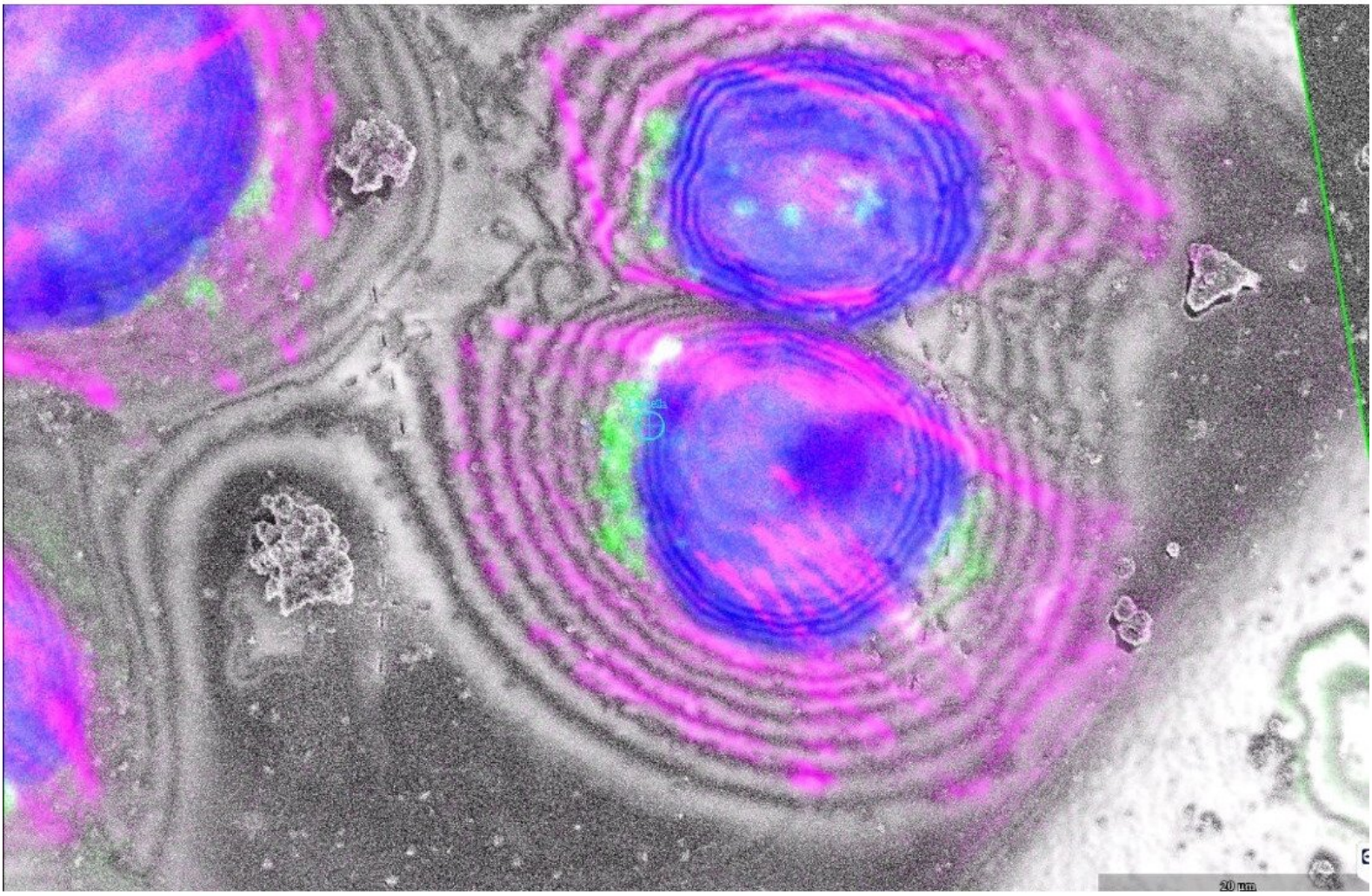
FIB-SEM - TFS Hydra Bio: Simple CLEM workflow

This guide aims to provide guidance for CLEM with the available hardware and software tools on the Hydra.

