

# Journal of Visualized Experiments

## Targeted studies using serial block face and focused ion beam SEM

--Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE59480R2
<b>Full Title:</b>	Targeted studies using serial block face and focused ion beam SEM
<b>Keywords:</b>	Volume Electron Microscopy, Scanning Electron Microscopy, 3D Electron Microscopy, Microwave Assisted Specimen Preparation, focused ion beam SEM, serial block face SEM
<b>Corresponding Author:</b>	Christopher J. Guerin Vlaams Instituut voor Biotechnologie Ghent, BELGIUM
<b>Corresponding Author's Institution:</b>	Vlaams Instituut voor Biotechnologie
<b>Corresponding Author E-Mail:</b>	chris.guerin@irc.vib-Ugent.be
<b>Order of Authors:</b>	Christopher J. Guerin Christopher J. Guérin Anna Kremer Peter Borghgraef Saskia Lippens
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Ghent, East Flanders, Belgium

**TITLE:**

Targeted Studies Using Serial Block Face and Focused Ion Beam Scan Electron Microscopy

**AUTHORS AND AFFILIATIONS:**

Christopher J. Guérin<sup>1,2,3</sup>, Anna Kremer<sup>1,2,3</sup>, Peter Borghgraef<sup>1,2,3</sup>, Saskia Lippens<sup>1,2,3</sup>

<sup>1</sup>VIB Bio Imaging Core, Ghent, Belgium

<sup>2</sup>VIB Inflammation Research Center, Ghent, Belgium

<sup>3</sup>UGent Department of Molecular Biomedical Research, Ghent, Belgium

Corresponding author:

Saskia Lippens (Saskia.Lippens@irc.vib-Ugent.be)

Email addresses of co-authors:

Christopher J. Guérin (chris.guerin@irc.vib-Ugent.be)

Anna Kremer (anneke.kremer@irc.vib-Ugent.be)

Peter Borghgraef (Peter.Borghgraef@irc.vib-Ugent.be)

**KEYWORDS:**

volume electron microscopy, scanning electron microscopy, 3D electron microscopy, microwave assisted specimen preparation, Serial Block Face Scanning Electron Microscopy, Focused Ion Beam Scanning Electron Microscopy.

**SUMMARY:**

Here, we present a protocol for efficiently combining serial block face and focused ion beam scanning electron microscopy to target an area of interest. This allows for efficient searching, in three dimensions, and locating rare events in a large field of view.

**ABSTRACT:**

This protocol allows for the efficient and effective imaging of cell or tissue samples in three dimensions at the resolution level of electron microscopy. For many years electron microscopy (EM) has remained an inherently two-dimensional technique. With the advent of serial scanning electron microscope imaging techniques (volume EM), using either an integrated microtome or focused ion beam to slice then view embedded tissues, the third dimension becomes easily accessible. Serial block face scanning electron microscopy (SBF-SEM) uses an ultramicrotome enclosed in the SEM chamber. It has the capability to handle large specimens (1000 µm x 1000 µm) and image large fields of view at small X,Y pixel size, but is limited in the Z dimension by the diamond knife. Focused ion beam SEM (FIB-SEM) is not limited in 3D resolution, (isotropic voxels of ≤5 nm are achievable), but the field of view is much more limited. This protocol demonstrates a workflow for combining the two techniques to allow for finding individual regions of interest (ROIs) in a large field and then imaging the subsequent targeted volume at high isotropic voxel resolution. Preparing fixed cells or tissues is more demanding for volume EM techniques due to the extra contrasting needed for efficient signal generation in SEM imaging. Such

protocols are time consuming and labor intensive. This protocol also incorporates microwave assisted tissue processing facilitating the penetration of reagents, which reduces the time needed for the processing protocol from days to hours.

## **INTRODUCTION:**

This protocol describes a workflow for the efficient targeting of high-resolution, three-dimensional electron microscopy (EM) to a specific region of interest (ROI). Since its beginnings in the 1930s, EM has been an essentially two-dimensional technique. The first published images were of whole tissues or cells but that soon gave way to sections that were cut by hand using an ultramicrotome and imaged using a transmission electron microscope (TEM). TEM produces very high-resolution micrographs where even the smallest of cellular structures are clearly discernable. However, the thinness of section necessary for the tissue to be imaged by the electron beam made information in the Z dimension minimal. Since cells are three-dimensional structures, interactions between cell structures and cell surfaces had to be inferred from limited data. This raised the potential for misinterpretation, especially in structures that were complex. Some microscopists managed to obtain more accurate 3D structures by serial-sectioning cells and tissues and then painstakingly reconstructing them from individual TEM images<sup>1</sup>. This was a very labor-intensive process and before the advent of digital imaging and computer rendering the results were also difficult to visualize. In recent years two techniques have been introduced which have become collectively known as volume electron microscopy (volume EM)<sup>2</sup> that have made EM in three dimensions accessible to more laboratories.

The idea of obtaining a stack of images from an embedded block inside an electron microscope can be traced back to 1981 when Steve Leighton and Alan Kuzirian built a miniature microtome and placed it in the chamber of a scanning electron microscope<sup>3</sup> (SBF-SEM). This prototype was eventually copied and improved 23 years later by Denk and Horstmann<sup>4</sup> and subsequently commercialized. At roughly the same time biological scientists became aware of another technology used primarily in materials science, the focused ion beam. This technique uses an ion beam of some kind (Gallium, plasma) to remove a very small amount of surface material from a sample (FIB-SEM)<sup>5</sup>. Both techniques employ sectioning followed by imaging providing a series of images which can be combined into an X, Y, Z, stack. Both techniques provide 3D information but at different resolution scales. SBF-SEM is limited by the physical properties of the diamond knife to slices no thinner than 50 nm for long serial imaging runs; however the sample block size that can be sectioned is large, up to 1 mm x 1 mm x 1 mm. Due to the large digital acquisition format of the back scattered electron detector (32k x 32k pixels) that receives a signal from the block face, image pixel sizes can be as small as 1 nm. This results in non-isotropic voxels where the X,Y dimension is frequently smaller than the Z. Because of the precision of the ion beam, FIB-SEM has the ability to collect images with isotropic voxels  $\leq 5$  nm. However, the total area that can be imaged is quite small. A summary table of various samples and volumes imaged with the two techniques has been published previously<sup>3</sup>.

Tissue preparation for volume EM is more difficult than for standard TEM or SEM because samples must be stained to provide adequate signal generation in the SEM. Frequently, stainings need to be optimized not only for the particular tissue type but also for adding contrast to certain cellular structures to make identification and reconstruction easier. The protocol used here is based on the NCMIR standard<sup>6</sup>. Additional staining usually means additional protocol steps. Thus for volume EM, standard protocols need to be extended to ensure sufficient time for reagents to penetrate the sample. Microwave assisted processing can reduce the time needed for staining from hours to minutes and makes volume EM sample preparation more efficient<sup>7</sup>. This method is applicable to all cell and tissue types<sup>8</sup> and to research questions where the inhomogeneity of the tissue makes sampling of a specific area essential<sup>9</sup>.

Once a data stack is obtained it can be aligned and the structures of interest segmented from the rest of the data and modeled in 3D. Although the automation of imaging many slices of tissue has made image acquisition relatively straightforward, the process of digitally reconstructing and visualizing the data is a time-consuming task. Software for this purpose is not yet integrated nor fully automated. Since much of the early work using volume EM was directed towards neuroscience, the techniques for staining and digitally segmenting structures such as axons is fairly far advanced compared to other cells and organelles. While the literature on other non-neuronal tissues is growing quickly, nonlinear or irregular structures require more manual input.

Using both SBF-SEM and FIB-SEM is a useful approach for targeting and imaging specific, nonhomogeneous, tissue structures at high resolution in 3D. Combining that with microwave assisted tissue processing that vastly decreases the time needed for sample preparation. Together this workflow will make generating high-resolution isotropic voxel image datasets of fine structures an efficient and more rapid process.

