

# Remote EPU operation guide for eBIC microscopes

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Revision summary: 1) Plasmon peak imaging added  
2) Improved 'Prepare all' function in 'Hole Selection' task added

Comments to: 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 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

## 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. The 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, email, 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

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## Quick start guide

- 1) Receive handover email from your local contact detailing microscope parameters
- 2) Optimise display settings for remote access (see section 1.2)
- 3) Check your magnification presets and adjust if required (see section 3.2)
- 4) Check image shift calibrations (see section 3.5)
- 5) 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

- 6) Create your EPU session (single grid - see section 5) (multi grid - see section 6)

<b>Naming:</b>	Supervisor_YYYYMMDD_XXXXXX_visit-ID_positionX_EPU
<b>Grid type:</b>	Holey Carbon / Lacey / Holey Gold
<b>Geometry type:</b>	Square / Hexagonal / Unknown
<b>Session type:</b>	Manual
<b>Acquisition Mode:</b>	Faster (AFIS on all Krioses)
<b>Tilted Acquisition:</b>	Tick or untick as required
<b>Image format:</b>	MRC
<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 section 3.3.2.1 for full details)
<b>Output folder:</b>	Z:\your session ID, i.e., biXXXXXX

- 7) Set up your target parameters (square selection, hole targeting, ice filter, template definition) (see sections 5 (single grid) or 6 (multi grid))
- 8) Add additional squares as required
- 9) Define template: acquisition areas, defocus series, autofocus area
- 10) Perform final checks in EPU (AutoFunctions and check alignments) (see section 7.1)
- 11) Start data collection (see section 7.2)
- 12) Inform your local contact you have started data collection
- 13) 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

- 14) Return to add more squares as necessary

# 1 Preparation

## 1.1 Communication

Prior to your session, you should have informed your local contact of parameters for your session and information about the grids you have shipped, including at least:

- How many grids have you sent? (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 micrograph ( $\dots \text{e}^-/\text{\AA}^2$ ) ?
- What is your experience with remote EPU sessions?
- If you require guidance in use of EPU, 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 squares selected	by 2 pm
Have hole targets setup	by 3 pm
Start data collection	by 4pm

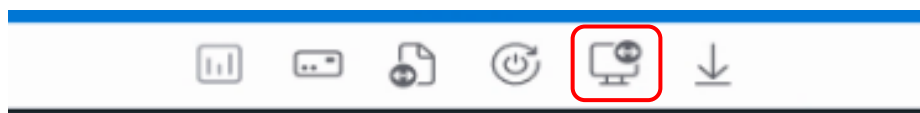
### Day 2

Add further acquisition targets  
(as required for session length)

## 2 User interface summaries

### Team Viewer

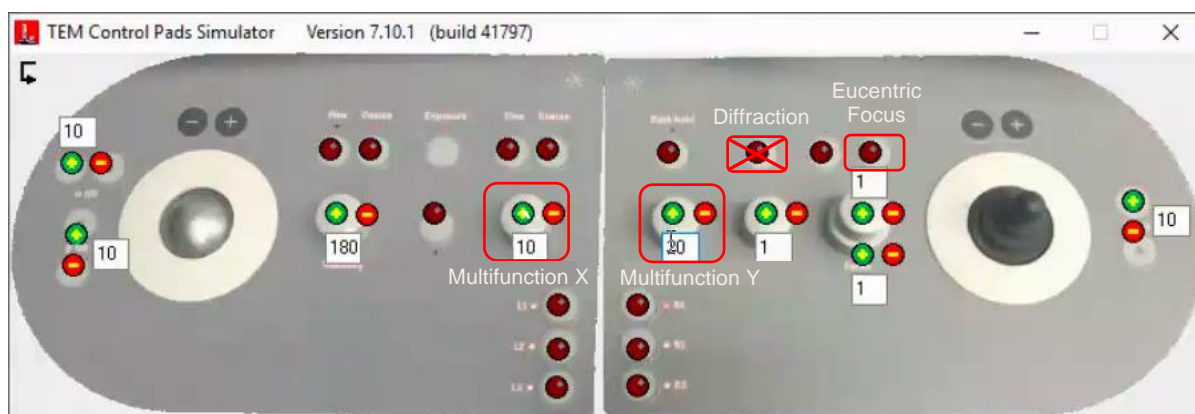
To switch between the virtual monitor hosting the TEM user interface and EPU, use the Team Viewer Monitor button.



### 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.

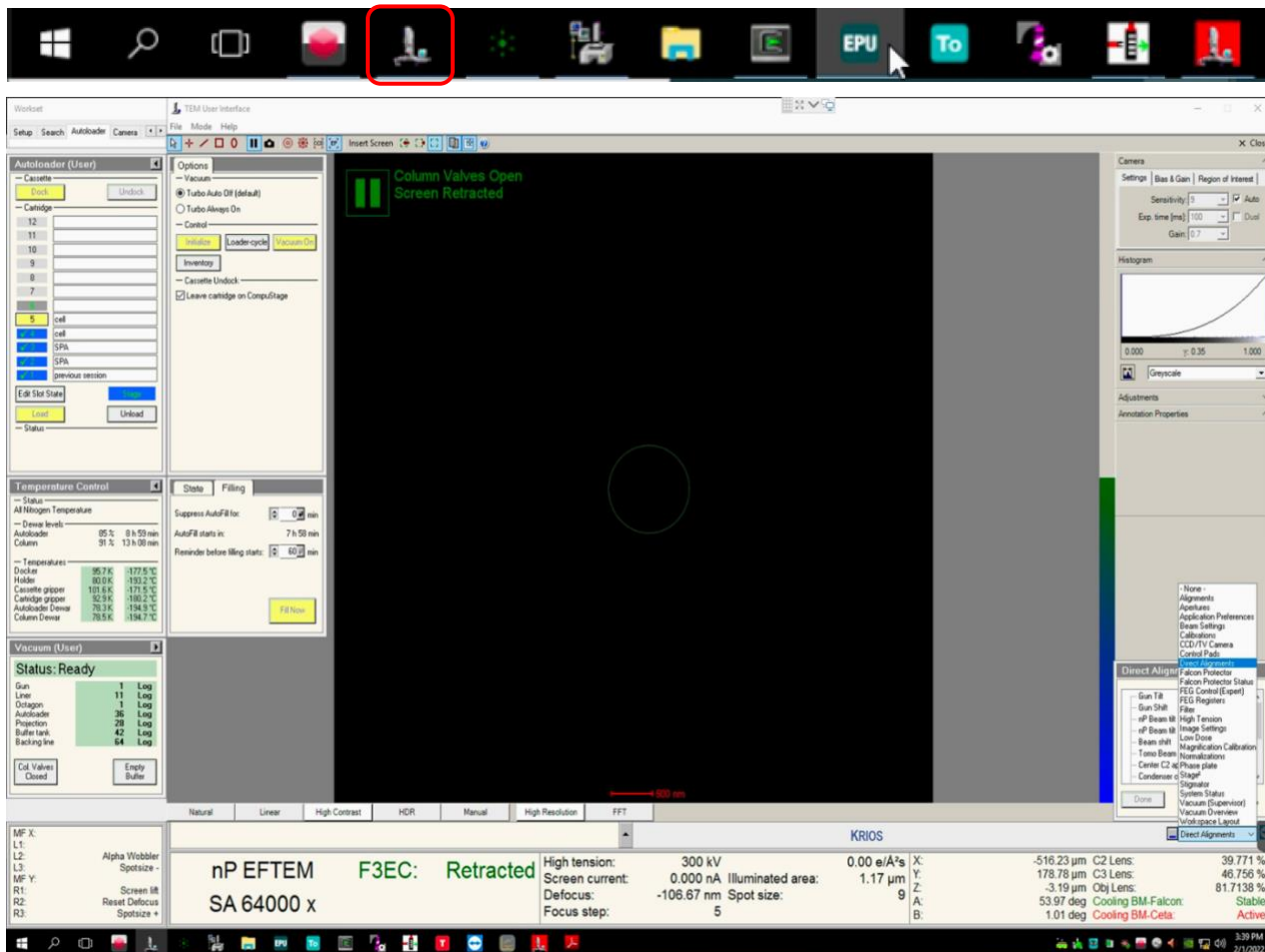
**! WARNING: Take care not to accidentally press the 'Diffraction' button !**





## TEM User Interface (TUI)

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 EPU software

You can generally navigate through the single particle workflow by working left to right through the 'Workflow tabs', and top to bottom through the various 'Workflow tasks'.

E.g., Imaging conditions (Presets) are first configured under the 'Acquisition and Optics Settings'.

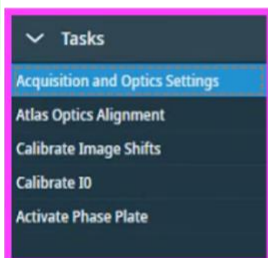
This guide presents a suggested sequence through the EPU workflow.



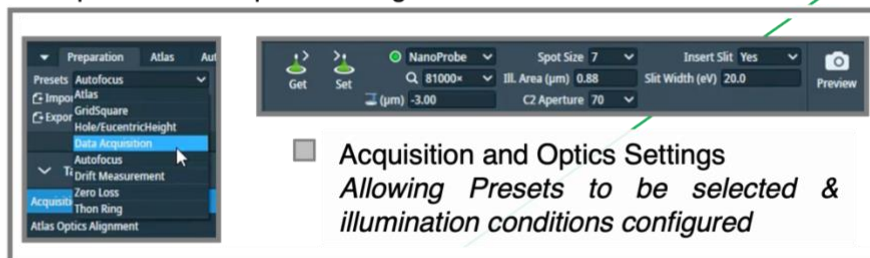
### Workflow tabs: Preparation, Atlas, Auto Functions, EPU



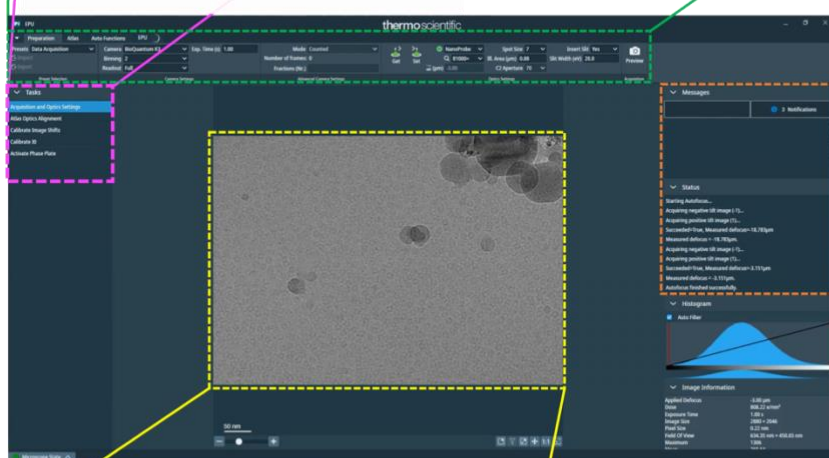
#### Workflow tasks



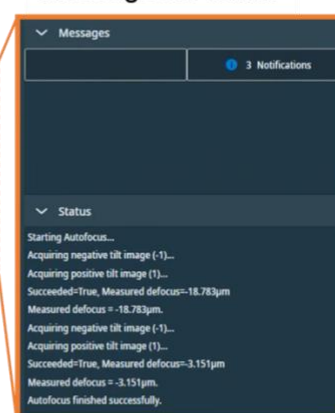
#### Acquisition and Optics Settings: Presets



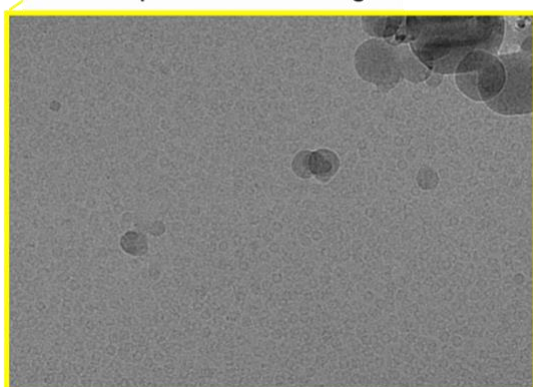
#### Main user interface for TFS EPU



#### Messages & status



#### Last acquired Preset Image



- **Workflow tabs**  
Preparation, Auto Functions, Atlas, EPU
- **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, preset checks and image shift calibrations

### 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 an EPU window is not open, begin by starting this software from the TEM PC



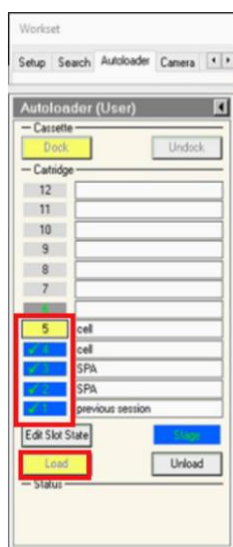
Session set-up can be controlled by users directly through the EPU interface. Changes to beam settings should all be controlled directly through the EPU imaging presets (EPU: Preparation > Presets) and navigation around the grid can be done via right-click options over the images in Preparation, Atlas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

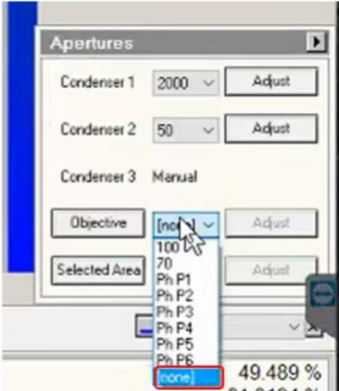
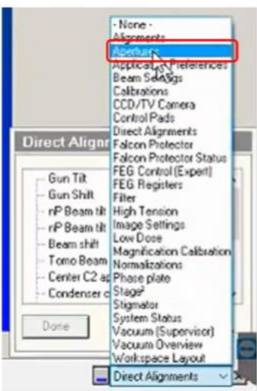
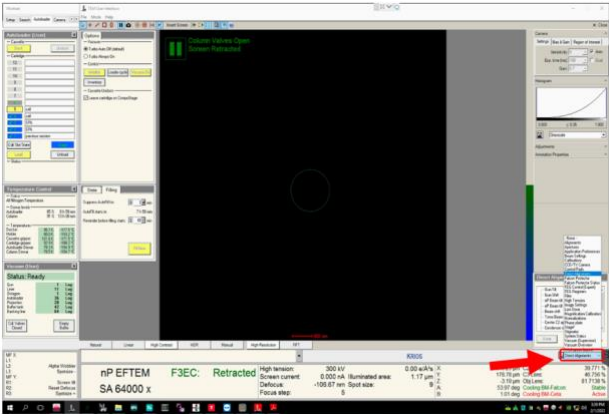
If no grid is loaded then select one of the slots and press 'Load'

**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, Grid square, Hole/eucentric) before moving on to your high magnification presets (Data Acquisition, Autofocus, Drift, Zero-Loss).

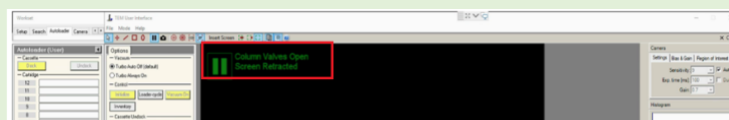
### 3.2.1 Check and adjust Low Magnification Presets (Atlas, Gridsquare, Hole/Eucentric)

- ☐ Take an image using the 'Atlas' preset

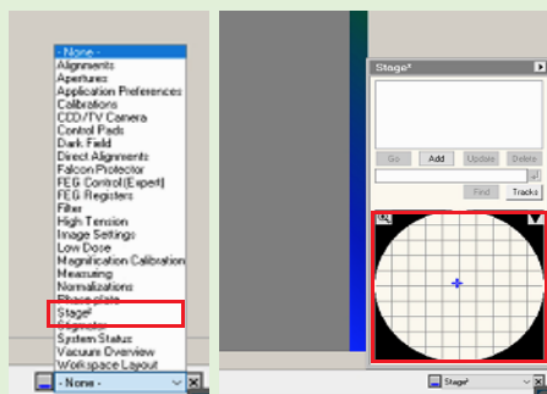
(EPU: Preparation > Tasks > Acquisition and Optics Settings > Presets > select the 'Atlas' preset > press 'Preview')

**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)  
(EPU: right-click on grid square on image > 'Move stage here')
- ☐ Go to the 'Preparation' tab and check each low magnification preset (Grid Square, Hole/ Eucentric Focus)

**In turn:**

- 1) Select the 'preset'  
(EPU: Preparation > Acquisition and Optics Settings > Presets > select 'Grid Square/Hole Eucentric')
- 2) Collect a preview image using that preset  
(EPU: Preparation > Acquisition > 'Preview')
- 3) Adjust your parameters for each magnification preset in EPU so that:

**Atlas**

We do not recommend changing the Atlas preset **magnification**

### Grid square

Image encompasses one grid square

### Hole / Eucentric Focus

Image encompasses one hole and sufficient surrounding carbon for placing an autofocus

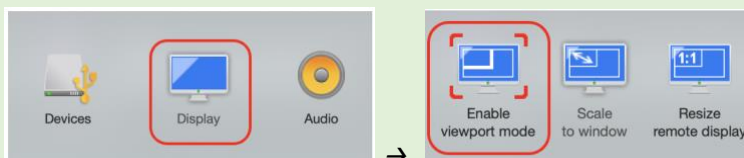
#### A note on other parameters in low magnification presets:

You should adjust various parameters to suit your experiment, including magnification, exposure time, defocus and whether to use plasmon imaging. Plasmon imaging is useful for grids with a gold film (e.g., HexAuFoil, UltraAuFoil etc.) to enhance low image thickness contrast, however you should ensure you inform your local contact that you want to use this setting before the start of your session so they can prepare the microscope.