Written By: z mbstaff

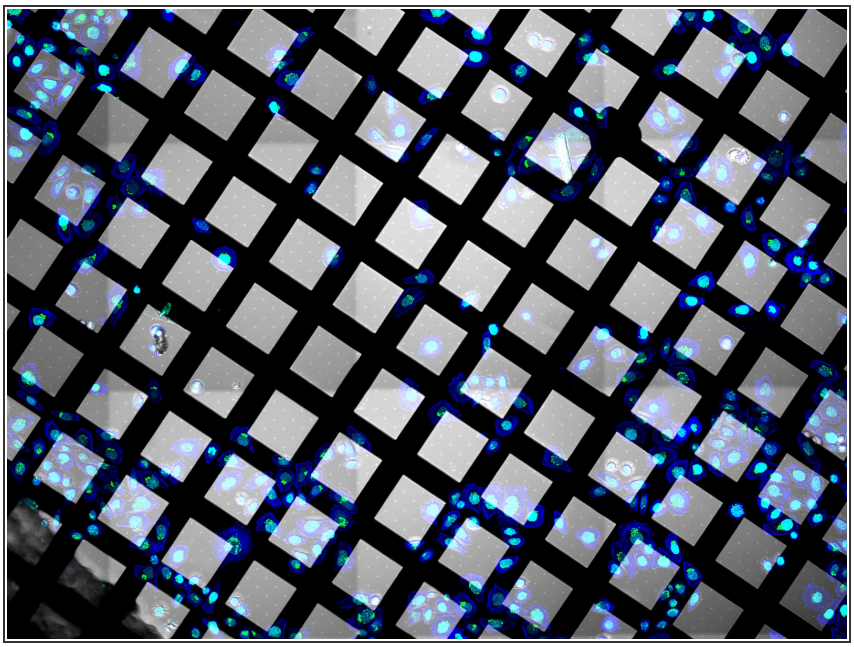


INTRODUCTION

This guide describes a simple cryogenic correlative light- and electron microscopy workflow (cryo-CLEM), that utilizes the iFLM module for in situ correlation. It works with the default software provided by Thermo Fisher Scientific with the Hydra Bio CX instrument.

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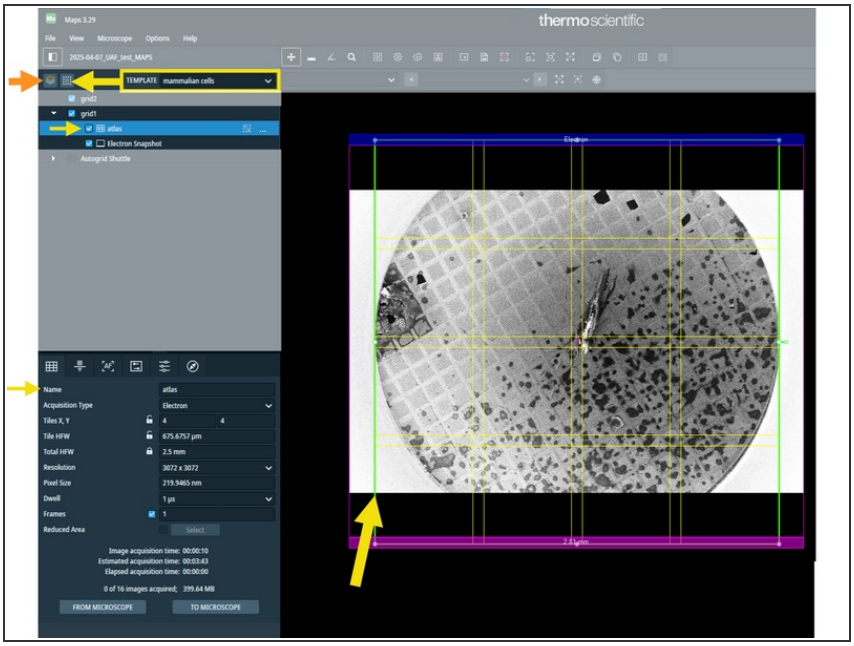
Step 1 — Optional prerequisites



ⓘ Ideally, you collect information about your grids before your milling session, to use the instrument efficiently.

