Tilman Franke and Irina Kolotuev

tilman.franke@thermofisher.com

irina.kolotueva@unil.ch

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Sample loading
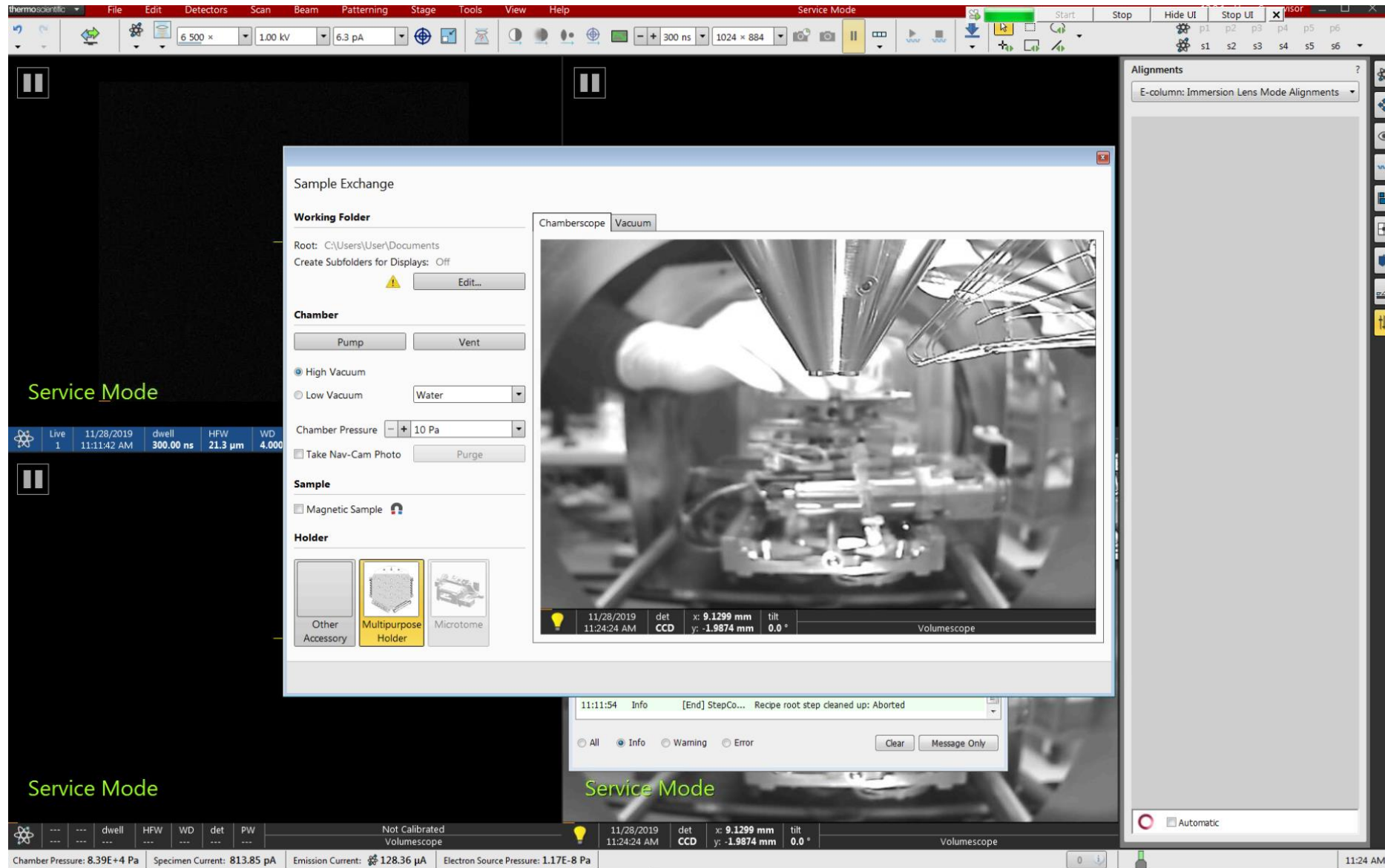
- The workflow starts with sample loading.
- Use the sample exchange dialog of the XT microscope control software to vent and open the vacuum chamber.
- Choose a suitable SEM holder to attach the Array Tomography sample and install it on the microscope stage.
- While closing the chamber door, observe the live camera view to make sure there is no collision with a detector.

