

# VolumeScope Set Up (High Vacuum)

## 1. CHECK MICROSCOPE PRECONDITIONS:

- Open the sample exchange dialogue in the XT-UI
- Make sure that the microtome is selected as accessories.

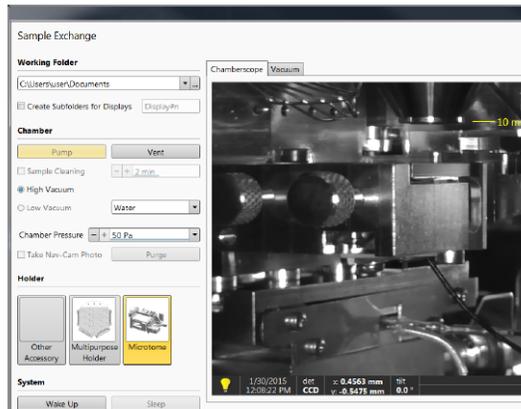
### Stage movement restrictions

When the ultramicrotome is mounted on the stage, there are some stage movement restrictions applied to the stage. This is applied when the *Sample Exchange* window / *Holder* area / *Microtome* item and the appropriate PLA Accessory (see below) are selected.

#### Stage module movement range

- X-axis: from 2.5 to 10.5 mm
- Y-axis: from 44.5 to 52.5 mm

When a sample is loaded to the ultramicrotome, it is expected to be find approximately in the middle of these ranges.



### Caution!

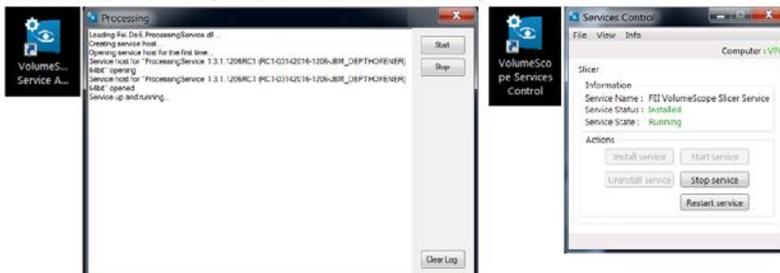
*Be sure to select these options! Otherwise the correct restrictions will not be applied. A collision with the final lens or any mounted accessory will be possible!*

- Make sure the Acquisition service(MPC), Processing service(VPC), Slicer service(VPC) are all running. (Do not close it)

2. Make sure that the Acquisition service (MPC) is running.



3. Make sure that the Processing and Slicer services (VPC) are running.



## 2. MICROTOME INSTALLATION:

- Create a new project in MAPS and click the VS plugin. (eye ball icon)

- Click “**Slicer Montage**” -> “**Vent Chamber**” -> “**Prepare Stage for Mounting**”

\* if “Prepare Stage for Mounting” does not work, manually input the stage position in the XTUI. The X axis: from 2.5 to 10.5mm, Y axis: 44.5 to 52.5mm, Z axis: 45 mm.

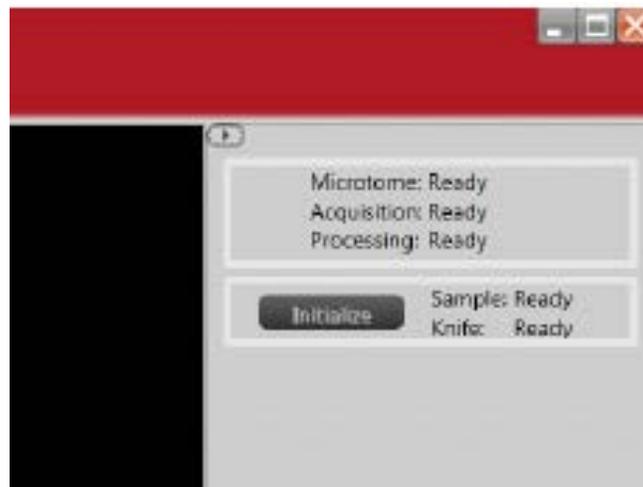
- Remove Multipurpose holder if installed.

- Mount the ring filter (HiVac) or VS-DBS (LoVac) if the vacuum mode can be pre-determined based on the sample type since there is more room for installation.

- Slide the Microtome onto the stage and secure it with two brass screws.