For the low magnification presets, some parameters that are predictable that you might reasonably expect are:

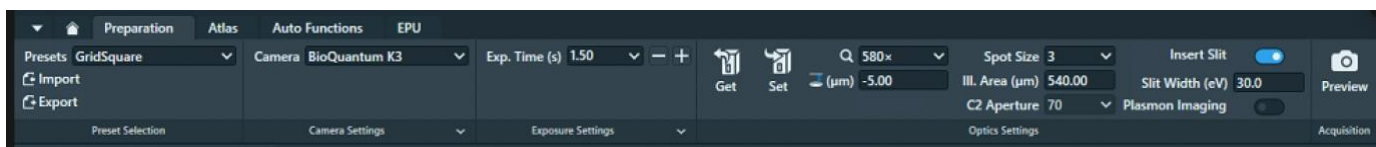
Preset	Magnification	Exposure (s)	C2	Defocus ( $\mu\text{m}$ )	Plasmon imaging
GridSquare	360–940X (Square size dependent) (suggested ~ 740X for HexAufoil grids)	~ 1.0	50	- 100 to - 500 (Grid dependent) (suggested ~ -50 for HexAufoil grids)	Grid type dependent (useful for gold film grids, e.g. UltraAuFoil, HexAuFoil)
Hole/Eucentric	6500–11500X (Hole size dependent) (suggested ~ 33,000 X for HexAufoil grids)	~ 1.0	50	- 20.0 (Grid dependent) (suggested ~ -5 for HexAufoil grids)	N/A

**A note if your struggling to see the values:** You must ensure all of your values are negative, if you are struggling to see the text open the NoMachine settings (Mac: `ctrl + shift +0`) (Windows: `ctrl + alt +0`), open the display settings and go to viewport mode to zoom in.

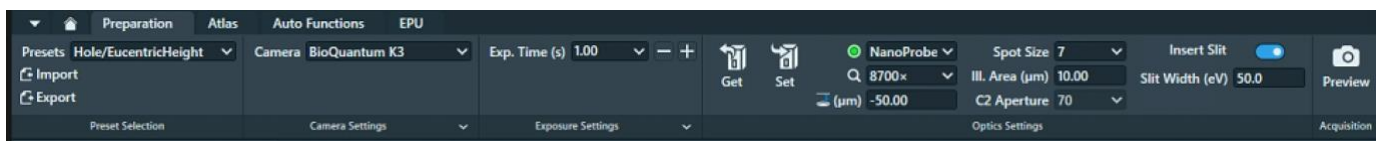


The following screenshots further indicate how you may expect to set your low magnification presets:

### GridSquare



### Hole/Eucentric Height





### 3.3 Check and adjust your Data Acquisition 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 Error! Reference source not found.**

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

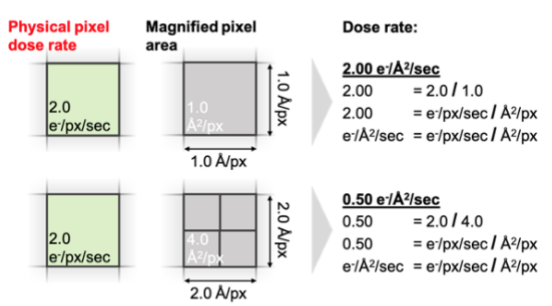
### 3.3.1 (Gatan K3 instructions) Checking and adjusting your Data Acquisition preset

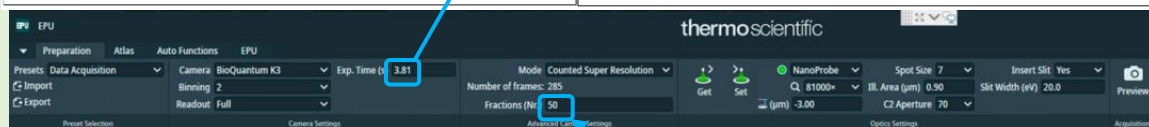
On a microscope equipped with a **Gatan K3 Direct Electron Detector**, the dose fraction output is set during your EPU session set up and but your dose is set in the data acquisition preset settings.

- ☐ Calculate the exposure time based on the **physical pixel dose rate (e<sup>-</sup>/px/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 (e<sup>-</sup>/Å<sup>2</sup>). You may find these [online dose calculators](#) helpful.

Example exposure time calculation:	Visual representation of calculation principle:
<p><b>Physical pixel dose rate:</b> 15.076 e<sup>-</sup>/px/sec</p> <p><b>Pixel size (magnified):</b> 1.072 Å/px</p> <p><b>Pixel area (magnified):</b> 1.149 Å<sup>2</sup>/px</p> $\frac{e^- / px / sec}{\text{Å}^2 / px} = e^- / \text{Å}^2 / sec$ <p><b>Dose rate:</b> 13.121 e<sup>-</sup>/Å<sup>2</sup>/sec</p> <p><b>Target total dose:</b> 50 e<sup>-</sup>/Å<sup>2</sup></p> $\frac{e^- / \text{Å}^2}{e^- / \text{Å}^2 / sec} = sec$ <p><b>Exposure time per tilted image (sec): 3.81</b></p>	



In this example the target total dose is 50 e<sup>-</sup>/Å<sup>2</sup>, and so the **number of fractions** is set to 50 to achieve a final total dose of 1 e<sup>-</sup>/Å<sup>2</sup> per frame of the movie.

- ☐ Under the 'Data acquisition' preset tab enter the calculated exposure time and frame number  
(EPU: Preparation > Preset Selection > Presets > 'Data Acquisition' > enter 'Exp. Time' and 'Fractions (Nr.)')
- ☐ Check that the correct imaging mode and binning value is selected (we recommend using 'counted super resolution mode' with '2' times binning on the K3)  
(EPU: Preparation > Advanced Camera Settings > Mode > select 'mode')  
(EPU: Preparation > Camera Settings > Binning > select value)



**'Mode'** controls the binning behaviour of EPU

**'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 EPU.*

**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 EPU.*

**Example 3:** 'Counted' mode **with** '1' times binning

*The K3 will collect in its default super resolution format and transfer super resolution movies to EPU, but 2-fold binning will be performed by EPU to obtain standard resolution movies.*

*As in example 1 the outcome is standard resolution movies however binning in EPU is slower than binning on the camera).*

☐ Check all other parameters are correct

### 3.3.2 (Falcon IVi instructions) Checking and adjusting your Data Acquisition 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)

*(EPU: Preparation > Preset Selection > Presets > 'Data Acquisition' > 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'.

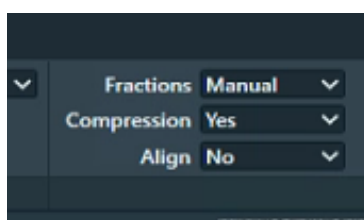
**'EER'**: Electron Registration Mode - If you select **'EER'**, your dose fraction output will be in EER format. In EER mode, the camera records the coordinates for the individual electrons that hit the camera. This may be useful for some experiments where you want to have fine control over how your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

*(EPU: Preparation > Preset Selection > Presets > 'Data Acquisition' > 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

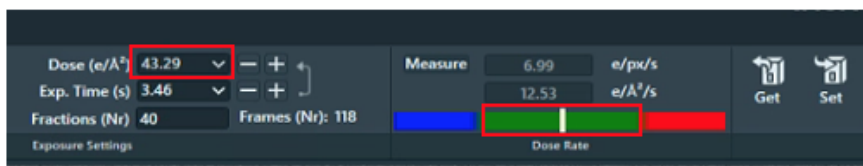
Your Local Contact will have set your Dose parameters for you over vacuum before handing over but you should double check that these values work for your sample.

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)

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

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



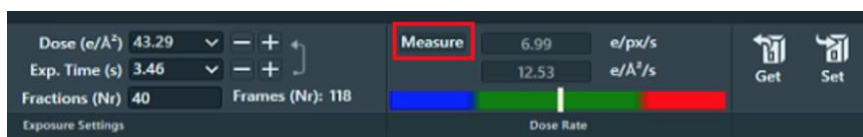
- ☐ Navigate to an area over your sample
- ☐ Ensure that the arrow between 'Dose (e/A²)' and 'Exp. Time' is pointing **away from** 'Exp. Time', **towards** 'Dose (e/A²)' (if not, press the arrow to reverse the direction – this ensures that the Exp. Time your local contact has set over vacuum does not 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

(EPU: Preparation > Preset Selection > Presets > 'Data Acquisition' > Dose Rate > press 'Measure')



- ☐ Check that the 'Dose (e/A²)' 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 (**autofocus**, **drift measurement** and **thion ring**) should be 'linked' to your **data acquisition** magnification settings but you should double check the rest of the settings for these presets are appropriate for your experiment.

For the high magnification presets, some parameters that are predictable that you might reasonably expect are:

Preset	Magnification	Exposure (s)	Probe Mode	C2	Defocus (µm)
Data Acquisition	Set by Local Contact as per request	Dose dependent	Nano	50	- 3.0 (Sample dependent)
Autofocus	Copy of Data Acquisition	~ 1.0	Nano	50	- 3.0
Zero Loss	Copy of Data Acquisition	~ 1.0	Nano	50	- 3.0
Thon Ring	Copy of Data Acquisition	~ 2.0	Nano	50	- 1.0 to - 2.0

The following screenshots further indicate how you may expect to set your high magnification presets (these images are taken on a K3 so if you are using a Falcon IVi the 'Camera Settings' will appear different):

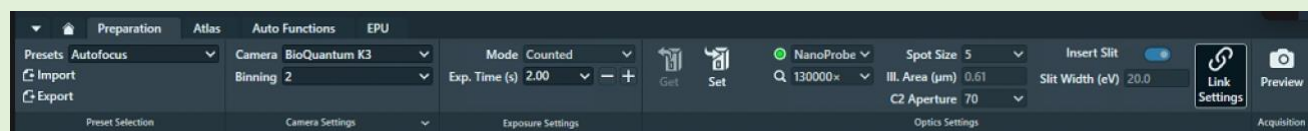
**Note:** the sections 'Preset Selection', 'Camera Settings', 'Advanced Camera Settings' and 'Optics Settings' sections. Optics Settings, in particular, will be microscope and session dependent.

**Note:** Do not change the Optics Settings for the high magnification presets except when indicated in this guide. The high magnification Optics Settings will have been optimised by your local contact to match your target magnification and the camera optimal dose rate.

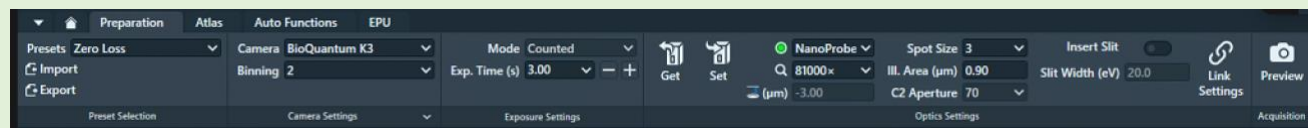
### Data Acquisition



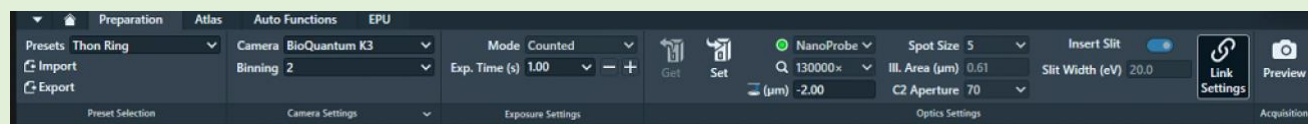
### Autofocus



### Zero Loss



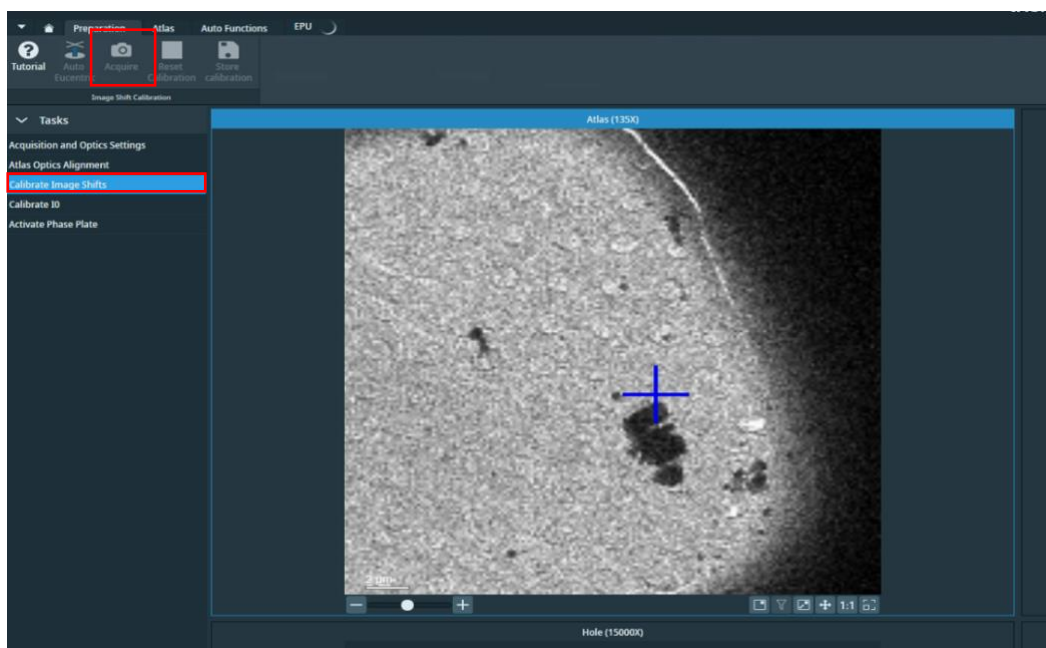
### Thon Ring



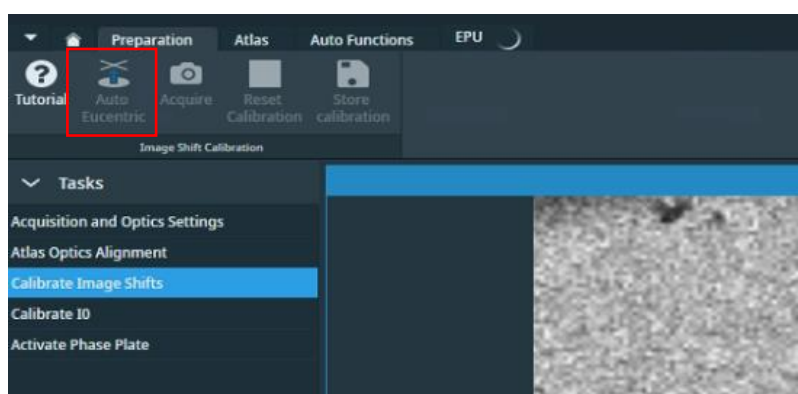
## 3.5 Image shift calibrations

### 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)  
(EPU: right click on image and 'Move stage here')
- ☐ Go to Image shift calibration and acquire an image at Atlas magnification  
(EPU: Preparation > Tasks > Calibrate Image Shifts > press 'Acquire')



- ☐ Perform Auto Eucentric Function over carbon  
(EPU: Preparation > Tasks > Calibrate Image Shifts > press 'Auto Eucentric')



### 3.5.2 Target a feature for Image Shift calibrations and track it through the magnifications

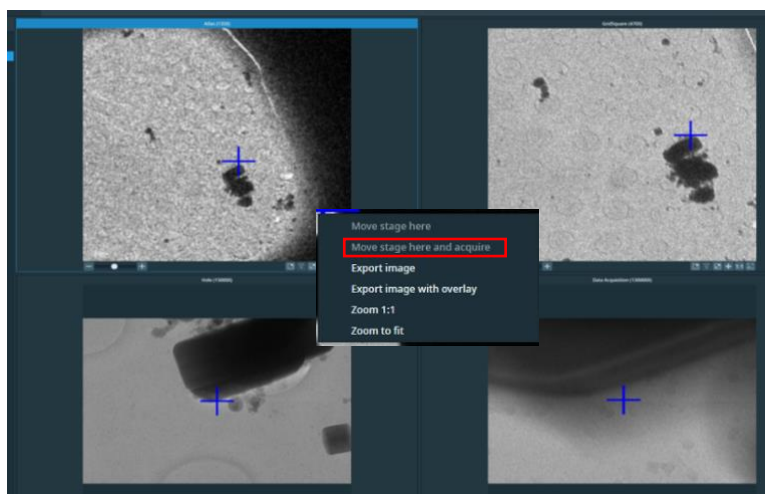
Prior to performing Image Shift Calibrations we systematically target a feature at low and up to high magnification. This will ensure that your feature is identifiable at all magnifications, provide indication of whether the Image Shift Calibrations need to be performed and importantly, centre a target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

☐ Find an identifiable feature which will be recognisable at all magnification presets (try to choose something which is fairly central)

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

☐ Starting on the top left quadrant (atlas magnification), centre the stage on your recognisable feature of choice in each quadrant of the Image shift calibration window:

- 1) Atlas magnification (top left quadrant)  
(EPU: Right click on your feature > Move stage here and acquire)
- 2) GridSquare magnification (top right quadrant)  
(EPU: Right click on feature > Move stage here and acquire)
- 3) Hole magnification (bottom left quadrant)  
(EPU: Right click on feature > Move stage here and acquire)
- 4) Data Acquisition magnification (bottom right quadrant)  
(EPU: Right click on feature > Move stage here and acquire)



There is a 180° rotation between the 'Gridsquare' and 'Hole/Eucentric' magnification presets on some K3 systems. You may find it helpful to take a photo on your phone and manually rotate this to orientate yourself.