## **PROTOCOL:**

### **1. Sample fixation and processing for electron microscopy**

1.1. Fix seedlings of *Arabidopsis thaliana* grown on agar plates in 0.5% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 6.8 for 2 h at room temperature (RT).

CAUTION: Aldehydes are irritants and corrosive and have carcinogenic, mutagenic and teratogenic potential. All solutions must be handled with appropriate protective equipment and in a fume hood.

1.2. Cut root tips of the plant grown in step 1.1 and put 2-3 tips in 0.5 mL tubes containing the same fixative overnight at 4 °C.

NOTE: The volume of this and any solutions in the remaining steps is determined by the sample volume; a minimum ratio of sample to solution is 10:1. Samples larger

than 1 mm in any dimension will be difficult to stain, so working with larger tissue blocks is more difficult. Not all tissues have the same characteristics; for example, plant leaves and stems can be difficult to stain. If larger samples or difficult tissue types are desired, optimization of sample processing must be performed before moving on to data collection.

1.3. Prepare the thiocarbohydrazide (TCH) solution, needed fresh and available before step 1.7. Add 0.1 g of thiocarbohydrazide to 10 mL of double distilled water (ddH<sub>2</sub>O) and dissolve by heating to 60 °C in the oven for 1 h. Before use, filter TCH solution using a 0.22 µm syringe filter.

1.4. Remove fixative from the tubes and replace with 0.1 M PB pH 6.8. Put the tubes on an orbital shaking table at 100 rpm and wash for 10 min. Repeat the washing using fresh PBS 5 times.

1.5. Post fix the root tips by replacing PB with 2% osmium tetroxide (OsO<sub>4</sub>) and 0.2% ruthenium red in 0.1 M PB pH 6.8. Put the tubes in the microwave with the lids open and start program 9 (**Table 1**).

CAUTION: Osmium is extremely hazardous in case of ingestion, very hazardous in case of inhalation, and hazardous in case of skin contact. Always handle using appropriate protective equipment and in a fume hood.

NOTE: Throughout the protocol, the lids of the tubes are always open during the microwave steps.

1.6. Wash the root tips twice with ddH<sub>2</sub>O for 5 min each on the benchtop. For the third and fourth ddH<sub>2</sub>O wash use program 15 on the microwave (**Table 1**). After the first 40 second ddH<sub>2</sub>O wash, take samples out of the microwave and replace the buffer with fresh ddH<sub>2</sub>O. Put samples back in the microwave and continue the program.

NOTE: The microwave will sound an alarm when buffer needs to be refreshed. Make sure the lid for the vacuum chamber is replaced correctly every time.

1.7. Incubate samples in previously prepared TCH solution at RT for 2 min on the bench and for further incubation use the microwave program 8 (**Table 1**). Do not change solution between bench and microwave.

1.8. Wash samples as described in step 1.6.

1.9. Place samples in 1% OsO<sub>4</sub> in ddH<sub>2</sub>O for microwave program 9 (**Table 1**).

1.10. Wash samples as described in step 1.6.

1.11. Incubate samples in 1% uranyl acetate in ddH<sub>2</sub>O using microwave program 16 (**Table 1**).

CAUTION: Uranyl acetate is toxic, an irritant and has carcinogenic, mutagenic and teratogenic potential. Always handle using appropriate protective equipment.

1.12. Wash samples as described in step 1.6.

1.13. Prepare Walton's lead solution for use in step 1.14. First make a stock solution of L-aspartic acid by adding 0.998 g of L-aspartic acid to 250 mL of ddH<sub>2</sub>O and adjusting pH to 3.8 with 1 M KOH. Next, dissolve 0.066 g of lead nitrate in 10 mL of L-aspartic acid stock solution and adjust pH to 5.5. Leave the solution in the oven at 60 °C for 30 min.

NOTE: No precipitates should form.

1.14. Incubate samples in Walton's lead solution for 30 min in the oven at 60 °C.

1.15. Wash samples as described in step 1.6.

1.16. Dehydrate samples in EtOH in graded steps of 50%, 70%, 90% in ddH<sub>2</sub>O, and then 2x in 100% EtOH. Use microwave program 10 (**Table 1**) and the microwave will prompt users every 40 s to replace solution with the next EtOH step. This is the last step done in the microwave.

1.17. Further dehydrate in 100% propylene oxide 2x for 10 min each at RT on the bench, replacing the solution between steps.

NOTE: Propylene oxide can dissolve some plastics such as polystyrenes; either use glass vials for this step or pre-test plastics for resistance.

CAUTION: Propylene oxide is highly flammable. Always handle using appropriate protective equipment and in a fume hood.

1.18. Start infiltration of root tips by incubating in 50% Spurr's resin in propylene oxide (min 2 h).

CAUTION: Spurr's resin components are irritants. Always handle using appropriate protective equipment and in a fume hood.

1.19. Replace solution with 100% Spurr's and leave overnight at RT.

1.20. Change to fresh 100% Spurr's resin 2 times (min 2 h incubations).

1.21. Place samples in an embedding mold, again containing fresh 100% Spurr's resin and polymerize in an oven at 65 °C for 36–48 h.

NOTE: The embedding mold used depends on the type of tissue and the approach used for imaging. Here, a flat silicone embedding mold was used (**Figure 1A**).

## 2. Prepare embedded samples for imaging

2.1. Remove samples from the oven, remove resin from the embedding mold (**Figure 1B**).

2.2. Using a razor blade, roughly trim the sample to a block of maximum 0.5 mm x 0.5 mm x 0.5 mm (**Figure 1C**).

NOTE: To prevent charging in SBF-SEM, it is important to trim away as much bare resin as possible and make the sample as flat/thin as possible. Ideally all sides of the block contain tissue already, but most importantly the side which will be attached to the metal pin (see step 2.3) has to contain exposed tissue so that the tissue is in direct contact with the conductive metal.

2.3. Remove the sample from the extraneous resin and attach it to a metal pin (**Figure 1D**) with conductive epoxy resin, making sure that part of the tissue is touching the metal pin. Let the epoxy cure overnight in the oven at 65 °C.

NOTE: Make sure the sample is positioned in the center of the pin, because the movement of the stage in the SB-SEM is limited. Removing the very small resin encased sample from extra resin can be difficult as the small sample will have a tendency to fly away when detached. A simple and effective solution to this is to cover the sample with a sheet of paraffin film as shown in the supplementary movie of reference<sup>10</sup>.

2.4. Fasten the carrier in the holder for the ultramicrotome. Use a razor blade to remove any excess epoxy and use the ultramicrotome and a diamond knife to smooth the face and sides of the block, forming a pyramid. Make sure that at least some of the tissue is already exposed on the block-face.

NOTE: The extra step of using a diamond knife is optional but it makes the resulting block easier to approach in the SBF-SEM because the shadow of the knife on the block face is clearer thus making estimating the distance between knife and block face easier to determine.

2.5. Place the trimmed sample block in the sputter coater and coat the sample with a thin layer (2–5 nm) of platinum (Pt).

NOTE: The platinum on the block-face will be cut away during the approach in the SBF-SEM (see below), but the platinum on the sides of the pyramid will provide additional conductivity. In this example, the sample was coated with platinum, but gold, or gold/ palladium is also effective; however, coating with gold resulted in increased debris on the block-face during an imaging run.

## 3. Imaging in the SBF-SEM

3.1. Insert the carrier in the SBF-SEM microscope and bring the knife close to the sample surface. Using the diamond knife trim off the upper portion of the sample so that the Pt layer has already been removed and at least part of the tissue is exposed.

NOTE: Since this process is different for each SBF-SEM microscope, not every step is specified here. As long as the sample surface is free from Pt and ready for imaging, the next steps will be possible.

3.2. Start imaging at low resolution and short dwell times to get an overview of the sample and locate a region of interest (**Figure 1E**).