- Connect the Microtome feedthroughs in the order from X1 to X5. (Red dots on the connector male/female parts must match)

- Click “**Initialize Slicer**”. (Microtome should move around if the connections are good. The other way is to check the upper right side of the MAPS window, where the list of items related to the VolumeScope can be found. All the status should be “**Ready**”. If an item is active, the status is “**Busy**”. If the item is not available or need to be checked, the status is either “**Not Connected**”, “**Not Homed**” or “**Error**”.)



### 3. MECHANICAL SLICING SETUP used to set the sample to slice position

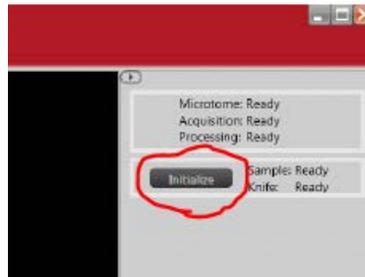
In this procedure the order of setup is:

1. Knife
2. Dinolite Camera
3. Sample

- In “**Mechanical Slicing Setup**”, select the “**Sample Exchange**” tab and click “**Park Sample and Knife**” in “**Sample Exchange Controls**”. (Make sure the axis position for both sample and knife is around zero)

- Mount the knife. **Make sure not touch the knife edges at any time !!!**

- Mount the camera tool on the microtome at 0° (top-down). (Sometimes the mounting procedure can knock off the communication. Just click “**Initialize**” on the right side of the MAPS main window)



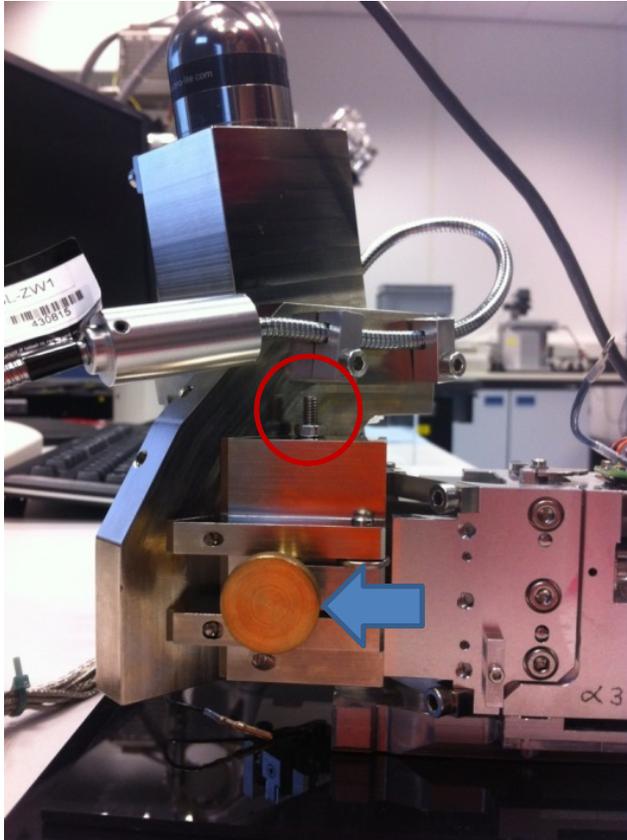
- Open the DinoLite Software on SPC.

- Move the knife to the camera center by clicking “**Go To**” in the “**Knife Setup And Controls**” panel.

- Check the eucentric height of the camera tool in the Dinocapture UI

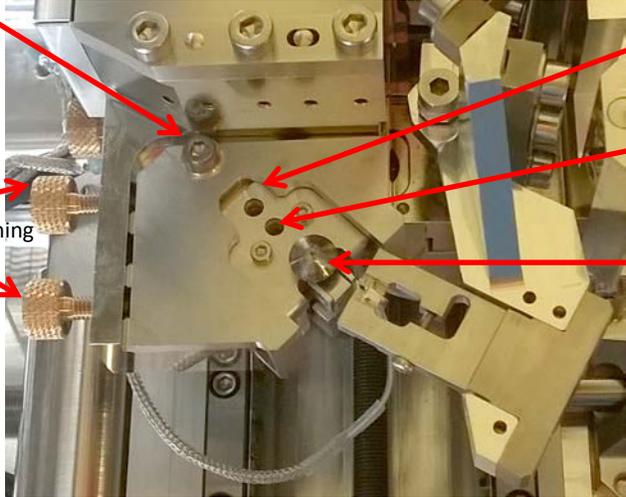
- Set camera to top down position, draw red line along the knife edges.