If you have any difficulties or you are struggling to find your feature seek assistance from your Local Contact.

#### **A note if using HexAuFoil grids**

The image shift calibrations need to be absolutely spot on. Worth doing a couple of iterations. Hole eucentric and data acquisition need to be perfect.



### 3.5.3 Calibrate the Image Shifts

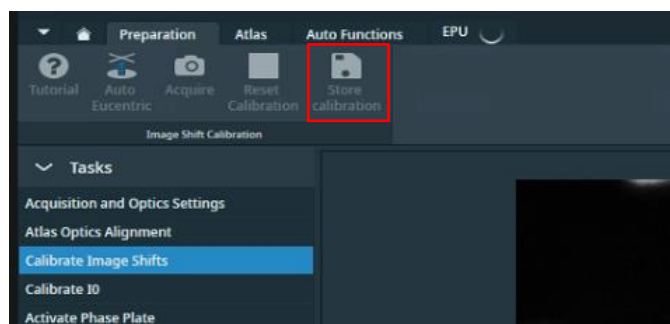
During image shift calibrations you will realigning the position of the stage to the same point on your target feature at each magnification.

- ☐ When all four quadrants are centred on the same recognisable feature use the left mouse button to reposition the blue cross (stage position) to the same point on the feature in each quadrant

*(EPU: Double left click on feature > the blue cross should move)*

- ☐ When you are happy that the blue cross is in the same position in all four quadrants press 'Store Calibration'

*(EPU: Preparation > Tasks > Calibrate Image Shifts > press 'Store Calibration')*





## 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, particle densities etc., now is an appropriate time to perform some quick screening.

There are several strategies, we will show you how to set up an atlas session and manually acquire images of different areas. These images can be saved for your own record keeping, as will be described in this section.

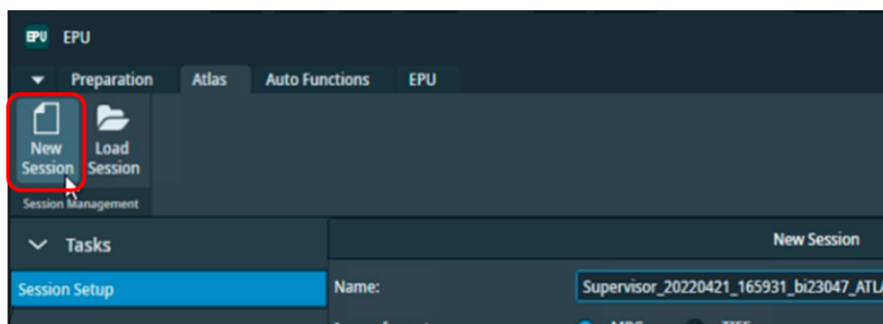
You will need to later manually configure your data collection if you wish to avoid re-exposing screened areas.

### 4.1 Atlas session setup and collection

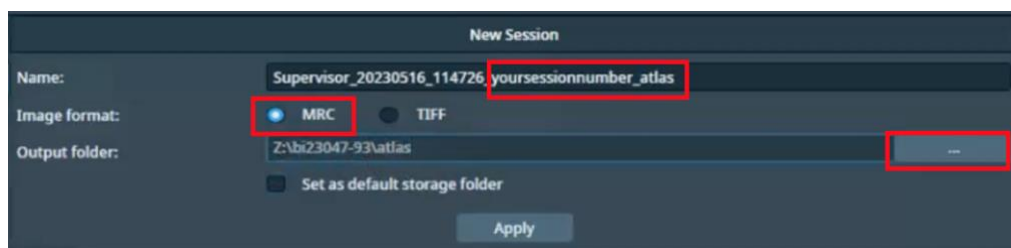
#### Atlas Session setup:

- ☐ Start a new session for taking an Atlas

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



- ☐ Setup the new session parameters



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

**Naming:** Supervisor\_YYYYMMDD\_XXXXXX\_visit-ID\_ATLAS

**Image format:** MRC

**Output folder:** Z:\[your session ID, i.e., biXXXXXX]\atlas

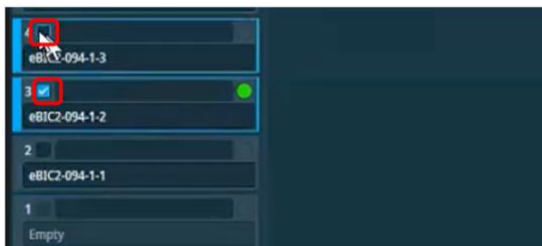
**Note:** If you do not see a folder with your session ID and atlas in the Z:\ drive (i.e. :Z\biXXXXXX/atlas) ask your LC to initiate 'murfey' before you set up your Atlas session

- ☐ Press 'Apply'

### Atlas collection:

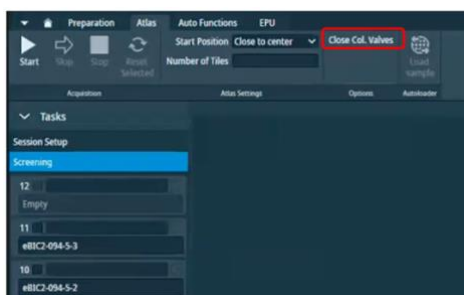
- ☐ Select which grids you'd like to acquire an atlas of

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



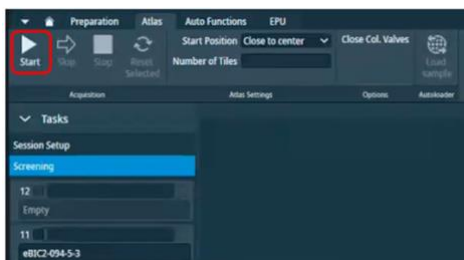
- ☐ Select 'close the column valves' to ensure EPU closes column valves when the Atlas is complete

*(EPU: Atlas > Screening > Close Col. Valves)*



- ☐ Press 'Start' to start acquiring atlases of your selected grids.

*(EPU: Atlas > Screening > Start)*

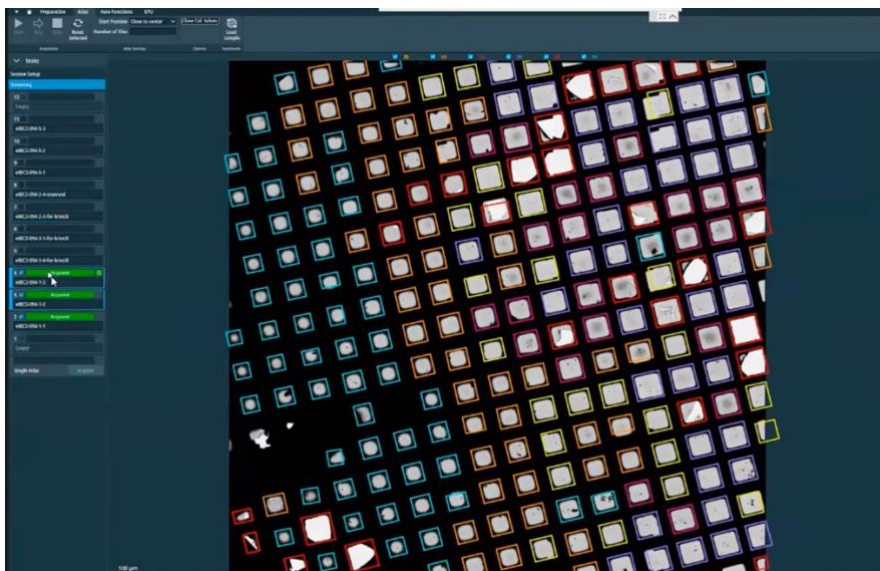


## 4.2 Navigation and targeting

### 4.2.1 Inspect your atlases and navigate to a square you want to screen using the Atlas

- ☐ Click on each atlas in turn to view the overall ice distribution on each grid

(EPU: Atlas > Screening > select grid to view acquired atlas)



**A note on atlas montage 'stitching':** On each Atlas, EPU 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 will not affect navigation or data collection.

**A note on square colouring:** EPU will colour squares according to ice thickness (*i.e.*, it will colour 'similar' squares purple or yellow, or orange etc.). You may find this helpful or you may not. If you do find this helpful it is important to note that the colourings between grids from different atlas sessions may not be the same (*i.e.*, purple may correspond to thin ice in one session and thick ice on another).

If you don't find this helpful you can turn the square colouring off using the tick boxes at the top of the atlas image.

**A note on selecting coloured squares for data collection:** Any colours that are 'ticked' will be automatically applied during your square selection in EPU. You may find this useful if you want to target certain ice thicknesses during your data collection.

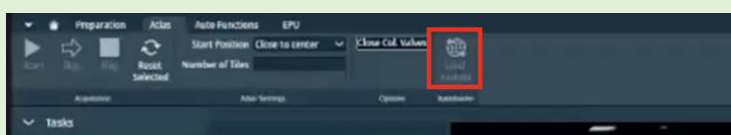
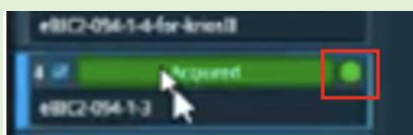
- ☐ Choose a grid to screen

(EPU: Atlas > Screening > select grid to view acquired atlas and load if required)

**A note on loading grids in EPU:** the grid currently loaded on the stage will be indicated by a green circle in EPU.

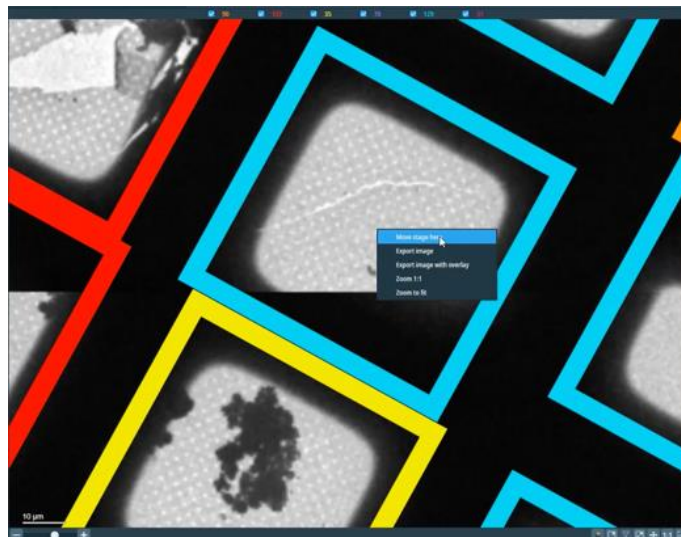
If you want to load a different grid to screen you must load this through EPU to ensure any shifts and rotations that occur to the grid during loading and unloading are measured and accounted for:

(EPU: Atlas > Autoloader > press 'Load sample' and wait a few minutes).



- ☐ Navigate to a square in your Atlas

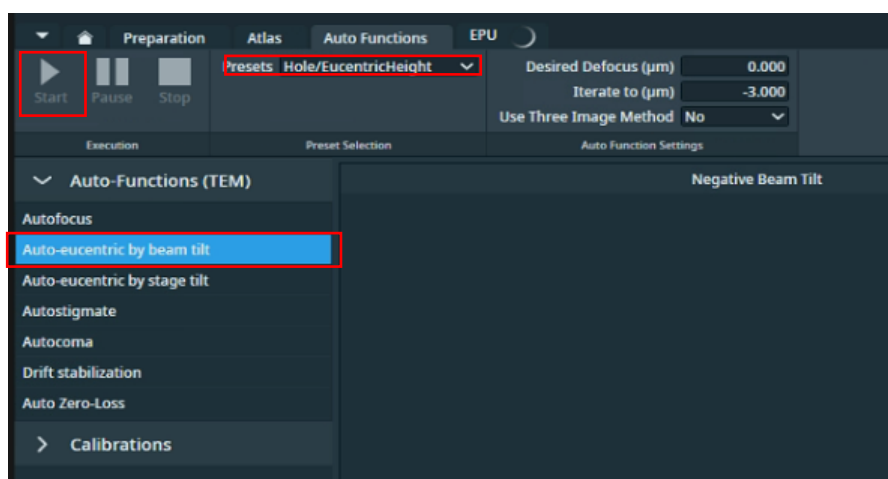
(EPU: Atlas > Right-click > Move stage here)



#### 4.2.2 Ensure you are at eucentric height

Each time you navigate to a new grid square, you must ensure that the stage is at Eucentric Height

- ☐ Navigate to centre of your selected grid square
- ☐ Take an image of the area using the 'GridSquare' magnification preset  
(EPU: Preparation > Acquisition and Optics Settings > select the 'GridSquare' preset > press 'Preview')
- ☐ Recentre the square
- ☐ Set the stage to Eucentric height using the **autoeucentric by beam tilt routine**:
  - 1) Select Auto Eucentric by beam tilt in the autofunctions tab  
(EPU: Autofunctions > Auto Eucentric by beam tilt)
  - 2) Set to 'Hole Eucentric' magnification preset in autofunctions  
(EPU: Autofunctions > Preset 'Hole/Eucentric Height')
  - 3) Press 'Start'



**A note on monitoring eucentric height progress:** Keep an eye on the Status window in EPU (right hand side) and look for decreasing Z values. On the EPU view you should see matching images on the left and a sharp 'peak'

**Notes on troubleshooting if EPU fails to find eucentric height:**

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

- 1) Try again with a smaller stage tilt of 5°, then if this succeeds try again with 15°
- 2) Try changing the 'GridSquare' preset to a smaller defocus offset and/or a shorter exposure time
- 3) Try Auto Eucentric by beam tilt at hole/eucentric magnification instead

*(EPU: Auto Functions > Auto-eucentric by stage tilt > Presets: Grid Square > press 'Start')*

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

## 4.3 Manual image acquisition using Presets

### 4.3.1 Grid Square image and positioning

- ☐ Select the GridSquare preset and acquire a Preview

*(EPU: Preparation > Acquisition and Optics Settings > select the 'GridSquare' preset > press 'Preview')*

- ☐ Move to an area of carbon

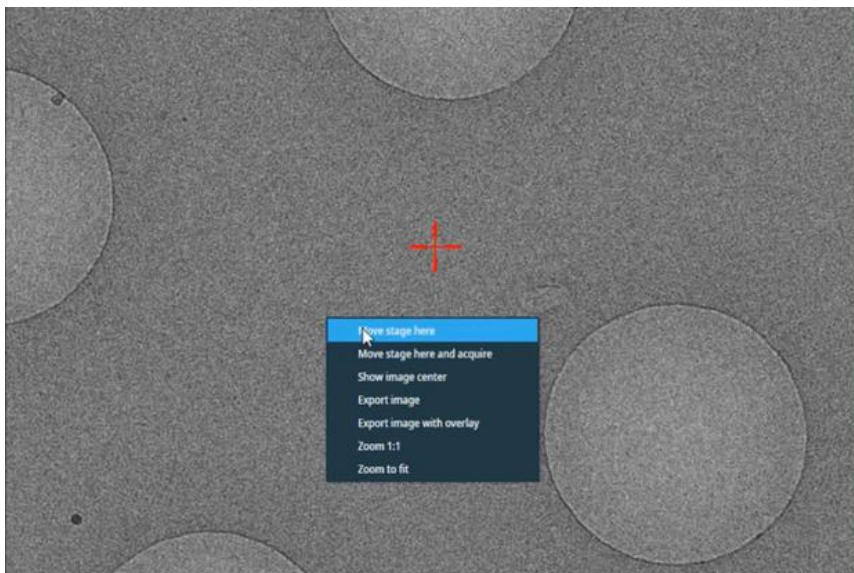
*(EPU: Right click > 'move stage here')*