CAPTION: The screenshot shows the live camera view taken from within the vacuum chamber while the sample is installed.
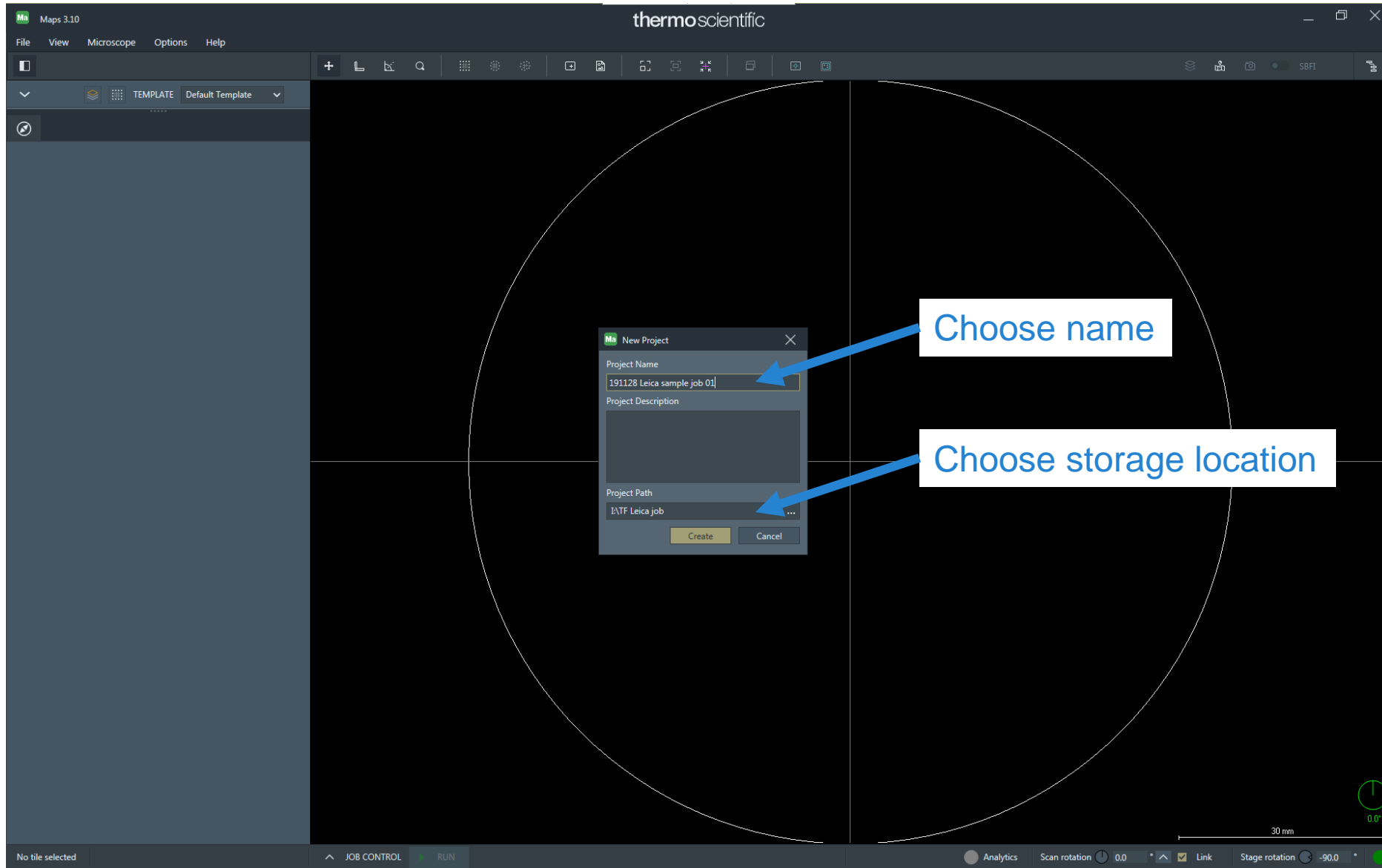
Acquisition of Navigation image
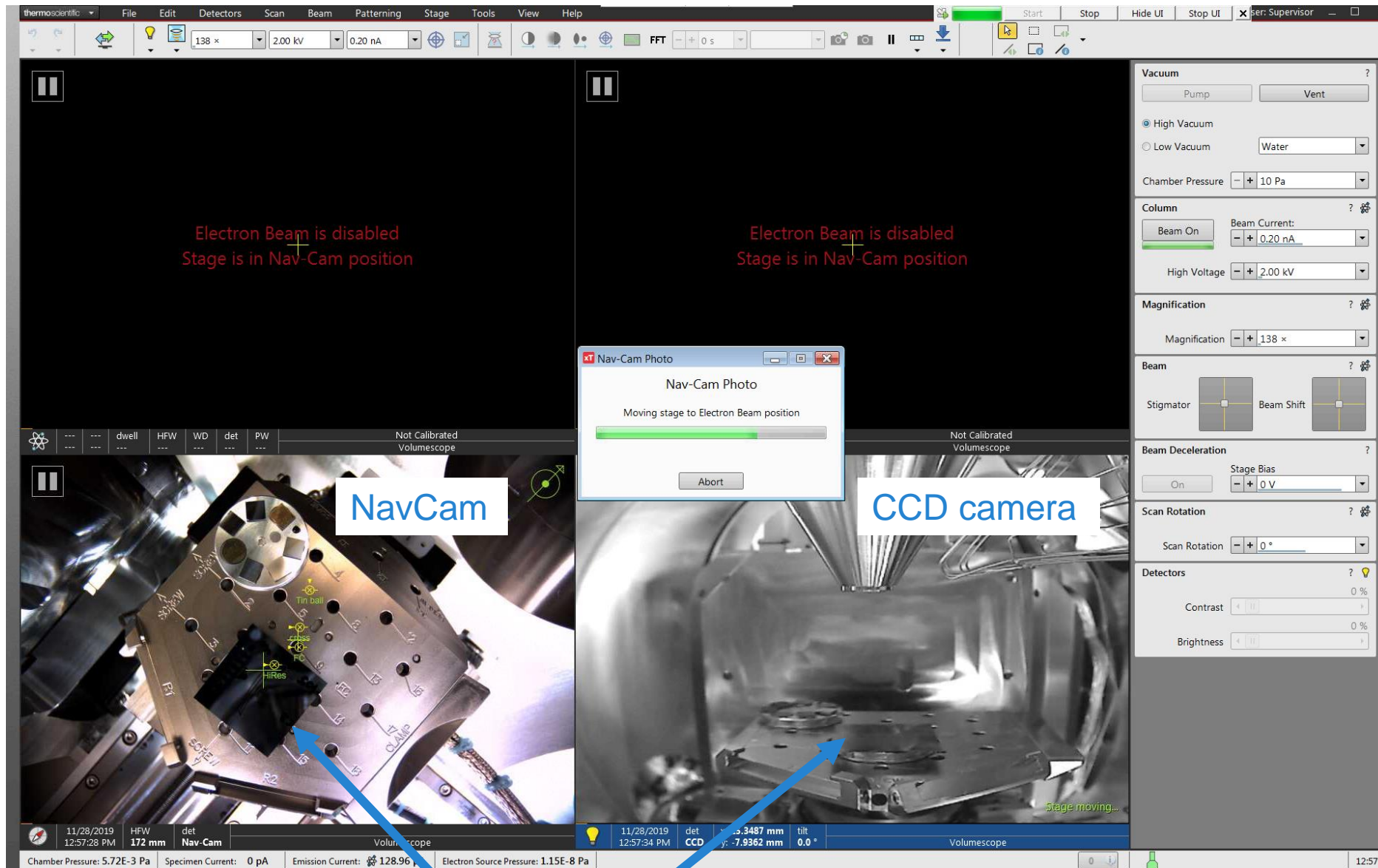
Most Thermo Fisher SEMs have two cameras. All systems are equipped with the "CCD camera" which looks at the sample from the side.

The optional NavCam is a color camera that can take a top down image of the sample holder and the sample.

- If available, acquire a NavCam image while pumping the vacuum. Alternatively, proceed to the next step.

New Maps project


Choose name

Choose storage location

- Launch Maps software which runs alongside the XT microscope control software.

  Maps has a zoomable viewer that shows any image recorded on a sample. Maps also automates workflows like tiling & stitching, CLEM, and array tomography.

- Directly after the start of the program, Maps asks the user to create a new Project. Do this by typing a project name and defining a storage location.

NavCam image in Maps

Three ribbons
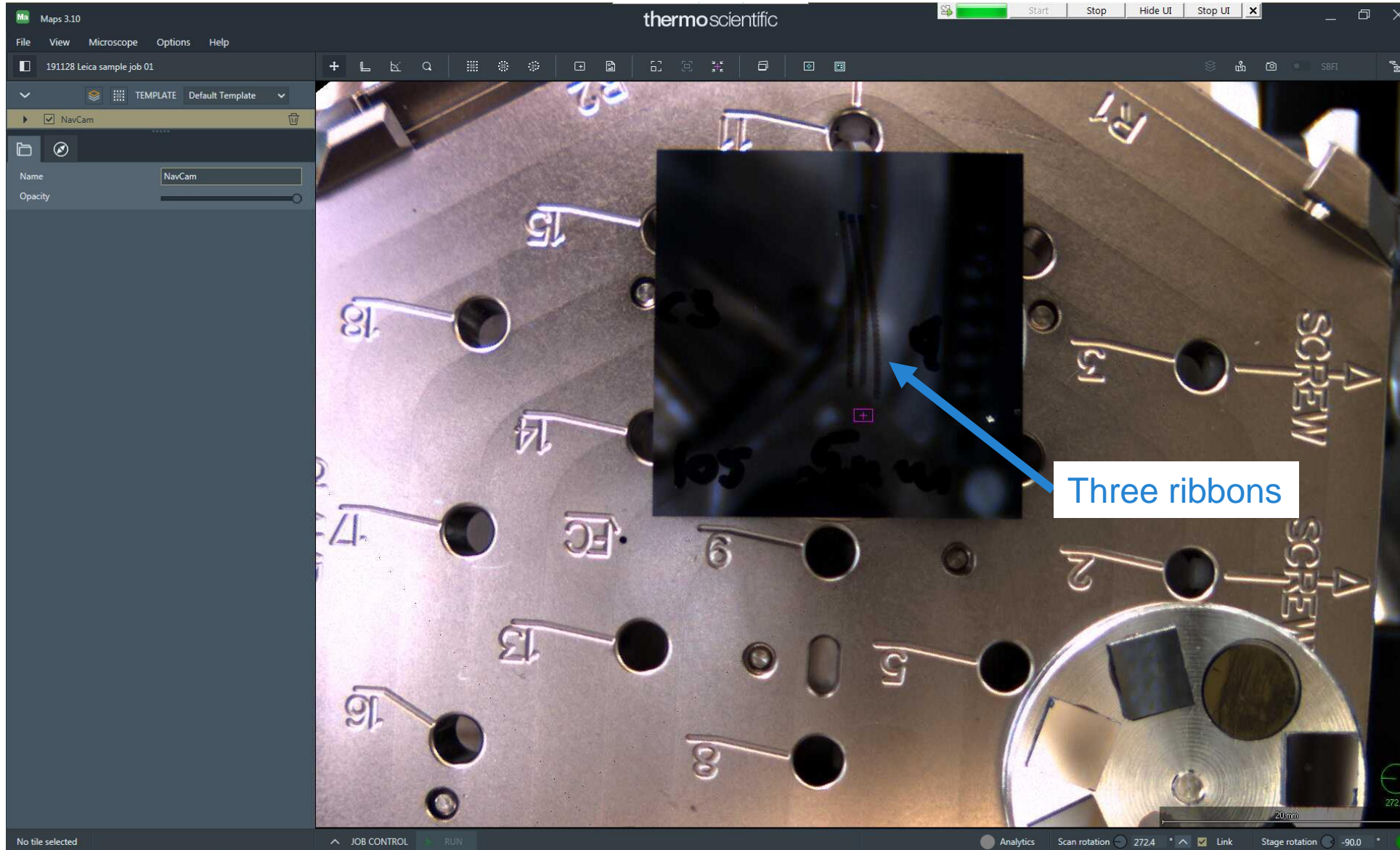
- Load the NavCam photo into Maps using the "Import from NavCam" feature in the Microscope menu.