- Move camera to 45° and see if the knife edge is still at the position aligned with the red line; if this is not the case adjust the camera using the screw depicted in red circle in the figure 1. The camera fixing screw depicted by blue arrow needs to be loosened as well.



- The knife **edges** should be in focus between the top down and 45° camera angle if the eucentric position has been established.
- Move the knife to “**Park Position**” in “**Knife Controls And Setup**”; press “**Go To**”. (or “**Park Sample and Knife**”)
- Press the “**Prepare Sample For Exchange**” button in “**Sample Exchange Controls**”; the sample axis position moves up to 0.6 mm.
- Loose the Z-fixing screw and set the mechanical lever to zero using the Z-axis positioning screw depicted in flowing figure.
- Mount the sample. **Do not touch the knife edge**
- Tighten the sample at some degree using the Z fixing screw. (Not too tight so that you can still use tweezer to rotate the stub to align the sample and not too loose either)

X / Y-axis fixing screw



Sample holder clamp (Z) fixing screw

Z-axis positioning screw

VS sample holder

X / Y-axis positioning

- Put the camera at the top down position, loose the X-Y fixing screw, align the sample to the knife edge in x and y direction if needed using the knobs for X/Y-axis positioning. Tighten the X-Y fixing screw after the adjustment.

- Bring the camera to the 45° and move the sample up by adjusting the Z-axis positioning screw so that the sample is at eucentric position when moving the camera between 45° and 0°.

- Make sure the sample is fixed (tighten the Z fixing screw) after setting the sample to the eucentric position. (Remember there is residual movement after tightening. It usually moves the sample slightly towards left.)

- Press “Park Sample” button in “Sample Exchange Controls”. **The knife now can move over the sample without touching it.**

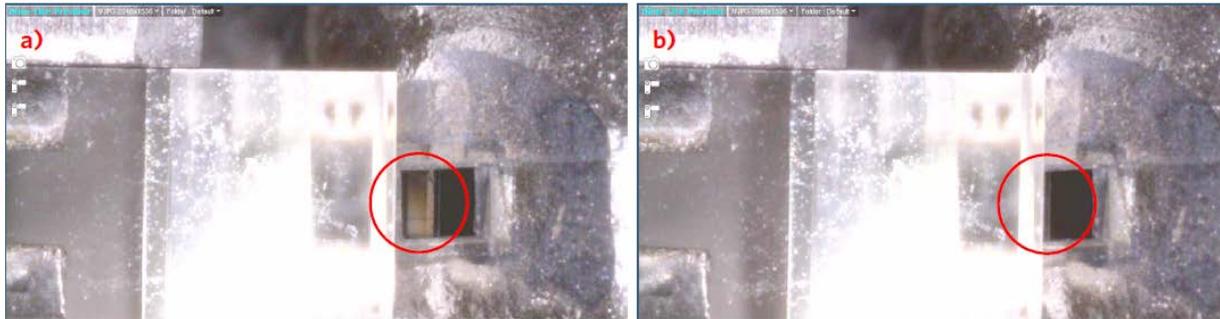
**Go to SAMPLE APPROACH AIR** for coarse approach of sample to slicing position

- Set the cutting window by updating the “Before Slice” and “After Slice” position in the “Knife Setup And Controls” by moving the knife over the sample using the arrows in “Axis controls” panel. (camera at 0°, top down)

- Connect the backlight to the correct position and turn off the camera light.

- Bring the camera to 45° and move the knife over the sample (between the before slice position and sample) to see the backlight reflection. (reflection can be best-viewed when the block has been faced and the knife has a clean edge without debris; **be careful if the reflection is not parallel to the knife edge, which means some part of the sample is higher.** )

**Figure 3-7:** *Sample Approach Air process. Sample is further from / closer to the knife*



- Move the sample up with the arrows until the backlight reflection is hardly visible anymore. (Be conservative for precious samples)
- Press the **"Set Current"** button in the **"Sample Setup And Controls"** box to update the **"Next Sample Slice"**. **!!!!**
- Set the knife to the **"Before Slice"** position.
- Remove the backlight.
- Camera at top down position (0°) and adjust auto exposure to better viewing the knife edge.