- ☐ Select the Hole/Eucentric Height preset and acquire a Preview

*(EPU: Preparation > Acquisition and Optics Settings > select the 'Hole/EucentricHeight' preset > press 'Preview')*

- ☐ Recentre on an area of carbon

*(EPU: Right click > 'move stage here')*



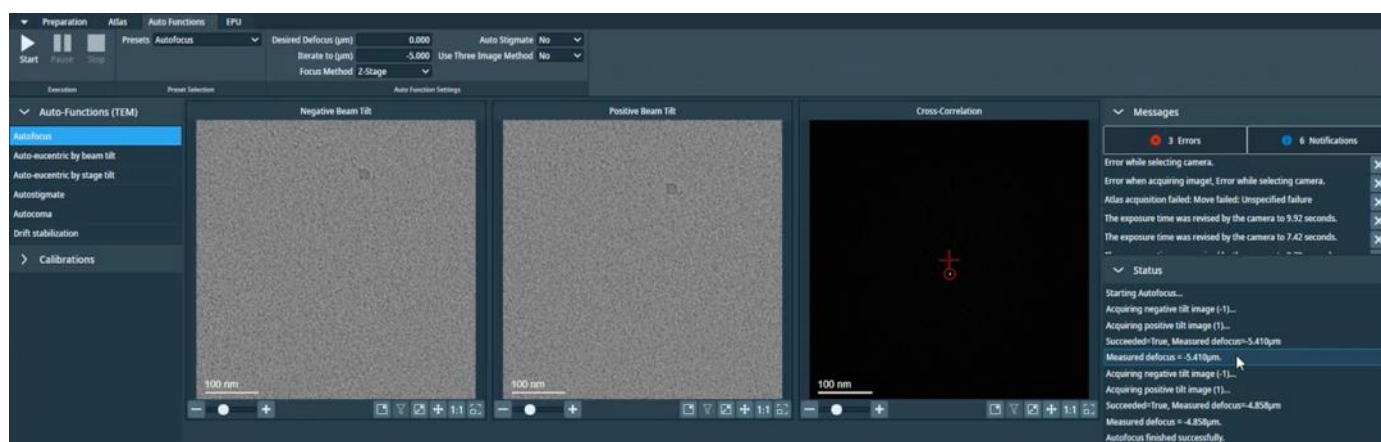


### 4.3.2 Autofocus

☐ Run the **autofocus routine** on carbon

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

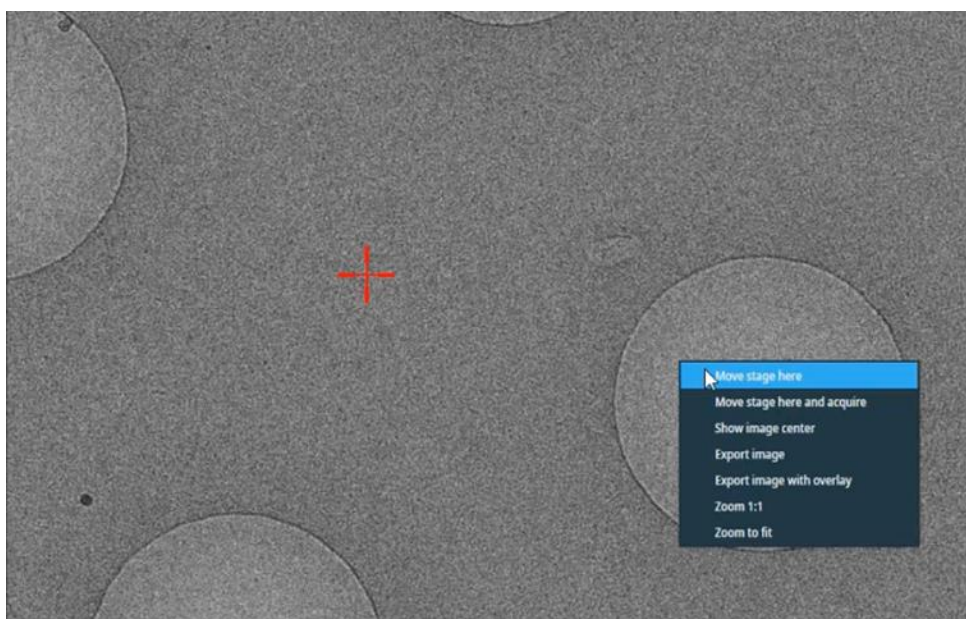
**Note:** Keep an eye on the Status window in EPU (right hand side) and look for a sharp 'peak' on the right



### 4.3.3 Acquire a screening image at Data Acquisition

☐ Return to the Hole/Eucentric Height preset and reposition the stage on a target area in a hole

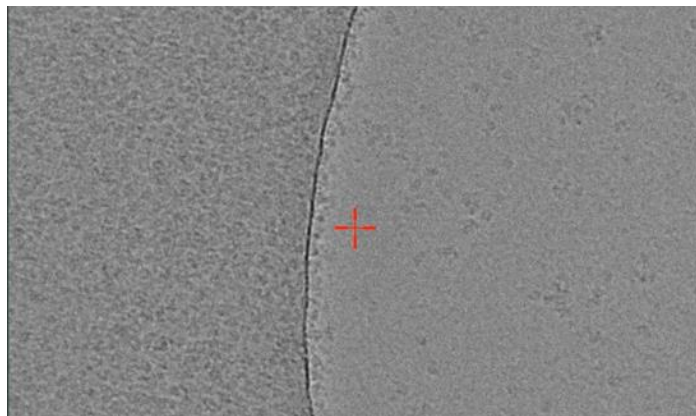
(EPU: Preparation > Acquisition and Optics Settings > select the 'Hole/EucentricHeight' preset > press 'Preview')



☐ Take an image at Data Acquisition preset



(EPU: Preparation > Acquisition and Optics Settings > select the 'Data Acquisition' preset > press 'Preview')



#### 4.3.4 Document your findings

Images are **not** automatically saved from this procedure so you may wish to save your images manually.

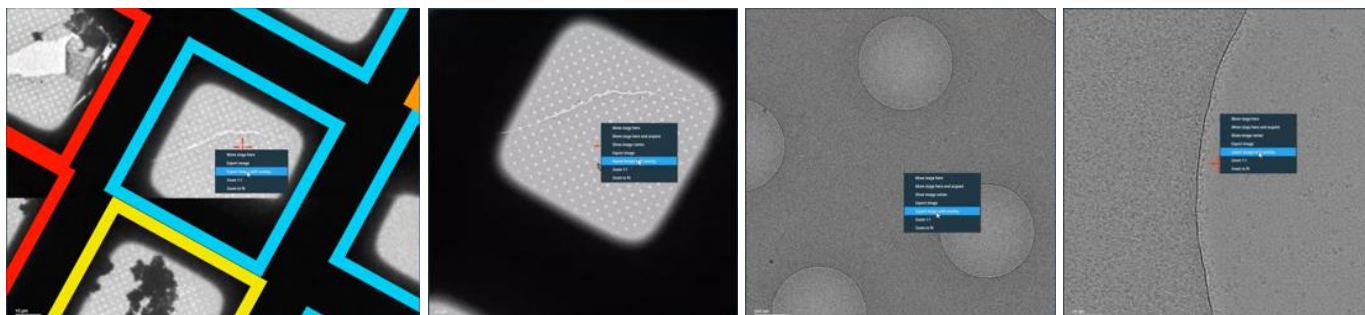
- ☐ Select one of the preset magnifications

(EPU: Preparation > Acquisition and Optics Settings > select a preset)

Unless you press 'Preview' again, EPU will display the last image that was acquired using the selected magnification preset

- ☐ Export the image with overlay

(EPU: Right click on image > press 'Export image with overlay')



- ☐ Save the image to an appropriate area in your session directory

**A note on where to save images and naming:** Ensure you are definitely in the right directory (i.e., Z:/[your session ID]) and ensure your naming convention makes sense to you and provides detail about the location of the image on the grid

E.g.,     Grid1\_Square1\_Hole1\_DA  
            Grid1\_Square1\_Hole1\_HE  
            Grid1\_Square1\_Hole1\_GS  
            Grid1\_Square1\_Atlas

## 5 EPU session setup

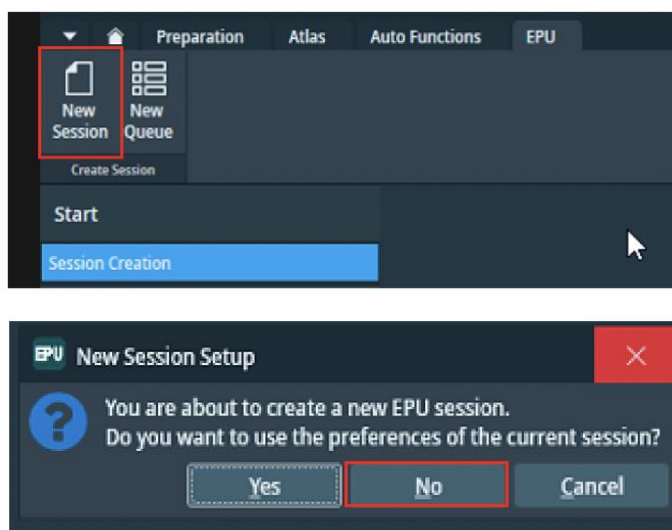
The following method covers setup for a single EPU session, for the EPU multigrid option (to queue multiple EPU sessions) see **section 6: The EPU multigrid option**

In this guide we will demonstrate a linear workflow on a quantifoil grid however if you have a different type of grid (e.g., Lacey, HexAuFoil etc.) seek advice from your LC.

### 5.1 Session Setup

- ☐ Set up a new EPU Session

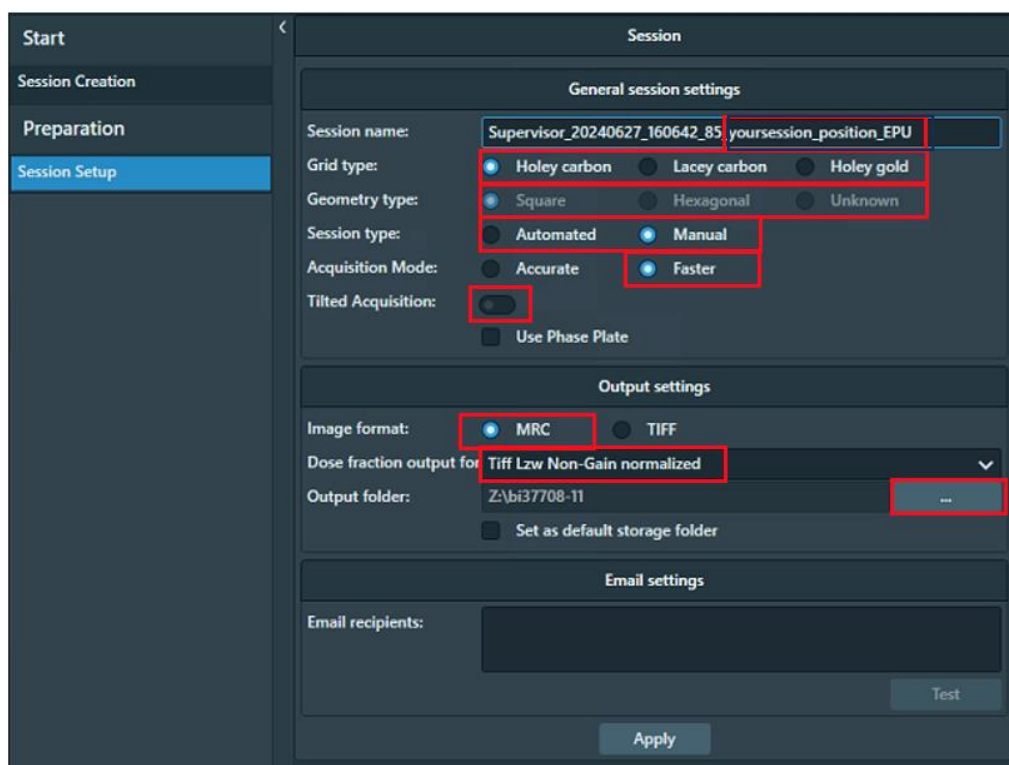
(EPU: EPU > New session > Session Setup)



#### **A note on using 'current session' preferences**

When you set up a new session, EPU will ask you if you want to use parameters from the current EPU session (this includes hole sizes, ice filters, image formats etc. as well as any **mistakes** made by the **last user** during setup). **DO NOT** use settings from a previous session unless you are 100 % sure that the parameters used were correct and also apply to your own experiment (i.e., if the previous session was your own).

- ☐ Setup the new session parameters



<b>Naming:</b>	Supervisor_YYYYMMDD_XXXXXX_visit-ID_positionX_EPU
<b>Grid type:</b>	Holey Carbon / Lacey / Holey Gold
<b>Geometry type:</b>	Square / Hexagonal / Unknown
<b>Session type:</b>	Automated (automatically detects suitable areas) / Manual (suitable areas hand picked)
<b>Acquisition Mode:</b>	Faster (AFIS on all Krioses)
<b>Tilted Acquisition:</b>	This option is available from EPU 3.8 onwards. If you tick this you will get the option to specify a tilt during Hole Selection
<b>Image format:</b>	MRC ( <b>Note:</b> These are the Atlas, GridSquare, Hole and Data images stored in the EPU 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 EPU 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 'murphy' before you set up your Atlas session)

☐ Click 'Apply'

## 5.2 Setting up a representative square and hole targeting

Square selection, hole finding and hole filtering can be done entirely manually however we usually employ a semi-automated method in which a single representative square is set up to establish your hole finding and ice filter conditions, you may then proceed to add more squares and utilise an automated 'Prepare all squares' function in EPU which will tell EPU to process all selected squares, find eucentric height for each square, find holes and apply your refined ice filter.

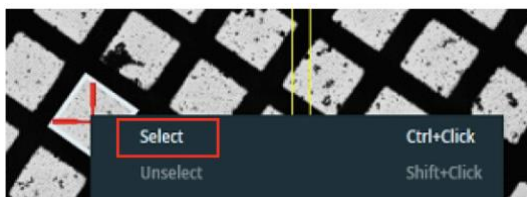
For particularly challenging samples, or where the sample is not flat, you may choose to repeat this manual process for all of your squares (i.e., repeat **section 5.2.1** for all squares) however this is quite time consuming.

### 5.2.1 Square selection

**A note on square selection by colouring/ice thickness:** Any colours for gridsquares that were 'ticked' in your Atlas session will be automatically applied during your square selection in EPU. To remove these, go to the top left of the screen and press 'unselect all'.