NOTE: Here, 512 x 512 pixels and 1  $\mu$ s dwell time were used for quick scanning and positioning of the stage and 2000 x 2000 pixels with 1  $\mu$ s dwell time were used for optimizing the imaging window and adjusting focus.

3.3. Using an accelerating voltage of 1.5-2.0 kV at a current of 80–100 pA, capture an image of the tissue.

NOTE: The example shown here was imaged on a high vacuum system where the beam current needs to be adjusted to minimize charging, which is very sample dependent. Typically, the electron beam is set to 1.5–2.0 kV but this will be sample dependent. At higher magnifications (usually > 10,000x) the resin is too much affected by the beam to guarantee smooth sectioning, so typically pixel-size is set to 8–20 nm at an image size of 8000–10000 x 8000–10000 pixels with a corresponding magnifications of 430 – 1400x and field sizes of 64 X 64  $\mu$ m and 200 x 200  $\mu$ m respectively.

3.4. Determine a region of interest and decide how many sections are needed to cover the volume of interest and start the imaging run, using the back scattered electron detector.

NOTE: In the example presented here, 500 sections of 80 nm were imaged at 10 nm pixels and 10,000 x 10,000 pixel images (dwell time 1  $\mu$ s). The microscope was set to 1.6 kV and 100 pA. In general, the number of sections is dependent on sample and size of the ROI and can vary from 100 s to 1000 s of consecutive sections. The resulting dataset consists of single images of every section. These images need to be converted to a 3D stack.

#### 4. SBF-SEM data processing

4.1. Using Fiji, select file-import-image sequence and locate the image stack to load the images. Depending on the size of the dataset, check the 'use virtual stack' box.

NOTE: If the dataset is really big, first convert it to 8 bit (if collected at 16 bit) and if necessary bin the data until it has a workable size.



4.2. Using the play button at the bottom of the image scroll through the dataset to see if the imaging run was successful. Check for typical imaging artefacts for SBF-SEM, such as sections falling off the knife on the block-face, charging in areas of bare resin, cutting artefacts of the knife (horizontal lines on the image).

4.3. Using the command image-properties adjust the pixel size and voxel depth (i.e., section-thickness) used during the run. If the data is already binned, take this into account.

4.4. Using the command plugins-registration-linear stack alignment with SIFT to register the data.

NOTE: Registration of SBF-SEM data is needed because there might be slight sample movement during the imaging due to charging or drift of the sample. As this is only a minimal movement in XY, only translation is needed.

4.5. Check the alignment by scrolling through the dataset and if OK use the save command under the file menu to save the aligned dataset as a 3D-tif file.

4.6. Analyze the dataset carefully to see if ROI is included and contains the information that is needed for the biological question. On the last image of the stack (= the current block-face), select a new ROI for FIB-SEM imaging.

NOTE: If there is no good region for FIB-SEM imaging present on the current block-face, more sections can be cut from the block (which is still in the SBF-SEM) until a ROI appears. There is a limit to the volume that can be imaged with the FIB. The ROI on the SBF-SEM image can be maximum in X,Y of 30-40µm x 15-20µm.

## 5. Imaging in the FIB-SEM

5.1. Take overview images of the sample in the SBF-SEM, ideally including one or more edges of the sample that are then recognizable in the FIB-SEM (**Figure 2A,C**).

NOTE: In this example the SBF-SEM overview images were taken at 10 nm pixel size, 8,000 x 8,000 pixels at 1 µs dwell time. The microscope was still set at 1.6 kV and 100 pA.

5.2. Remove the sample from the SBF-SEM and place in the sputter coater. Coat the sample with ≥20 nm platinum for FIB-SEM imaging.

5.3. Load sample into FIB-SEM and using secondary electron detector at 15 kV, 1 nA locate the ROI identified in the SBF-SEM on the block-face (**Figure 2B,D**).

NOTE: Imaging at 15 kV is necessary to see through the platinum coating.

5.4. Bring the ROI on the sample into the coincidence point of the FIB and SEM beams, by moving and tilting the stage (**Figure 3A**).

NOTE: The FIB column is usually mounted under an angle (**Figure 3A**). This means that any sample needs to be tilted in such a way that the surface to be imaged and sectioned is positioned parallel to the FIB beam. The surface to be imaged is now tilted with respect to the SEM beam and a trench of tissue needs to be removed before the SEM is able to image the ROI (**Figure 3E**)

5.5. Using the FIB beam and gas injection system, deposit a 1  $\mu\text{m}$  protective layer of platinum on the surface above the ROI (**Figure 3C**). Next, using a low milling current (50-100 pA), mill fine lines into the platinum deposition for auto-focusing and 3D tracking during the imaging run (**Figure 3D**). Using the FIB column and carbon gas injector, cover these lines with carbon deposition.

NOTE: The size of the ROI here corresponds to the size of the ROI on the SBF-SEM image and the maximum size can thus be 30-40  $\mu\text{m}$  x 15-20  $\mu\text{m}$ . In this example a ROI of 17  $\mu\text{m}$  x 8  $\mu\text{m}$  was imaged. Carbon deposition is needed for protection of the lines and to create a black-white contrast between the carbon and platinum which is ideal for auto-focusing. The milling currents used for each step can be found in **Table 2**.

5.6. Using a high milling current, mill a trench of 30  $\mu\text{m}$  in front of the ROI, creating the imaging surface for the SEM beam (**Figure 3E**).

NOTE: The FIB beam is inherently destructive, even more so at high currents. Make sure to keep imaging at high currents to a minimum and image at low magnification and fast scan speeds to avoid melting the resin at the ROI.

5.7. Smooth the imaging surface with a milling current closer to the current used during the imaging run. Stop polishing when all auto-focus and 3D tracking marks are clearly visible on the imaging surface (**Figure 3F**). The progress of the FIB can be followed by imaging the surface with the EM (using a back scattered electron detector) while polishing.

5.8. Determine the area to be imaged on the newly created surface and set the imaging parameters. Make sure the electron beam is focused on the surface, set brightness and contrast and set the pixel-size and section thickness. Keep the imaging time below 1 minute by adjusting dwell time and line average.

NOTE: It is important to use a low voltage for imaging with the electron beam to ensure that only the surface of the block is imaged (i.e., that no electrons from deeper into the sample are imaged). This is done by keeping the voltage below 2 kV and using a back scattered electron detector with a grid voltage, allowing only high energy electrons to be imaged. In this example, the electron beam was set at 1.5 kV and 1 nA with a grid voltage of 1.2 kV on the back scattered electron detector. Also here, a pixel-size of 5 nm was used with 5nm sections to result in a dataset with isotropic voxels. An area of 17  $\mu\text{m}$  x 10  $\mu\text{m}$  was imaged at 6.5  $\mu\text{s}$  dwell time and a line average of 1.0.

5.9. Set the windows for auto-tuning and 3D tracking, using the same pixel size, dwell time and line average as used for the imaging.

NOTE: This process will differ for different systems, therefore only the step is mentioned without specifying the different actions.

5.10. Start the imaging run and monitor the stability of the process during the first 50–100 sections. Once the system is running smoothly, leave the room and ensure that there is as little disturbance to the room as possible.

NOTE: The duration of the run and the number of sections will depend on the size of the ROI and the section thickness. In FIB-SEM the Z axis is actually the height of the ROI box on the SBF-SEM image (max 15–20  $\mu\text{m}$ ; **Figure 3E**).

5.11. Register the FIB-SEM data in the same way as described above for the SBF-SEM data.

#### REPRESENTATIVE RESULTS:

Images from the SBF-SEM provide an overview of the tissue, giving insight into the spatial orientation of cells and intercellular connections (**Figure 4A**). The subsequent FIB-SEM imaging on a new region, which is usually a region of interest determined after inspection of the SBF-SEM run, adds high-resolution detail of specific cells and/or structures (**Figure 4B**).