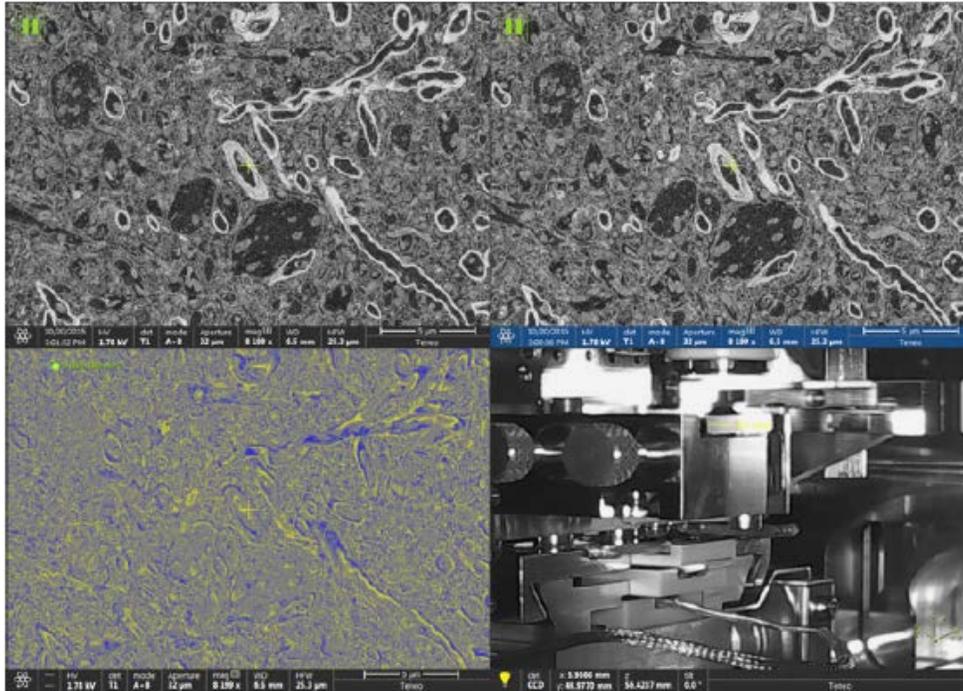
**Go to SAMPLE POLISHING IN AIR** to approach the sample to slicing position driven by the software.

- Set up a slicing job in **"Slicing Setup And Controls"** by putting 100 slices/slice thickness of 100 nm/cutting velocity of 0.8 mm/s; **do not exceed a slice thickness > 150nm.**
- Press **"Start Slicing"** button.
- If the whole block face is exposed, press **"Stop After Slice"** button; the knife goes to park position. (For precious sample, stop the job once part of the block face was cut. Start another job if the block face was not approached after the first 100 slices.)
- Remove the slices/debris from the knife/sample with an eyelash or by using compressed air and the vacuum cleaner. **Make sure not to touch the sample or the knife.**
- Close the DinoCapture SW and remove the camera tool.
- Mount the PLA if you have not done this part at the beginning. (Ring filter for HiVac, VS-DBS for lowVac)
- Document the **"Sample Axis Position"** by using snipping tool for screenshot.

- Before closing the chamber, make sure there is enough room between the final pole piece and the microtome by monitoring the CCD camera in the sample exchange window. (Make sure the wire on the microtome does not tangle on the stage)
- Pump the system and select the right PLA accessories. (Ring filter or VS-DBS)
- Wait for vacuum to stabilize. Under Hi-Vac, it usually takes about 2-3 hours to reach mid level of -6 mbar, if planning for a long job, leave the system pumping overnight is preferred. Under low-vac, wait for the pressure stabilized at the desired value. (You can optimize the imaging condition when the beam is turned on, you do not have to wait for the final vacuum.)