- Zoom in until you can see the ribbons of sections on the silicon wafer.

The image quality is usually suficient for the next step, which is positioning an SEM overview mosaic which will show the sections more clearly.

Creating an overview mosaic
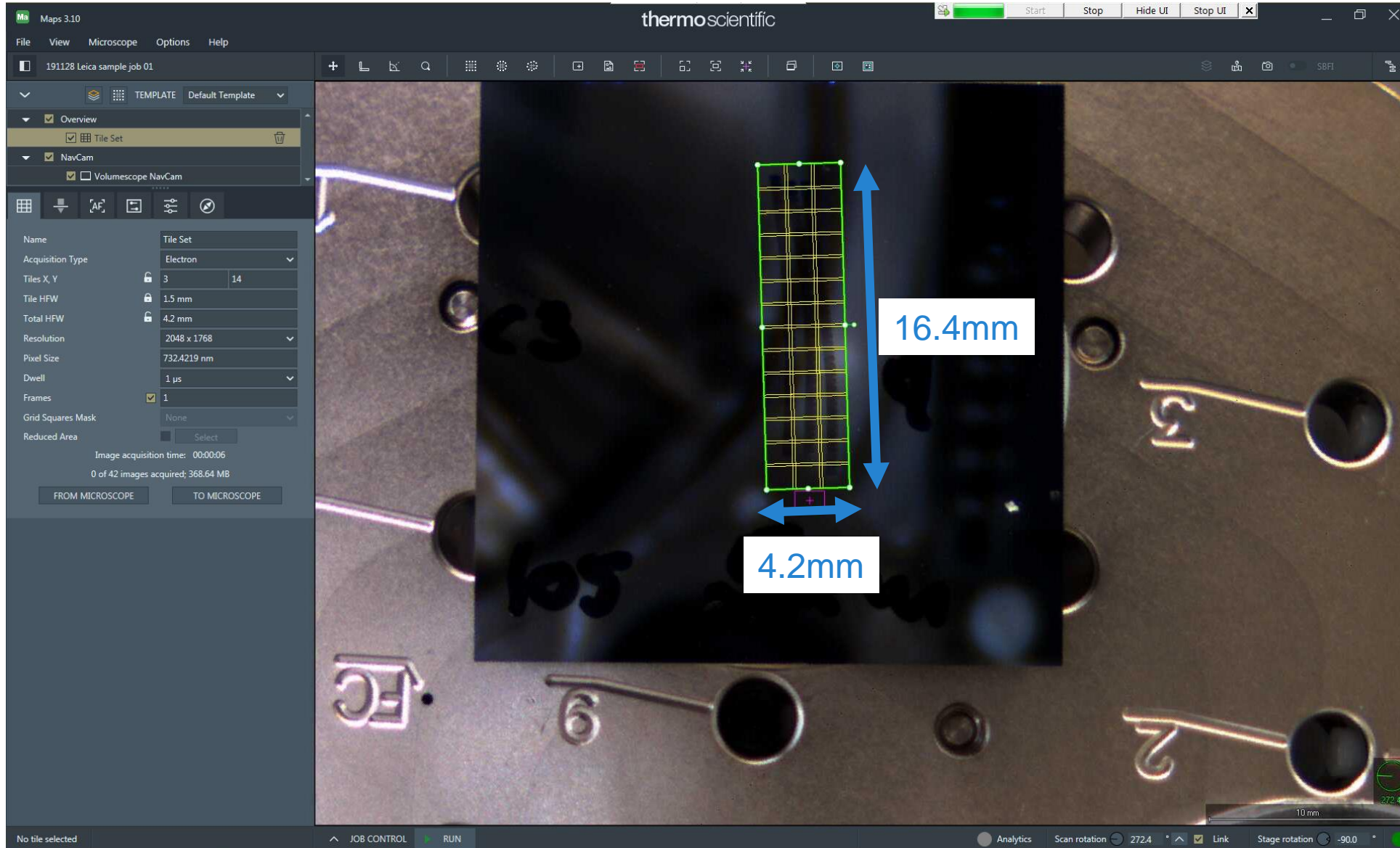
- Create an image mosaic by drawing a rectangle while holding down the ALT key.

- Once created, the tiling layout can still be changed by dragging the corners of the mosaic.

- To fit the ribbons of sections optimally, it may be necessary to rotate the mosaic. To do this, grab and move the "lollipop" handle at the right-hand side of the mosaic.

CAPTION: A single overview mosaic of 3x14 tiles of 2048x1768 pixel images was defined to cover all three ribbons.

