

Supplementary methods

Workflow for SEM imaging on serial ultra sections in Maps

Tilman Franke and Irina Kolotuev

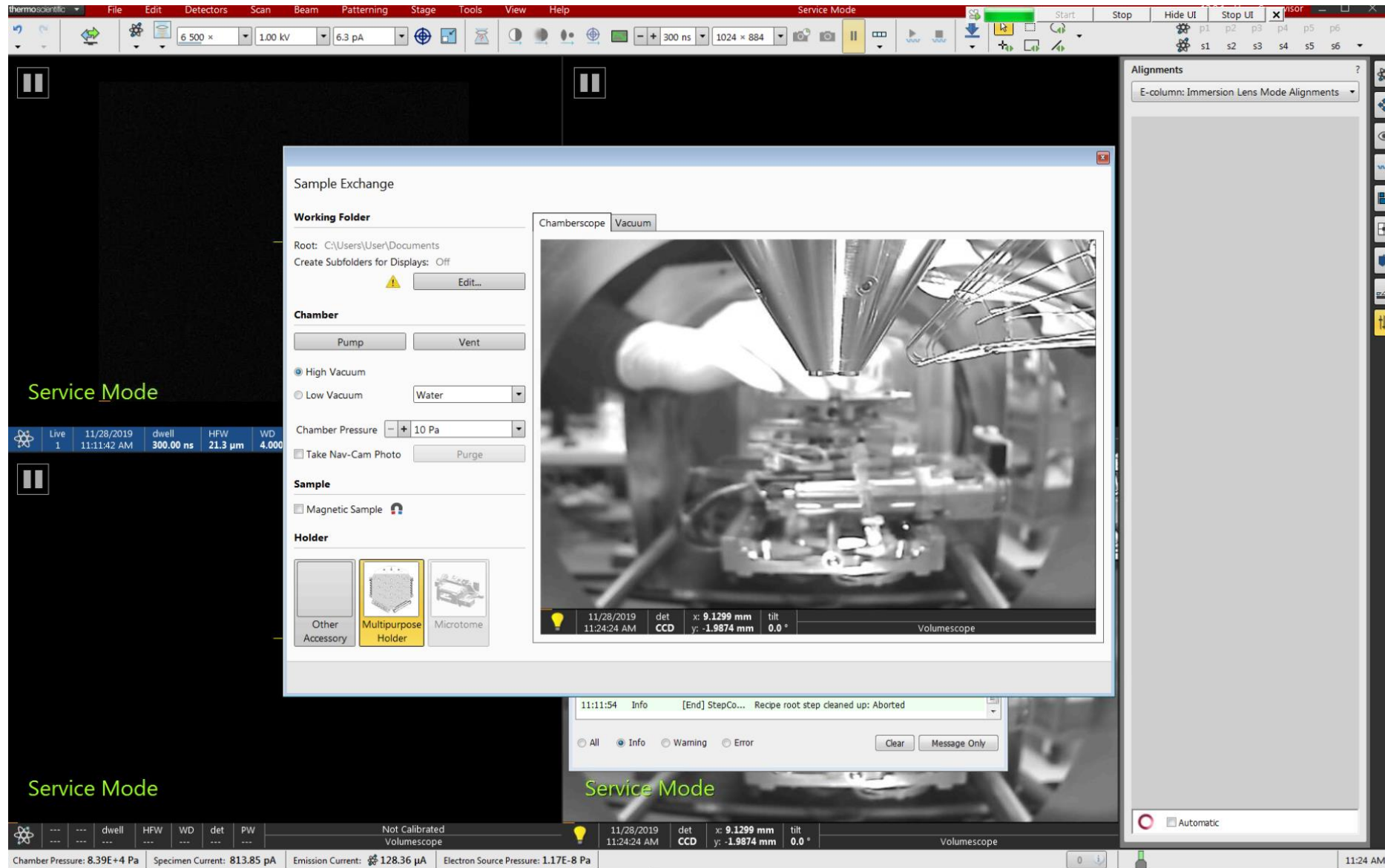
tilman.franke@thermofisher.com

irina.kolotueva@unil.ch

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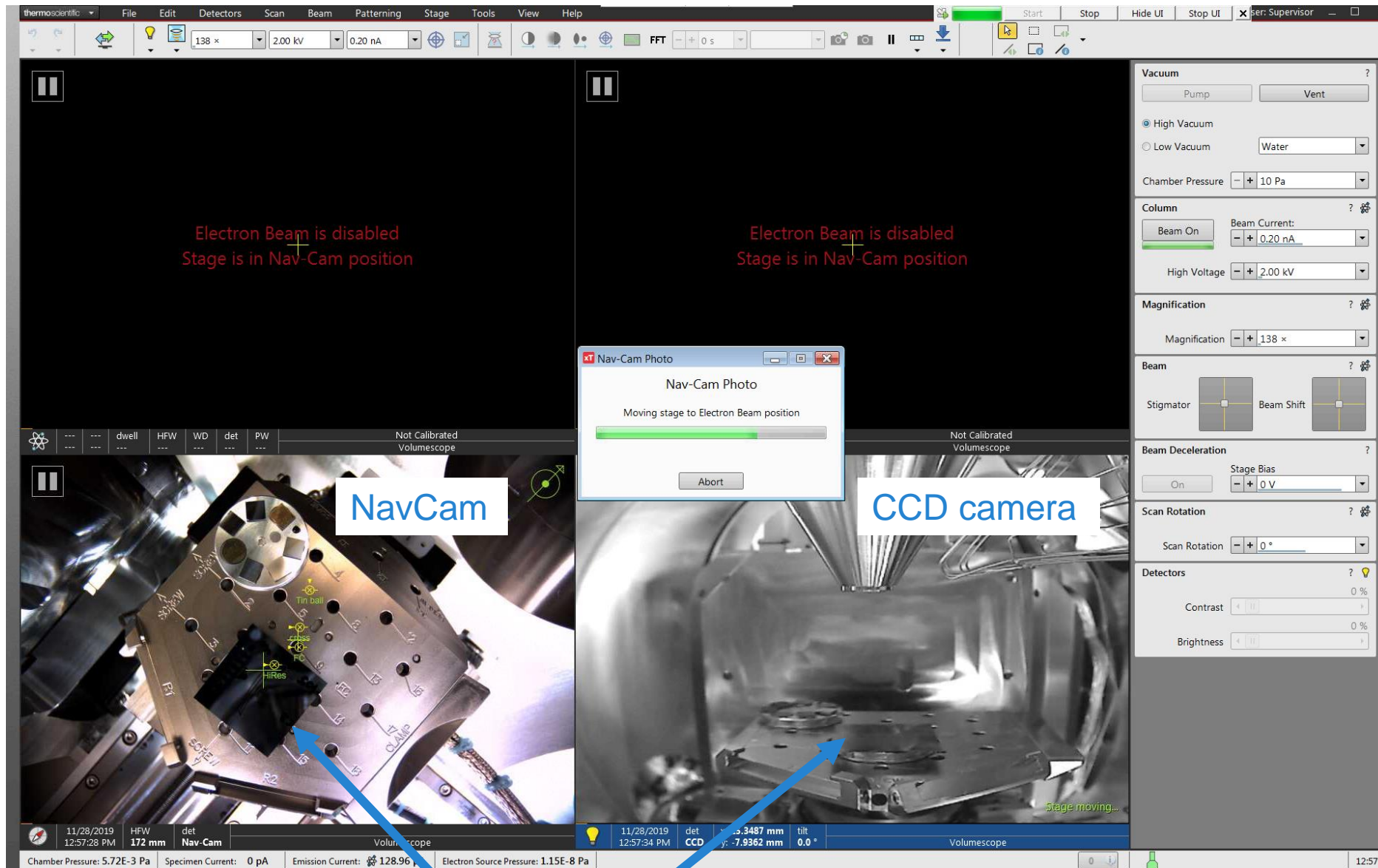
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Sample loading



- 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.