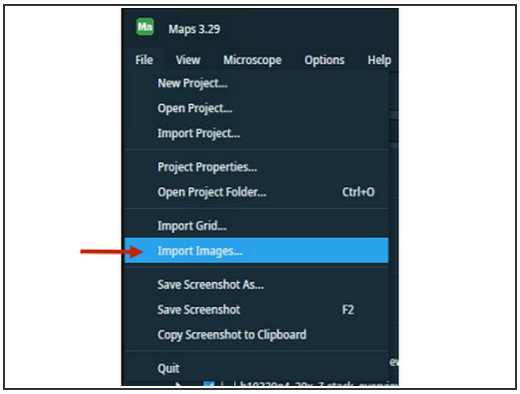
- One example, is a fluorescent atlas overview of our sample.

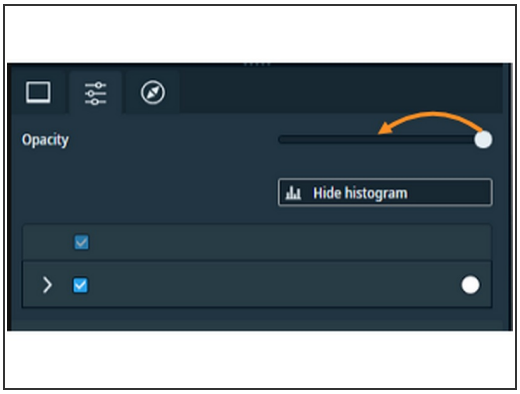
Step 2 — Acquire SEM Atlas

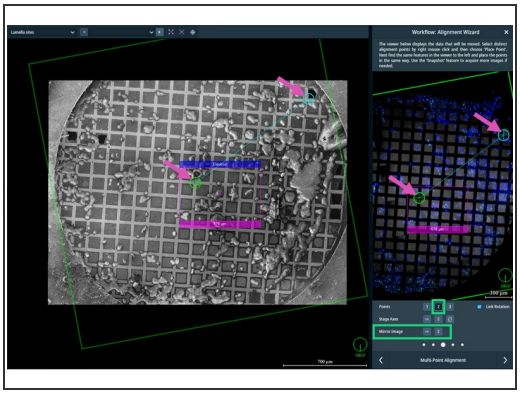


- Load your sample in the Hydra.
- Follow steps 1-5 in the basic lamella milling protocol.
  - Briefly: i) open Maps, ii) create a new project, iii) take SEM snapshot of your grid, iv) add and acquire atlas tileset