Start acquisition - The job cue
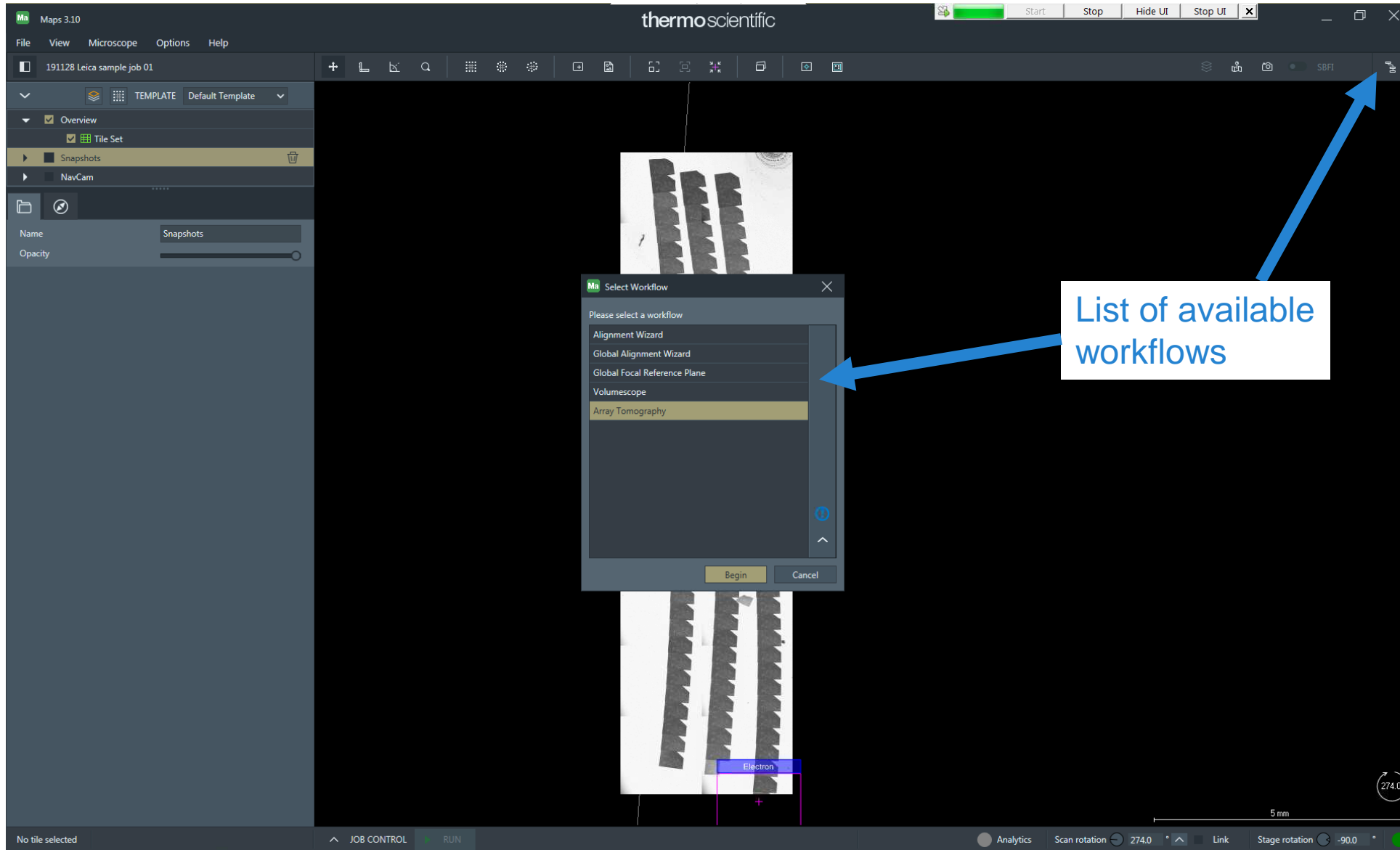

Each added tile set appears in the job queue. After one tile set is finished, the next in queue will be launched automatically. The job queue also calculates the total time for all image recordings.

- Start recording by clicking the RUN/STOP button.

Job queue

RUN/STOP button

Remaining time

CAPTION: Job cue shows that the recording time for the overview is 3:34 minutes.

Starting the Array Tomo workflow

List of available workflows

Once the SEM overview is recorded, one can hide the NavCam image.

The SEM overview mosaic shows the sections clear enough for the Automatic Section Finder.

- Launch the Array Tomography workflow by selecting it in the list.

The workflow panel

The Array Tomography Workflow consists of 11 steps, each represented by a Panel on the right-hand side of the screen.

- In each step, follow the instructions from top to bottom.

