Always check for the most recent document on the eBIC website.

https://www.diamond.ac.uk/Instruments/Biological-Cryo-Imaging/eBIC/User-Guide/User-guide-downloads---draft.html

Please submit feedback about this document *via* the session feedback in the UAS.

User responsibilities

As a user, you are responsible for managing several key aspects to ensure a smooth and productive experience. Below are the main responsibilities:

FedID and password

You are expected to know your Federal ID (FedID), for example, abc12345, along with your associated password. You may be required to log into Diamond's computing systems either onsite or remotely. If you need help with your FedID or password, please contact useroffice@diamond.ac.uk for guidance.

Session numbers

Each experiment will be assigned a unique session number, such as bi36379-42. You should record this session number, as it will be necessary for saving your data and accessing it on Diamond's servers later.

Shipping

Your samples must arrive onsite before the start of your experimental time. Samples should be shipped in containers that maintain them in a vitreous state. Experiments cannot proceed if samples arrive in a non-vitreous state. If there are any delays or concerns about the timely arrival of samples, notify your local contact (LC) as soon as possible. Misleading the LC regarding sample delivery status is strictly prohibited, as instrument time is valuable and can be reassigned if needed.

After your experiment, ensure that the shipment vessel is returned to your institution. If the space is needed, your shipper might be returned without notice.

Data Storage and File Paths

It is essential to save your data in the designated file locations specified by your LC or as outlined in this document. If you are unsure about the correct location, consult your LC for assistance.

Sample quality

The quality of your sample is the most critical factor in obtaining reliable, high-quality results. Samples that do not meet the necessary standards may lead to inconclusive or unusable data, and the experiment could be terminated at the LC's discretion. Below are some common issues that can result in poor sample quality:

- Incorrect Clipping: For FIB lamella preparation, clip the TEM grid with the support film facing away from the C-ring. Grids clipped upside down cannot be used, as this misalignment will interfere with lamella placement and preparation. Ensure each grid is clipped securely to prevent disruptions.
- Bent or Damaged Grids: Grids that are bent or otherwise damaged can obscure the milling target, blocking the ion beam from the intended area. Ensure grids are flat and undamaged to allow for precise milling.
- Excessive Thickness: If a sample is too thick, ice below lamellae positions cannot be removed. Samples should be prepared to the appropriate thickness. Screening using a TEM helps identify suitable regions to operate on.
- Contamination: Contaminants on the sample surface, mainly ice particles and occasionally fibers, can obscure the milling targets. Even low levels of contamination can significantly reduce success.

If the LC assesses that any of these issues will likely prevent the sample from producing viable data, the experiment may be canceled to avoid wasting valuable instrument time. To prevent such issues, ensure that each sample is prepared, handled, screened and stored appropriately.

Monitoring and LC Notifications

You are responsible for monitoring your experiment. Remote monitoring can be performed using the NoMachine software (see the “Remote connection” section below). Keep the LC informed of any critical observations or issues that arise during the monitoring (more details can be found in the “Milling/thinning” section). Between 9 AM and 5 PM UK time, inform the LC of any critical issue directly. Outside of these times, call the Experimental Hall Coordinators (EHCs):

- Phone +441235 77 8787 or 8787 from a phone at Diamond
- Say you’re on the Aquilos, m11
- Say you need to get in touch with the LC
- You might be asked for your session number
- You might be asked for the name of the LC
- Be concise in reporting the problem to the EHC

Requests of the LC

Your LC is available to provide guidance and facilitate the success of your experiment. LCs are often assigned several months in advance, and their advice may vary based on their experience. Please be mindful that last-minute requests for complex experimental adjustments cannot be accommodated.

Remote connection

To remote control the instrument you'll need the software NoMachine (https://www.nomachine.com/).

Connect after the session start time.