**Go to FINE APPROACH VACUUM** the sample is moved to slicing position inside the vacuum

- Compare the sample axis position before and after the pumping. In general, the after-pumping value is lower than the before-pumping value by  $\sim 1.5\mu\text{m}$ . If the after-pumping value is higher, select "**Fine Approach Vacuum**" panel, click "**Decrease Next Sample Slice**" by selecting an appropriate step size. (ie, "1 $\mu\text{m}$ ").
- Turn the beam on (ie: 1.78 kV, 100pA, T1/VS-DBS, usecase: volumescope), find the sample and link the working distance after focusing the image. If the stage is too far off (difficult to focus), use Standard Mode and the ETD detector first. The other option is go to the coordinate box in the XTUI, type 55mm (Z axis) to drive the stage close when the working distance is not linked. (Press "ESC" at any time if you feel the microtome is too close to the pole piece)
- Set the WD to 6.5mm if using the filter ring in HiVac and WD to 7mm if using the VS-DBS in LowVac. (Focus/stig needs to be tuned after stage move/working distance change. After every adjustment, make sure you re-link the working distance. )
- Apply  $\sim 135$  degree **scan rotation** in the XTUI. The cutting direction is then from left to right.
- Set up an appropriate HFW (horizontal field width). i.e., for standard brain sample, 15 $\mu\text{m}$  is a good start.
- Focus and acquire exactly the same image in quad one and two; third quad should have mixed image of 1<sup>st</sup> and 2<sup>nd</sup> quad. In the "**Processing Tab**" on the right hand site in the XT UI, the mixed image can be set to grayscale or progress visualization. Check the invert box for either quad 1 or quad 2. (The other option is to import the display presets. )



- Set a reasonable amount of slices to be cut in the **“Fine Approach Vacuum”** For example, putting 30 slices/slice thickness of 100 nm/cutting velocity of 0.8 mm/s. (One slice/slice thickness of 0 nm/cutting velocity of 0.8 mm/s can be started first to make sure the knife has not approached the sample yet.)

- Press **“Start Slicing”**. Imaging is alternated between quad 1 and quad 2. The mixed image in quad 3 remains structure-less until the cutting starts. (Need to differentiate if the difference is due to image drift or there is true cut; This procedure can speed up by using **“Sample Polishing in Air”** without imaging alternatively after each slice, not recommended for precious samples. )

- If there is a structural difference in the quad3, stop cutting by clicking the **“Stop After Slice”** button.

The **MECHANICAL SLICING WORKFLOW** is now finished and an experiment can be set up in the **OPTICAL SLICING SETUP**

## OPTICAL SLICING SETUP

When the Mechanical Slicing Setup is finished, define all the properties of the tile sets within this workflow. The middle chain of the buttons represents the core of the workflow for optical slicing. The buttons to the right cover the needed functionality directly related to the given action in the middle chain. The buttons to the left represent advanced settings for experienced users.

The screenshot shows the VolumeScope software interface. The left panel, titled "Optical Slicing Setup", displays a vertical workflow of buttons: "Tile Sets Definition", "Z-Stack Definition", "Column Alignment", "Data Set", "Processing", and "Volume Rendering". To the right of this central chain are buttons for "Acquisition Parameters", "Autofunction Selection", "Autofunction Selection", and "Processing Setup". To the left are buttons for "Autofunction Area", "Advanced Setup", and "Autofunction Selection". At the bottom of the left panel are navigation buttons: a left arrow, "MAPS VS", and a right arrow.

The right panel, titled "Tile Sets Definition", contains explanatory text and a table. The text states: "Now when the sample is prepared for imaging, ThruSight 3D volume reconstruction process has to be setup. First step is defining alignment and data tile sets inside the main Maps application. Alignment tile sets are the ones marking all the places where base alignments can be done. These areas will be highly exposed to the electron beam scanning so it is highly probable that the sample quality will be degraded there at the end of the process. Data tile sets are the ones where the high quality images (possibly later used for the 3D volumetric information reconstruction) will be grabbed. Please use the main application window to create at least one alignment tile set on the place that can be destroyed and some data tile sets on the places you are interested in. If you have selected previously that you are interested in data both from physical cutting only and from the combination with the ThruSight, you have to create alignment and data tile sets of both the physical cutting only and the combined types."

	Alignment tile sets		Data tile sets	
	Physical	ThruSight	Physical	ThruSight
Total	0	1	3	2
Selected for automated processes	0	1	3	2

In MAPS two tile sets can be created: alignment tile set and data tile set

In the "Optical Slicing Setup" each tile set can be created/modified using the guided workflow.