The first panel asks the user to acquire an SEM overview of the section set. As this was done already one can go directly to the next step.
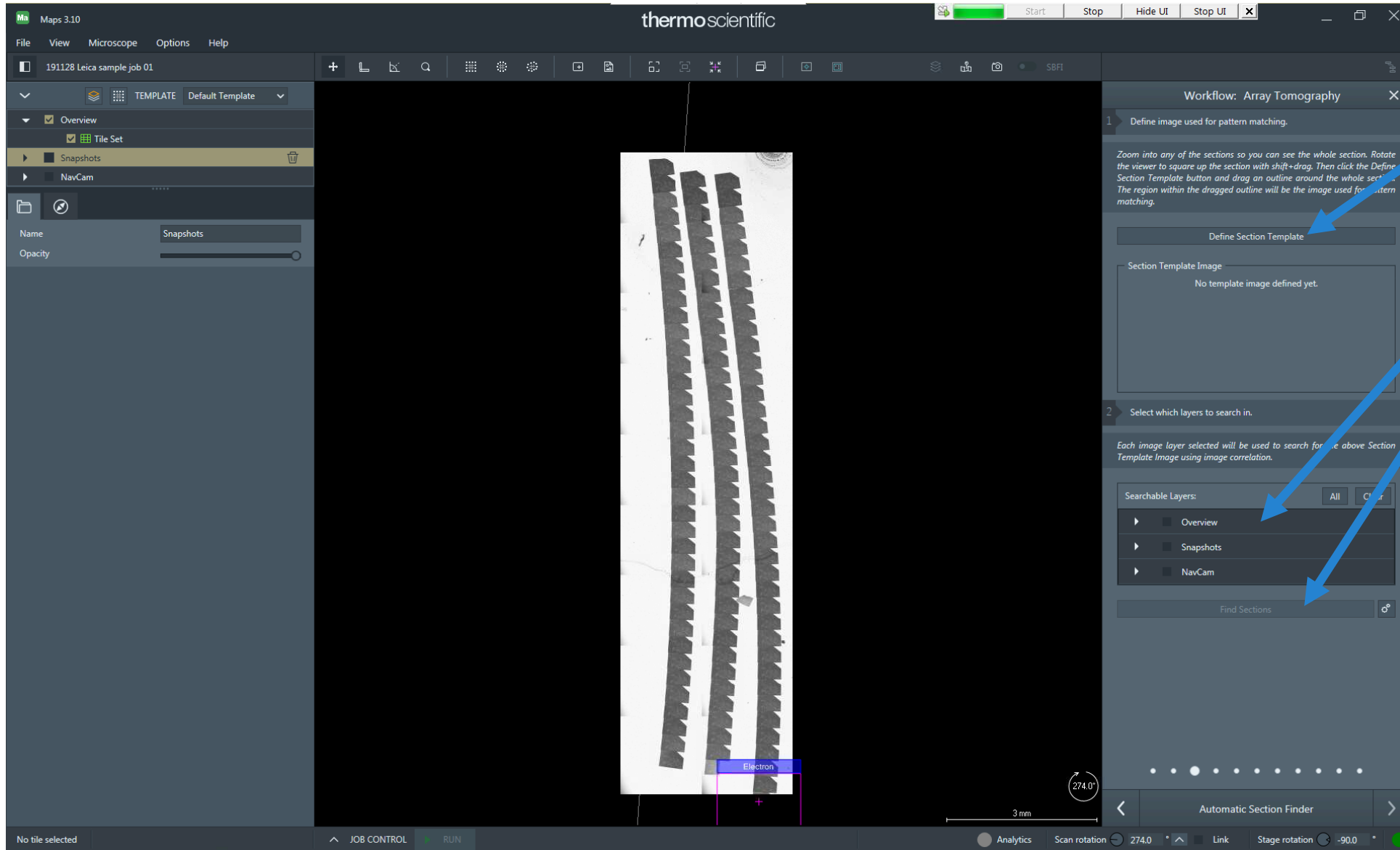
CAPTION: The first step in the AT workflow.
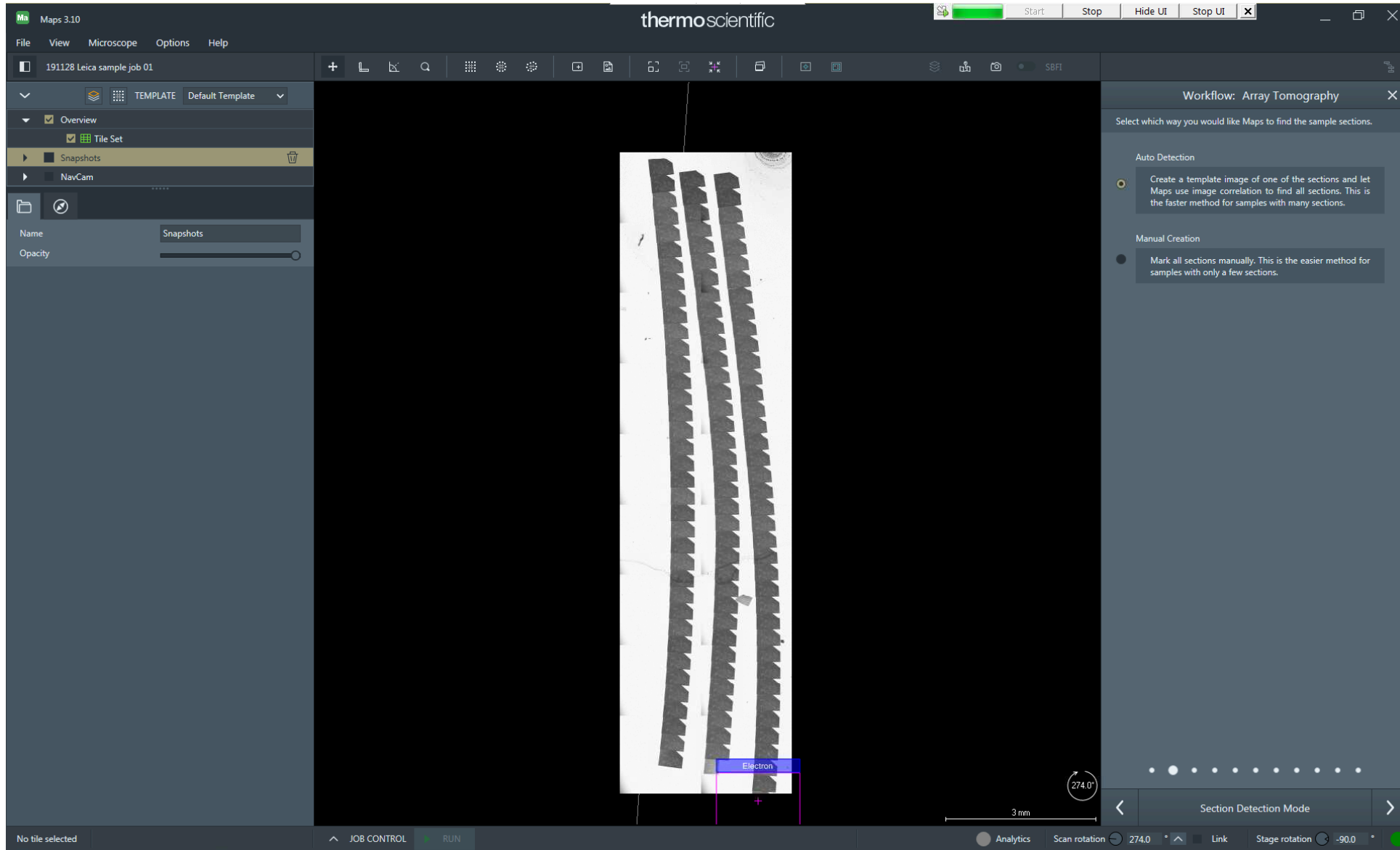
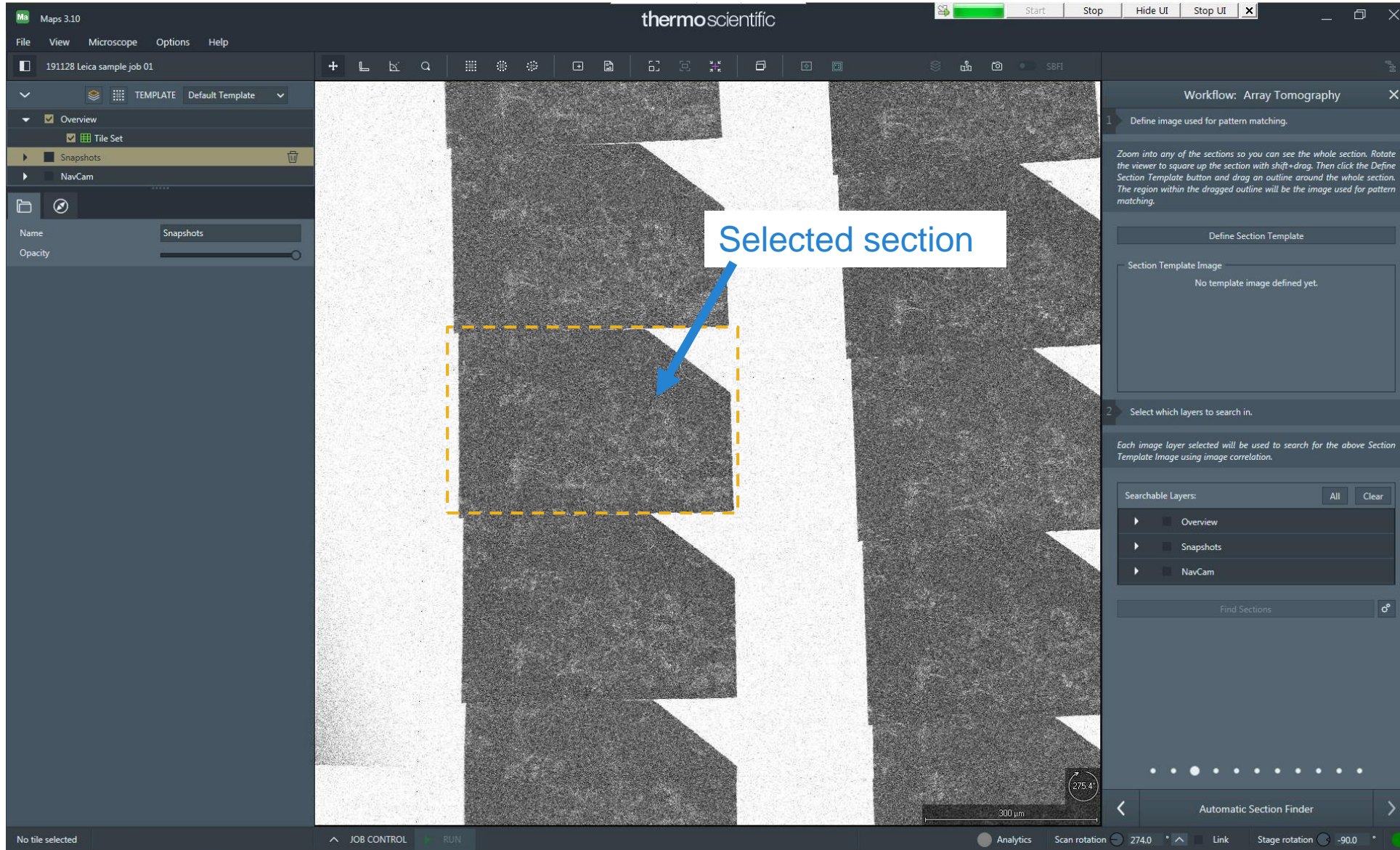*In this step the user can decide between manual outlining of sections and the Automatic Section Finder.*

- Always choose „auto detection"!

