Select 'Autostigmat' in the autofunctions tab  
(TOMO: Autofunctions > Autostigmat)
- 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



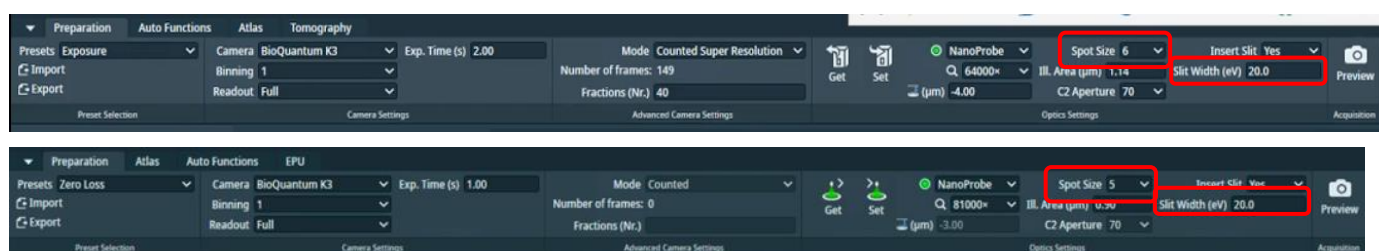
## 6.1.4 Zero-loss peak

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 autofunctions tab  
(TOMO: Autofunctions > Auto Zero-Loss)
- 2) Set to 'Zero Loss' magnification preset **in autofunctions**  
(TOMO: 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 3.3** 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 3.3**)

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



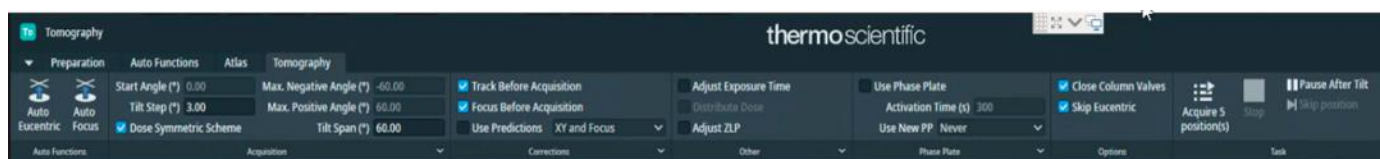


☐ Go to your Tomography Data Acquisition settings and double check your parameters

(TOMO: Tomography > Tasks > Data Acquisition)

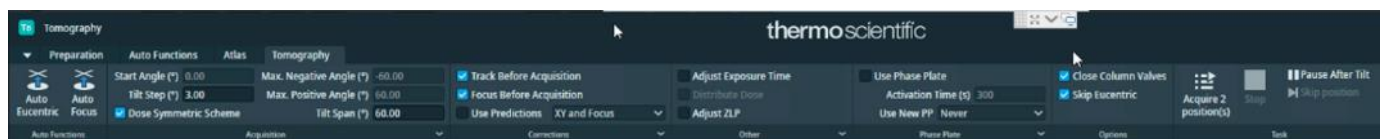
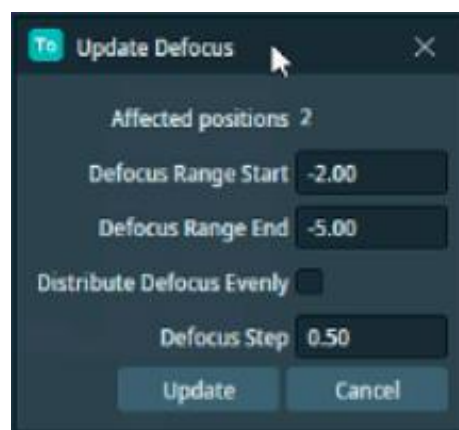
Check:

- **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

Batch Positions				
	Name	Defocus (µm)	Status	Start Angle (°)
<input checked="" type="checkbox"/>	Astro_1	-2.00	Acquired	0
<input checked="" type="checkbox"/>	Astro_2	-2.50	Acquired	0
<input type="checkbox"/>	Astro_3	-3.00	Initialized	0
<input checked="" type="checkbox"/>	Astro_4	-3.50	Acquired	0
<input checked="" type="checkbox"/>	Astro_5	-4.00	Acquired	0
<input checked="" type="checkbox"/>	Astro_6	-4.50	Refined	0
<input checked="" type="checkbox"/>	Astro_7	5.00	Refined	0

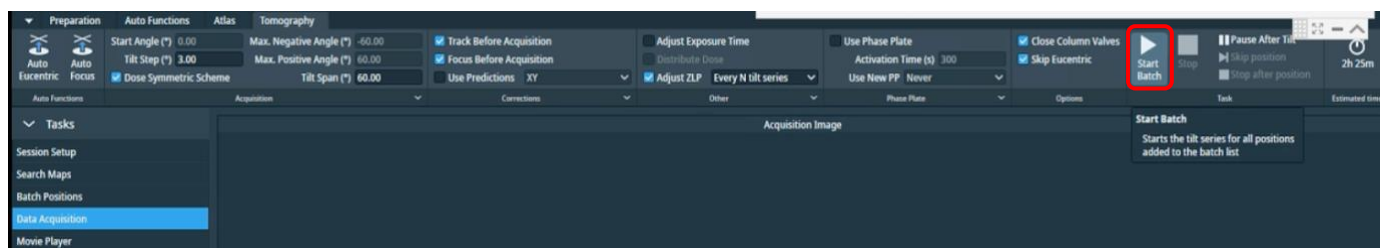


## 6.2.2 Start tomography acquisition

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

☐ Start the automated acquisition

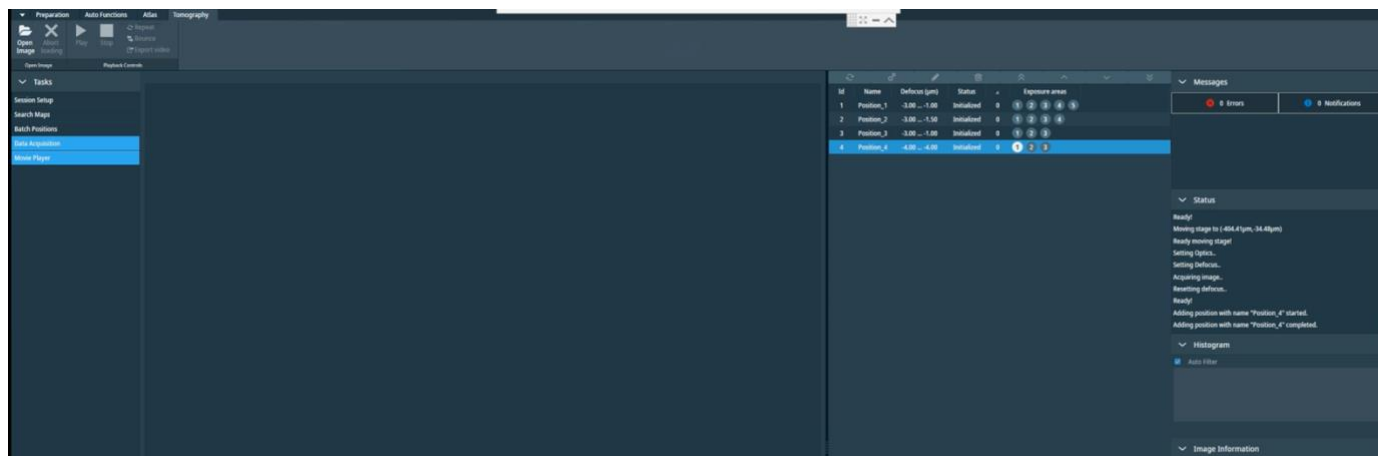
(TOMO: Tomography > Data Acquisition > Task > press 'Start Batch')



☐ 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



☐ Add more batch positions as required

### 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 Set up on-the-fly processing

Please refer to the '[Remote access, analysis and data download:](#)' for further instructions.