Step 3 — Optional: correlate fluorescent and SEM atlases

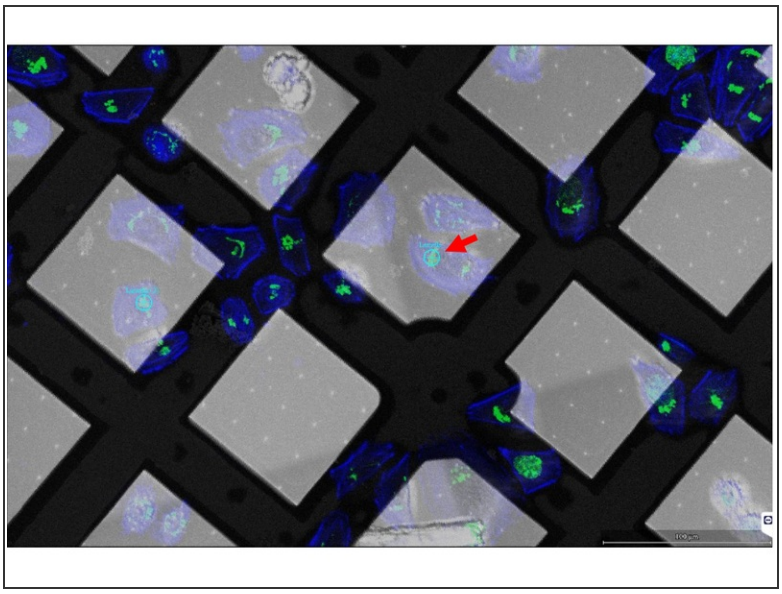


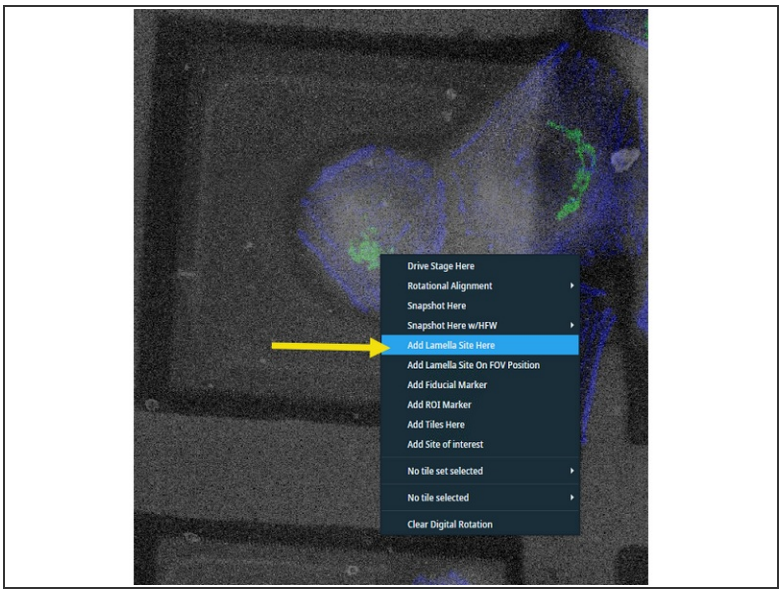




- Import previously acquired fluorescent atlas of your grid.
- Adjust opacity of this image to 50%.
- If Alignment Wizard doesn't appear automatically, right-click on the imported image -> Alignement -> Fine Alignment
- Use 2-point alignment, if necessary apply mirroring/flipping to your fluorescent atlas.
- To select matching features between SEM atlas and imported image, right-click -> Place Point. Select the same feature on both images.
- ⓘ Using the fluorescent atlas you can already select areas of interest and place lamella site over them.