- Zoom in on a section (any)
- Click „Define Section Template"
- Drag a rectangle around one of the sections (see also next slide)
- Select overview image(s) to search in
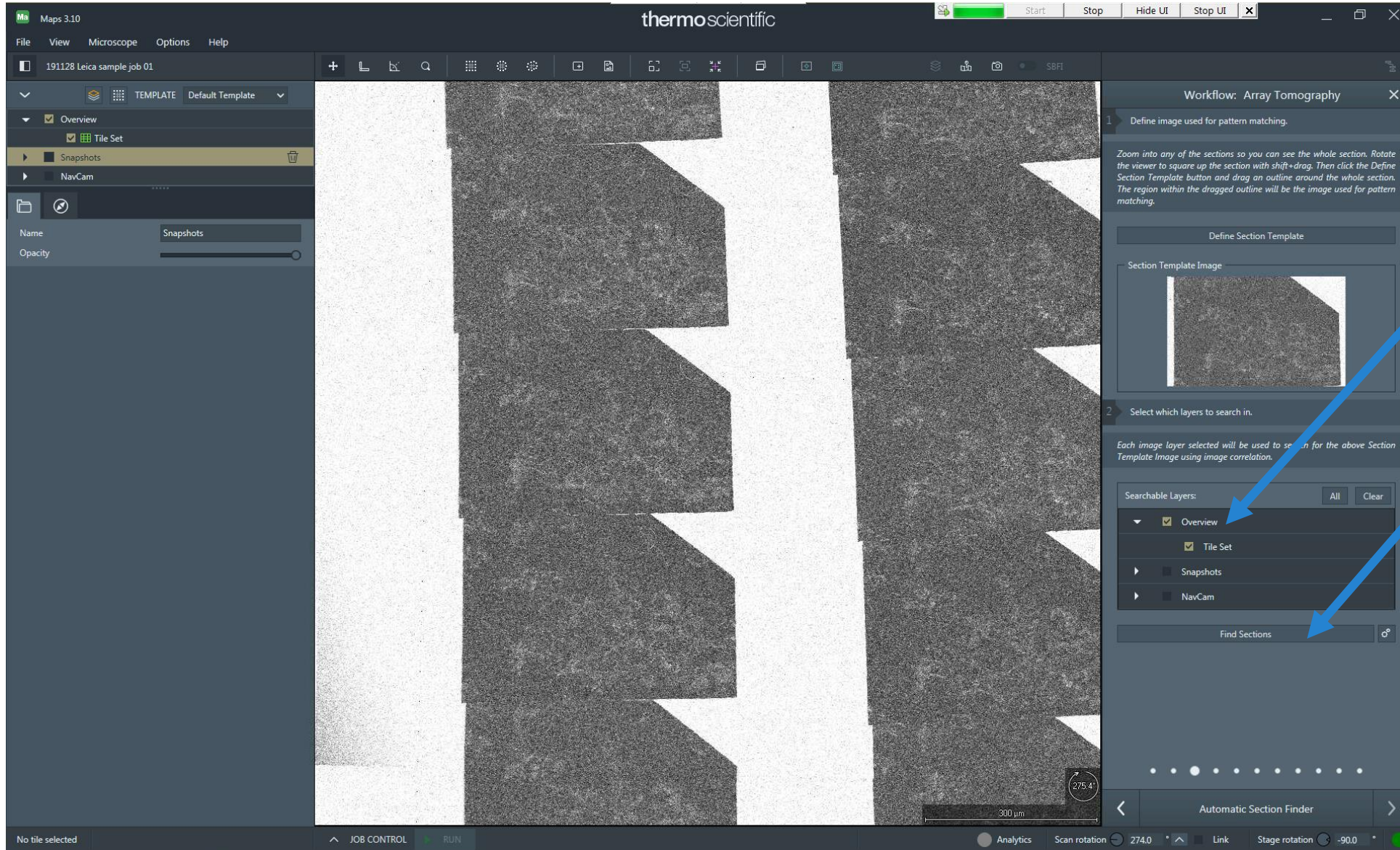- Click „Find Sections".

Step 3: The Automatic Section Finder

- Outline a section after zooming in by click+drag with the left mouse button.

- Choose a section in the middle of any of the ribbons.

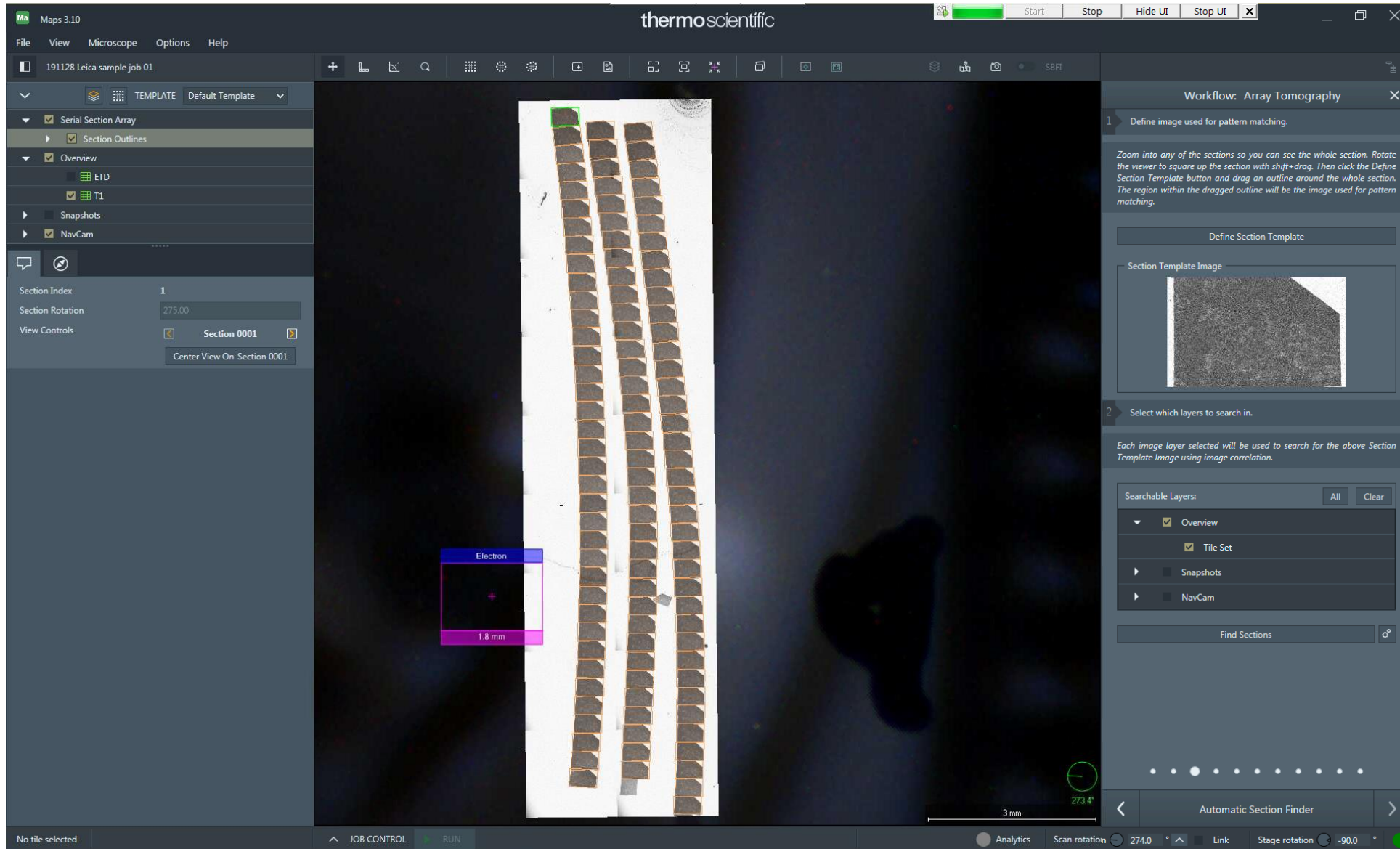*Avoid sections with obvious artefacts (dust, folds).*

Step 3: The Automatic Section Finder

The outlined section is used as a search template which allows Maps to find all section positions.