- Acquire an overview image of the whole block face in the XT UI. Prepare the overview preferably at an energy you would like to use for acquisition of a physical (cutting-only; one energy) tile set. In MAPS in the "Microscope Tab", select "Get Image From XT UI" and use the correct quad. Check the "Link Box" to link the scan rotation (bottom of MAPS UI). In "Options Tab" select "Prefer Scan Rotation". (The overview image is to help locating the area of the interests. The image does not need to be super-high resolution, but good enough to identify potential area of interests. )

## SET UP OF AN ALIGNMENT TILE SET (NON-MED)

- Using the overview image, choose an area to create a VS alignment tile set, best is to place it behind/on the right side of the region of interest. (We recommend the alignment tile set does not overlap with the data tile set)
- In the **“Z-Stack Definition Tab”**, click **“Change Tile Set Type to Physical Only”** and type in the preferred accelerating voltage.
- In the **“Acquisition Parameters Tab”**, type in the imaging conditions and make sure select the right detector and beam current. Typical parameters for alignment tile set is summarized below:

Dwell Time	1 $\mu$ s	Scan Interlacing	1
Image Resolution	2048 X 1768	Drift Corrected	Unchecked
Horizontal Field Width	20.48 $\mu$ m	Detector	T1
Pixel Size	10 nm	Beam Current	100 pA
Number of Frames	1	Use Case	VolumeScope
Line Integration	1	Use Reduced Area	Unchecked

- In the **“Autofunction Selection Tab”** of the **“Column Alignment Tab”**, Click **“Select All”** for the standard selection. (The lazy man’s trick)
- Go back to the **“Column Alignment Tab”**, Click the **“Align”** button.
- If the alignment is successful, the **“AF OK”** will be displayed for each item. Then you can use **“From SEM”** and **“To SEM”** to fine tuning the focus, stigmation, contrast/brightness if needed.

A successful alignment depends on a few things: make sure that the sample is at cutting position; the condition of the column is also a determination of the success of the alignment; alignment might struggle if the sample is charging. Do not just rely on the software to do all the lens alignment, you can do better.

You do not have to select all the Autofunctions at the beginning. You can simply just select **“Apply Alignment”**, **“Contrast and Brightness”**, **“Focus”**, **“Final Position Alignment”**. Click **“Align”**. After the auto alignment finished, click **“To SEM”**. After manually adjusting all the lens alignment, click **“From SEM”** to update all the information. (If there is no value stored, the XTUI will pick the default value. This value might not be the one you want. Therefore, adjusting the imaging conditions yourself and click **“From SEM”** to update it. )

\* Do not move the stage during imaging condition adjustment. You can zoom in and out.

## SETUP OF DATA TILE SET

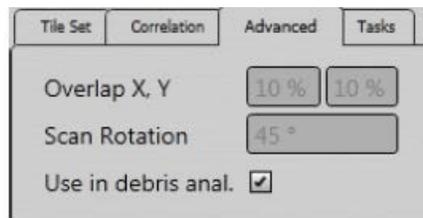
We recommend setting up at least two Data Tile Sets to check the sample and the cutting quality. First set up an Overview tile set and second for a detailed tile set on the ROIs.

- Simply right click on the alignment tile set and select “**copy grids**”, then select “**No**” to create a data tile set using this particular alignment condition.

- In the “**Acquisition Parameters Tab**”, input the desired final imaging conditions such as horizontal field width, pixel resolution, dwell time.

- In the “**Autofunction Selection Tab**” of the “**Data Set**”, select the typical autofunctions such as “**Apply Alignment**”, “**Final Position Alignment**”. (For overview tile set, please deselect the “**Final Position Alignment**”. Other common autofunctions such as “**Contrast Brightness**”, “**Focus**”, “**Stigmator**” are optional. For detailed explanation of each autofunction, please refer to the Teneo VolumeScope user menu.)