**Figure 4C,D** show the difference in rendering of the non-isotropic voxels of the SBF-SEM data (**Figure 4C**) and the isotropic voxel FIB-SEM data (**Figure 4D**). The z thickness used in SBF-SEM means that the rendering clearly shows the sections, resulting in a 'staircase' effect in on the surface. In the FIB-SEM data the 5 nm sections ensure that the rendering appears much smoother and individual sections blend into the surface completely.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Creation of the block face from a resin embedded sample.** (A) A root-tip embedded in resin. (B) Using a razor blade the excess resin is trimmed away until a block of 0.5mm<sup>2</sup> remains. (C,D) The trimmed block is glued onto a metal pin and after a night in the oven, the sides of the block are trimmed and the surface smoothened with a diamond knife using an ultramicrotome. (E) Inside the SBF-SEM, the sample is oriented so that the blockface and ROI can be recognized, scale bar = 20  $\mu\text{m}$ .

**Figure 2: Correlation between SBF-SEM and FIB-SEM.** Overview images of the block-face using the SBF-SEM (A) and the FIB-SEM (B), scale bar = 5  $\mu\text{m}$ . (C,D) Zoom on the ROI. Red box delineates the region to be imaged with FIB-SEM, scale bar = 5  $\mu\text{m}$ .

**Figure 3: FIB-SEM scheme and preparation steps.** (A) Scheme showing the orientation of the FIB beam, SEM beam and sample. The sample needs to be positioned to the coincidence point of the FIB and SEM beams to be able to mill and image on the same region. (B) Schematic drawing of the trench needed for SEM imaging of the sections removed by the FIB. (C) Image taken with the FIB beam showing Platinum deposition on the ROI, scale bar 5  $\mu\text{m}$ . (D) Image taken with the FIB beam showing the lines used for auto-focus and 3D tracking. The lines in the middle are used for auto-focus and the outside lines provide 3D tracking. Carbon deposition on top of the lines provides the needed contrast (platinum vs carbon) to perform these tasks, scalebar 5  $\mu\text{m}$ . (E) Image taken with the FIB beam after milling of the trench, scalebar 5  $\mu\text{m}$ . (F) Image taken with the SEM beam showing the region of interest imaged during the FIB-SEM run, scale bar 2  $\mu\text{m}$ .

**Figure 4: SBF-SEM and FIB-SEM results before and after segmentation.** (A) 3D view of SBF-SEM dataset (100 x 100 x 40  $\mu\text{m}$ , scale bar = 10  $\mu\text{m}$ ), (B) 3D view of FIB-SEM dataset (17 x 10 x 5.4  $\mu\text{m}$ , scale bar = 2  $\mu\text{m}$ ), (C) Rendered vacuoles segmented from SBF-SEM data by thresholding, scale bar = 2  $\mu\text{m}$  D. Rendered granules segmented from FIB-SEM data by thresholding, scale bar = 2  $\mu\text{m}$ .

**Table 1. Detailed protocol for microwave processing.**

**Table 2. FIB milling currents used for sample preparation and imaging run**

**DISCUSSION:**

Volume electron microscopy is more challenging and time consuming than conventional SEM or TEM. Because of the need to stain tissues or cells en bloc, processing steps must be long enough to ensure penetration of reagents throughout the sample. Using microwave energy to facilitate penetration makes for shorter, more efficient processing and improves staining. Because preparation for EM is much more stringent than for light microscopy all solutions and reagents must be quality controlled strictly. Changes in pH, tonicity, the use of impure reagents, and introduction of contaminants due to poor technique can all have deleterious effects on the final image.

Volume EM also requires individually tailored protocols for each different sample type. Mammalian tissues of different types: plants, single cells such as yeast, trypanosomes, *C. elegans*, etc., all need their own variations to achieve optimal results. Fixation and staining must be designed so as to preserve structural integrity and keep the sample as close to its in vivo morphology as possible. Fixation of tissues at physiological temperature, pH and tonicity is critical to making the sample as life-like as it can be. High-pressure freezing (HPF) of samples may help to preserve a more life-like situation, (or perhaps just yield different artifacts), but for other than cells and very thin tissues HPF will fail as vitreous ice can only be generated in small volumes. Therefore for many questions chemical fixation is the only option. No matter if the fixation is HPF or chemical, in any EM experiment the structural results need to be carefully compared to similar results from live cell or tissue imaging to see if they are consistent. Staining must also be optimized while considering the

specific question that needs to be answered and the protocol that will be used for visualization of the digital images.

Having both an SBF-SEM and FIB system in close proximity is a great advantage in many experiments. The large field of view and high X,Y resolution of SBF-SEM makes finding individual structures/cells/events straightforward and provides an overall spatial orientation of cells in tissues. In addition, its ability to allow imaging through a sample in Z is very powerful; however, reconstructions that require fine geometric detail can fail or produce artifacts using this technique due to the non-isotropic voxels it generates. The FIB is limited by the physics of the process to a smaller imaging field but its 3D resolution is sufficient for very accurate reconstructions. Combining the two techniques is straightforward as samples can move from SBF-SEM to FIB without further processing or preparation. We acknowledge that using the SBF-SEM for searching through a sample to find a particular area is a very expensive use of a much more capable tool. However, the ability to immediately see the new blockface and determine whether the ROI has been reached is a great advantage. Additionally, the alternatives of using serial semi-thin (0.5  $\mu\text{m}$ ) LM sections may remove small structures before they are detected, and inspecting a block using single TEM sections which have to be cut, put on a grid and then viewed in an equally expensive TEM is not as efficient as the method presented.

Because many programs exist to segment and render the data, and the needs of a given structure may not be best served by a single application, no standard workflow can be proposed. Some simple structures may be segmented with a thresholding algorithm if they fall within very narrow grey scale values. Neuronal structures can be semi-automatically segmented using a program such as Ilastik<sup>11</sup> but it will be less useful on more random or complex shaped organelles such as ER. Microscopy Image Browser is a very flexible program that can align, segment, and render volume EM data, but requires significant user interaction<sup>12</sup>. As a general rule the amount of time needed to digitally visualize the results will greatly exceed the time for preparing the sample and imaging.

Volume EM techniques have opened up the third dimension to ultrastructural analysis. Other methods of obtaining 3D EM have limitations in their volume (TEM tomography), or their efficiency (serial section TEM). Although for the most part volume EM techniques are too complex and costly to be implemented in individual laboratories, the number of shared core facilities offering them has been growing and the number of sample types successfully imaged has increased rapidly. For those with a specific question and a particular tissue it is likely someone will be able to offer advice and instructions on its preparation and imaging. Volume EM equipment can be improved to include the capacity to handle larger samples in the SBF-SEM and the capability of milling larger ROIs with the FIB. Software which is able to segment out structures of interest in a more automated way will vastly simplify the process of analyzing the data and improvements in computing speed will reduce the time needed to do so. Despite its current limitations, volume EM is still a useful tool and combining SBF-SEM and FIB-SEM provides an efficient workflow for identifying rare events and imaging them at high resolution.

**ACKNOWLEDGEMENTS:**

The equipment for volume EM was provided by a generous grant from the Government of Flanders.