- Check the boxes next to all images in which Maps should search for sections. It is possible to search in multiple overview mosaics at once!

- Click „Find Sections".

The Search usually takes 1-3min to complete. The quality of the recognition depends on the quality of the ribbons and type of the sample.

With tidy, high contrast samples as the one shown in this example the Finder usually does not miss sections and does not falsely detect other structures as sections.

In case not all sections are recognized correctly, one can manually add or remove sections or switch to the manual workflow and locate all sections manually (not shown here).

CAPTION: In this example the finder took 35 seconds and found all sections. No sections were missed, no non-sections were found.

(Steps 4 and 5 are not shown, as no sections needed to be added or removed.)

Sections are found in random order, so Maps needs the user to define their true sequence.

- Define the section sequence by clicking „ORDER" and then drawing lines along the ribbons (left mouse click and drag).

The arranged sections turn green in the process.

Step 7: Record "Section Previews" - Rationale

- Section Previews are SEM images with higher resolution than the SEM overview. Typically, SEM overviews are acquired with a pixel size of 1-2µm, while Previews should average about 500nm per pixel. This increased resolution image set can be obtained within reasonable time as the software knows the section positions by now. Therefore, previews are only acquired on sections (one per section).

- Previews are needed in the following workflow steps for:

  - Navigation: The SEM overview mosaic is perfectly suitable for Section Finding but does not have high enough resolution for localizing areas of interest.

  - Image quality: Low signal-to-noise ratio is acceptable for overview mosaic and does not hinder the success rate of the Section Finder. Preview images, however, should be less noisy, as they will be used by the operator for locating ROIs. As Preview images are recorded in a much smaller total are (only on sections, not the empty space in between), it is possible to increase the pixel dwell time (for example from 1µs to 3µs) while maintaining an acceptable recording time.

  - Precision: Image mosaics can have discontinuities on tile borders, especially if the tiles are large. Preview images, on the other hand, are acquired without tiling. Each Preview is centered on one section. This minimizes the impact of geometric imperfections that SEMs can have in large fields of view.

  - Position Refinement: Section Previews are the basis for the Position Refinement step, which follows in the workflow.

- Use live imaging in the XT software to find good imaging parameters and to adjust contrast and brightness.

These parameters are typically used:

- *1.5-2kV*
- *100-800pA*
- *4mm working distance*
- *1-3µs pixel dwell time*
- *Field size sufficient to contain one section*
- *Pixel size ~500nm*

- Back in Maps, click three buttons:
- „Go To First Section", which moves the stage and the Maps viewer to the first section,
- „Acquire First Section Preview", which gives the user a chance to review the imaging settings before acquiring all remaining Previews.
- „Acquire All".

- Ultimately, we want to outline an imaging region in (any) one section, clone it to exactly the same relative location in all sections, and record the data as an image stack. We want all images to be well aligned.
- In reality this is very hard: Stage precision of +/-1µm, geometric distortions of the images, and variability of the sections themselves (stretching, compression, folds) cause at least 10µm, often 100µm of variability of imaging region placement.
- Position Refinement is a function that reduces the position variability of imaging regions.
- It allows the user to select a local area in one of the Section Previews, which is then used by Maps as a search template. This templates is matched in all the Section Preview images (it is also periodically updated in the process). From the matching positions, offsets are determined which are then used to improve imaging region placement.
- In case of variable compression/distortion, the correction is most precise in and around the selected template area. For acquisition of images in other, far away areas, it is possible to run Refinement a second time.

- Zoom in on the first section and outline an area around one or several regions of interest (left mouse button).

The template is shown in the workflow panel.

- Click „Start position Refinement".

- Observe the updating template while the refinement is continuing through the section set: there should not be jumps or drift.
- In case of failure, choose larger search template.

The process takes approximately 2 minutes.

If it fails consistently, in 99% of cases the section sequence is not correct! A jump (missing sections) or a mix-up in the section sequence can make it impossible to find a match.

Multiple different Array Tile Sets can be set up, each with individual size, position, rotation, and imaging settings (including mosaics).

- Create one or several Array Tile Sets (imaging regions which are repeated on many or all sections) using the button in the panel.

Position, rotation, and imaging parameters can be changed at any time; changed parameters are always cloned to all images that constitute the series.

- Define start and end section for each Tile Set using the Section Span Slider (default: tile set will be recorded on all sections).

22 CAPTION: Two Array Tile Sets were created - (a) a single image for auto functions (b) a large mosaic which covers an area of interest.

This is the last step in the workflow. It gives control over the acquisition sequence.

- Set the acquisition sequence to "By Section".

- Make sure the auto functions tile set is acquired first on each section.

Images are then acquired in this sequence:

- *Section 1: Auto focus, then Mosaic*

- *Section 2: Auto focus, then Mosaic*

…

- *Section 105: Auto focus, then Mosaic*

During acquisition
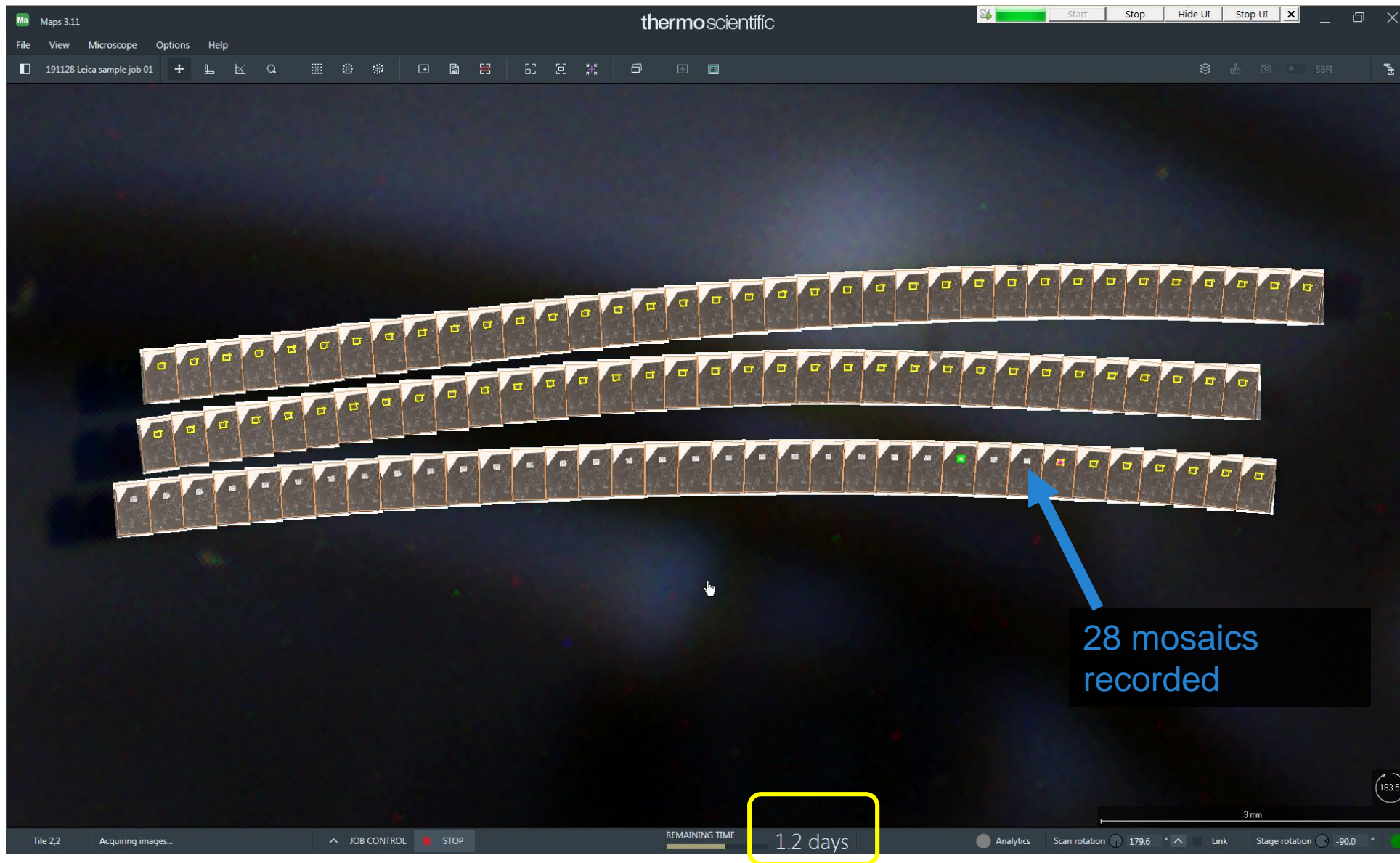
- *It is possible to add more Array Tile Sets at any time! Just pause the acquisition and continue from Step 10.*

- *Likewise, image positions can be changed by click-drag. This can be useful for tracking of features through the 3D volume.*

- *In case of change in focus, brightness etc during the acquisition, the workflow can be paused and re-initiated after the application of changes.*

- *Failed or mispositioned images can be reset during or after acquisition. Doing so automatically queues them for re-acquisition.*

CAPTION: Screenshot taken when 28 of 105 mosaics had been recorded. The remaining time shows 1.2 days left.

Data saving and the Job Queue
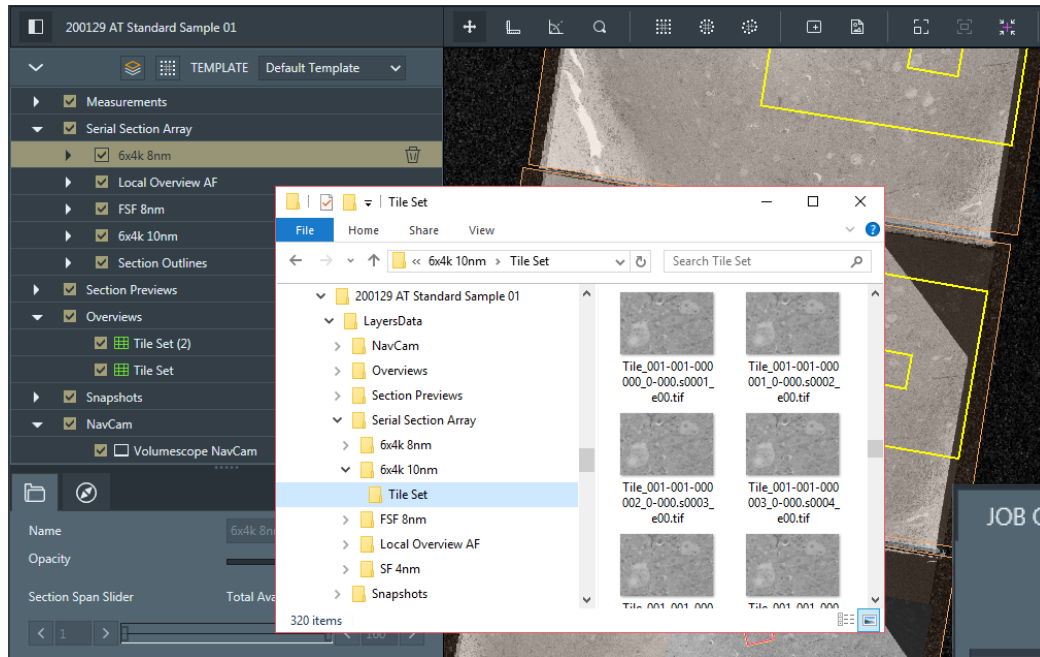
- The Job Queue shows remaining time in the status bar and the time per tile set when expanded. It also aloows reviewing or setting post-acquisition functions like „switch off beam when done".

- Data are saved into folders on disk in just the same way as arranged in the Layer tree.

- Images are saved in .tif format.

- Mosaics acquired in multiple sections can be stitched into fused .tif images in batch mode.

Troubleshooting and general advice

- Ribbons are not visible in the NavCam image / no Navcam installed.
  - SOLUTION: Find corners of sample holder by navigating in live SEM view and acquire snapshots. Then, use snapshots for orientation while setting up low-resolution SEM overview mosaics of 2x2mm tiles. Even very large areas can be recorded in acceptable time, for instance 5x5cm can be covered within 20 minutes! (28x31 tiles of 1024x864 pixels, 10% overlap, 1µs dwell time, 0.5s per tile for stage move).
  - In even larger samples, record several long 1 x N mosaics crisscrossing the stage motion area until sufficient fractions of ribbons or sections are visible. Then, set up one or several SEM overview mosaics that show all sections.
  - In many cases, such low-resolution SEM overview are sufficient for running the Section Finder!
- Section Finder finds less than 90% of sections or more than 10% of false-positives.
  - In case false-sections are detected on tile borders: Use an in-lens detector for acquiring SEM overviews.
    The Section Finder falsely recognizes sections on tile borders in SEM overview mosaic(s) where the images have gradients. ETD is known to produce such gradients, while in-lens detectors often produce very flat, gradient-free images.

- If too few sections are recognized, or if the result is overall imprecise, make sure the section-to-background contrast in the image mosaic is higher than the contrast between any other structures in the overview. This can be achieved by choosing suitable accelerating voltage: 2kV is perfect for sections of 50-70nm thickness; reduce the high tension if sections are thinner or weakly stained. Increase to >2kV if sections are thicker. Exclude areas in which false sections are detected from the SEM overview by acquiring several local SEM mosaics instead of one big one.
- I deleted an image (or other object) in Maps. How can I get it back?
  - Not at all. Be careful when selecting items in the layer tree or in the viewer for deleting them! Maps does not have undo, and it doesn't have a recycle bin either.
- I want to run the Section Finder once more in an existing project. What should I be aware of?
  - This can be desirable for example when the array acquisition was set up on only a part of all ribbons of a sample. Be very careful, though: when running the Section Finder a second time in an existing project, all previously found sections are deleted including all images recorded in them! The SOLUTION is to export the SEM overview mosaics into a new Maps project by right-clicking them (or the layer they reside in) in the layer tree and selecting "Export" in the context menu.