Only one nx-cloud.diamond.ac.uk connection can be made at a time. The node is shared between two systems. **Please sign out and disconnect when you are not using it:**

> To do this: on the Windows 11 desktop > Start menu > [your name] > Sign out > press ctrl+alt+0 (zero) > Connection > Disconnect

Open NoMachine and:

1. Add a new connection (or open the connection you have already made)
2. Set:

Name	Diamond NX cloud
Host	nx-cloud.diamond.ac.uk
Port	4000
Protocol	NX

3. Add
4. Double click the icon you've just made
5. Enter your FedID as the Username in the format: YOUR_FED_ID@fed.cclrc.ac.uk
   e.g. abc12345@fed.cclrc.ac.uk
6. Enter your password
7. Check "Save this password in the connection file"
8. Click OK
9. Select the server that is: M05/M11

   M05/M11, ED,
   Windows 10
   Running 0

10. Check "Don't show any more for this connection" and "OK" all the display options
11. Double click the blue background
12. Log in to the Windows 10 desktop with your FedID and password (FedID is in the standard format). Be aware that the keys corresponding to you keyboard might not show correctly in the input box.

    > Note: You can bring up the "On-screen keyboard" by clicking the "Ease of access" icon in the bottom right corner of the screen. Use this to enter characters that you can't see on your physical keyboard.

13. When you see the Windows 11 desktop, search for and open the program: Radmin Viewer 3
14. Add a new connection

15. Set:

Name of entry	Aquilos PC
IP address	10.185.8.11

16. Select the viewing mode:

Full control	 Monitor, mouse and keyboard icon
View only	 Monitor icon

17. Double click the connection that has just been made
18. Enter:

User name	user
Password	user
Domain	(leave blank)
Save as default	✓

19. Wait at least 60 seconds, and then you should see the Aquilos PC desktop
20. Move mouse cursor to the very top of the monitor to see viewing options
21.  Adjust view settings – hover the pointer over the icon to read the description. Try to select an option that suits your viewing mode – full screen stretch is *probably* the best.
22.  Switch between the two monitors (Display 1 or Display 2). Find the one you need. Do not use “Entire Desktop”.
23. Consider changing the settings to show the remote pointer.  Display settings > Full control > check “Draw the remote cursor in the remote screen window”
24. When you’re not actively working or monitoring, disconnect from the NoMachine session. Do ctrl+alt+0 (zero) > Connection > Disconnect to disconnect fully.

Introduction

This document is meant as a guide for preparing lamellae using autoTEM on the Aquilos (beamline: m11) at eBIC. Always check for the most recent document on the eBIC website.

https://www.diamond.ac.uk/Instruments/Biological-Cryo-Imaging/eBIC/User-Guide/User-guide-downloads.html

Please submit feedback about this document *via* the session feedback in the UAS.

The instrument will generally be ready for loading samples an hour after the session start time.

The local contact (LC) will be available for guidance between 9 am and 5 pm. Outside of these times, the LC should only be contacted for experiment critical reasons.

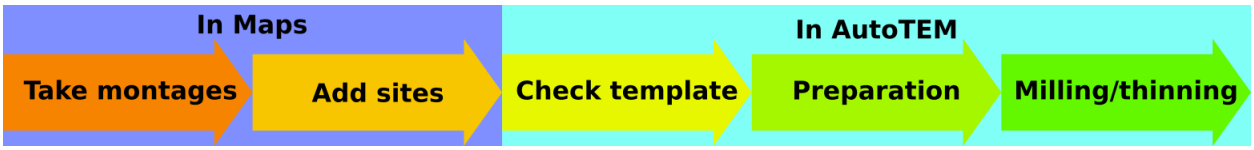
Protective and conductive coatings will be performed by the LC.

The instrument control will be handed to you when montage imaging using the SEM is running.

You are expected to make decisions and operate at sufficient speed. You should have the automatic milling started by 5 PM.

It helps if you understand the principles behind on-grid lamella milling and secondary electron image generation and interpretation.

The workflow is as follows:

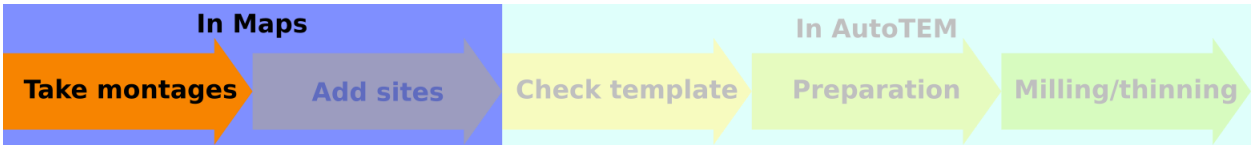


For each stage in the workflow information is provided. This information falls into these categories:

- **General** – important descriptions and information
- **Do this** – you must do this to proceed
- **Do not do this** – doing this will lead to a poor outcome
- **For best results** – doing this will lead to a better outcome
- **Points to note** – helps you to understand and operate

Ensure you *read all categories* in a section before proceeding. There are important **Do not do this** items that occur after the **Do this** items.