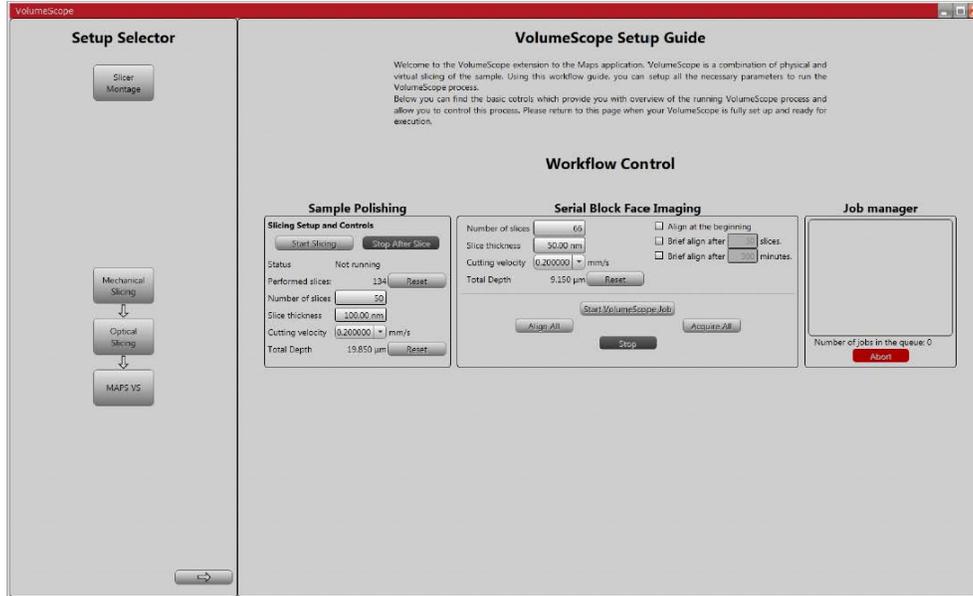
- Select the debris analysis for the overview tile sets.



- Check the order of the job by going to “**Options Tab**” in the MAPS UI, select “**VolumeScope Job Settings**” (Overview goes first, then the area of interest)

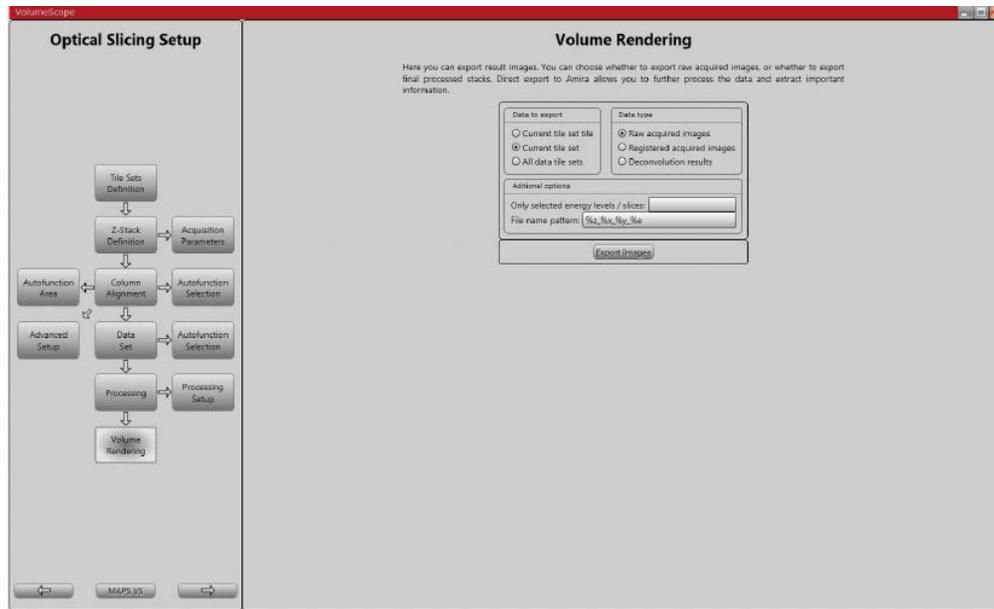


- Under the **“Workflow Control”** page, using **“Serial Block Face Imaging”** to set up the acquisition using 40 nm as slice thickness and a 0.2mm/s cutting speed. (Before starting the real job, it is better to polish the surface for couple slices (using **“Sample Polishing”**) to remove the “damaged area” when you adjust the imaging parameters and tune the focus.)



- Go to **“Options Tab”** in the MAPS UI, select **“Turn off Beam when done”** for HiVac job and add **“Pump to HiVac When Done”** for LowVac job if the job is finished at a time with no one around.