Acquisition of Navigation image



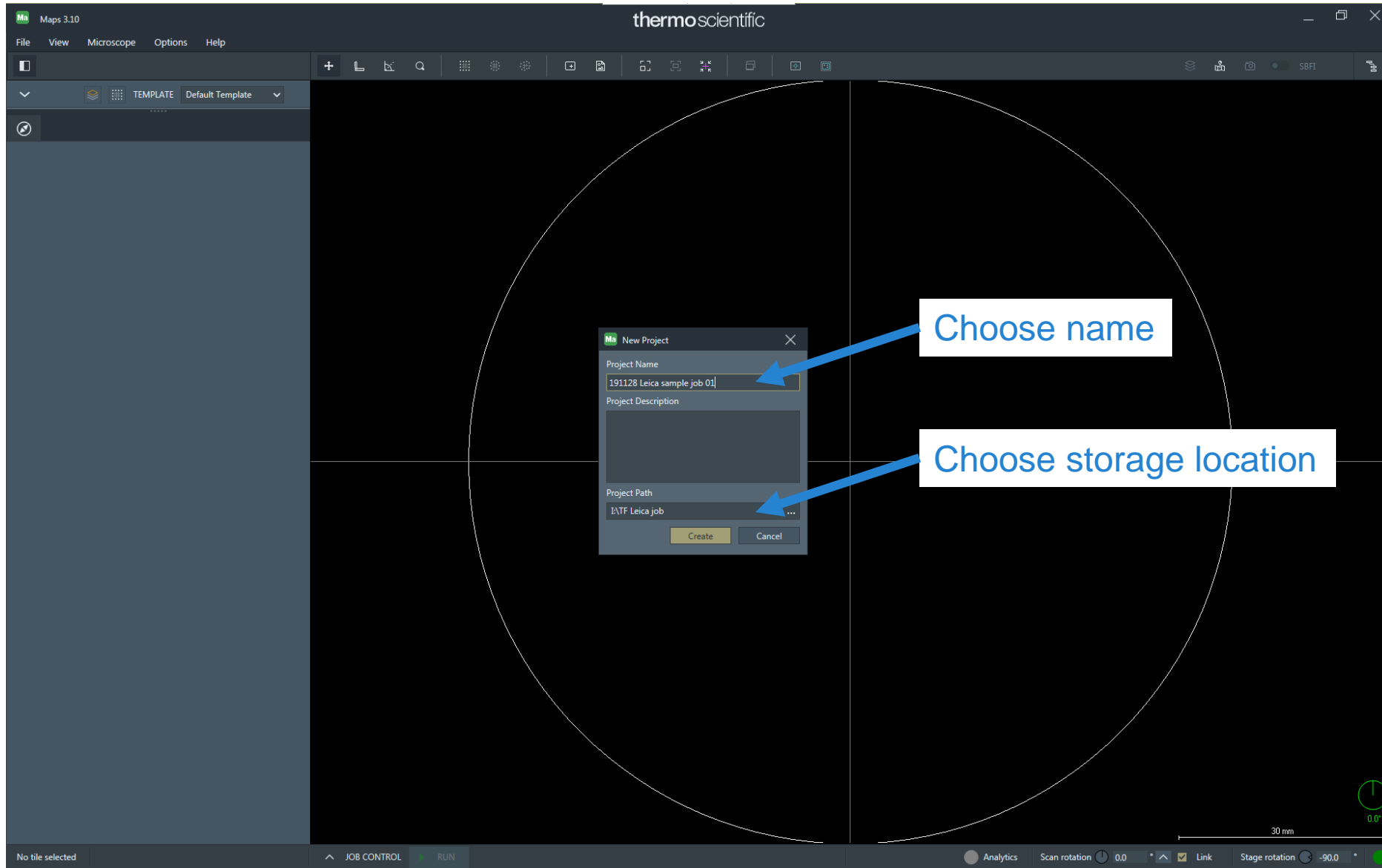
Most Thermo Fisher SEMs have two cameras. All systems are equipped with the “CCD camera” which looks at the sample from the side.

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.

Array Tomography sample

New Maps project

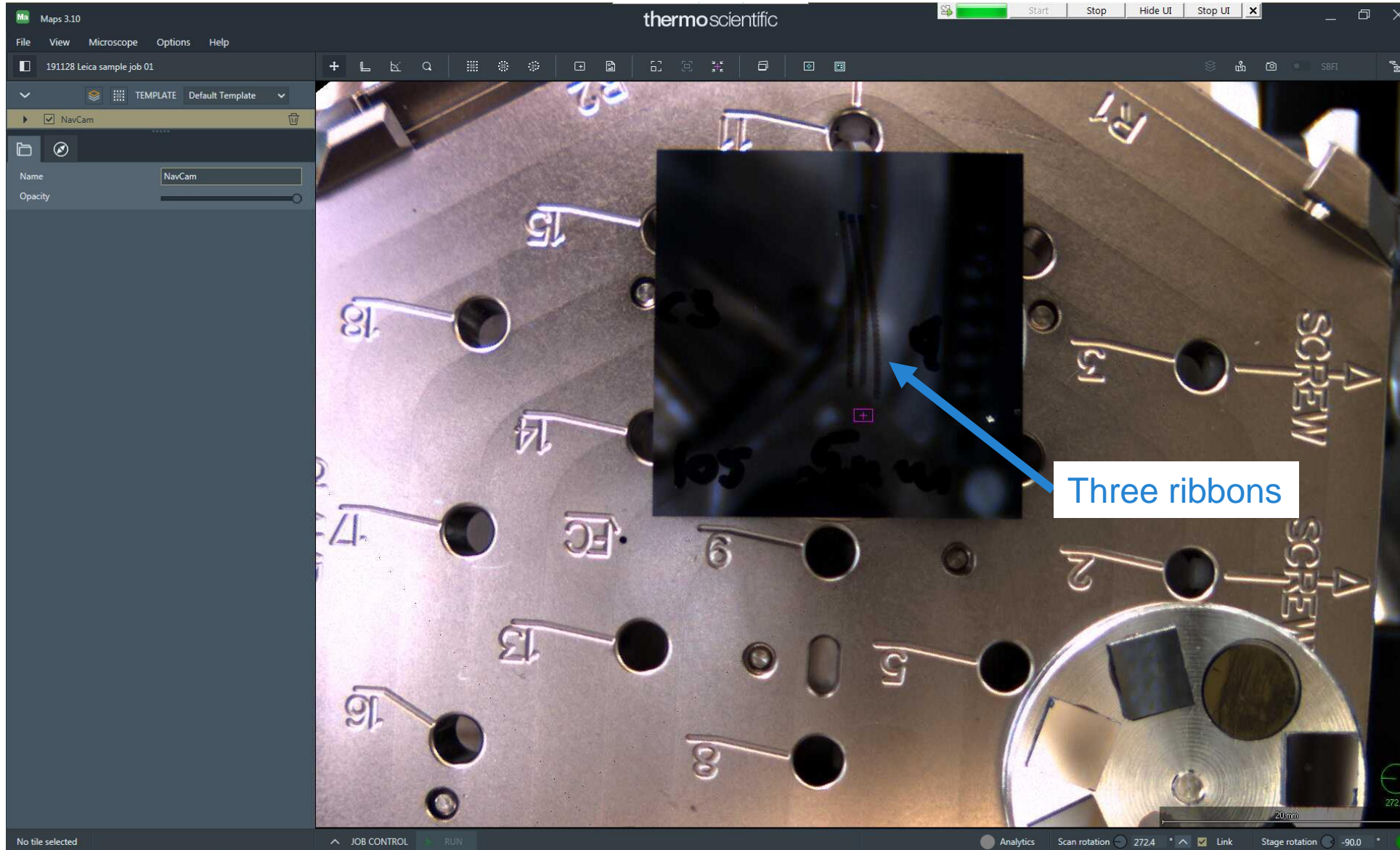


- Launch Maps software which runs alongside the XT microscope control software.