Take montages



General

Maps is software that allows easy interaction with a global view of your grids. The LC will start the SEM montages of your grids. Each standard montage takes about 25 minutes to run. Making changes to the SEM settings can dramatically impact the speed and quality of the montage collection. Sites for lamellae are added to the montage in the next section.

Do this

- Inform the LC of specific SEM requirements e.g. voltage, pixel size, dwell time. Standard conditions are used otherwise.
- If you can, identify regions of the grid which you don't want to work on – ask for these to be deselected.
- Familiarise yourself with moving around the Maps workspace with the mouse (see **Points to note** below)

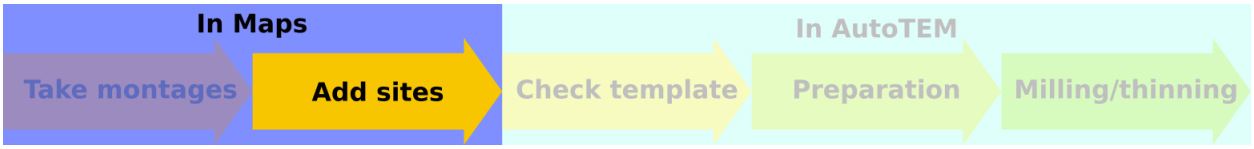
For best results

- While the montage is collecting identify the locations you want to work on
- If TEM atlases have been collected and sent to the LC before the session, these might be aligned to the SEM montage. It is not always easy or possible to align the TEM atlas with the SEM montage.

Points to note

- To move around the workspace left click-hold and drag
- To zoom in and out of the workspace use the mouse wheel
- Grid 2 is on the left Grid 1 is on the right (this is the reverse of the loading orientation)
- Features at tile edges often do not align

Add sites



General

On the SEM montage, you will add the sites where you want lamellae to be prepared. There are better and worse places to make lamellae. In the SEM images it will help if you identify:

- grid squares
- grid bars
- milling sites
- ice meniscus *below* the support film
- very large pieces of contamination
- broken support films pointing up from the grid-face

For every site added, about 10 minutes will be spent preparing the site in AutoTEM.

Adding sites in Maps is *not* precise – you will place milling boxes on these sites later.

Do this

- Add sites according to the SEM image in the montage
- Keep within the green area of the grid (Figure 1)

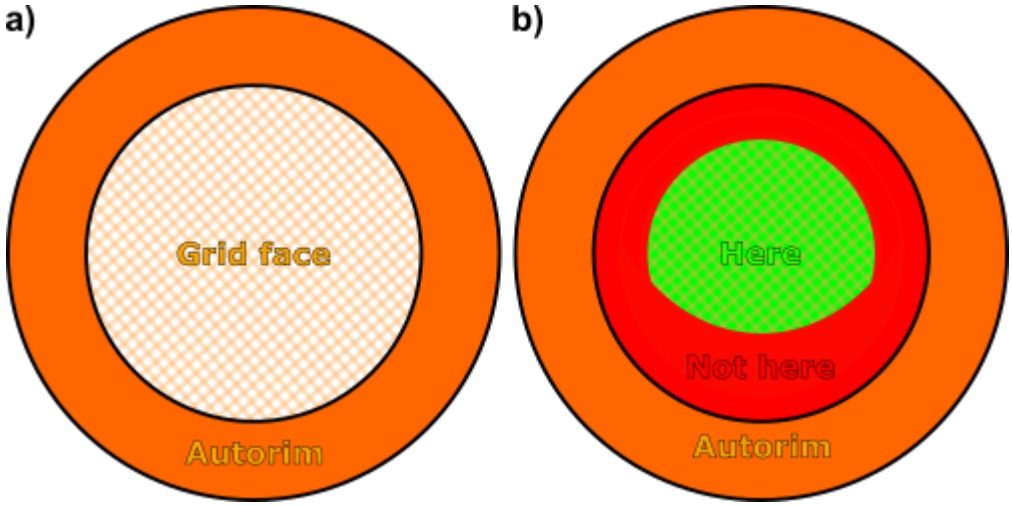


Figure 1 Schematic of grids showing a) the whole grid face and b) the region for lamellae milling