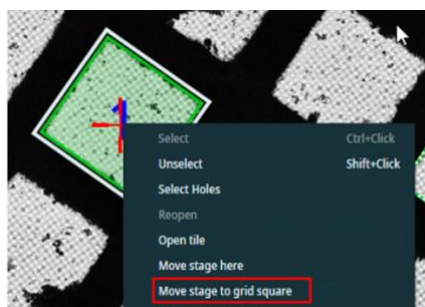
- ☐ Select a square in the Atlas for setting up acquisition targets

(EPU: EPU > Preparation > Square Selection > Right-click on square and press 'Select')



- ☐ Navigate to the selected gridsquare

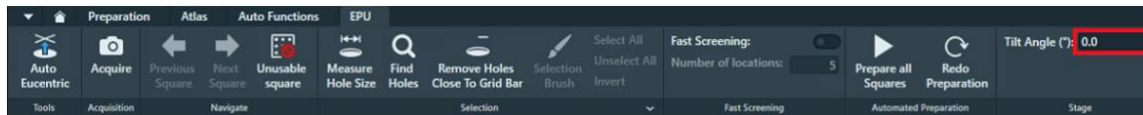
(EPU: EPU > Preparation > Square Selection > Right-click on square and press 'Move stage to grid square')



## 5.2.2 Hole selection (for holey carbon grids)

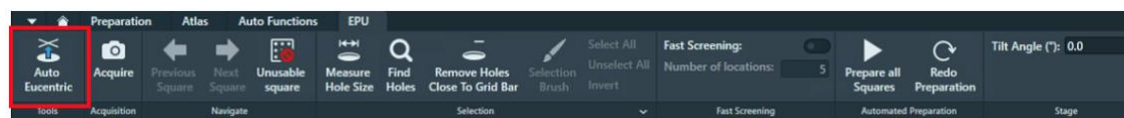
### ☐ (Option: If doing a tilted data collection)

Specify a tilt angle for the grid if you wish to perform a tilted data collection



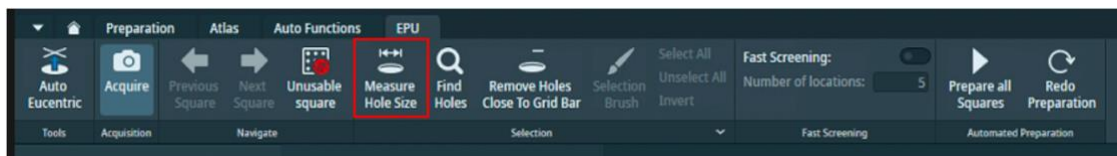
### ☐ Set eucentric height for the selected gridsquare

(EPU: EPU > Preparation > Hole Selection > press 'Auto Eucentric')

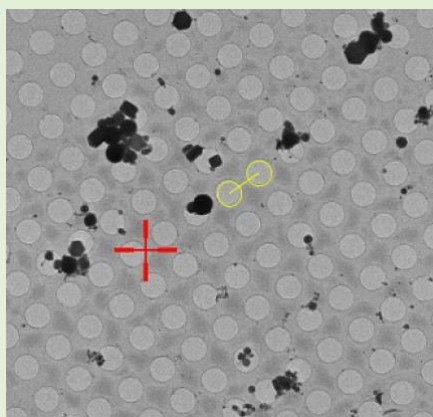


### ☐ Following auto eucentric an image of the gridsquare should be acquired. Measure hole size on the resultant image

(EPU: EPU > Preparation > Hole Selection > press 'Measure Hole Size')

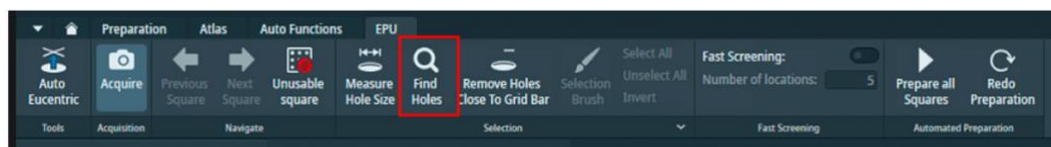


**A note on measuring hole size:** Left-click on the yellow hole outline circles to reposition them and resize around two adjacent holes. You only need to do this on the first square you set up.



### ☐ Find holes

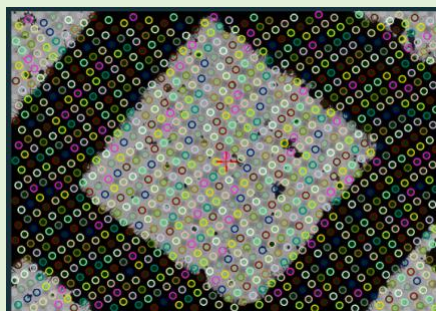
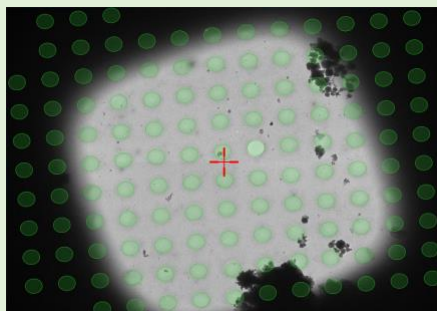
(EPU: EPU > Preparation > Hole Selection > press 'Find Holes')



**A note on hole finding:** Inspect quality of hole targeting using middle-scroll to zoom, left-click + drag to navigate.



**A note on hole colouring:** Ordinarily selected holes will appear green however on some of our Krioses selected holes are coloured by AFIS clusters (rainbow coloured) these will initially be coloured at random but once data collection session is started clusters will be assigned.



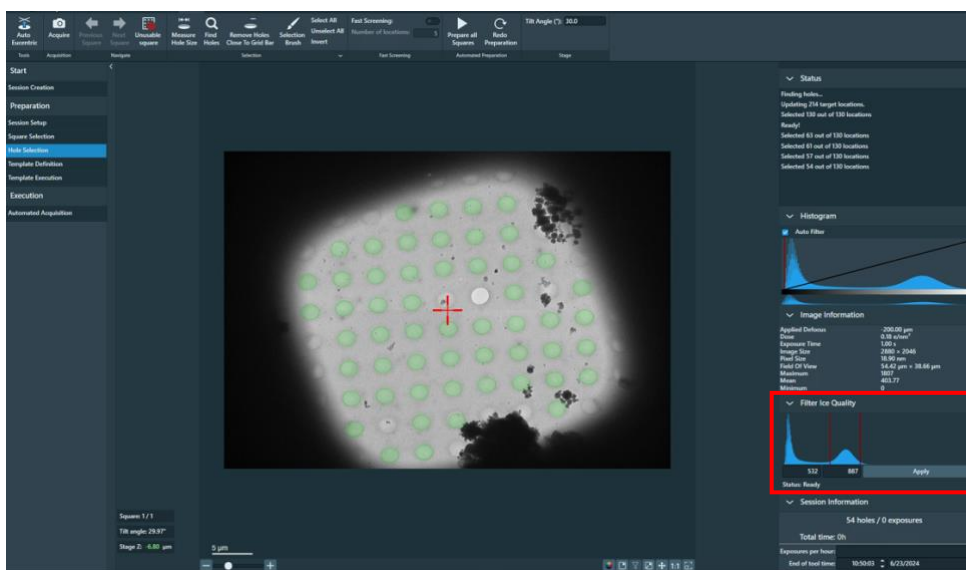
### A note if using HexAuFoil grids

The hexagons need to be flat. Try not to attempt a set up on a hexagon with a crack in it.

## 5.2.3 Filter the hole selection

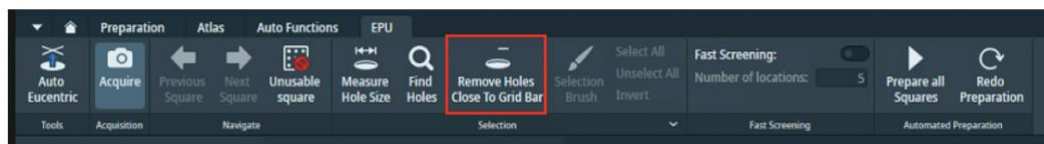
- ☐ Adjust the ice filter to remove contaminated holes, dry holes and grid bars

(EPU: EPU > Preparation > Hole selection > 'Filter Ice Quality' (right hand side of EPU window, bottom Histogram))

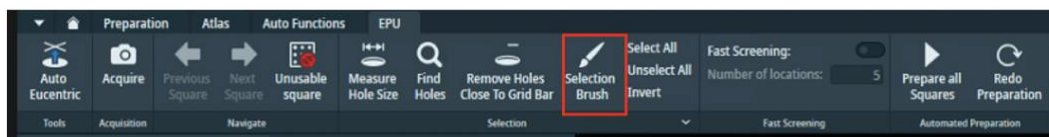


- ☐ Remove holes close to grid bar (optional)

(EPU: EPU > Preparation > Hole Selection > select 'Remove Holes Close to Grid Bar' (optional))



- ☐ Use the Selection Brush to edit the hole targets on the square



**Selection brush controls:**

**Left-click** to remove

**ctrl+left-click** to add

**shift+middle-scroll** to adjust brush size



### 5.3 Additional square selection and hole target set up (manual)

If proceeding manually, repeat **section 5.2** for each square you wish to set up then proceed to **section 5.5** to set up a template for data collection. If proceeding automatically, continue on to **section 5.4**

## 5.4 Additional square selection and hole target set up (automatic)

If you wish to proceed automatically, we strongly recommend that users select and prepare a small subset of ~ 5 squares initially to ensure that automatic square preparation is functioning as expected (i.e., finding the correct z-height consistently etc.). After ensuring that the 'prepare all squares' tool is functioning as expected on a subset of squares, you may continue adding additional squares. Users may stop data collection to add more targets as required during their session.

### 5.4.1 Square selection

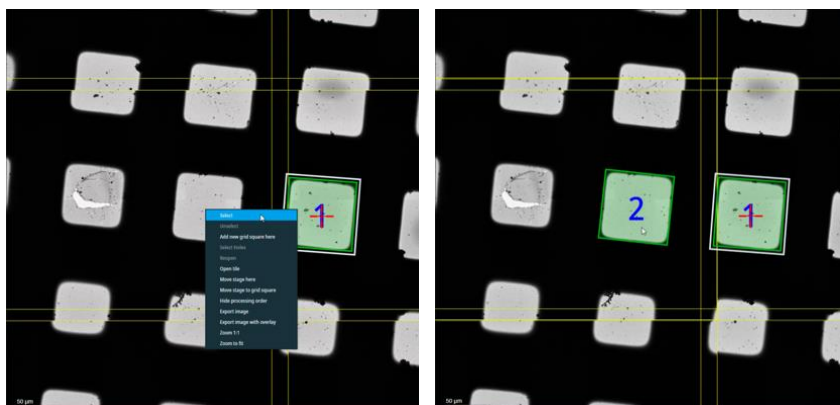
- ☐ Navigate to a new square you would like to collect on

(EPU: EPU > Preparation > Square Selection)

Right-click on a grid square in the image and press 'Move stage to grid square'

- ☐ Add the square to your selected squares

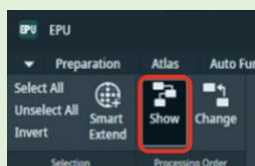
Right-click on the grid square and press 'Select'



**A note on the order of collection:** To minimise any issues EPU may encounter with refining eucentric height for your selected squares, try to ensure that the order of collection minimises large stage movements (i.e., squares are ordered close together, not on opposite sides of the grid).

Check the 'Show' box to label selected squares in the order they will be acquired.

(EPU: EPU > Preparation > Square Selection > Processing order > 'Show')

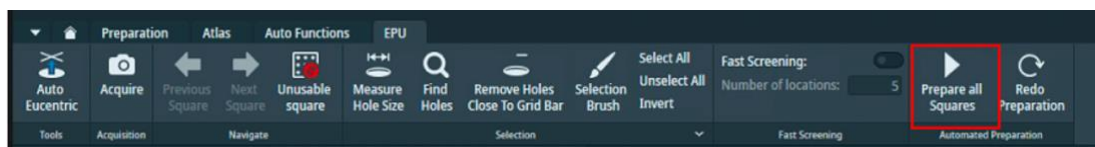


You can activate the 'Change' button to reorder squares if required (clicking on a square will move that to the top of the collection order)

### 5.4.2 Automated square preparation

- ☐ Continue adding squares as in 5.4.1 until you reach an appropriate number of squares
- ☐ 'Prepare all Squares' using the hole filtering you set up in section 5.2.3. EPU will attempt to find eucentric height, 'Find Holes' and apply the Ice Filter to automatically set up hole targets

(EPU: EPU > Preparation > Hole Selection > Automated Preparation > 'Prepare all Squares')

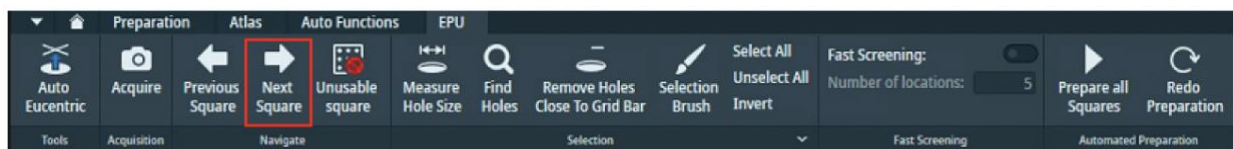


**A note on Z-heights:** You should keep an eye on the 'z-height' displayed in the bottom left corner for each square during square preparation and ensure this is consistent with what you would expect in different areas of the grid. If the eucentric height appears to vary significantly from square to square speak to your LC

- ☐ Inspect the quality of the automated square preparation

Switch between the squares to inspect the quality of the hole target setup and ensure that the z-height for each square is as expected (z-height displayed in bottom left of image)

(EPU: EPU > Preparation > Hole Selection > Automated Preparation > Navigate > 'Next Square')



- ☐ Refine hole target selection as required using the Selection Brush as described in section 5.2.2

## 5.5 Template definition

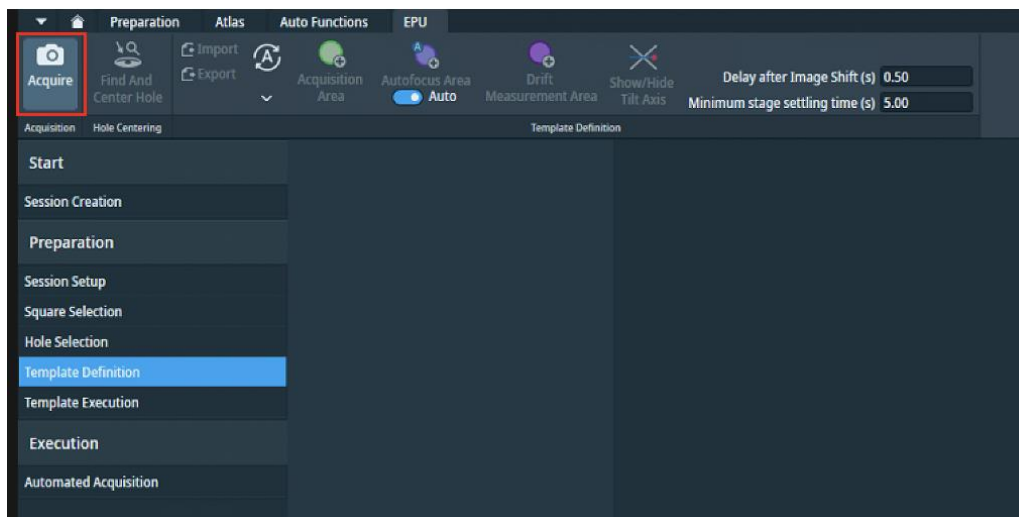
After you have set up a representative square (**section 5.2**) and prepared more squares either manually following **section 5.3** or automatically following **section 5.4**, you can set up a template for your data acquisition areas.

- ☐ Move to a hole on your gridsquare

(EPU: EPU > Hole Selection > Right-click on a hole > 'Move stage to location')

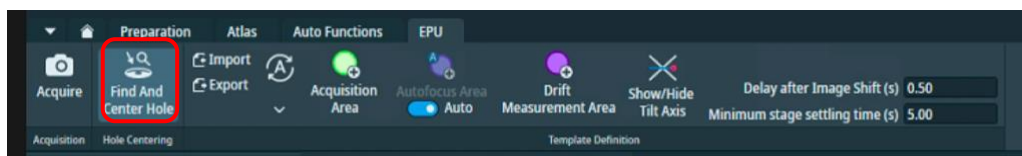
- ☐ Acquire an image of the hole

(EPU: EPU > Template Definition > Acquisition > press 'Acquire')



- ☐ Centre the hole in the field of view

(EPU: EPU > Template Definition > Hole Centring > Find and Center Hole)



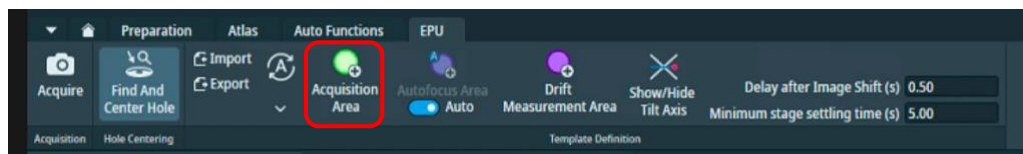
- ☐ After you have done 'Find and Center Hole' an image of the hole should be acquired.

**A note on checking hole centring accuracy:** You should examine the Hole centring accuracy. The hole targeted for template definition is expected to be found after 'Find and Center Hole'. You may want to check on several holes with identifiable features to confirm consistent behaviour.