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.

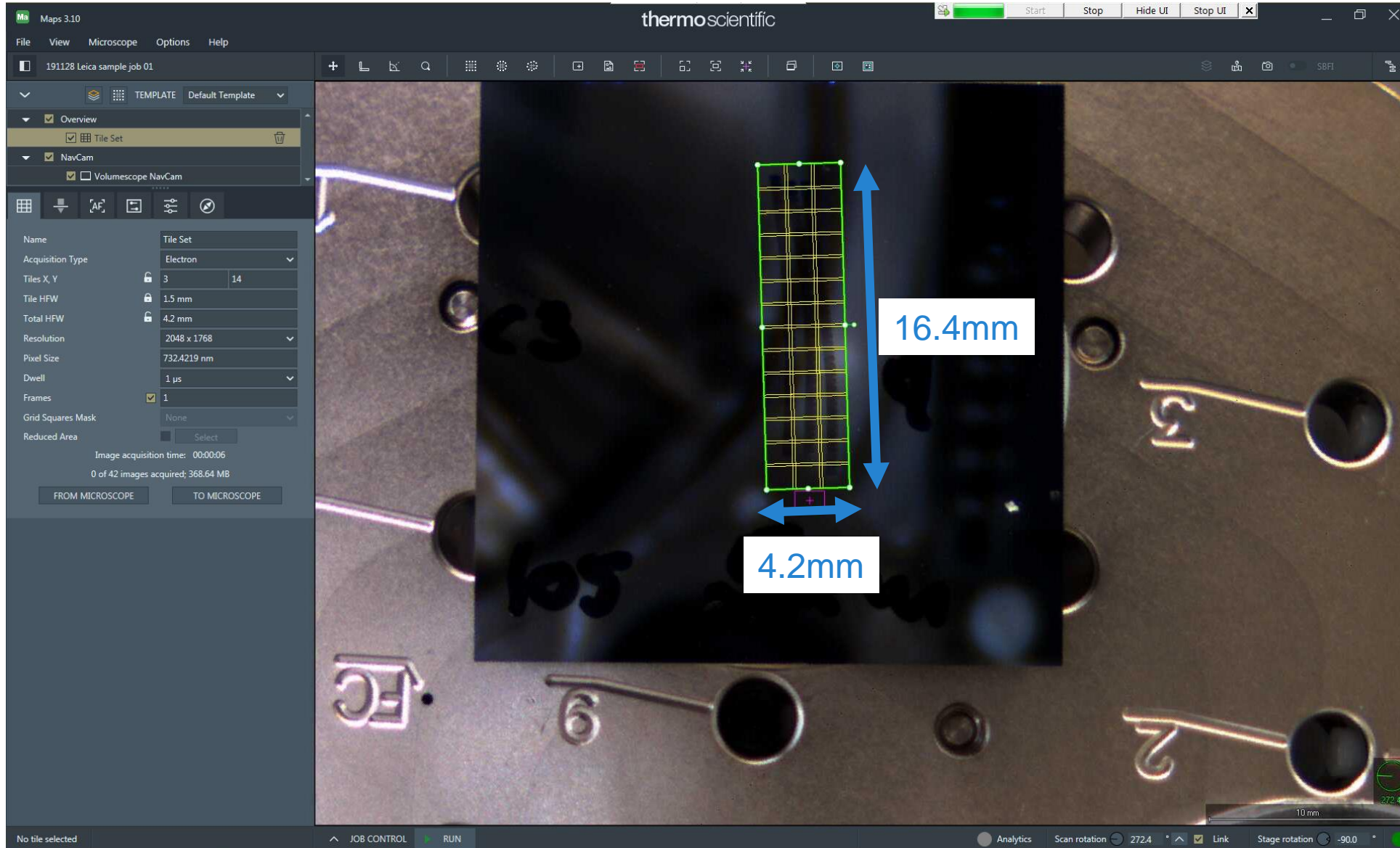
NavCam image in Maps



- 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 sufficient 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.

Start acquisition - The job cue

The screenshot displays the Thermo Scientific Maps 3.10 software interface. The main window shows a microscope view with a grid overlay. A 'JOB QUEUE' window is open, showing a single job in progress. The job name is 'Tile Set', and it is currently 'Acquiring images...' with a progress bar at 4%. The remaining time for this job is 00:03:34. The interface also shows acquisition parameters such as 'Acquisition Type: Electron', 'Tiles X, Y: 3 x 14', 'Tile HFW: 1.5 mm', and 'Total HFW: 4.2 mm'. A 'RUN/STOP' button is visible in the bottom left corner of the job queue window. A 'REMAINING TIME' display at the bottom of the software shows 00:03:34.

Job queue

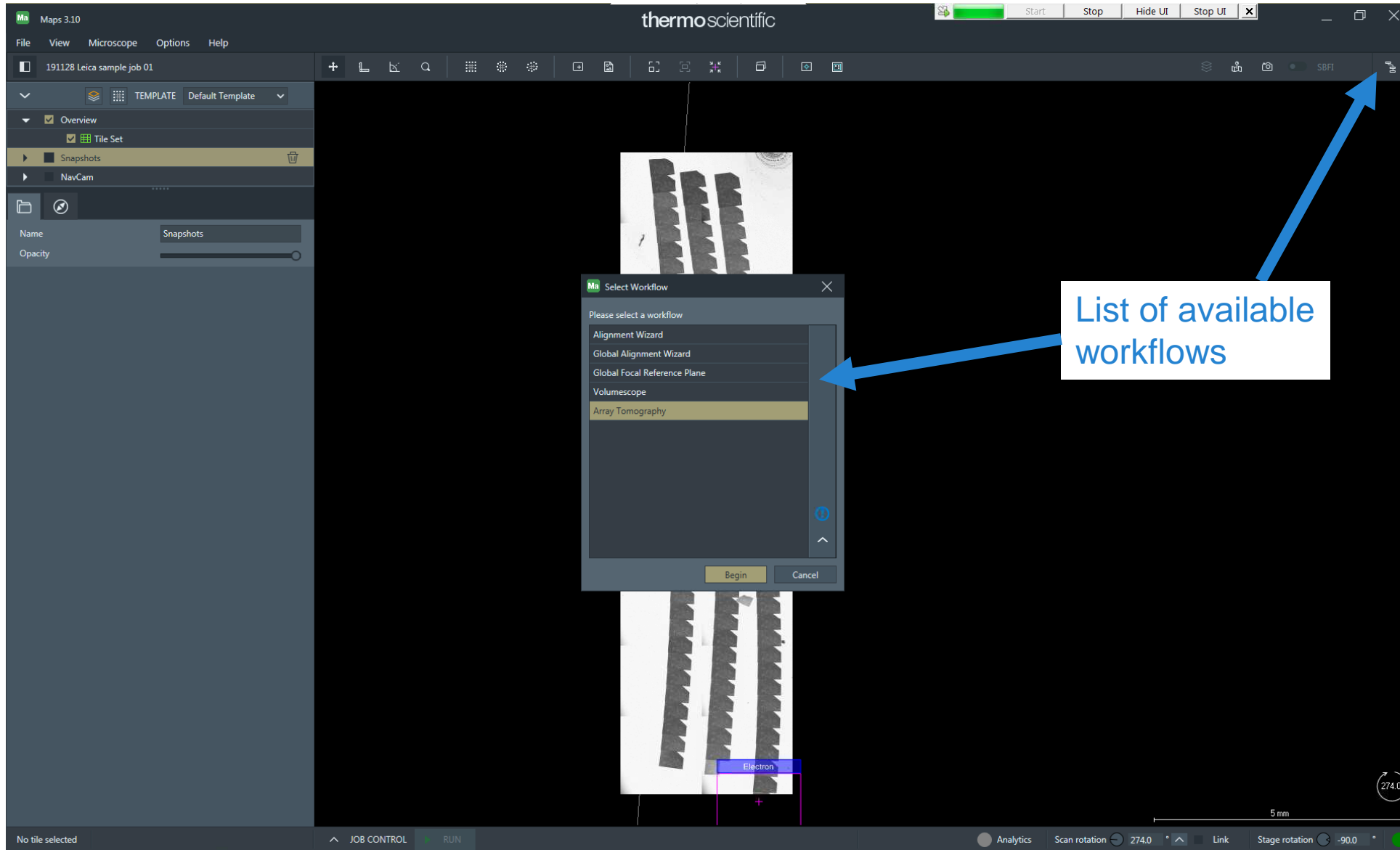
RUN/STOP button

Remaining time

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.