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

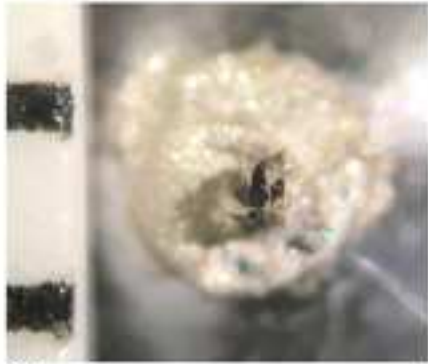
- 
- <sup>1</sup> Linberg K.A., Fisher S.K. An ultrastructural study of interplexiform cell synapses in the human retina. *Journal of Comparative Neurology*. **243**, 561-576 (1986).
  - <sup>2</sup> Peddie C.J., Collinson L.M. Exploring the third dimension: volume electron microscopy comes of age. *Micron*. **61**, 9-19 (2014).
  - <sup>3</sup> Leighton S. SEM images of block faces, cut by a miniature microtome within the SEM – A technical note. *Scanning Electron Microscopy*. (pt 2) 71-76 (1981).
  - <sup>4</sup> Denk W., Horstmann H. Serial block-face scanning electron microscopy to reconstruct three dimensional tissue nanostructure. *PLoS Biology*. **11**, 329 (2004).
  - <sup>5</sup> Heymann J.A., Hayles M., Gestmann I., Giannuzzi L. A., Lich B., Subramaniam S. Site specific 3D imaging of cells and tissues with a dual beam microscope. *Journal of Structural Biology*. **155** (1), 63-73 (2006).
  - <sup>6</sup> Deerinck T.J., Bushong E.A., Thor A., Ellisman M.H. NCMIR Methods for 3D EM: A new protocol for preparation of biological specimens for serial block face scanning electron microscopy. V7\_01\_10 <https://ncmir.ucsd.edu/sbem-protocol> (2019).
  - <sup>7</sup> Giberson R.T., Austin R.L., Charlesworth J., Adamson G., Herrera G.A. Microwave and digital imaging technology reduce turnaround times for diagnostic electron microscopy. *Ultrastructural Pathology*. **27** (3), 187-196 (2003).
  - <sup>8</sup> Kremer, A. et al. Developing 3D EM in a broad biological context. *Journal of Microscopy*. **259** (2), 80-96 (2015).
  - <sup>9</sup> Vanslebrouck B, Kremer A., Pavie B., van Roy F., Lippens S., van Hengel Three-dimensional reconstruction of the intercalated disc including the intercellular junctions by applying volume scanning electron microscopy. *Journal of Histochemistry and Cell Biology*. **149**, 479–490 (2018).
  - <sup>10</sup> Russel M.R., et al. 3D correlative light and electron microscopy of cultured cells using serial blockface scanning electron microscopy. *Journal of Cell Science*. **130** (1), 278-291 (2017).
  - <sup>11</sup> Sommer C., Strähle C., Köthe U, Hamprecht F. A., ilastik: Interactive Learning and Segmentation Toolkit in: *in Eighth IEEE International Symposium on Biomedical Imaging (ISBI). Proceedings*, 230-233 (2011).
  - <sup>12</sup> Belevich I, Joensuu M, Kumar D, Vihinen H, Jokitalo E. Microscopy Image Browser: A Platform for Segmentation and Analysis of Multidimensional Datasets. *PLoS Biology*. **14** (1), 1002340 (2016).