### 5.5.1 Acquisition area template

- ☐ Add Acquisition Area(s) on the resultant image and position them where you want on the hole

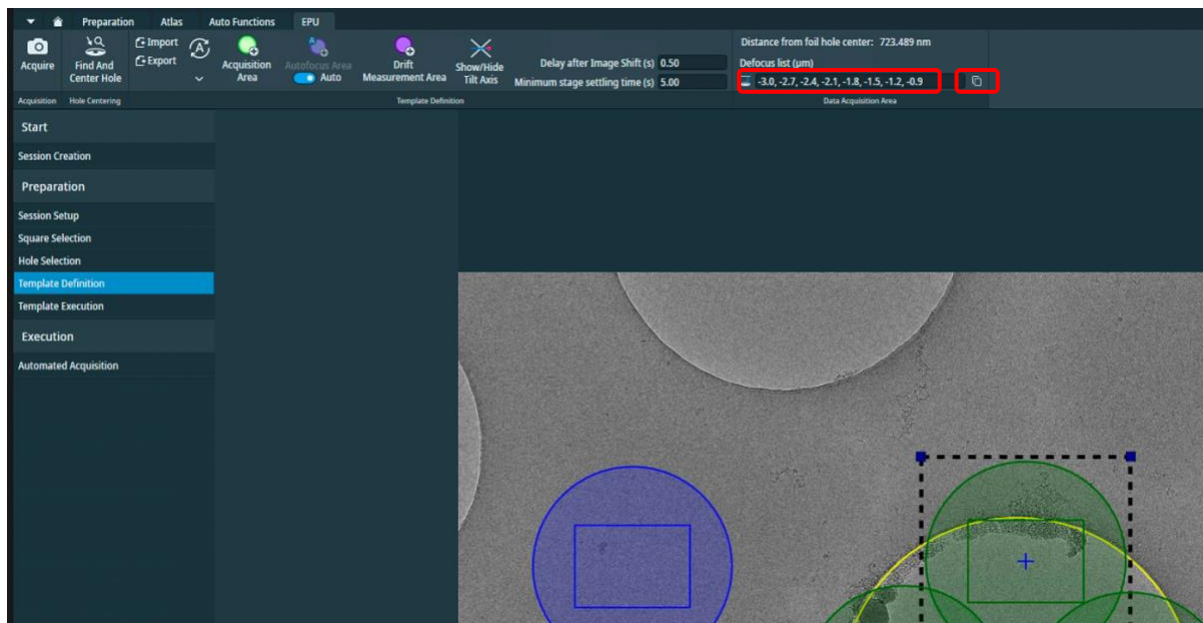
(EPU : EPU > Template Definition > Template Definition > press 'Add Acquisition Area')



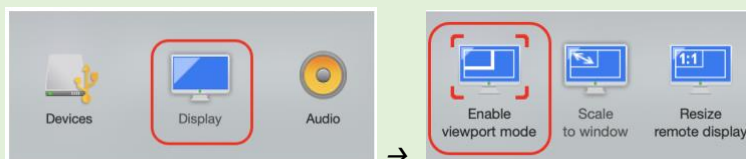
**A note on positioning acquisition areas:** Left-click to position the acquisition area and drag to reposition. For multishot strategies you may overlap the beam areas (circle) a little but **do not** overlap beam with the image acquisition area (rectangle)

- ☐ Select the Acquisition area and enter a defocus series you want to use

(EPU: EPU > Template Definition > Data Acquisition Area > Defocus list ( $\mu\text{m}$ ) > enter a list and press the 'copy' button to copy to all acquisition areas)

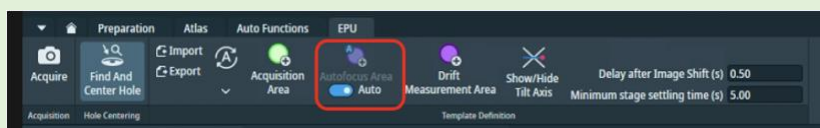


**A note if your struggling to see the values:** You must ensure all of your values are negative, if you are struggling to see the text open the NoMachine settings (Mac: cntrl + shift +0) (Windows: ctrl + alt +0), open the display settings and go to viewport mode to zoom in.



## 5.5.2 Autofocus area template

**A note on automatic AutoFocus areas:** You may wish to enable automatic autofocussing.  
(EPU : EPU > Template Definition > Template Definition > tick 'Auto' under 'AutoFocus Area')



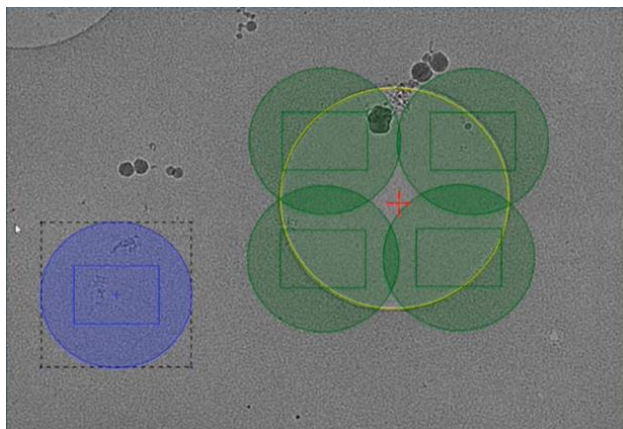
- ☐ Add an autofocus area

(EPU : EPU > Template Definition > Template Definition > press 'AutoFocus Area')

- ☐ Position the autofocus area on carbon near the hole

(EPU: Left-click+drag to reposition)





- ☐ Select the AutoFocus area and enter a focussing regime

(EPU: EPU > Template Definition > AutoFocus Area > select parameter for 'Recurrence')

**A note on AutoFocus Recurrence:**

Always

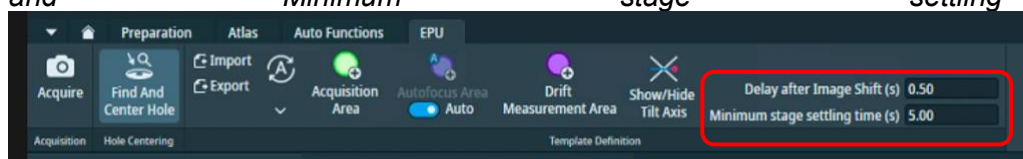
After Centering (suggested when using Faster Acquisition - see 5.1)

Never

After Distance (suggested when using Accurate Hole Centering - see 5.1)

- ☐ Select the AutoFocus area and setup settling times for stage and image shift (suggested)

(EPU: EPU > Template Definition > Template Definition > add parameter for 'Delay after Image Shift' and 'Minimum stage settling time')



**A note on AutoFocus settling times:**

Delay after Image shift (s): 0.50 – 1.00

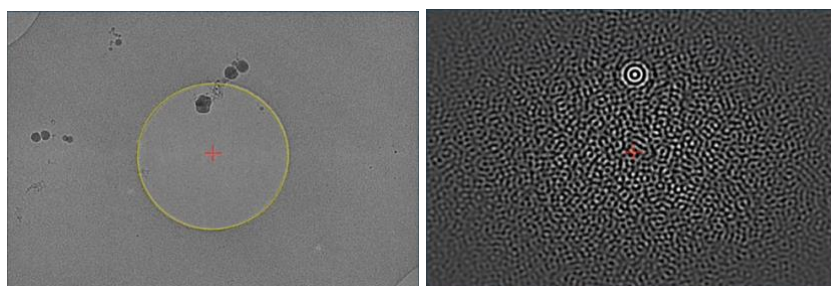
Minimum stage settling time (s): 3.00 – 5.00

### 5.5.3 Template execution

This will run a trial automated acquisition on the hole that is currently centred on the stage, you can check for the success of hole centring, autofocus and acquisition areas. Note that the first defocus value in the defocus series setup will be used. This function can also be helpful if you need to test whether your template definition is targeting the best area of the hole for your particle distribution.

- ☐ Execute the template and inspect the image and status report to assess template execution

(EPU: EPU > Template Execution > 'Start')







☐ You may proceed to section 7 to perform final checks and begin data collection

## 6 The EPU multigrid option

The multigrid functionality of EPU allows you to set up a queue of multiple EPU sessions and then start data acquisition for the entire queue. If the queue involves multiple grids then changing from one autoloader position to another will be performed automatically.

In this guide we will demonstrate a linear workflow on a quantifoil grid however if you have a different type of grid (e.g., Lacey, HexAuFoil etc.) seek advice from your LC.

**A note on prerequisites:** This section assumes a familiarity with EPU session setup (**section 5**) for some specific operations but also troubleshooting. Please ask your Local Contact if you should require assistance.

### 6.1 Preparation

Please keep in mind that EPU multigrid is a relatively new feature to the EPU software.

Further considerations on eBIC systems are that:

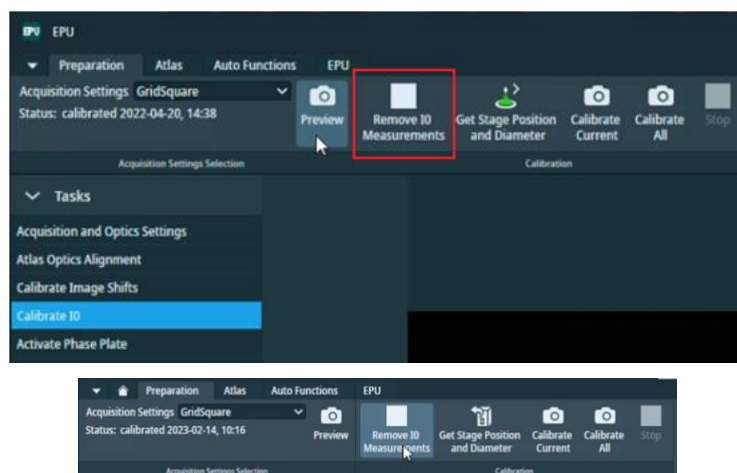
- 1) A single DataAcquisition magnification must be used for all grids in the Queue
  - a. As for all eBIC visits, the DataAcquisition magnification is set by your Local Contact
- 2) Different grid types may be used but it might help to group Sessions by their grid type
- 3) The I/O calibration must be removed
- 4) We strongly advise to monitor the data collection, particularly during grid changing
  - a. To check the grid translation and rotation calibration is successful on grid loading
  - b. To check automatic on-the-fly GridSquare set up is functioning correctly

☐ Perform all tasks in **section 3** (Microscope setup, preset checks and image shift calibrations) and **section 4** (Screening) as required

#### 6.1.1 Remove I0 calibrations

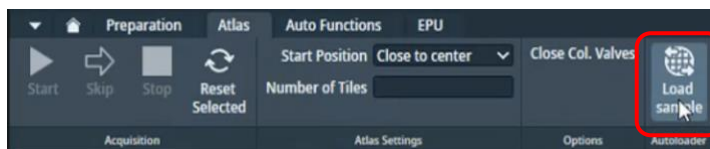
☐ Remove the I0 calibrations

*(EPU: Preparation > Calibrate I0 > press 'Remove the I0 Measurements')*



### 6.1.2 Load a grid and adjust presets

- ☐ Ensure the grid you want to set up has been loaded onto the microscope stage from within the Atlas tab



- ☐ Ensure that the Status window reports a sample displacement has been measured



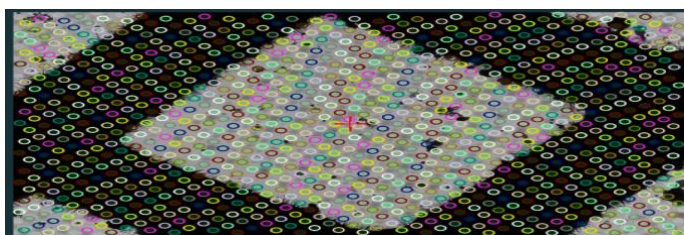
- ☐ Adjust your Presets if required as in **section 3.2**

Bear in mind that if you change your imaging presets substantially at any point you will need to repeat image shift calibration (**section 3.5**) and reacquire your atlas

### 6.1.3 Creating a new EPU queue

- ☐ Create a new multigrid queue under the EPU tab

(EPU: EPU > Create Session > New Queue)



**A note on using 'current session' preferences:** When you set up a new session or queue, EPU will ask you if you want to use parameters from the current EPU session (this includes hole sizes, ice filters, image formats etc. as well as any **mistakes** made by the **last user** during setup).

**Press 'No'** - If you are setting up a new queue **DO NOT** use settings from a previous session unless you are 100 % sure that the parameters used were correct and also apply to your own experiment (i.e., if the previous session was your own).

**Press 'Yes'** – If you are setting up a new session in the same queue, you may wish to keep the current parameters

☐ Proceed to Manual (section 6.2) or Automatic (section 6.3) queuing of data collection sessions

## 6.2 Manual queuing of grids

This approach follows the concept of loading a grid from inside EPU, adding the first grid to the Queue as a Session and establishing session parameters on it. Subsequent grids are also loaded onto the stage from inside EPU and added to the Queue as Sessions, and each may be set up individually, thus allowing a unique set of session parameters to be established per grid. Unique session parameters have been successfully tested at eBIC for the following:

- 1) Hole/Eucentric preset magnification and defocus
- 2) Template definition (shot number, pattern and defocus series)
- 3) Ice filter user defined boundaries
- 4) Data Acquisition energy filter slit width (eV) settings

However, due to the requirement for Local Contact intervention for magnification dependent energy filter tuning, only a single DataAcquisition magnification may be used.

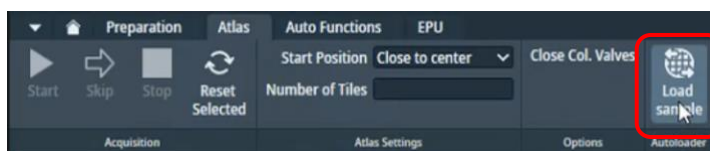
Each grid loaded onto the stage and added to the queue can have a custom set up. However, note that any subsequent grid left unmodified in the Queue will use the session parameters of the most recently collected grid. Therefore, you may choose to set up the first grid of each grid type manually on and automatically queuing (**section 6.3**) the rest of the same type, (i.e. all 1.2/1.3 first and then all 2/2 grids).

**Important note on folder destinations:** For each session in a queue, you must ensure that all settings have been selected correctly, in particular note the Output has been directed to **your** session folder and not the default

### 6.2.1 Set up the first grid

#### 6.2.1.1 Grid loading

- ☐ Ensure the grid you want to set up has been loaded onto the microscope stage from within the Atlas tab



- ☐ Ensure that the Status window reports a sample displacement has been measured

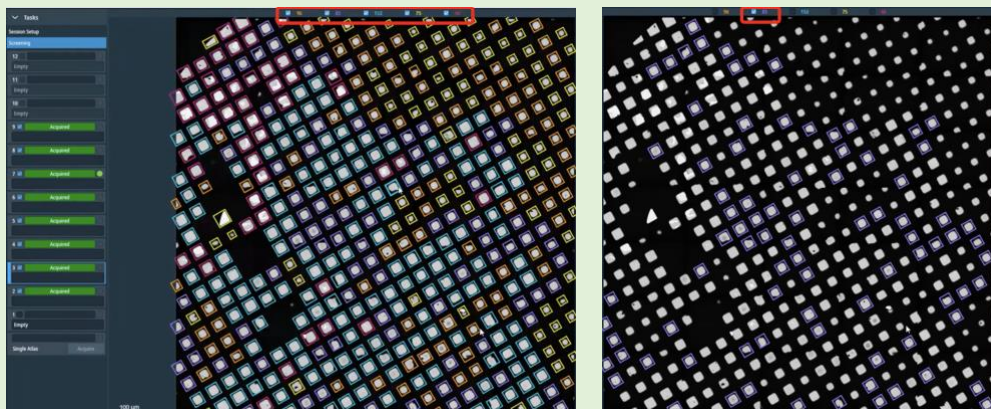


### 6.2.1.2 Initial square selection and session setup

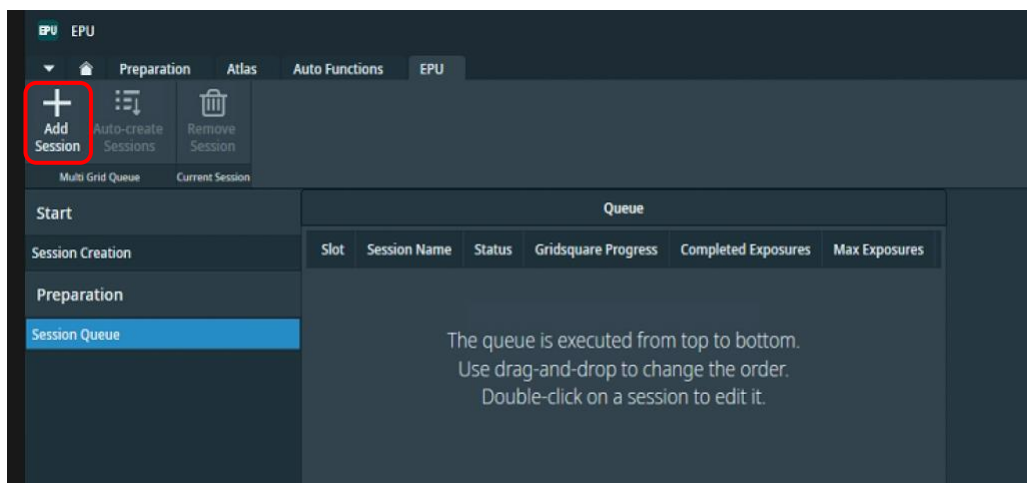
- ☐ Adjust the square selection under the Atlas tab
- ☐ Leave only the Grid Squares you wish to collect on selected

**A note on square selection by colouring/ice thickness:** Any colours for gridsquares that are 'ticked' in your Atlas session will be automatically applied during your square selection in EPU. Before you begin you may wish to adjust this in the Atlas tab and select only the gridsquares with ice thickness you would like to collect on.