Starting the Array Tomo workflow



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

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Maps 3.10

File View Microscope Options Help

191128 Leica sample job 01

TEMPLATE Default Template

Overview

Tile Set

Snapshots

NavCam

Name: Snapshots

Opacity

Workflow: Array Tomography

- 1 Create and acquire tile sets or import images that cover all sections.
Make sure these images have a high contrast between the sections and the substrate. This will help with the auto detection of sections.
- 2 Move all the tile sets and/or imported images from the above step into a single layer in the layer tree.

Instructions for current step

11 Workflow steps, use arrow buttons to go forward and backward through the steps at any time.

Electron

3 mm

274.0°

Overview Imaging

No tile selected

JOB CONTROL RUN

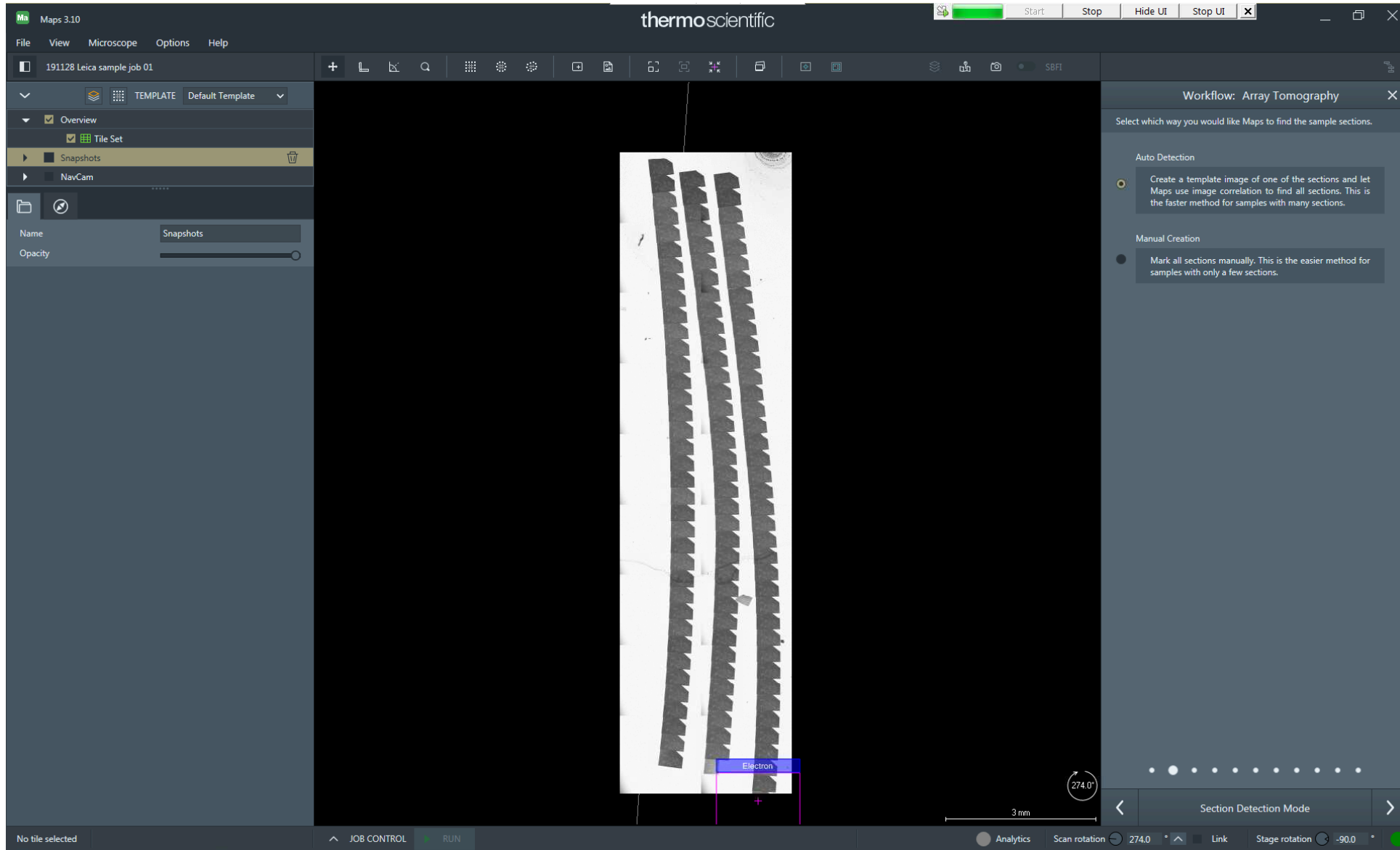
Analytics Scan rotation 274.0 Link Stage rotation -90.0

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.

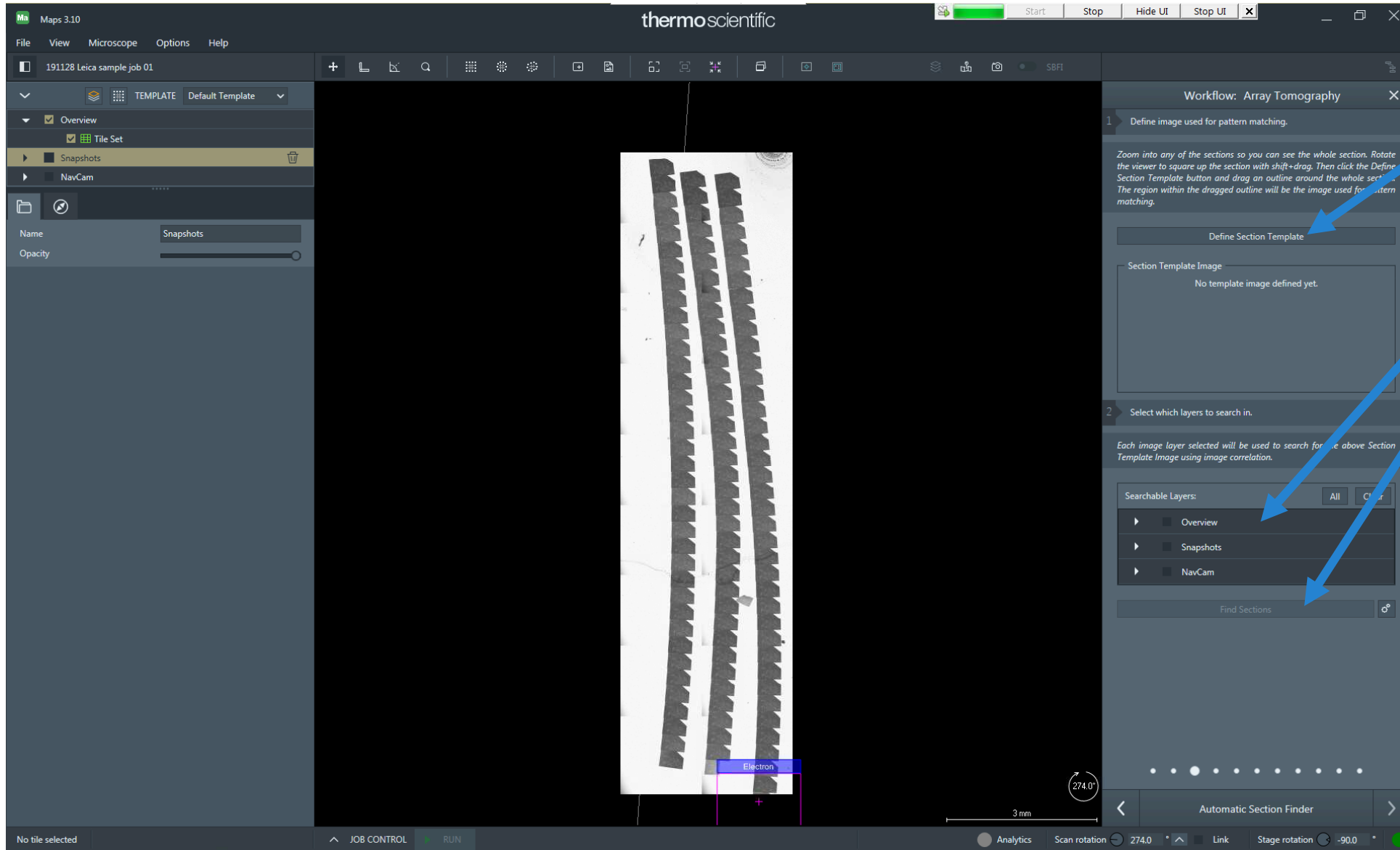
Step 2: Choose the Automatic Section Finder