- Set one site per grid square
- As best as you can, keep to the center *or* the top of the grid square. Avoid the bottom of the grid square (Figure 2)

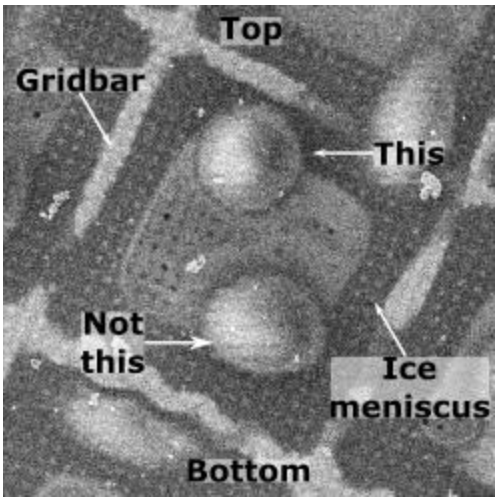


Figure 2 SEM image of a grid square highlighting the Top and Bottom, gridbars, the ice meniscus below the support film, and preferential positions for milling

- Right click where you want to add a lamellae site
- Select “Add lamella site here”

Do not do this

- **Do not set more than 23 sites**
    - **With no changes to the standard template, and prompt operation, you can prepare and mill 23 lamellae to electron transparency**
    - **The actual number of sites that can be processed depends on various factors (see below)**
    - **If you make changes to the standard template, you will need to adjust the number of sites accordingly**
- Do not add so many sites that you run out of experimental time before reaching electron transparency. The number of sites that can be prepared and milled depends on several parameters, principally:
    - Lamella width – wider lamellae take longer
    - Milling box dimensions – larger boxes take longer
    - Milling currents – milling with lower currents will take longer
    - The size of the object – larger objects take longer
    - 10 pA aperture quality – once this aperture is worn, we will switch to a lower current aperture with a longer dwell time. This significantly increases the amount of time for the preparation step in autoTEM.
- Do not add sites randomly
- Do not add sites on grid bars
- Do not add sites where the ice meniscus is thick – this can be very hard to know from SEM imaging. Using a low magnification TEM overview helps.
- Do not add sites close to the bottom of the grid square
- Do not add sites near large pieces of contamination
- Do not add sites to images from another aligned modes (fluorescent microscopy, TEM)

For best results

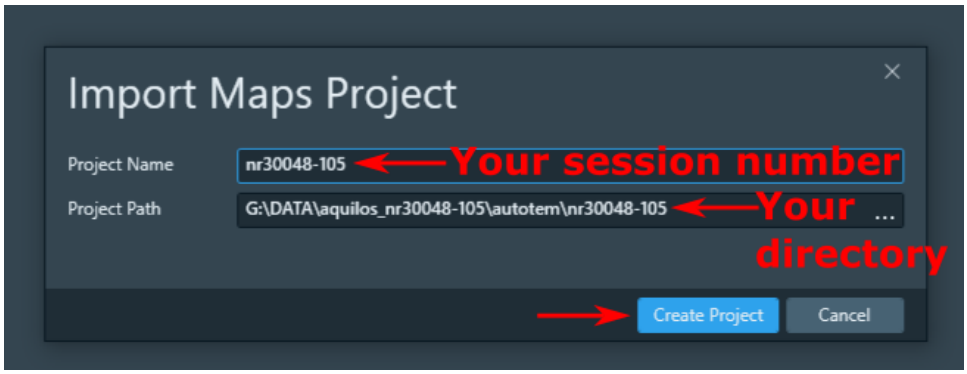
- Add sites in order from the top to the bottom of the grid
- Avoid (if you can) cracked grid squares

***

When you've finished adding lamella sites, open autoTEM 2.4 (icon with "AT" on the task bar). Double click the project with your session number the one highlighted green and "On-line".