(EPU: Atlas > Screening > select grid to view acquired atlas)

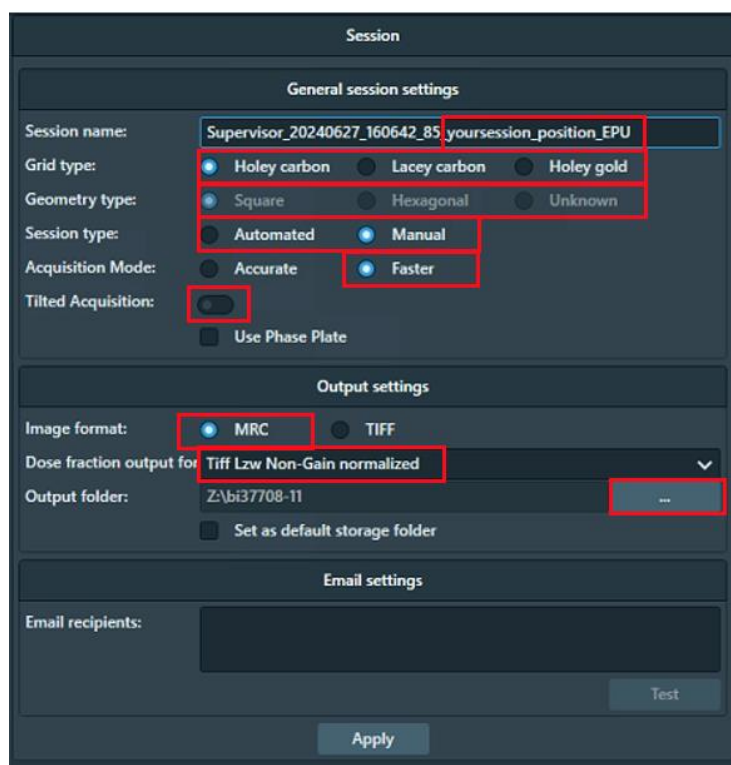


- ☐ Click 'Add Session' under the EPU tab to queue a new Session for the grid currently on the stage



- ☐ Set up the Session parameters





**Naming:** Supervisor\_YYYYMMDD\_XXXXXX\_visit-ID\_positionX\_EPU  
**Grid type:** Holey Carbon / Lacey / Holey Gold  
**Geometry type:** Square / Hexagonal / Unknown  
**Session type:** Automated (only option in multi EPU)  
**Acquisition Mode:** Faster (AFIS on all Krioses)

**Tilted Acquisition:** This option is available from EPU 3.8 onwards. If you tick this you will get the option to specify a tilt during Hole Selection

**Image format:** MRC  
**(Note:** These are the Atlas, GridSquare, Hole and Data images stored in the EPU folder. Using MRC is recommended to retain the extended MRC header information)

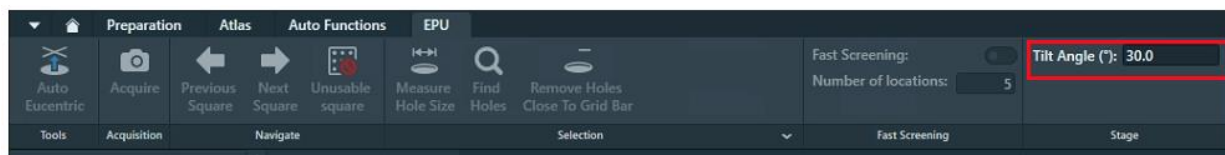
**Dose Fraction output (K3 only):** Tiff LZW non-gain normalised  
**(Note:** These are the raw movie stacks from the detector. Using Tiff LZW non-gain normalized is required by eBIC. Please note that if EPU crashes this will revert back to .mrc format so **always double check** 'Tiff LZW Non-Gain normalized' is selected)

**(Note:** on a Falcon IVi the Dose fraction output is set in the 'Data Acquisition' Preset – refer to **section 3.3.2.1** for full details)

**Output folder:** Z:\your session ID, i.e., biXXXXXX]  
**(Note:** Press the ... to navigate to your session ID folder. If you do not see a folder with your session

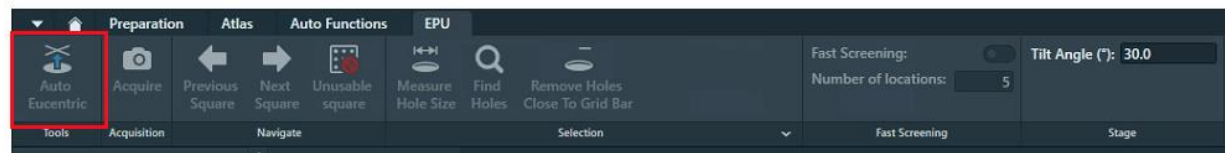
☐ Click 'Apply', your session will appear in the queue on the left





- ☐ Set eucentric height for the selected gridsquare

(EPU: EPU > Preparation > Hole Selection > press 'Auto Eucentric')



- ☐ Measure hole size, find holes and apply filtering as in section 5.2

**A note on ice filtering for multigrid:** The ice filter boundaries will be remembered for each session in the Queue, you should adjust for each queued session as required.

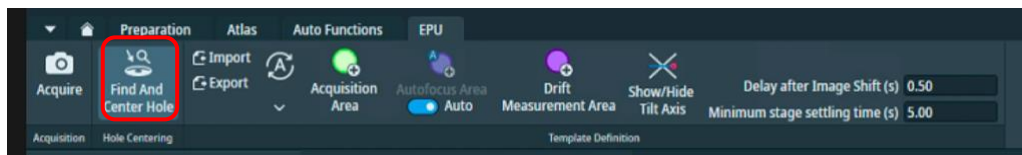
#### 6.2.1.4 Template definition

- ☐ Move to a hole

(EPU: EPU > Hole Selection > Right-click on a hole > 'Move stage to location')

- ☐ Centre the hole in the field of view

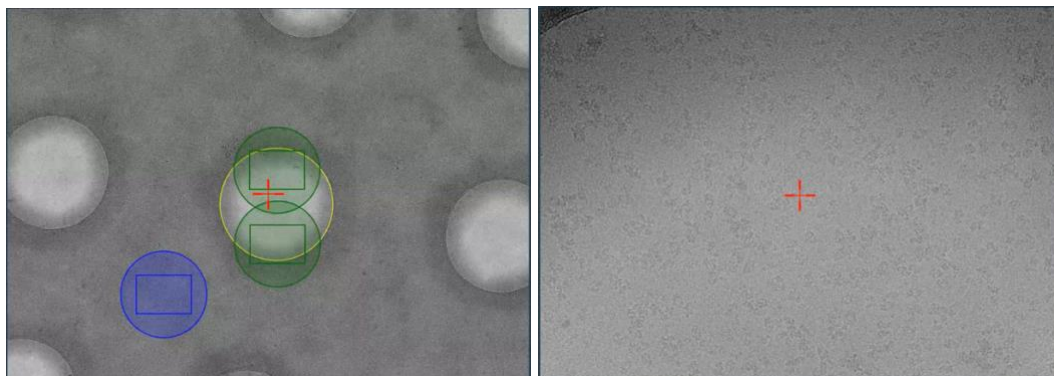
(EPU: EPU > Template Definition > Hole Centring > Find and Center Hole)



**A note on checking hole centring accuracy:** You should examine the Hole centring accuracy. The hole targeted for template definition is expected to be found after 'Find and Center Hole'. You may want to check on several holes with identifiable features to confirm consistent behaviour.

- ☐ Adjust your template, defocus strategy, and autofocus behaviour as in section 5.5

- ☐ Run a Template Execution as in section 5.5.3 to test your set up, remember this routine will run wherever the stage is currently positioned

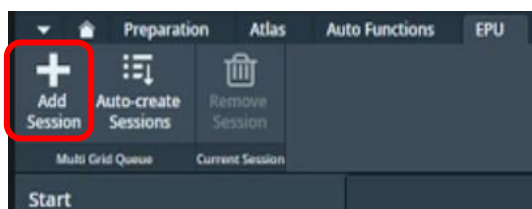


### 6.2.1.5 Finalise

**A note on automatic square set up:** It is only necessary to set up Grid Squares to test the square targeting, hole finding and filtering and the template execution. You may set up additional squares if you wish to further test the sessions behaviour but note that every square (including any manually set up squares) will still be set up again on-the-fly (auto-eucentric by beam tilt and hole finding) during data collection.

### 6.2.2 Load a subsequent grid

- ☐ Load the next grid onto the stage
- ☐ Ensure that the Status window reports a sample displacement has been measured
- ☐ Once on the stage, add the grid to the Queue manually by pressing Add Session under the EPU tab



### 6.2.3 Set up targeting and other custom Session parameters

- ☐ You may check whether the Presets are appropriate for the newly loaded grid as in **section 3.2**  
Remember to check your image shift calibrations if you change any Presets, as in **section 3.5**
- ☐ Set up the rest of the Session as in **sections 6.2.1.1– 6.2.1.3**
- ☐ You may adjust the ice filter (**section 6.2.1.3**) to be suitable to the currently loaded grid
- ☐ You may customise your template as in **section 6.2.1.4**

### 6.2.4 Repeat for all grids

- ☐ You may repeat these steps individually for each grid you want to collect on
- ☐ When all grids have been added, you may proceed to **section 7** to perform final checks and begin data collection

Remember in this workflow, since each grid is loaded onto the stage and added manually to the Queue, there is opportunity to appropriately customise session and preset parameters. If you are collecting on groups of similar grid types, you may set up the first grid manually as in this section and subsequent similar grid types automatically without screening their Session settings. Then for the next grid type, load and set up the first of the set manually.

Note: EPU will use the session setup for all subsequent grids in the Queue until it reaches a grid with a different session setup. Remember, every square (including any manually set up squares) will still be set up on-the-fly (auto-eucentric by beam tilt and hole finding) during data collection.

## 6.3 Automatic queuing of grids

This approach follows the concept of loading the first grid from inside EPU, adding to the Queue as a Session, establishing session parameters that will be used for this master grid and then all subsequent grids. Subsequent grids are added as Sessions to the Queue automatically and are set up on-the-fly. Where each subsequent grid in the queue is left unmodified it will use the master defined presets and template.

### 6.3.1 Set up a master grid

- ☐ Set up the grid you wish to use as the master grid as in **section 6.2.1**

These settings will be used by all subsequent grids in the Queue

- ☐ You may adjust the ice filter (**section 6.2.1.3**) but we recommend leaving lots of room in the boundaries to accommodate intensity changes in subsequent grids



- ☐ Set up your template as in **section 6.2.1.4**

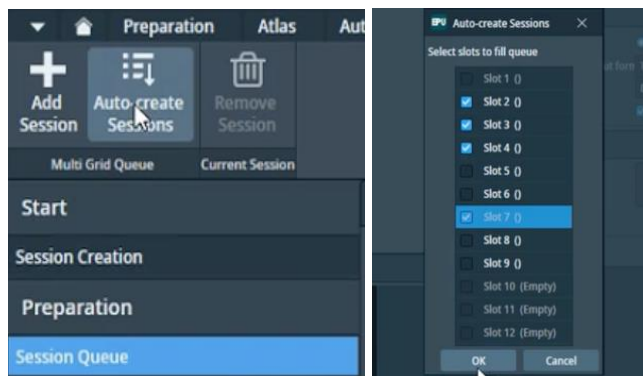
**Important not on carrying parameters over for automated queueing:** all parameters (including the ice filter settings and hole template) will be carried over onto subsequent grids. In particular, when using the automatic set up approach we recommend setting the ice filter boundaries conservatively since it may change from grid to grid.



### 6.3.2 Auto create additional Sessions for subsequent grids

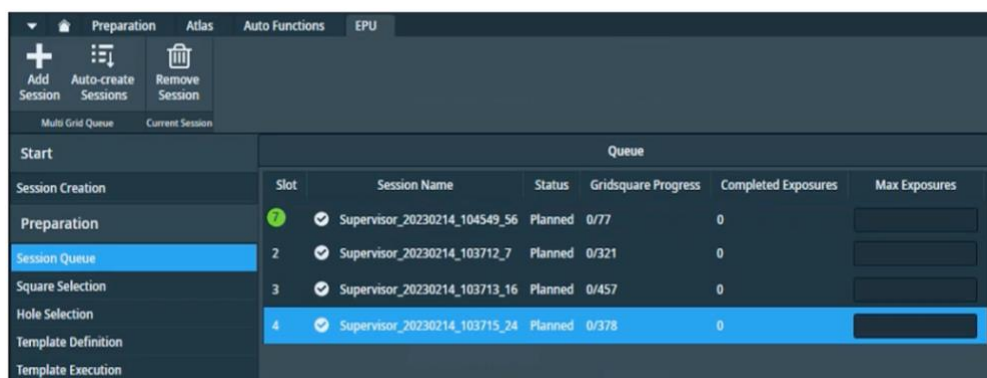
☐ If you are satisfied with the set up and that it will apply to all subsequent grids you may select Auto-create Sessions

☐ Select the additional grids you wish to collect data on



☐ Note that if no square selection is made in the atlas, all available Grid Squares will be queued for collection

☐ You may modify the targeted squares under the Square Selection tab and/or enter Max Exposure values for each grid



☐ You may proceed to **section 7** to perform final checks and begin data collection

Remember in this workflow, additional grids are not yet loaded onto the stage and so you will only be able to explore the grids from the available Atlas under the Square Selection task.

Note: EPU will use the entire session setup for all subsequent grids in the Queue. Remember, every square (including any manually set up squares) will still be set up on-the-fly (auto-eucentric by beam tilt and hole finding) during data collection.



## 7 Final checks and data collection

### 7.1 Auto Functions and alignment

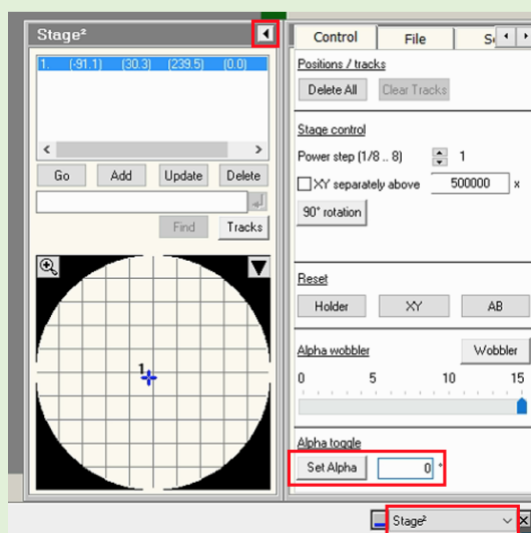
#### 7.1.1 Alignments via Autofunctions

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 beam tilt' in the autofunctions tab  
(EPU: Autofunctions > AutoEucentric by beam tilt)
- 2) Set to 'Hole EucentricHeight' magnification preset **in autofunctions**  
(EPU: Autofunctions > Preset 'Hole EucentricHeight')
- 3) Press 'Start'

#### Step 2: AutoFocus

- 1) Select 'AutoFocus' in the autofunctions tab

(EPU: Autofunctions > AutoFocus)

- 2) Set to 'AutoFocus' magnification preset **in autofunctions**

(EPU: Autofunctions > Preset 'AutoFocus')

- 3) Press 'Start'

### Step 3: Autostigmatize

- 1) Select 'Autostigmatize' in the autofunctions tab

(EPU: Autofunctions > Autostigmatize)

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

(EPU: 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

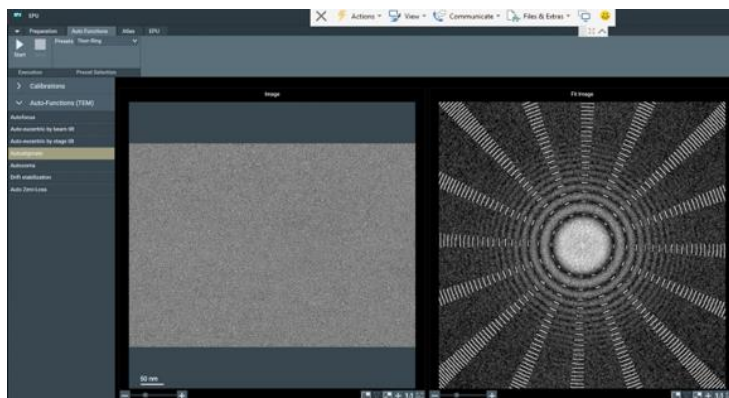
(EPU: Autofunctions > Autocoma)

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

(EPU: Autofunctions > Preset 'Thon ring')

- 3) Press 'Start'

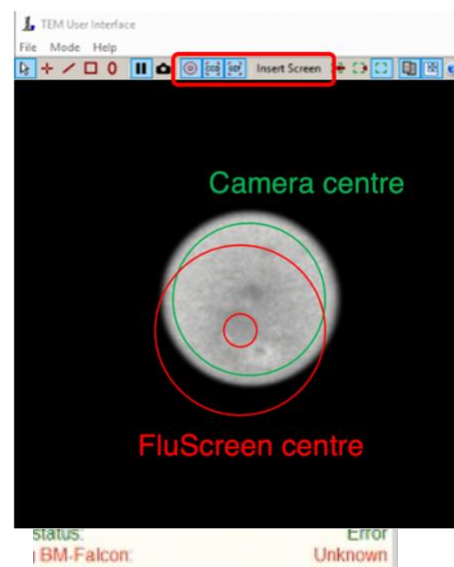
**Note:** The coma would typically be less than 200 nm on eBIC Krioses



### 7.1.2 Beam centring

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 7.1.1.