In this step the user can decide between manual outlining of sections and the Automatic Section Finder.

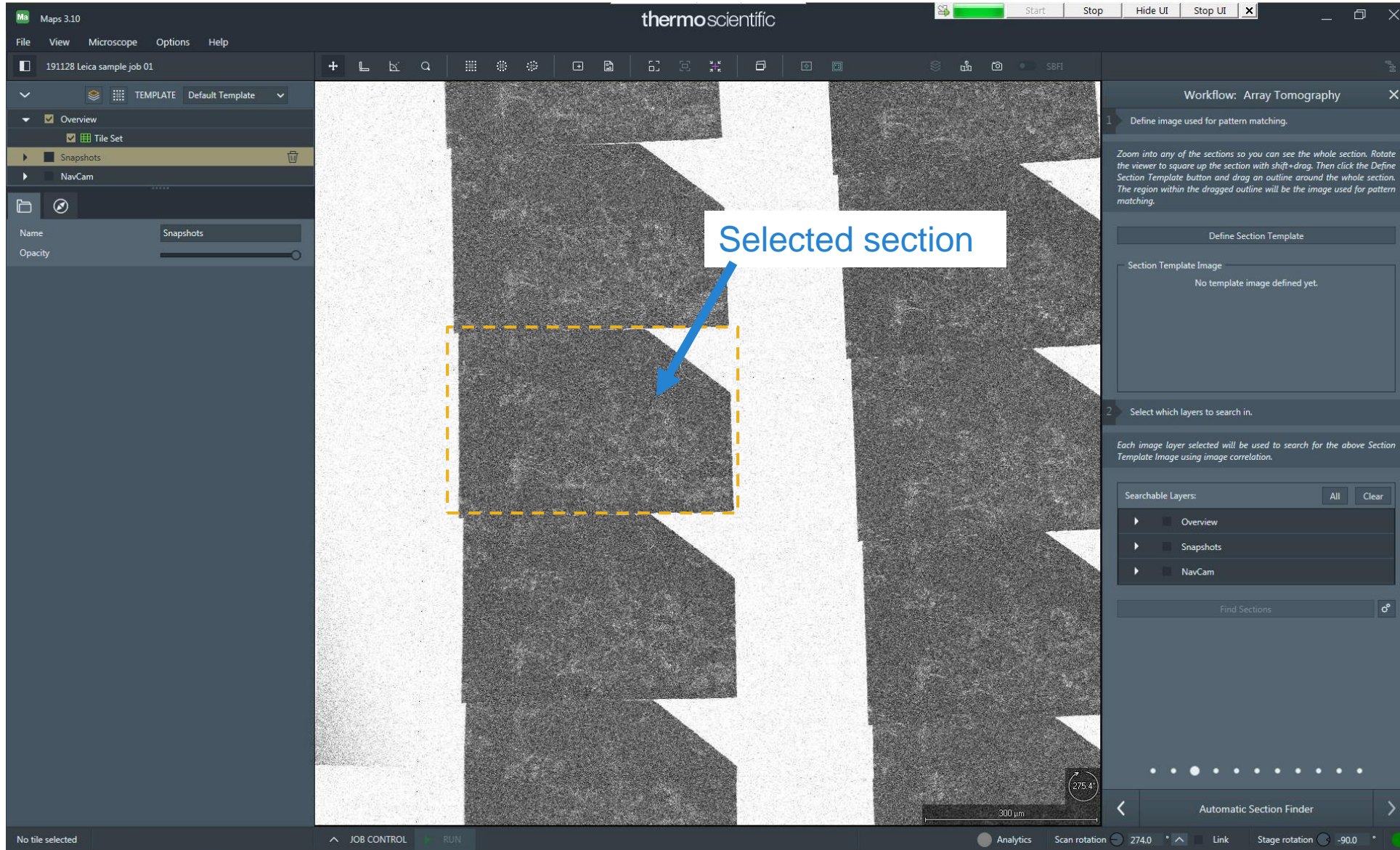
- Always choose „auto detection“!

Step 3: The Automatic Section Finder



- 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“.