Do this

- Check the name the project is your session number – by default it is (if the maps project is saved correctly)
- ***Change*** the project path to F:\DATA\aquilos_SESSION-NUMBER\autotem – by default it is *not*

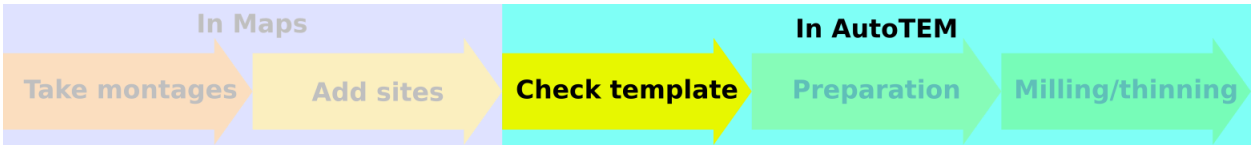


- Click "Create Project"
- When the lamella sites have finished importing, close Maps
- Close Maps

Do not do this

- ***Do not save data in other directories!***
- ***Do not leave Maps open!***

Check template



General

To mill the selected sites, a set of parameters are applied to each one. A standard template has been prepared. It is called "00_1nA_to_30pA". The template is applied to each lamella site and has three main sections which involve:

- "Preparation" – positioning the desired lamella site, acquiring the FIB view image, and positioning milling boxes
- "Milling" – generation of coarse lamellae
- "Thinning" – taking coarse lamellae to electron transparency

Parameters in the template can be changed as you see fit – however these changes can have a dramatic impact on the outcome of your experiment.

You will change some parameters in the upcoming steps.

Do this

- Open the Templates tab
- Find the standard template "00_1nA_to_30pA" on the left-hand side of the autoTEM window
- Right click on the template and select "Clone"
- Rename the template to your session number
- Find and click on your renamed template on the left-hand side of the autoTEM window
- Make the adjustments you require to the template. Pay attention to these parameters. Modify them if you understand the consequences:

  In Preparation

  - In "Milling Angle" the "Target milling angle" – this will be angular difference between the lamella plane and the grid plane. Our standard is 12°.
  - In "Milling Angle" choose whether to "Enforce Milling Angle" – checking this ensures that all lamellae will have the set milling angle. If the milling angle can't be achieved, the site will be abandoned. If not checked autoTEM might increase the milling angle by a couple of degrees.

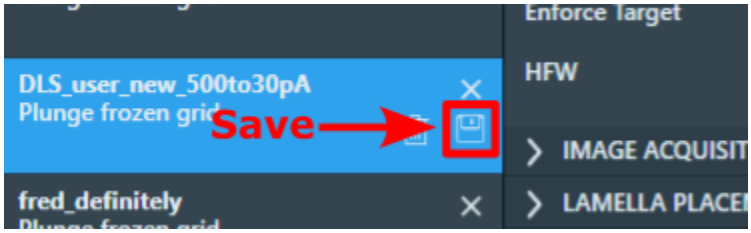
  In Milling

  - Decide if you want stress relief cuts. Consider selecting this if you have had problems with bending lamellae previously. If selected, you must also select "Reference Redefinition 1".
  - Please keep the "Finer Milling – Electron Image" on. Assessing this image determines the site is suitable for taking to electron transparency (in the Thinning section).