### 6.2.5 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.



## 6.2.6 Monitor 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](mailto: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 TOMO will pause collecting images when LN<sub>2</sub> is being refilled, or when GIF ZLP is being adjusted (for K3). These brief pauses in data collection should not last longer than 15-20 minutes. Additional time might be required for TOMO 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.

## 6.2.7 Closing Teamviewer and disconnecting NoMachine

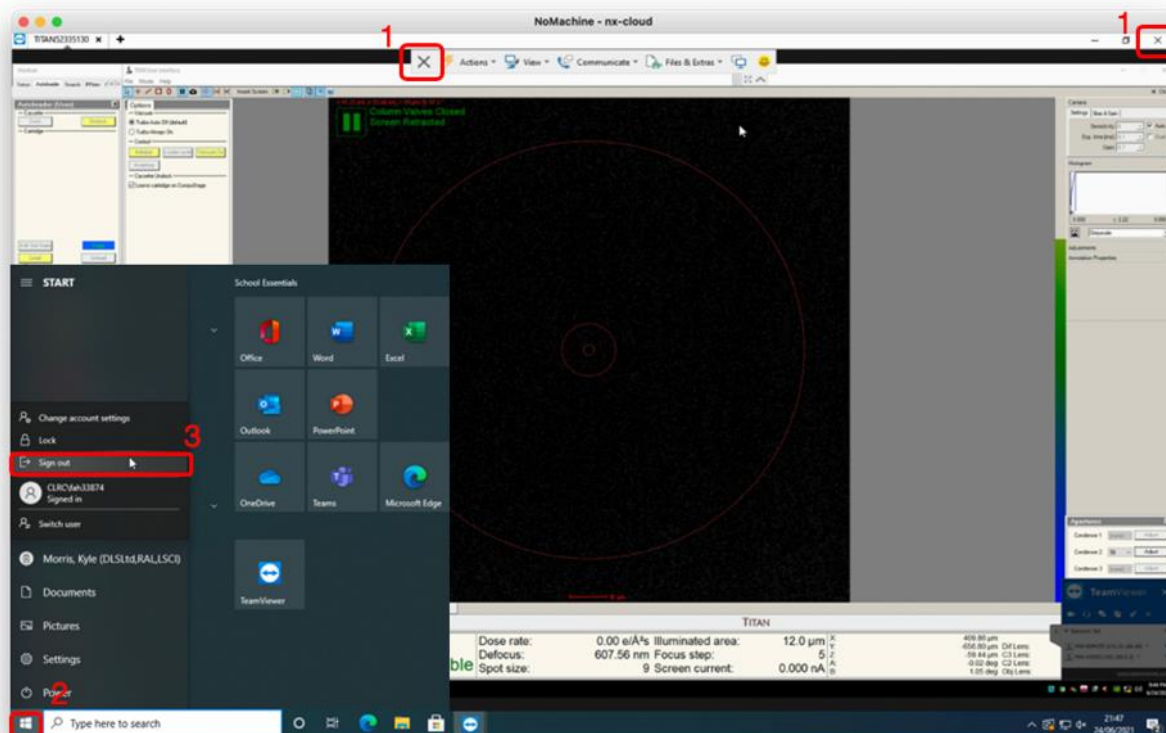
If at any point you leave the microscope unattended, please ensure you close the NoMachine connection in case your LC requires access (only one NoMachine connection is permitted at a time for security reasons).