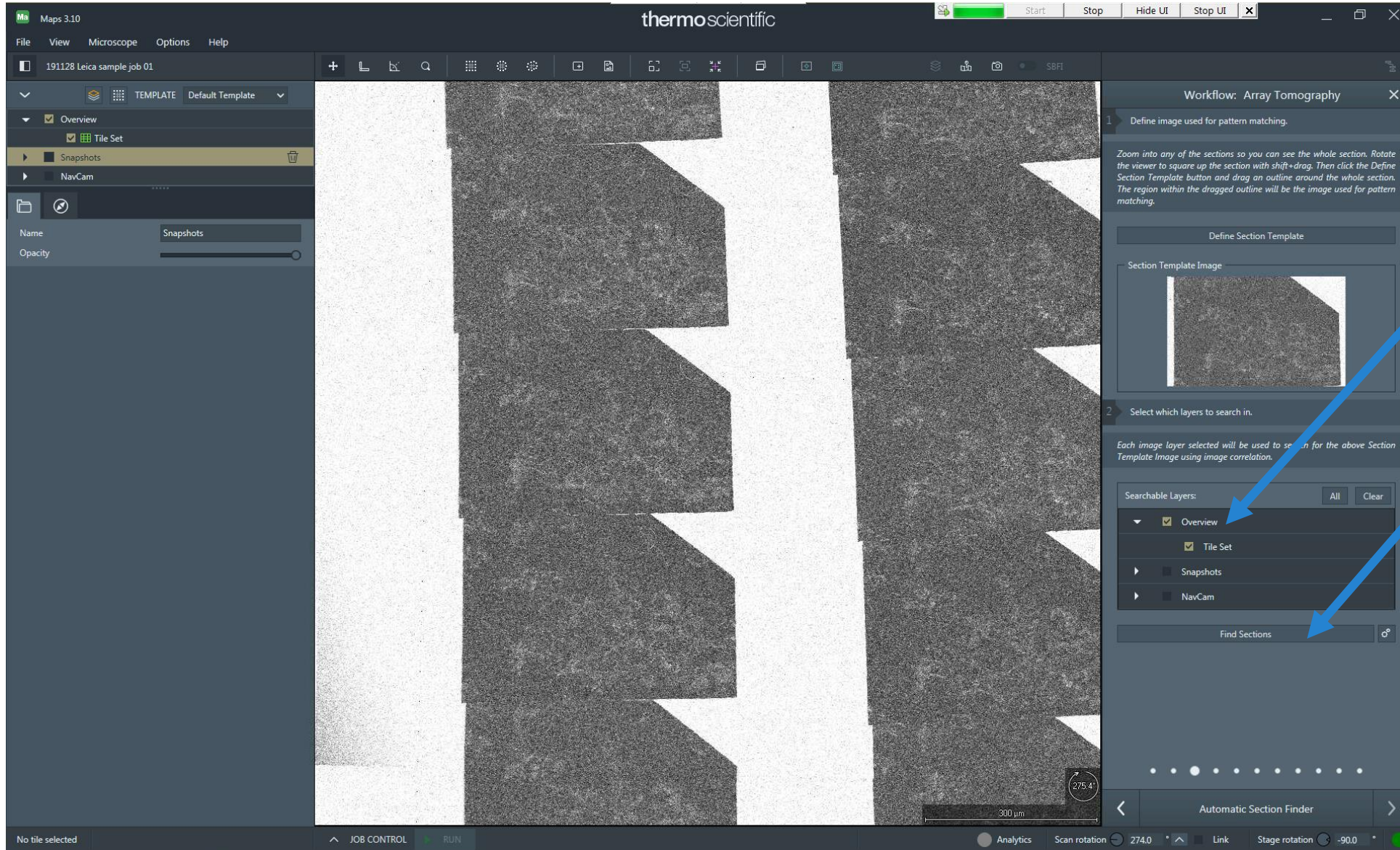
Step 3: The Automatic Section Finder



- 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).

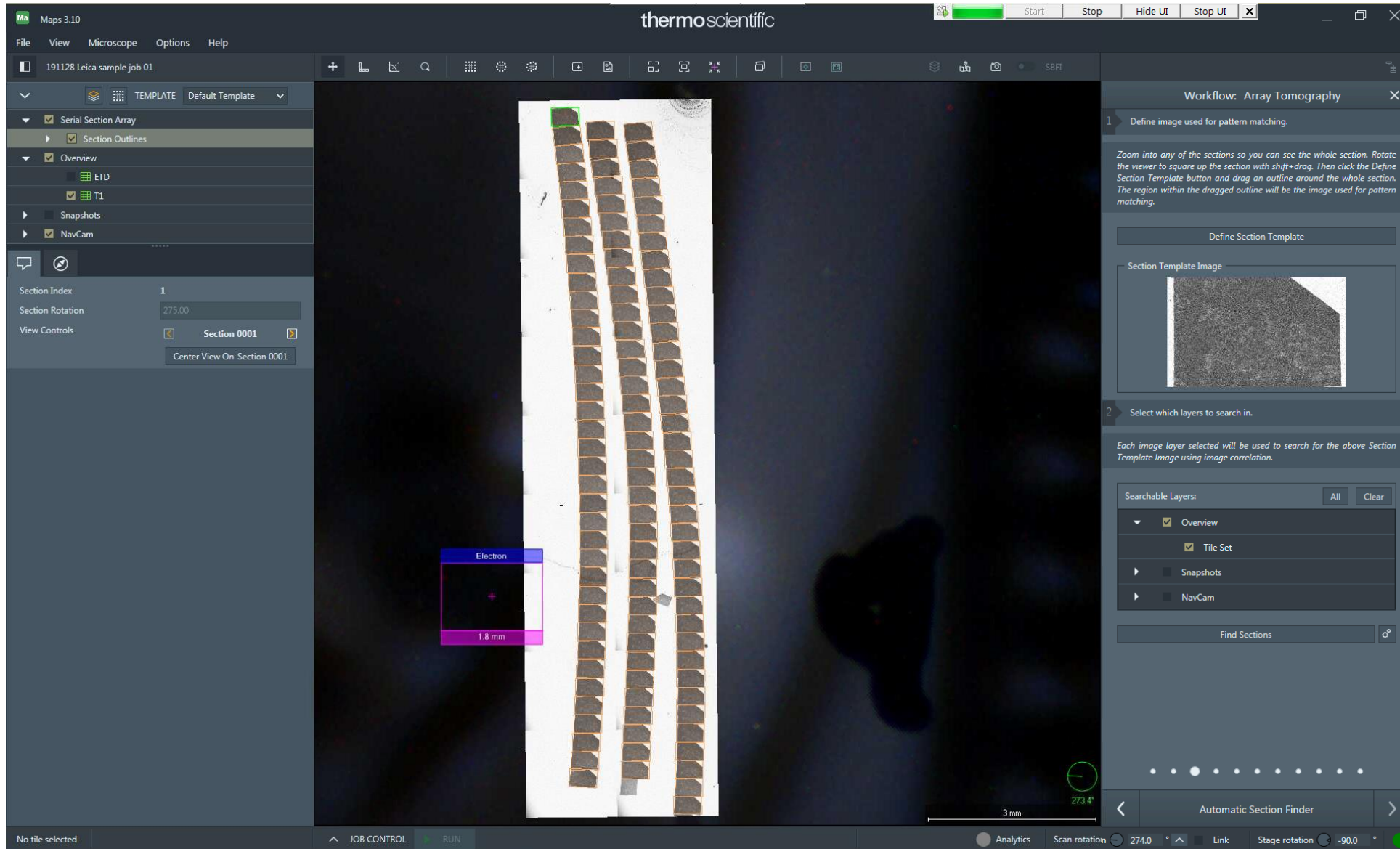
Step 3: The Automatic Section Finder



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“.

Step 3: The Automatic Section Finder - Result



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).

Step 6: Sort section sequence

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Maps 3.10

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Serial Section Array

Section Outlines

Overview

ETD

T1

Snapshots

NavCam

Section Index: 1

Section Rotation: 275.00

View Controls: Section 0001

Center View On Section 0001

Workflow: Array Tomography

Use the ORDER button to draw a set of lines in the viewer to indicate the sequence of Sections from first to last. Use the VIEW button to pan and zoom in the viewer between painting lines.

Ordered Section (Green)

Unordered Section (Brown)

ORDER VIEW

Ordered Sections:

- Section 0105
- Section 0104
- Section 0103
- Section 0101
- Section 0102
- Section 0100
- Section 0099
- Section 0098
- Section 0097
- Section 0096
- Section 0095

Reset Order

Section 37

Section 1

Electron

1.8 mm

3 mm

273.4°

Define Section Sequence

Analytics Scan rotation 273.4 Link Stage rotation -90.0

(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 1-2 μ m, 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 area (only on sections, not the empty space in between), it is possible to increase the pixel dwell time (for example from 1 μ s to 3 μ s) 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.

Step 7: Find good imaging settings for Section Previews

The screenshot shows the ThermoFisher XT software interface. The main window displays a live electron microscopy image of a biological specimen, likely a section of a cell, with a yellow crosshair indicating the current beam position. The interface includes a top menu bar (File, Edit, Detectors, Scan, Beam, Patterning, Stage, Tools, View, Help) and a toolbar with various icons for navigation and control. On the right side, there is a control panel with several sections: Vacuum (High Vacuum selected, Chamber Pressure 10 Pa), Column (Beam On, Beam Current 0.20 nA, High Voltage 1.80 kV), Compound Lens Filter (On, Threshold Energy 1.0 keV), Magnification (753 x), Beam (Stigmator and Beam Shift controls), Beam Deceleration (On, Stage Bias -4000.00 V), Scan Rotation (-86.0°), and Detectors (Contrast 32.47%, Brightness 22.26%). At the bottom, a status bar displays technical parameters: 11/28/2019 1:49:59 PM, HV 1.80 kV, curr 0.20 nA, dwell 3.00 µs, WD 4.1894 mm, HFW 550 µm, PW 358 nm, and a 100 µm scale bar. The status bar also shows Chamber Pressure: 1.88E-3 Pa, Specimen Current: 0 pA, Emission Current: 129.36 µA, and Electron Source Pressure: 1.57E-8 Pa.