Step 4 — Select lamella sites

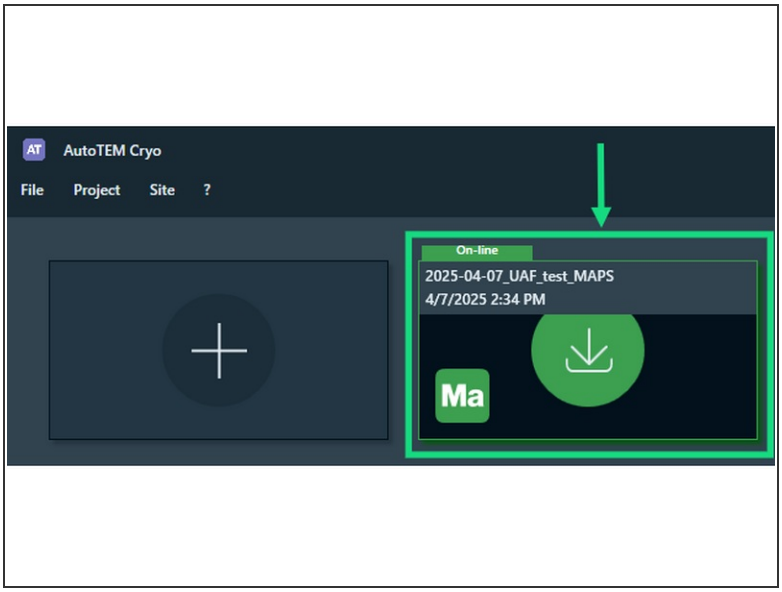


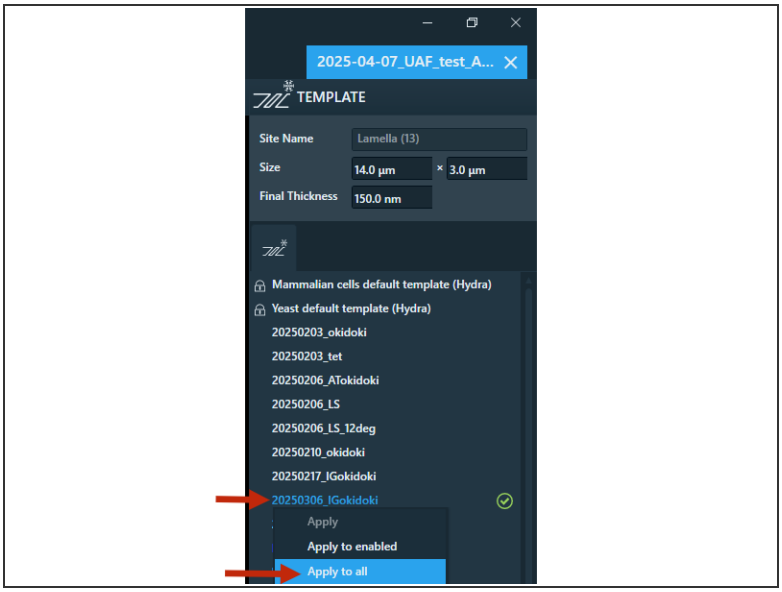


- Either based on the fluorescent atlas image (previous step) or based on SEM atlas, select cells for milling.
- Right-click on the cell -> Add lamella site here

ⓘ You may be able to fit 15-20 lamellae in one session.

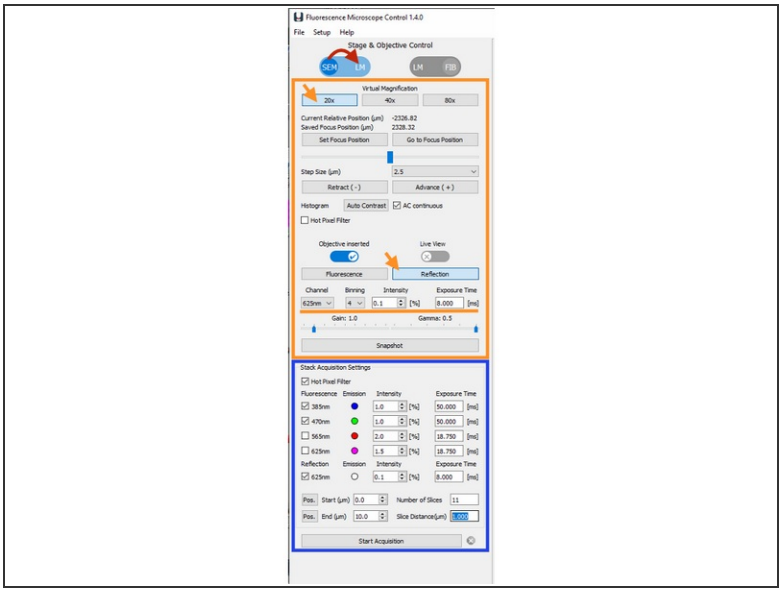
Step 5 — Create new AutoTEM Cryo project based on live Maps project





- Select live Maps project and import lamella sites.
- Apply your preferred template for the selected lamella sites and start lamella preparation.
  - ⓘ It is recommended to switch on **milling of artificial features**, that will not only enchance lamella targeting, but can **serve as CLEM fiducial** in the following step.
- Start lamella site preparation. (see basic workflow here)
- Revisit lamella sites in Maps software and take high magnification snapshot with all fiducials in the field of view.