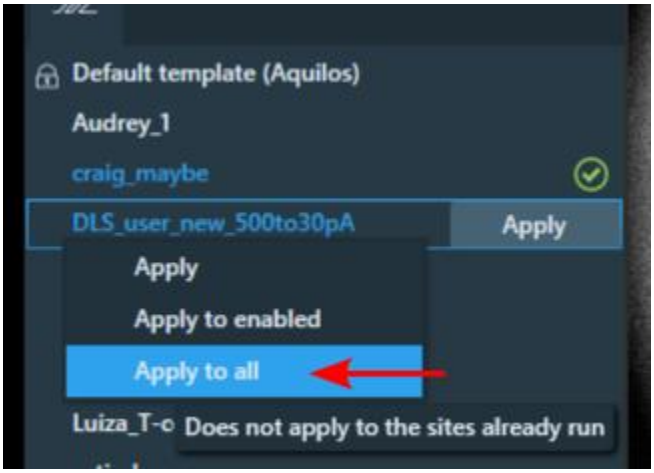
In Thinning

- Change the “Final thickness” to the desired value. You must understand that the final thickness is not the actual thickness of the lamellae.
  - A lower value will produce thinner lamellae.
  - A lower value will have a higher risk of breaking and loss.
- Typically, the value is between 120 and 150 nm

- Save the template – on the left-hand side, click the disk icon on your template



- Go back to your project (the tab with your session-number in the top right of the AutoTEM window).
- On the right-hand side of the AutoTEM window, right click on the template you have just made, select “Apply to all”



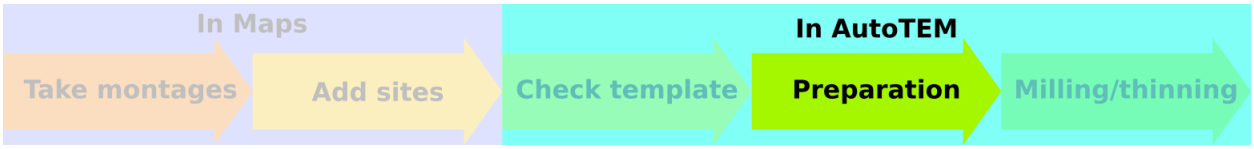
- Wait until it has finished
- ***Ensure Maps is closed***
- ***Ensure Maps is closed!***

For best results

Carefully consider if decreasing the milling angle is needed

- Lower milling angles are more challenging
- Lower milling angles risk fewer viable lamellae
- Changes to this parameter cannot be reversed once rough milling has started

Preparation

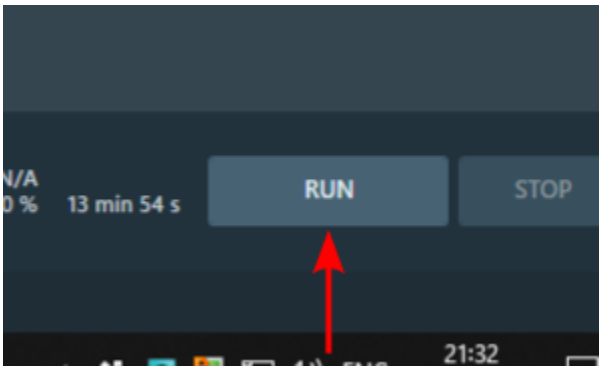


General

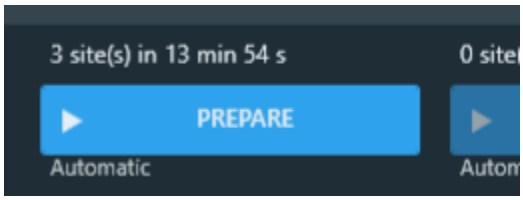
Preparation is the section of the workflow that puts the site to be milled at the e-beam-ion-beam coincidence point, and an image is take using the ion beam on which you will position boxes for milling lamellae.

Do this

- Click Run in the bottom right of the AutoTEM window



- Click “Prepare” to start the preparation step



- For each site the preparation step takes about 10 minutes
- Once started, ensure the procedure is running – you should see the microscope user interface taking images automatically and these images appearing in autoTEM.
- Once a site has been successfully prepared, position the milling boxes. Do this while the preparation process is still running.
- Preparation can take several hours – take a break at some point here.

Points to note

- AutoTEM will always switch to the latest site when a step has concluded. Simply re-select the site you are looking at.