- 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

Step 7: Record all Preview images

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Maps 3.11

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Serial Section Array

- Section Outlines
- Section Previews
 - Section 0001-preview
 - Section 0002-preview
 - Section 0003-preview
 - Section 0004-preview
 - Section 0005-preview
 - Section 0006-preview
 - Section 0007-preview
 - Section 0008-preview
 - Section 0009-preview
 - Section 0010-preview
 - Section 0011-preview
 - Section 0012-preview
 - Section 0013-preview
 - Section 0014-preview
 - Section 0015-preview
 - Section 0016-preview

Section 0001-preview

Acquisition Type: Electron

Tiles X, Y: 1, 1

Tile HFW: 550 µm

Total HFW: 550 µm

Resolution: 1.536 x 1024

Pixel Size: 358.0729 nm

Dwell: 3 µs

Frames: 1

Grid Squares Mask: None

Reduced Area: Select

Image acquisition time: 00:00:00

1 of 1 images acquired; 5.5 MB

FROM MICROSCOPE TO MICROSCOPE

Workflow: Array Tomography

In this step, high resolution images will be acquired at each section which will be used to increase the accuracy of the section outline placement in the next step.

- 1 Use the Go To First Section button to bring the viewer and stage to the first Section.
Go To First Section
- 2 Use the microscope UI to set up suitable acquisition settings and click the Acquire First Section Preview button to create and acquire first preview image using these settings.
Acquire First Section Preview
Tips: Try and encompass the whole section in the field of view if possible. This will reduce acquisition time. Make sure you can see a good contrast of details within the section. Make sure the sample is in focus. Try to have at least 2k resolution. These tips will all help for the refinement in the next step.
- 3 Use the Acquire All Section Previews button to have Maps record previews of all Sections.
Acquire All Section Previews
Make sure the first section preview is in focus and has visible sample details. The Acquire All Section Previews button will create and acquire an identical tile set as the first preview on the rest of the sections.

Acquiring Section Previews...

Reset Previews

Section Previews (optional)

278.0°

4 mm

REMAINING TIME 00:00:01

Analytics Scan rotation 278.0° Link Stage rotation -90.0°

- 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“.

Step 8: Position Refinement - Rationale

- 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 $\pm 1\mu\text{m}$, geometric distortions of the images, and variability of the sections themselves (stretching, compression, folds) cause at least $10\mu\text{m}$, often $100\mu\text{m}$ 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 template 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.

Step 9: Position Refinement

The screenshot displays the Thermo Scientific software interface for a serial section array. The main window shows a grid of electron microscopy images. A central image is highlighted with a blue dashed box, and a white mouse cursor is positioned over it. A blue arrow points from the 'Start Position Refinement' button in the workflow panel to this central image. The workflow panel on the right is titled 'Workflow: Array Tomography' and contains the following steps:

- Define the refinement region in the first section and begin refinement.
 - Bring View To First Section
 - Define Refinement Region
 - Start Position Refinement**
- Use the apply refinement button to apply the refinement results. WARNING: this action is not reversible.
 - Reset Position Refinement
 - Apply Refinements

The 'Start Position Refinement' button is highlighted in yellow. Below the workflow panel, there is a 'Refinement Results for Section 0001' section with fields for X Offset, Y Offset, and R Offset, all showing 'N/A'. Below that is a 'View Controls' section with buttons for 'Section 0001', 'RR', and 'Section'. At the bottom of the interface, there is a 'Position Refinement (optional)' section with navigation arrows and a status bar showing 'Scan rotation 278.0' and 'Stage rotation -90.0'.

- 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“.

Step 9: Position Refinement

Updating template shown here

Section outlines turn green once their position has been refined.

Workflow: Array Tomography

- 1 Define the refinement region in the first section and begin refinement.
Bring View To First Section
Define Refinement Region
Start Position Refinement
Refine region from section 0094
- 2 Use the apply refinement button to apply the refinement results.
WARNING: this action is not reversible.

Performing refinement on Section 0094

Progress:

Refinement Results for Section 0093
X Offset: -7.3676 μm Y Offset: -9.6741 μm R Offset: -2.33°

Stop Refinement

Reset Position Refinement

Apply Refinements

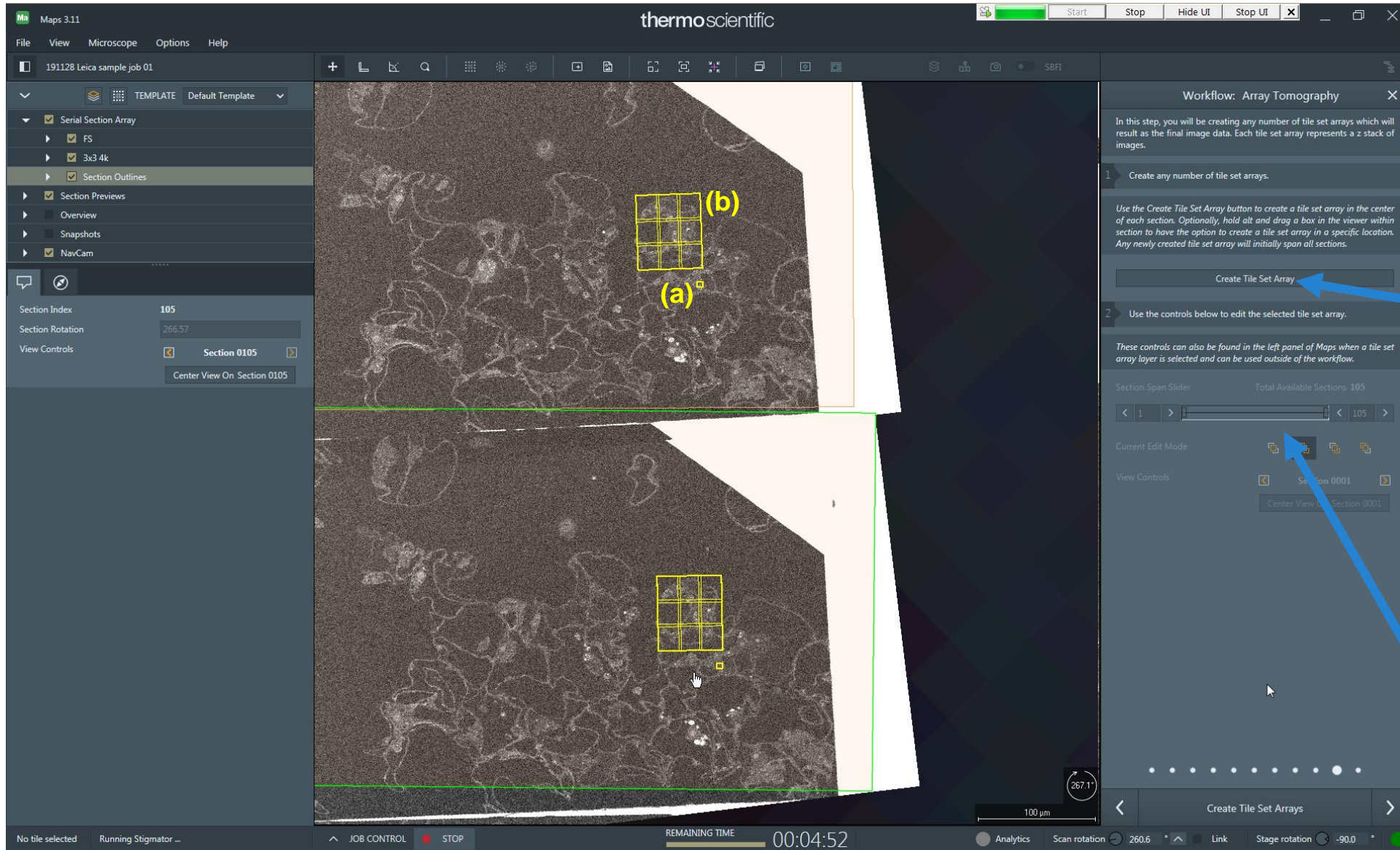
Position Refinement (optional)

- 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.