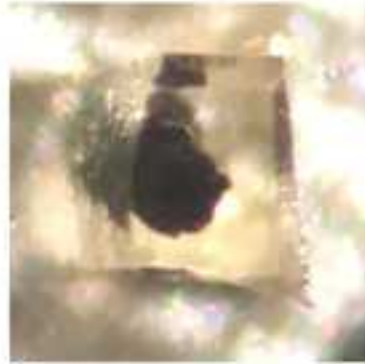
A



B



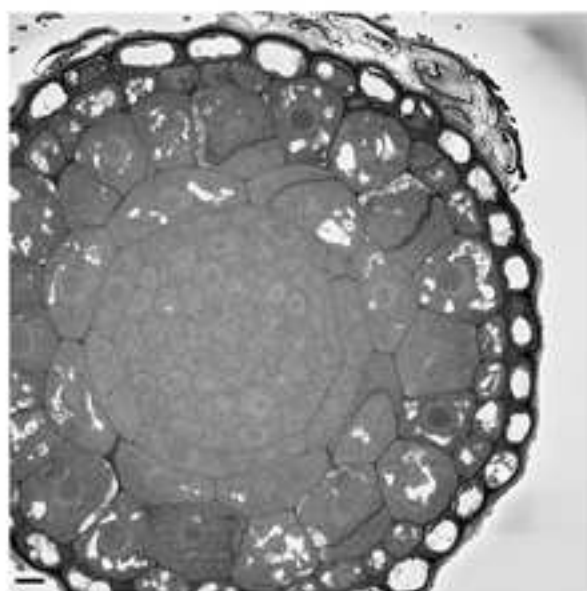
C



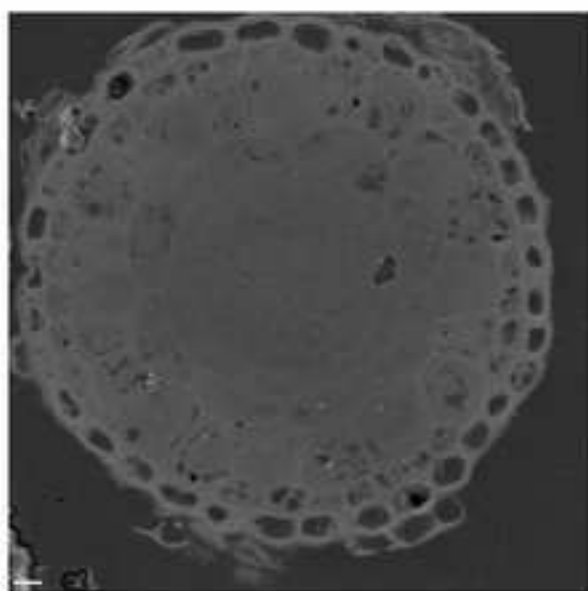
D



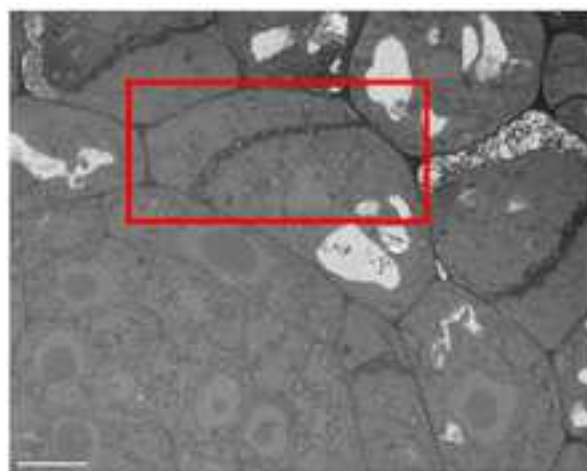
E



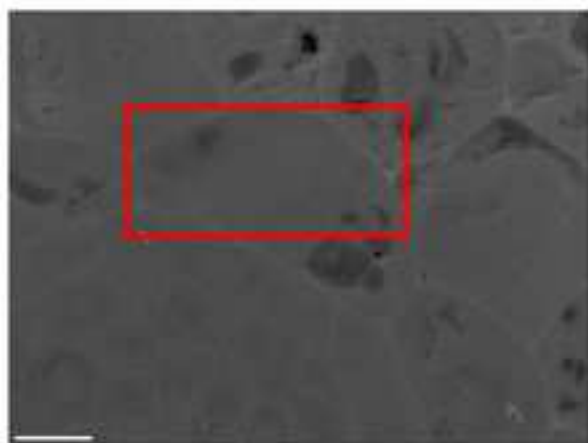
A



B

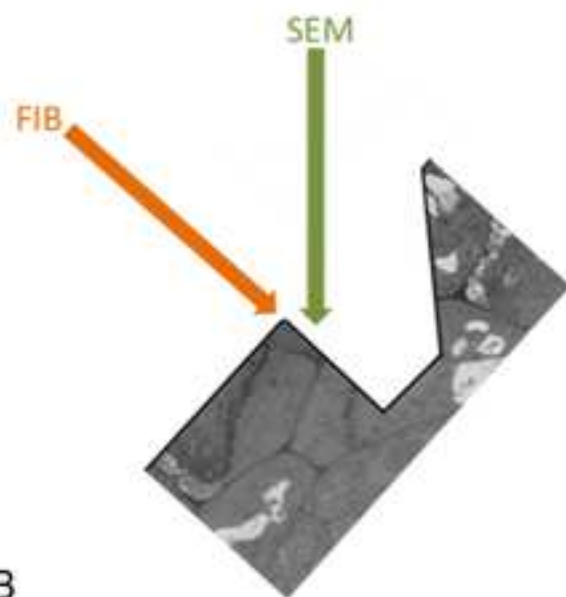
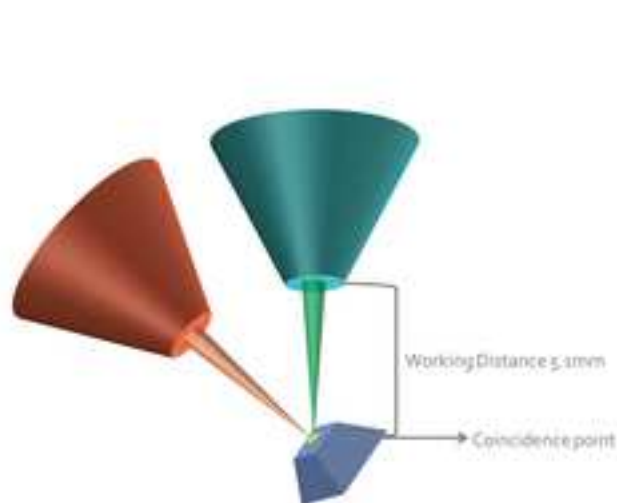