Step 6 — Acquire iFLM Z-stacks at prepared lamella sites





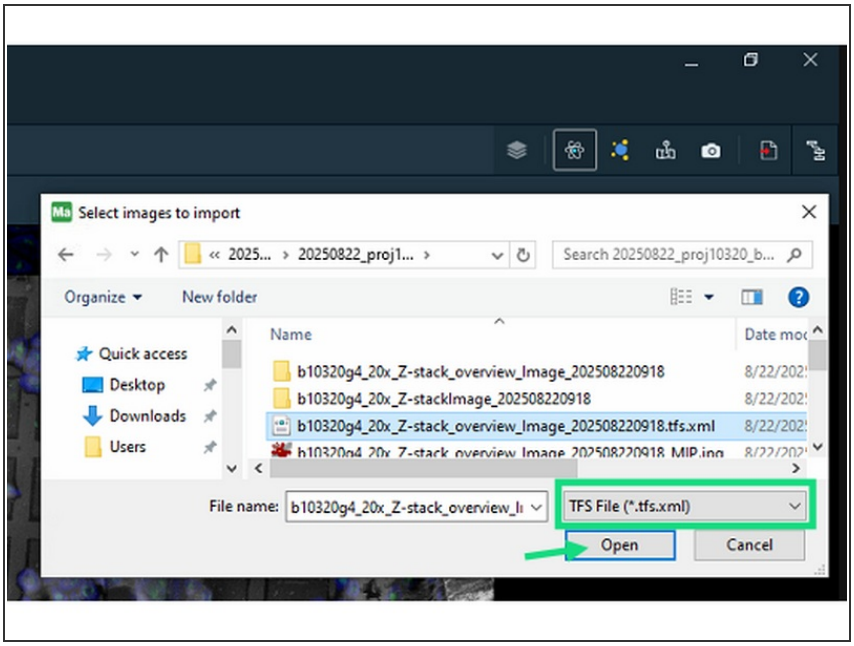
- Open iFLM software.
- Switch microscope to LM mode.

  ⚠ Make sure to switch to LM mode ONLY, when the stage is in mapping position (sample is perpendicular to SEM).
- Bring sample in focus (ca. 2500um) while using reflection mode, 20x magnification (bin 4x), 625nm wavelength, 0.1% LED power and 8 ms exposure time.
- Set focus distance and link Z-height to Y axis. Setup destination path for your Z-stacks and switch on auto-saving. If you need MIP or TIFF images as well, you will need to save them after each acquisition.
- Navigate to first lamella site, setup Z-stack imaging parameters and acquire Z-stack. Repeat this step for each lamella site.

  For a 20x, 10um Z-stack with GFP-labeled sample, these parameters work well. Fluorescence: 470nm, 1% LED power, 50ms exposure || Reflection: 625nm, 0.1% LED power, 8ms exposure || Start: 0um, End: 10um, Step: 0.5um

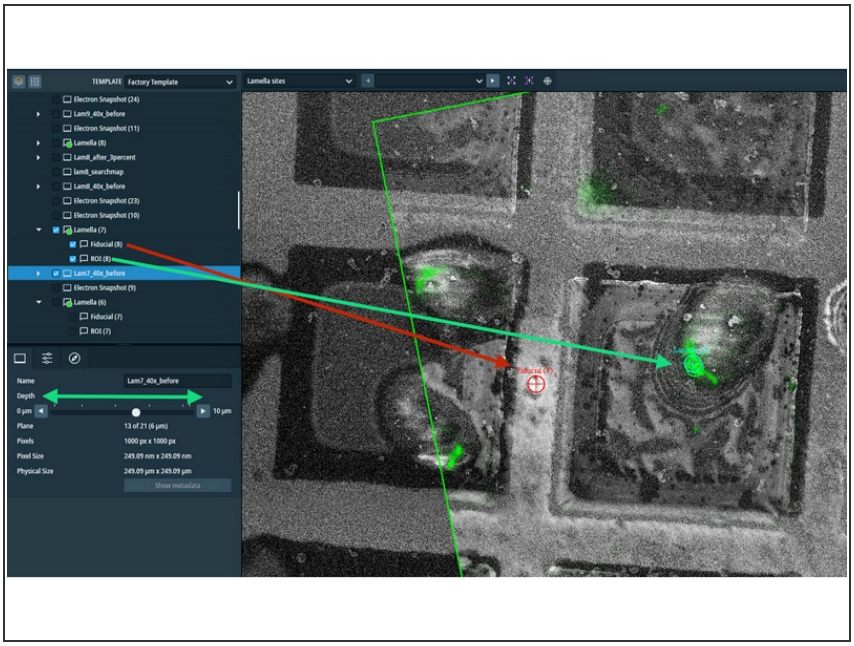
  ⓘ Make sure the previously milled artifical fiducials appear in focus somewhere in your Z-stack. This will be crucial for the next correlation steps.