- ☐ Click on (1) the highlighted cross to close TeamViewer

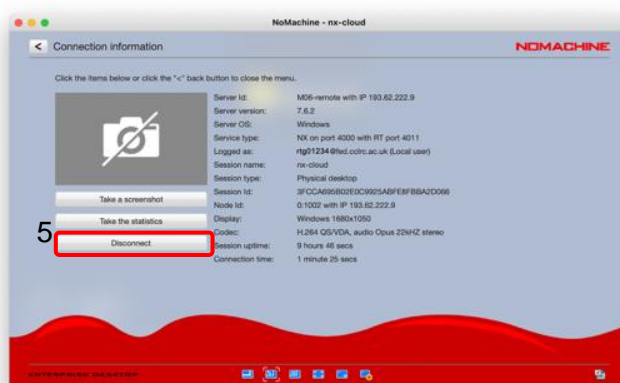
- **(Take care that you do not accidentally close the 'TEM User Interface')**

- ☐ 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:

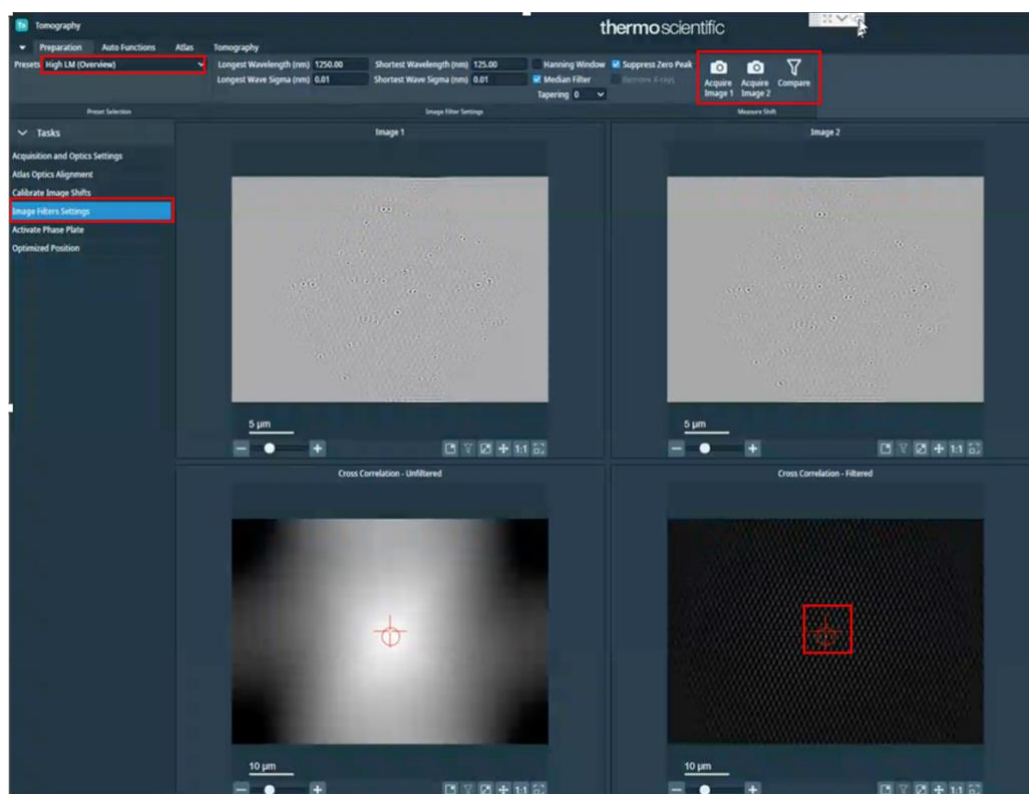
- 1) Re-run 'Auto-eucentric by stage tilt' with 'Presets: Overview' for courser eucentric optimisation. If this succeeds, subsequently re-run 'Auto-eucentric by stage tilt' with 'Presets: Eucentric Height' to increase precision.
- 2) Try again with a smaller stage tilt of 5°, then if this succeeds try 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) –  $\frac{1}{4}$  of the scale bar
  - Shortest wavelength (nm) –  $\frac{1}{40}$  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.