Step 10: Create “Array Tile Sets”: Imaging regions that are cloned to all sections



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).

Step 11: “Acquire all arrays”!

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Maps 3.11

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Workflow: Array Tomography

1. Choose an order of acquisition for the tile sets and sections.

By Tile Set Array

By Section

1. FS

2. 3x3 4k

2. Verify that all tile sets are placed correctly. Once verified, use the Acquire All Arrays button to acquire all tile set arrays created.

ACQUIRE ALL ARRAYS

REMAINING TIME 00:04:52

Array Tile Set Acquisition

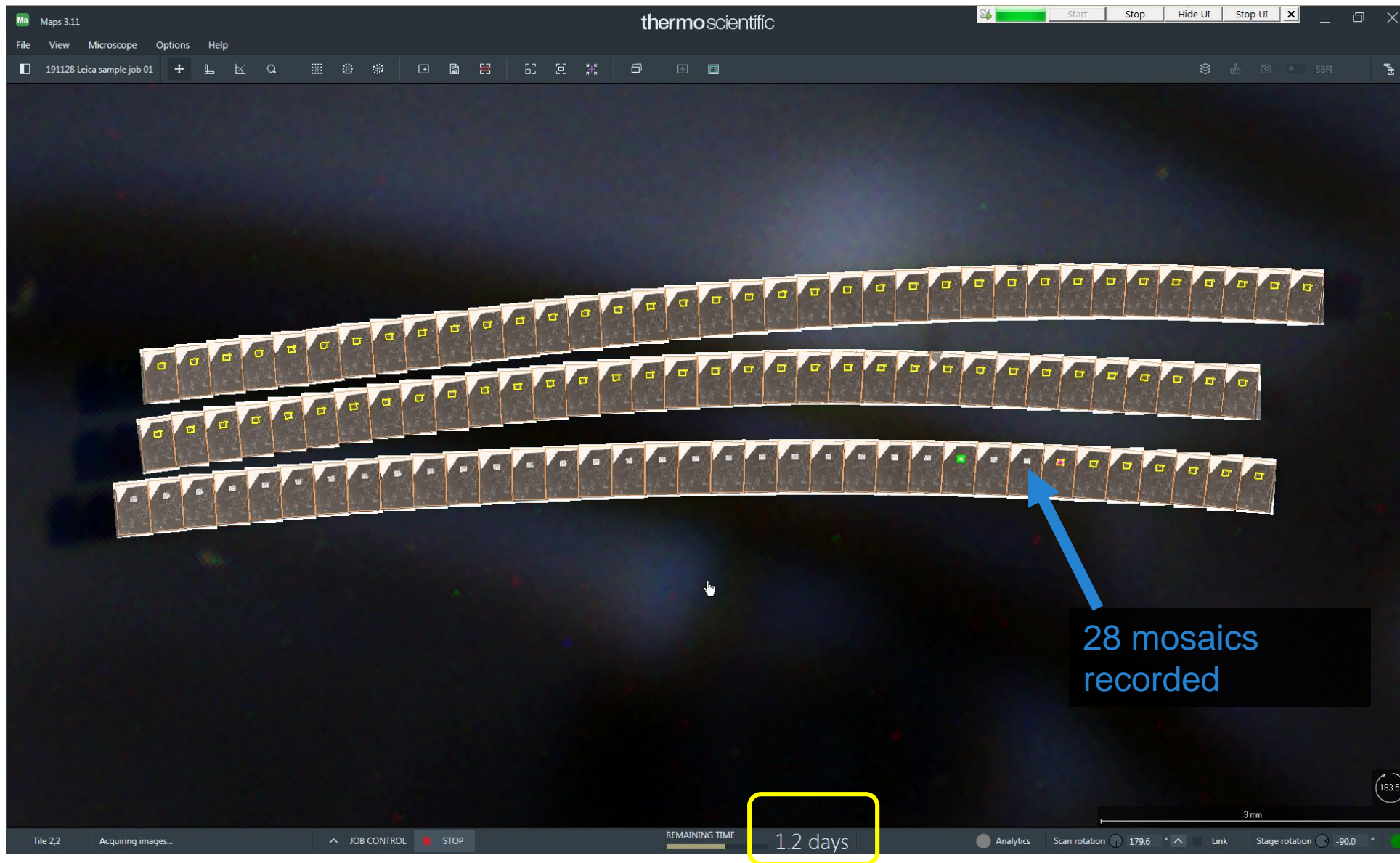
This is the last step in the workflow. It gives control over the acquisition sequence.

- 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:

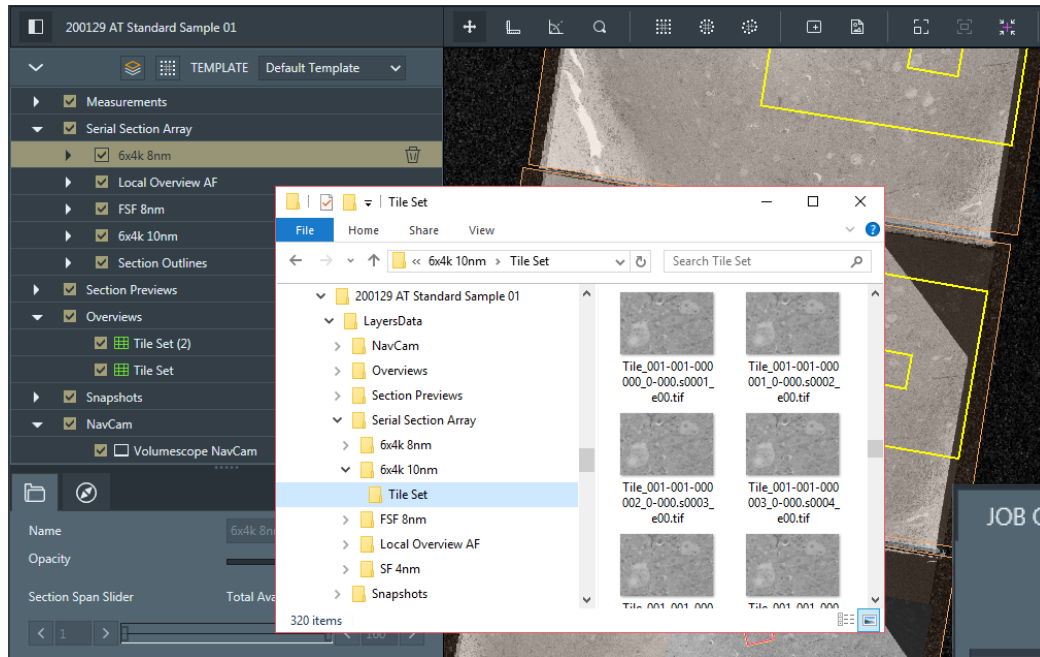
- Section 1: Auto focus, then Mosaic
- Section 2: Auto focus, then Mosaic
- ...
- Section 105: Auto focus, then Mosaic

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.*

Data saving and the Job Queue



JOB QUEUE PROCESSING ●

DESELECT ALL SELECT ALL

Show completed jobs

Local Overview AF-0152	00:01:18	✓
FSF 8nm-0152	00:00:05	✓
6x4k 8nm-0152	00:02:33	✓
6x4k 10nm-0152	00:01:18	✓
SE 4nm-0152		

ESTIMATION DISK SPACE
1.93 GB / 820.1 GB

▼ POST ACQUISITION

Turn Off Beam When Done

Pump to HiVac When Done

^ JOB CONTROL ■ STOP REMAINING TIME 00:49:07

- The Job Queue shows remaining time in the status bar and the time per tile set when expanded. It also allows 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, 1 μ s 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.