Step 7 — Correlate iFLM and SEM images



- Import the previous acquired iFLM Z-stacks from your project folder, by clicking on the import button and selecting the the **.tfs** files.
- Right click on the Z-stack and select fine alignment. Change Z-slice to set artificial or natural fiducials in focus and use them to correlate to SEM atlas.

Step 8 — Select ROI and fiducial



- Select your Z-stack and switch to the Z-slice where your **artificial or natural fiducial** is in focus.
- Right-click on fiducial -> Select as **fiducial** -> Select corresponding lamella site from the drop down list.
- Select your Z-stack and switch to the Z-slice where your **region of interest** is in focus.
- Right-click on ROI -> Select as **ROI** -> Select corresponding lamella site from the drop down list.

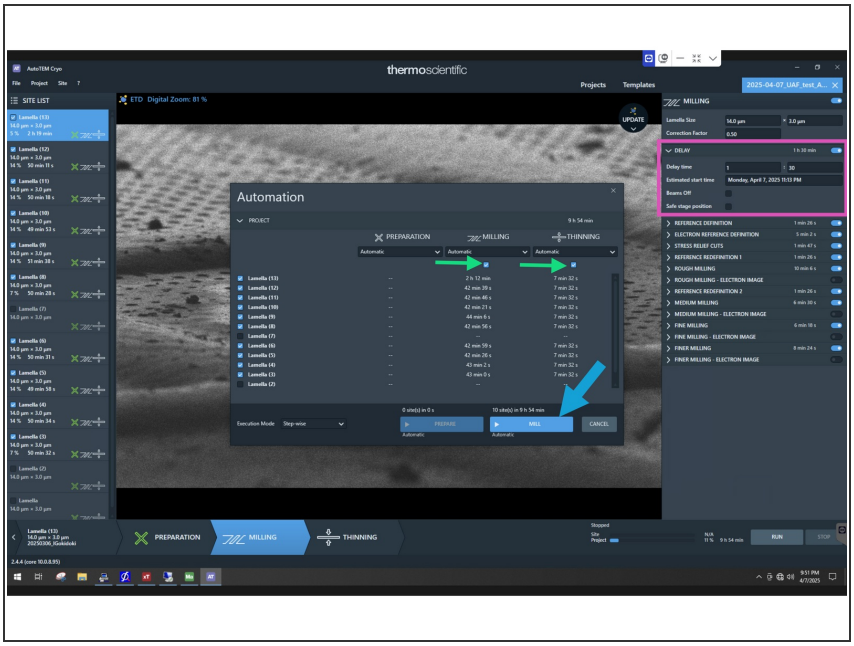
ⓘ In AutoTEM Cryo the correlation between fiducial and ROI will be calculated from X,Y coordinates in SEM atlas and the Z-coordinate based on the plane number in the Z-stack.

Step 9 — Reposition lamellas in AutoTEM Cryo



- Enable correlation in Preparation step.
- Place red cross-hair over you r selected fiducial. The calculated position of ROI will be marked by a green rectangle.
- Select **Correct lamella position**
- Adjust rough milling pattern height.

Step 10 — Start milling



- Continue lamella milling with milling and polishing steps.
- If needed, set delay time on the first lamella on top of the list.

⚠ **Safe stage position** and **Beams off** options cause an error, make sure to disable them if you use delay.