Box positioning

General

This sub-step requires interpretation of the ion beam image taken at the end of each preparation step. Positioning boxes on the object you want milled is arguably the most important choice you will make. There is a best position for these boxes. You must identify:

- Incorrect and out-of-focus sites
- The object to be milled
- Ice particles on and around the object to be milled
- Grid-bars
- Ice below the support film

Do this

On each FIB image from a successfully prepared site:

1. Drag the center of the yellow box to the center of the location where you want the lamella
2. Drag the left and right edges of the yellow box so they are securely inside the edge of the object to be milled
3. Drag the top edge of the top blue box past the top of the object to be milled
4. Drag the lower edge of the lower blue box far enough away from the yellow box to successfully clear material away from the lamella *but also not over a grid-bar*

Understand that increasing the width of the yellow box and the height of the blue boxes will increase the total time that spent milling.

- **You will be advised to stop the preparation if coarse milling will take too long**

For each unsuccessfully prepared site:

- Make a note of the lamella number for later

Points to note

- Zoom in and out of the image with the mouse wheel
- Pan around the image using left click-hold and drag
- The box positioning cursor is different from the panning cursor
- To drag box edges, click once on the box center first, then click-hold and drag the box edge
- You can't mill though a grid-bar

For best results

- Keep the yellow box width between 2 and 16 µm
- Ensure no ice particles are inside or immediately around the yellow box
- Keep as far away from cracks in the object as possible

Completing the preparation

Do this

While the preparation is running, position all of the milling boxes according to the instructions above.

If the site is wrong or undesirable (e.g. no object to be milled, auto-rim edge obscuring grid-square, too contaminated, too cracked, out of focus, etc.) make a note of the lamella number and proceed.

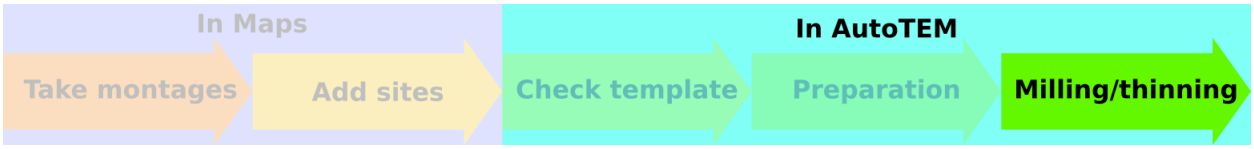
After preparation has completed:

- If the system has gone to “sleep” – alert the LC and they will restart the ion beam.
- Ensure your milling boxes have been positioned on all the site you want to be milled.
- On the left hand side, deselect the sites that you ***don’t*** want to mill

Points to note

- **The estimated time in autoTEM is inaccurate**

Milling/thinning



General

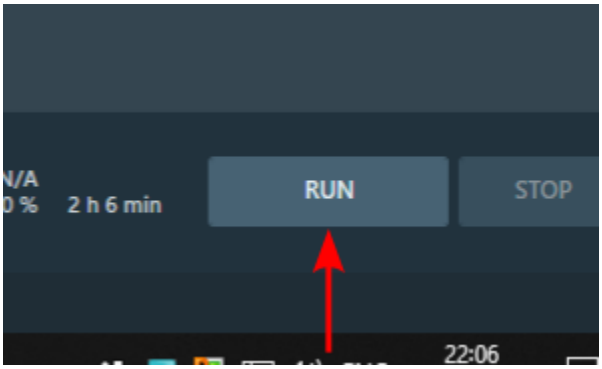
Starting the automatic milling is the last step for now. This should be done before the LC leaves at 5 PM. *Thinning should not follow sequentially from coarse milling*. Review each SEM image after the finer milling step and decide which sites should undergo the thinning step. See the "SEM check" step below.

You might have to start the "Thinning" step very early in the morning.

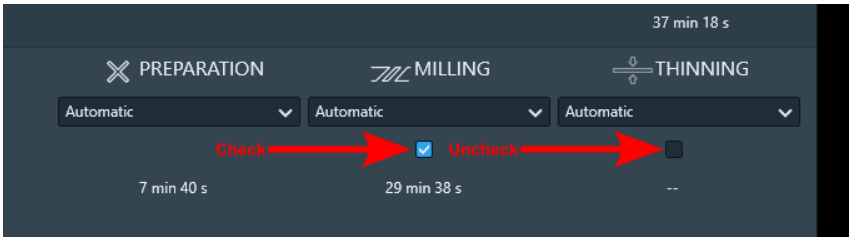
The grids should be extracted as soon as possible after the thinning has ended – this will be between 9 AM and 5 PM and before the session end time.