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



Eucentric Focus

Multifunction X/Y

### 7.1.3 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 'Beam Shift' from the Direct Alignments  
(TUI: Direct alignments (bottom right drop down) > Beam Shift)
  - 2) Use the **multifunction X/Y** on the simulator pads to centre the beam
  - 3) Click 'Done' on the Direct Alignment panel
- ☐ Repeat the Autostigmat Auto Function on a carbon area

#### Autostigmat

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

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

#### 7.1.4 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 'Data Acquisition' preset)

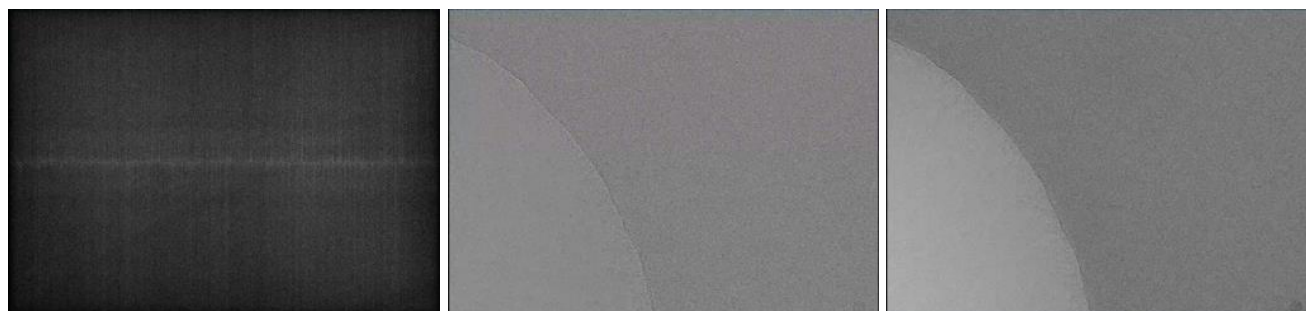
Note whether the ZLP preset uses a larger spot size (lower number) than the 'Data Acquisition' preset or the ZLP may fail.



☐ Perform Auto Zero-Loss Auto Function

#### Auto Zero-Loss

- 1) Select 'Auto Zero-Loss' in the autofunctions tab  
(EPU: Autofunctions > Auto Zero-Loss)
- 2) Set to 'Zero Loss' magnification preset in autofunctions  
(EPU: Autofunctions > Preset 'Zero Loss')
- 3) Press 'Start'



☐ Inspect the Status window for reporting the operation was Successful

## 7.2 Start automated acquisition

### 7.2.1 Double check your data acquisition parameters

- ☐ Go to Data Acquisition preset and check your exposure time is consistent with your dose calculation (refer to **section 3.3**)

(EPU: Preparation > Preset Selection > Presets > 'Data Acquisition')



- ☐ Go to your template definition and check your defocus list (check numbers all negative and definitely copied to all acquisition areas)

(EPU: EPU > Template definition > Left-click on your acquisition area then check Defocus List)



- ☐ Go to your template definition and check your stage and image shift wait/dwell times for autofocus are correct

(EPU: EPU > Template definition > Left-click on your autofocus area then check image shift wait/dwell times)

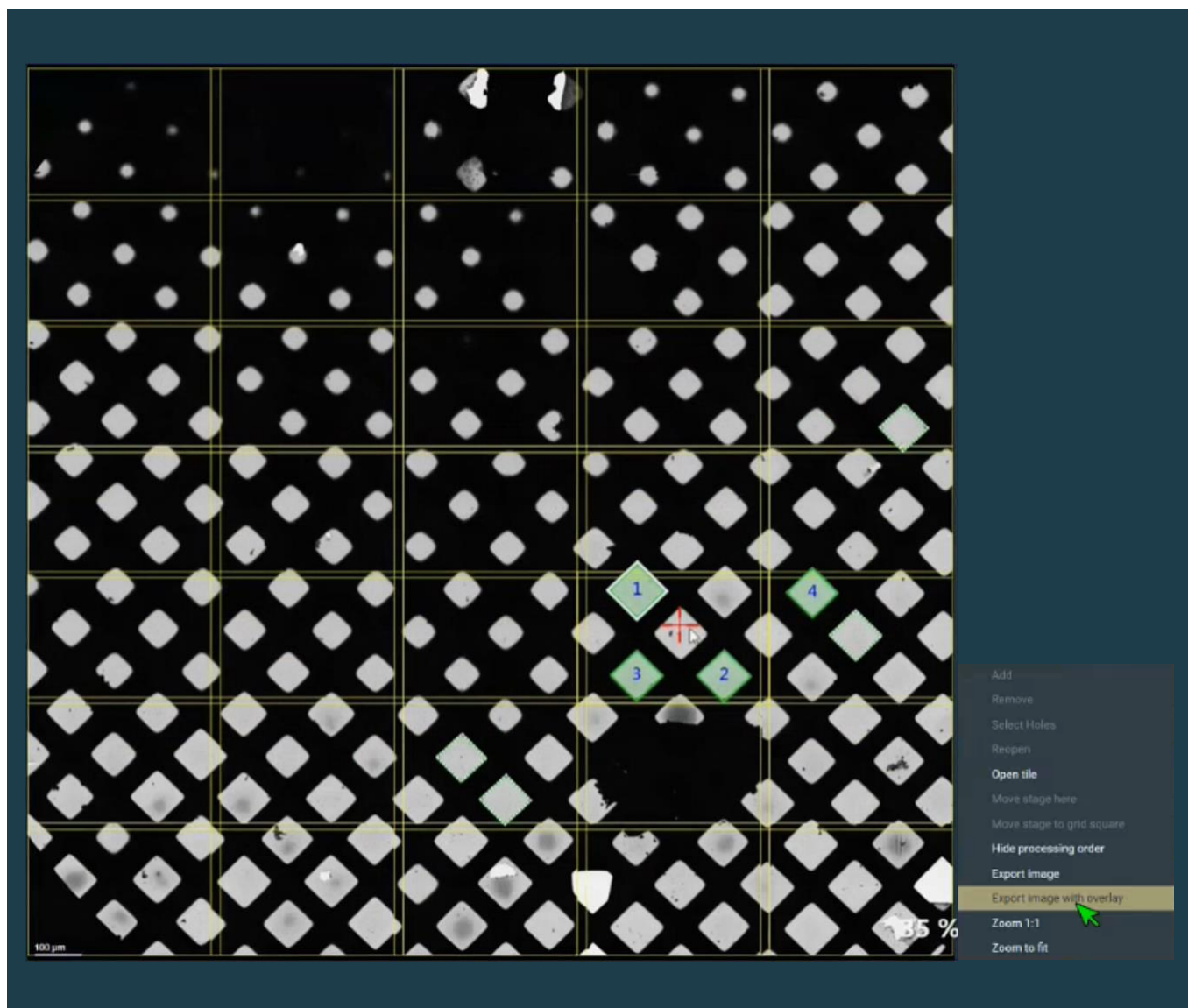


### 7.2.2 Record keeping

- ☐ Save an image of the Atlas with the squares you have set up

(EPU: EPU > Preparation > Square Selection > Right-click > 'Export image with overlay')





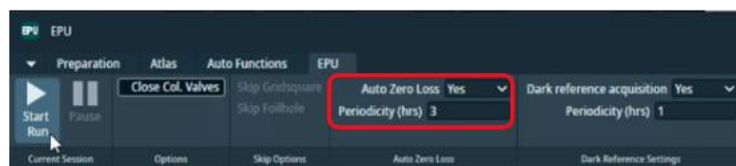
### 7.2.3 Data collection

☐ Enable Auto Zero Loss refinement and input periodicity

Suggested values:

- Between 3 – 8 hrs for a microscope equipped with a K3 and GIF
- Between 12 – 24 hrs for a microscope equipped with a Falcon IVi and Selectris

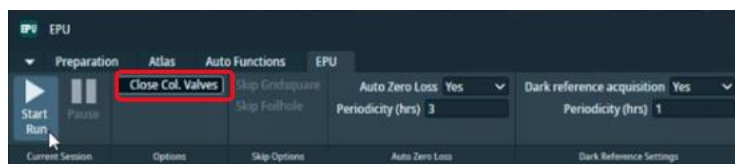
(EPU: EPU > Automated Acquisition > Auto Zero Loss > Auto Zero Loss > select 'yes' and add a value for 'periodicity')



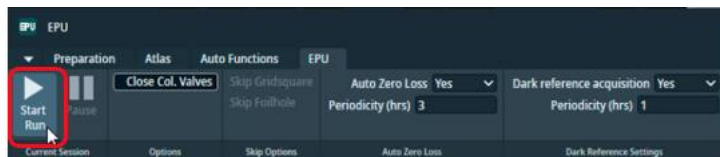
☐ Select Close Col. Valves to ensure column valves close once data collection is complete

(EPU: EPU > Automated Acquisition > Options > select 'Close column valves')

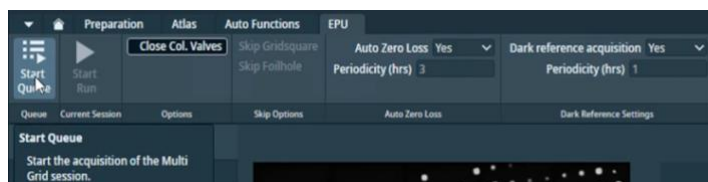




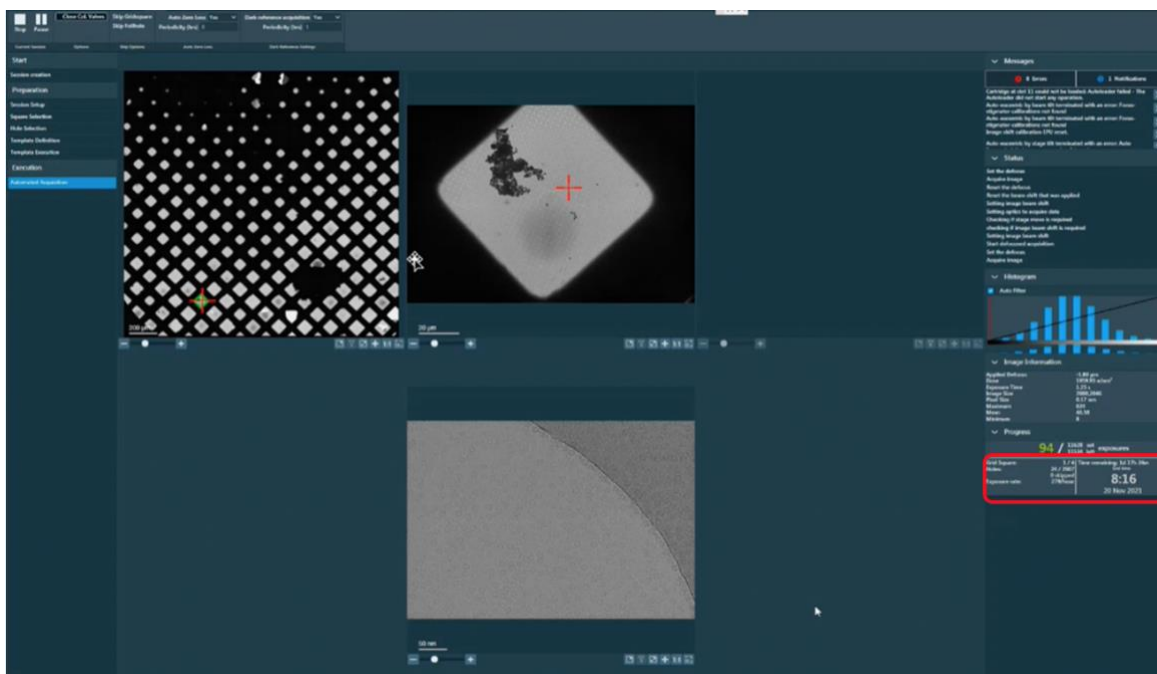
- ☐ Start the automated acquisition for SPA single grid  
(EPU: EPU > Automated Acquisition > press 'Start')



- ☐ Start the automated acquisition Queue for SPA multi grid



- ☐ Monitor the beginning of data acquisition and frequently during your session
- ☐ **Inform your local contact that you have started your data acquisition.**
- ☐ Observe the estimated time remaining and plan to add more squares as required



## 7.2.4 Communication with you 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.

## 7.2.5 Set up on-the-fly processing

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

## 7.2.6 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.

## 7.2.7 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 EPU 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 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.

## 7.2.8 Closing Teamviewer and disconnecting NoMachine

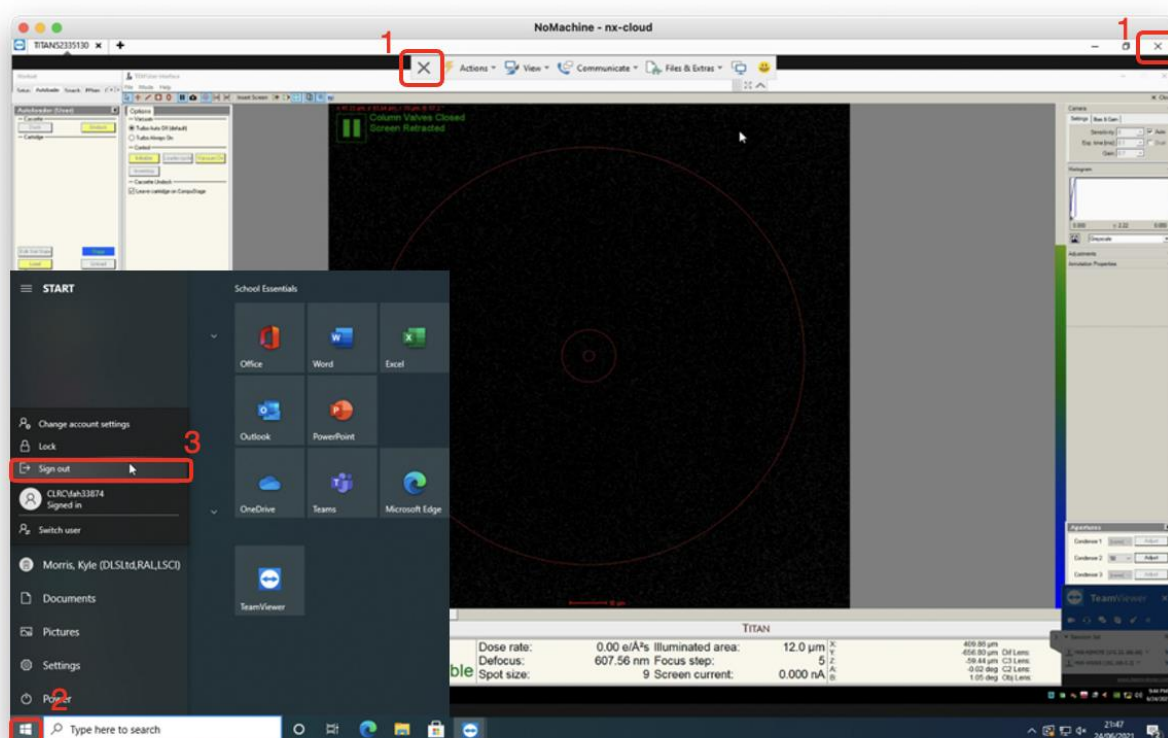
If at any point you leave the microscope unattended, please ensure you close the NoMachine connection in case your LC requires access (only one NoMachine connection is permitted at a time for security reasons).

- ☐ 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