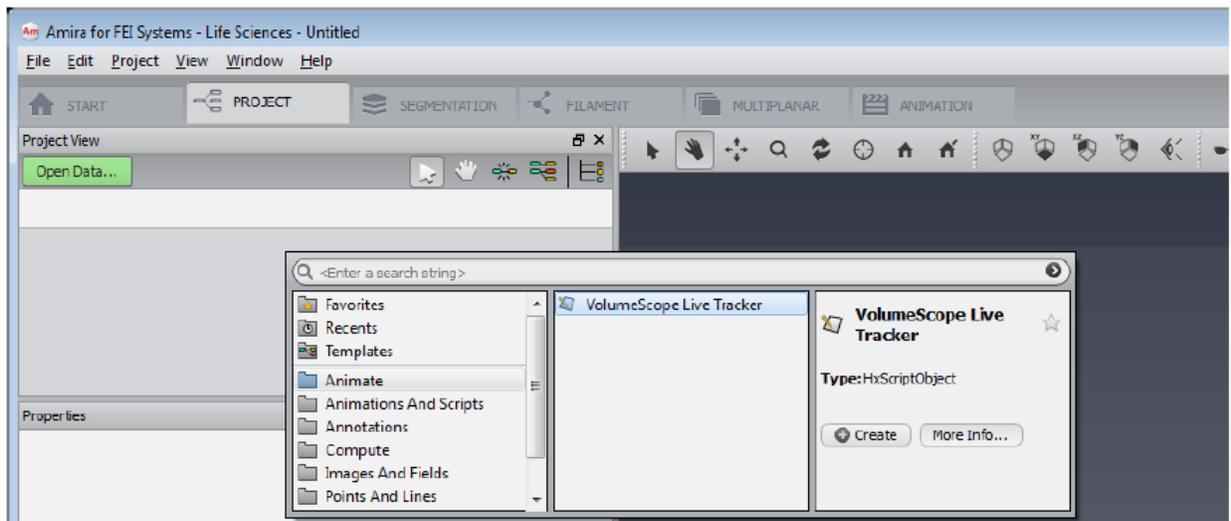
- Export the acquired data sets as **“Raw Acquired Images”** or **“Deconvolution Results”** (MED) in the **“Volume Rendering Tab”** after the acquisition.



## VISUALIZATION OF DATA TILE SET

Using Amira live tracker to monitor the cutting behavior. (Any missing or uneven cut, unexpected image shift, focus/stig stays consistent, the amount of debris.) Monitor at least 30 slices before leaving a long job unattended.

- Open Amira on the SPC, click the **Project** menu/**Create Object**/double-click the **VolumeScope Live Tracker** or click the **create** button to open the module.



Click the *More Info* button to access the help page describing basic functionality of the module.

- Select the tile sets you want to live track. The images will display after at least 2 slices have been performed.

## SAMPLE/MODE SWITCHING

- When switches samples, you do not have to take the microtome out, simply venting the chamber, vacuum clean or using compressed air to remove debris on the knife/sample/stage/chamber, remove the sample by loosening the Z-fixing screws, zero out the Z level and change PLA accessory (Ring filter or VS-DBS) if needed. Then you just start the approaching sample again.

- If switching to regular SEM mode, venting the chamber, clean the debris, remove the sample, remove the knife, take out the microtome and place it safely in its parking garage, put back the multipurpose holder, select microtome as the accessory in the sample exchange window.

#### More Tips:

- \* Ring filter and VS-DBS needs to be installed concentrically on the pole piece, otherwise, local image distortion might be observed.
- \* Before approaching the samples in vacuum, it is a good time to optimize the imaging conditions. (select the appropriate accelerating voltage, beam current and imaging conditions)
- \* Remember the balance game between dose and cutting behavior, a nice image with higher dose does not guarantee a consistent cutting behavior.
- \* A data tile set can be excluded from the VS job by unchecking the “ **Use in autom. proc** ” in its specific tile set tab of the MAPS UI.
- \* Autofunctions can be changed on the fly.
- \* A selected area imaging can be chosen to avoid getting an image without a double exposed focus area.
- \* Do not forget to clean the debris on the knife edge, samples, stage, wires.