C

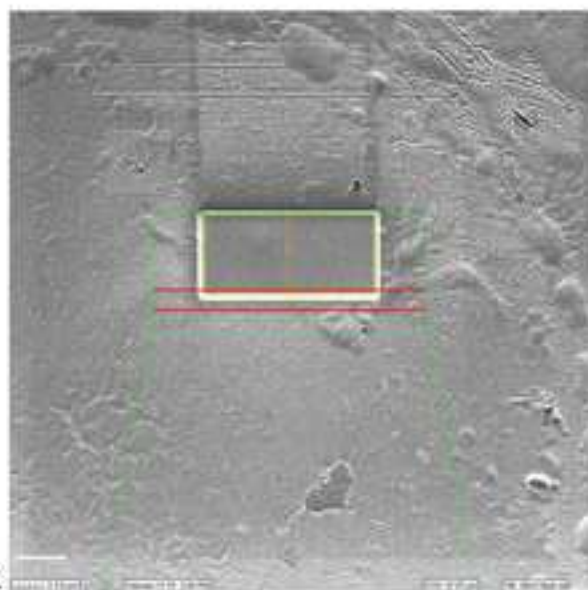


D

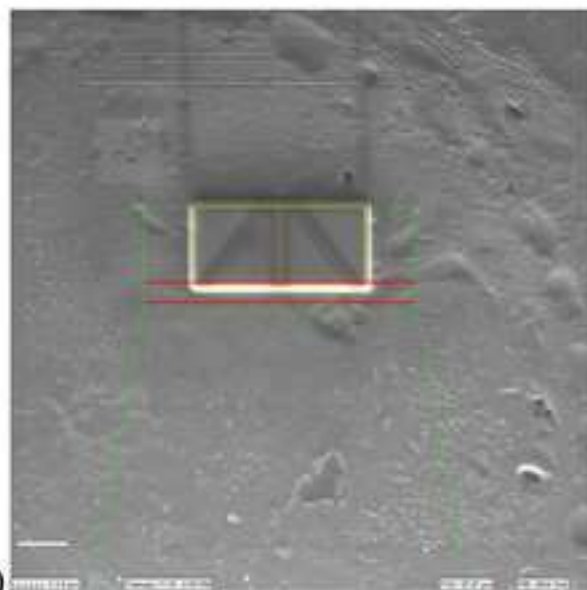




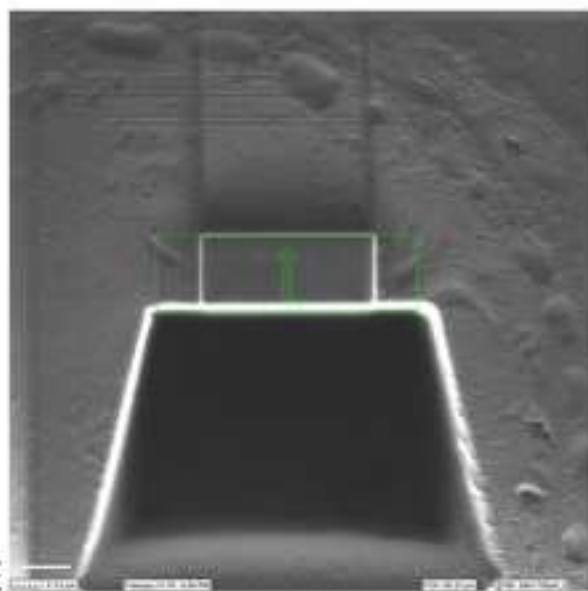
A



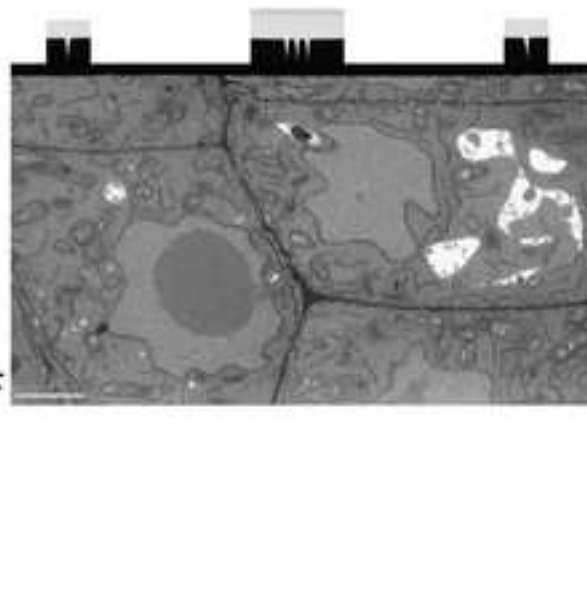
B



C



D

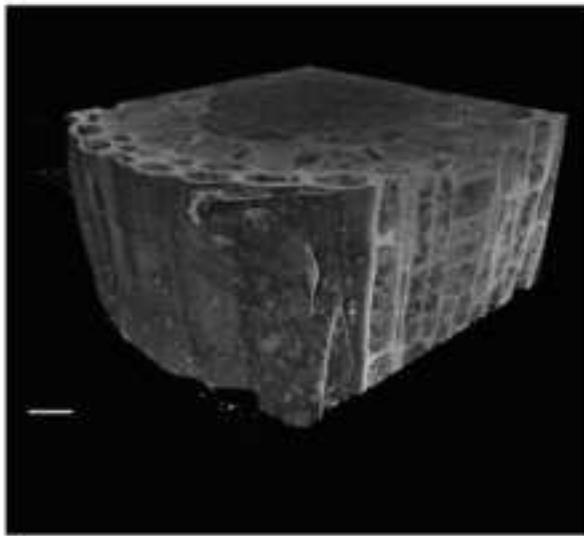


E

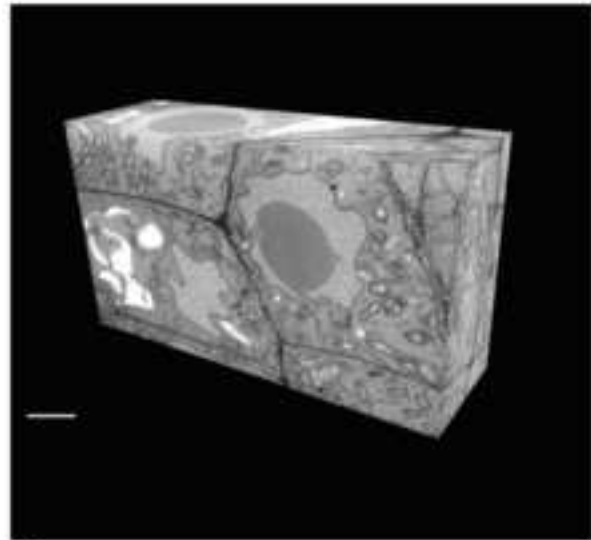


F

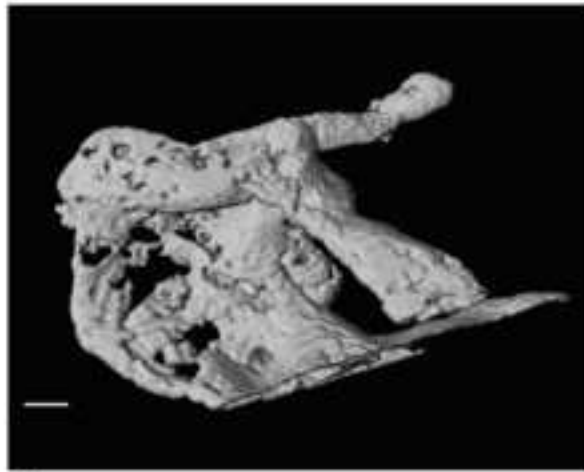




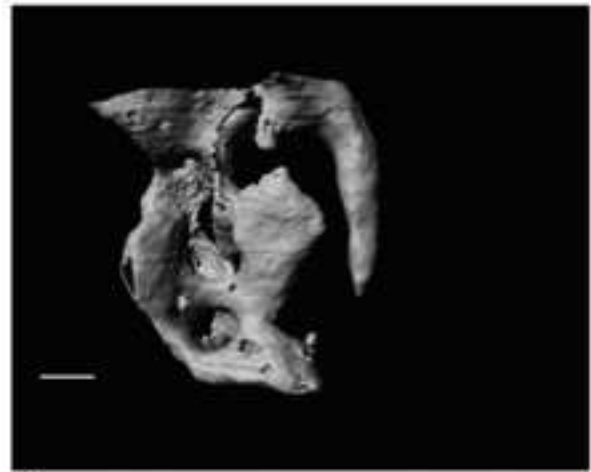
A



B



C



D

Protocol for Microwave Processing						
Program #	Description	User Prompt (on/off)	Time (hr:min:sec)	Power (Watts)	Temp (°C)	Load Cooler (off/auto/on)
8	TCH	OFF	0:01:00	150	50	OFF
		OFF	0:01:00	0	50	OFF
		OFF	0:01:00	150	50	OFF
9	OSMIUM	OFF	0:02:00	100	50	OFF
		OFF	0:02:00	0	50	OFF
		OFF	0:02:00	100	50	OFF
		OFF	0:02:00	0	50	OFF
		OFF	0:02:00	100	50	OFF
10	50% EtOH	ON	0:00:40	150	50	OFF
	70% EtOH	ON	0:00:40	150	50	OFF
	90% EtOH	ON	0:00:40	150	50	OFF
	100% EtOH	ON	0:00:40	150	50	OFF
	100% EtOH	ON	0:00:40	150	50	OFF
15	0.1M CACODYLATE	ON	0:00:40	250	50	OFF
	0.1M CACODYLATE	ON	0:00:40	250	50	OFF
15	ddH <sub>2</sub> O	ON	0:00:40	250	50	OFF
	ddH <sub>2</sub> O	ON	0:00:40	250	50	OFF
16	Uranyl Acetate	OFF	0:01:00	150	50	OFF
		OFF	0:01:00	0	50	OFF
		OFF	0:01:00	150	50	OFF
		OFF	0:01:00	0	50	OFF
		OFF	0:01:00	150	50	OFF
		OFF	0:01:00	0	50	OFF
		OFF	0:01:00	150	50	OFF

[illegible]

Step	Current	Estimated Time
Deposition Platinum	3n A	10-15 minutes
Milling Autotune and Tracking Marks	50-100 pA	4-6 minutes
Deposition Carbon	3 nA	5-10 minutes
Milling Coarse Trench	15-30 nA	30-50 minutes
Polishing Surface	1.5-3 nA	15-20 minutes
Imaging Run	700 pA- 1.5 nA	Hours-days

<b>Name of Reagent/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
3View 2XP	Gatan	NA
Cacodylate buffer 0.2M solution	EM Sciences	11652
Conductive epoxy resin (circuit works)	RS components	496-265
Diatome Histo 4.0mm diamond knife	EM Sciences	40-HIS
Digitizing tablet	Wacom	DTV-1200W
Eppendorf tubes	Eppendorf	0030 120.094
Flat Embedding Mold	EM Sciences	70900
Gluteraldehyde 25% solution	EM Sciences	16220
High MW Weight Tannic Acid	EM Sciences	21700
Lead Citrate	Sigma-Aldrich	22861
NaCl	Sigma-Aldrich	746398
Osmium Tetroxide 4% solution	EM Sciences	19170
Paraformaldehyde	Sigma-Aldrich	P6148
Pelco Biowave Pro +	Ted Pella	36700
Potassium Ferrocyanide	Sigma-Aldrich	P3289
Quorum Q150T ES sputter coater	Quorum Technologies	27645
Reichert-Jung Ultracut ultramicrotome	NA	NA
Sodium Cacodylate 0.2M	EM Sciences	11653
Spurrs Resin kit	EM Sciences	14300
Uranyl Acetate	EM Sciences	22400
Fiji (Image J)	Univ. Wisconsin-Madison	NA
Imaris	Bitplane	NA
Microscopy Image Browser	Univ. of Helsinki	NA

### **Comments/Description**

In chamber ultramicrotome for SBFi

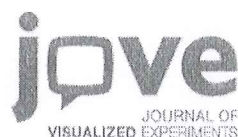
No longer available

No longer available

<https://fiji.sc/>

<http://mib.helsinki.fi/>





1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Using serial block face and focused ion beam scanning electron microscopy on the same sample.

Author(s):

Christopher J. Guérin, Anna Kremer, Peter Borghgraef, and Saskia Lippens

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/author>) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to Sections 4 and 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in Item 1 above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.



## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name:	Saskia Lippens, Ph.D.	
Department:	VIB Bio Imaging Core	
Institution:	VIB	
Article Title:	Using serial block face and focused ion beam scanning electron microscopy on the same sample.	
Signature:		Date: 03/12/2018

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

We thank the editor and reviewers for their helpful comments and submit the changes requested as described below.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

3. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

4. Please do not use in text citation in the Abstract section.

5. Please revise the Introduction to include all of the following with citations:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

6. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

**Commented [CG1]:** Done, completely rewritten.

**Commented [CG2]:** Done, please see lines 47-63.

**Commented [CG3]:** Removed

**Commented [CG4]:** Please see lines 47-48

**Commented [CG5]:** Please see lines 96-100

**Commented [CG6]:** Please see introduction paragraph 1

**Commented [CG7]:** Please see lines 105-114

**Commented [CG8]:** Please see lines 131-133

**Commented [CG9]:** Done

For example: 3 view, Merlin/3View, Pelco BioWave Pro, A Reichert-Jung Ultracut ultramicrotome, Quorum Q150T ES sputter, etc.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please number the subheadings as **well**.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique with all specific details (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used **sparingly**.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question in complete sentences., i.e., how is the step **performed**?

10. 22: where is the knife and how/ why are you blocking the **face**.

11. 23: What part of the sample is referred to as **face**.

12. 24: Close what and how do you pump down **SEM**?

13. 25: Image how? Do you perform any knob turns? Do you perform any button clicks? what is the blockface? What is the suitable **region**?