Do this

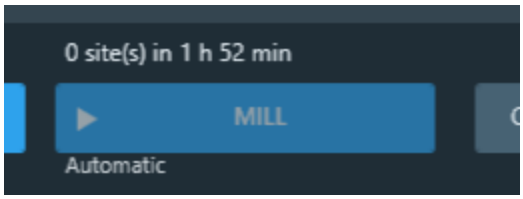
- Click Run in the bottom right of the AutoTEM window



- On the left-hand side of the pop-up window, deselect all the lamella positions that shouldn't be milled (review your notes from the preparation step)
- In the pop-up window, keep checked the box for "Milling", un-check the box for "Thinning".



- Click "Mill" to start the milling process



- Monitor the experiment. Raise non-urgent questions with the LC when the LC is free.
- Monitor the experiment. Inform the LC of any urgent problems between 9 am and 5 pm (UK time) as soon as they are noticed. Outside of these hours, contact the EHC's (see "Remote Monitoring and LC Notifications" in the "User Responsibilities" section above).
- Urgent problems can be, but are not limited to:
    - Stage temperatures above -178 C or below -188 C
    - Low vacuum warnings
    - Warning signs of impending warm-ups
    - Constant failures
    - AutoTEM completely stopping
    - Issues with monitoring remotely
    - Software crashes
    - Loss of imaging ability

Do not do this

- Do not mill badly prepared sites. Deselect the sites that should not be milled.

SEM check

General

It is important to check the SEM image that is produced after the "Finer" milling step. These can be found in the directory where the AutoTEM project is saved. If the project has been saved correctly, the images should be collected into a sub-directory called "lamellaevaluationimages" in the "autotem" directory.

If the above directory can't be found, navigate through the "autotem" directory until all the "Site" directories are located. Check in each one for the "LamellaEvaluationImages" directory and find the "Finer" milling step image.

Do this

- Examine all the images produced after the "Finer" milling step
- Identify lamellae which have ice below the lamella site (Figure 3) and note the lamella number

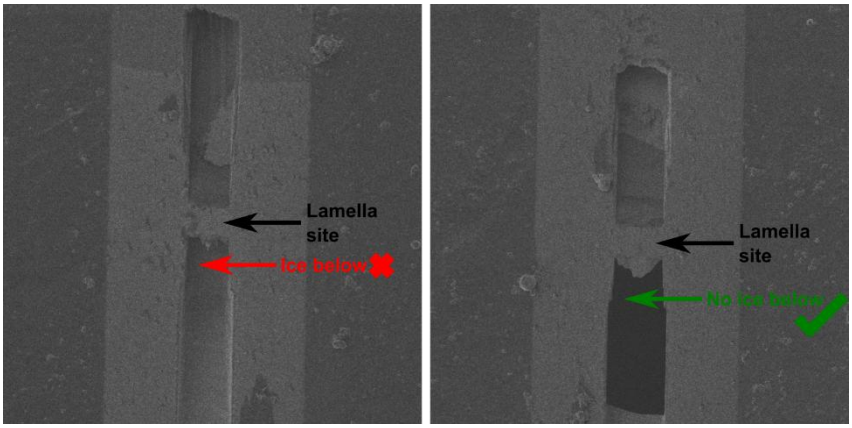


Figure 3 Two sites showing ice below the lamella (left) and clear space below the lamella (right)

- Identify lamellae which are broken and note the number
- Identify lamellae which have not completed all the coarse milling steps (no “Finer” image has been produced) and note the number

***

- Click Run in the bottom right of the AutoTEM window
- On the left-hand side of the pop-up window, deselect all the lamella sites that shouldn’t be milled (the lamella sites you have just noted)
- In the pop-up window, un-check the box for “Milling”, check the box for “Thinning”.
- Click Mill to start the thinning process
- Monitor the experiment as previously
- Alert the LC that you have started the thinning process

End of the experiment

General

Once the last thinning step has been completed, the samples should be extracted as soon as possible.

Do this

- If the LC is unaware that the experiment is finished, inform them
- Make a note of the storage details (if applicable)
- Thank the LC