14. 26-36: Please describe in detail what is being done and how? If performing any software steps, please provide the scripts if used any, button clicks, graphical user interface. Please be specific with respect to your **experiment**.

15. 37: Stacks of what? How many images per sample is **taken**?

**Commented [CG10]:** Done

**Commented [CG11]:** Noted

**Commented [CG12]:** The protocol was completely reworked, please see lines 163-490

**Commented [CG13]:** The knife is mounted in the in-chamber ultramicrotome, and the block face is the upper surface of the tissue sample

**Commented [CG14]:** See above

**Commented [CG15]:** The SEM chamber which must be kept under high vacuum, it is pumped down using an ion getter pump.

**Commented [CG16]:** This will all be clear in the video, and it will be obvious to anyone who has ever seen or used an electron microscope. There are many knob turns and button clicks required and they vary during acquisition. To include them all stepwise is impossible.

**Commented [CG17]:** Please see above. All software is commercial and no scripts have been added.

**Commented [CG18]:** The images are collected in series which is referred to as a stack, this is common nomenclature.

16. 38: How do you adjust the pixel size? To what?

17. 39: how do you do so? Do you have any scripting files for the same? Please upload as supplementary info. Also please move the hyperlink to the reference section.

18. 40-41: How do you do so? What is the level of Brightness and contrast adjustment in your experiment?

19. Please provide volume and concentrations of all the solutions used in your experiment.

20. The Protocol should contain only action items that direct the reader to do something. Please use complete sentences.

21. Please ensure that the highlight is no more than 2.75 pages including heading and spacings.

22. Please describe the result with respect to your experiment, you performed an experiment using the protocol described here, how did it helped you to conclude what you wanted to and how is it in line with the title.e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

23. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

24. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

**Commented [CG19]:** The pixel size is determined by the physical size of the imaging field divided by the number of pixels used to capture the digital image. The size of the structure of interest will determine the final pixel size needed to resolve it. By the Nyquist theorem the minimal pixel size must be approximately 0.41 times the size of the resolved structure.

**Commented [CG20]:** See above, the hyperlink was removed.

**Commented [CG21]:** This step has been removed in the new protocol.

**Commented [CG22]:** Please see lines 178-179

**Commented [CG23]:** Done.

**Commented [cg24]:** Done.

**Commented [CG25]:** OK

**Commented [CG26]:** OK, titles in bold.

**Commented [CG27]:** No reprinted figures are used



25. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

26. Please include Acknowledgment section as well.

27. Please ensure that the references appear as the following:  
[Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate the journal name.

28. Please alphabetically sort the materials table.

**Commented [CG28]:** Please see lines 834-838, 844-845, 847-848.

**Commented [CG29]:** Please see lines 847-849, 880-885, 930-931.

**Commented [CG30]:** Please see lines 943-944

**Commented [CG31]:** Please see lines 936-939

**Commented [CG32]:** Done

**Commented [cg33]:** Corrected

### Reviewer #1:

#### Manuscript Summary:

The manuscript of Guérin et al. presents an efficient protocol that combines the strengths of serial-block face (SB-EM) and focused ion beam (FIB-SEM) scanning electron microscopy for analysis of heart tissue. The protocol covers all steps of a typical workflow: starting from mouse perfusion and sample preparation, continuing to imaging with both of aforementioned techniques and finishing with image processing of the collected datasets and visualization of results.

There are two main aspects that make the protocol efficient:

1. Use of a microwave-assisted sample preparation routine, which decreases the time and workload of the sample preparation part from several days to about a day without compromising the staining quality.

2. Use of SB-EM as a guiding technique that can be used to solve "finding a needle in a haystack" problem and also supplements the high-resolution FIB-SEM imaging with overview volumes that place the high-resolution dataset into a context of the surrounding tissue.

My impression is that it would be beneficial for the community to have this workflow published as a video protocol. I have few comments that needs to be addressed during preparation of the final version of the protocol.

#### Major Concerns:

The title of the protocol is "Using serial block face and focused ion beam scanning electron microscopy on the same sample", which indicates that the presented protocol will be quite generic and applicable to various specimens. In reality, the protocol describes sample preparation of hearts and, as also pointed out by the authors, may not be good enough for other tissues. This makes the title a bit misleading. I think it would be nice to clarify this in the title and in the abstract.

#### Minor Concerns:

1. There is inconsistent use of acronyms in the manuscript: serial block face SEM is called: SB-EM, SBFI and sometimes 3View. 3View is a product name from Gatan, while there are other manufactures that produce SB-EM systems on the market.

2. It would be nice to get more clear information on selection of powers for the microwave oven during the sample preparation. For example, the step 21 (table 1) has 150 W, while the next step 100W. Also, normally, microwave-assisted steps are followed by steps without microwave to cool down the specimen, while in this protocol the MW-

**Commented [cg34]:** The protocol title has been changed and the tissue used from heart to root tip. Although still only a single sample type we feel including other tissues would make the protocol too complex. We still emphasize the need for fine tuning sample preparation to individual tissue types and include two references, Peddie and Collinson and Kremer et al to guide readers to additional information on diverse samples.

**Commented [cg35]:** We have changed all references to the acronym SBFSEM used in the paper by Denk and Horstmann.

steps are following each other (steps 21 and 22), which may heat the specimen too much.

**Commented [cg36]:** These details are now included in the protocol as [table 1](#).

3. Can the authors comment on selection of the vacuum mode for the MW-steps? Mostly, the 'vacuum cycle' mode is used, is it better than having the microwave under the vacuum all the time?

**Commented [cg37]:** We followed the manufacturers instructions and also were guided by other users. Admittedly an experiment should be done to determine if the vacuum cycle is beneficial, and for which steps, but it is outside the scope of this paper. We can state from experience that if used as described the protocol works well.

4. On lines 236-238 and also later in the manuscript, when the authors describe SB-EM they state that "their [synaptic vesicles] 3D structure when rendered becomes elongated because of the anisotropic voxels and appears tube-like" and "The ability of SB-EM to allow searching through a sample in Z is very powerful; however, reconstructions that require fine detail will fail or produce artifacts using this technique (Fig. 4A-B)."

These statements are misleading: the rendering looks elongated due to the fact that the voxel sizes were not correctly provided to the visualization program. As result, the visualization program assumes the isotropic voxels, while they are anisotropic. Providing correct physical sizes or stretching factors for voxels will fix this visualization artefact. Imaris package that was used for visualization has this feature. SB-EM does not produce artefacts; it just has slightly lower resolution in Z.

**Commented [cg38]:** We appreciate and agree with the reviewers expert comment. We have omitted this figure as it does not directly relate to the revised manuscript.

5. The authors were using Microscopy Image Browser for image segmentation and Fiji for the alignment. Is there any specific reason for that? On line 321, the authors state that MIB can also be used for alignment, is there any specific reason to use two programs instead of one?

**Commented [cg39]:** We have now used FIJI for all operations prior to visualization.

6. Could authors also provide the microwaving protocols as supplementary in the Ted Pella microwave oven compatible format, so that it would be easier for other users utilize them.

**Commented [cg40]:** We have done this in table 1.

**Reviewer #2:**

## Manuscript Summary:

The manuscript 'Using serial block face and focused ion beam scanning electron microscopy on the same sample' is a nice outline of the steps necessary for using serial block face electron microscopy as a tool for targeting regions of interest prior to isotropic high resolution imaging using the FIB. The authors use intercalated discs as a target structure, however the method description is general which will allow it to be applied to many sample types, which is useful for EM methods. The steps listed in the procedure are clear, though a bit brief in some instances, but should be able to be successfully followed with a few minor revisions. I have provided comments to help clarify some aspects of the procedure, though the authors may show many of these during the video however it is important to include in the text version also.

## Major Concerns:

1. The paper is about using the 3view as a tool for targeting regions of interest for imaging in the FIB. I suggest changing the title of the paper to reflect this. Currently the title can be interpreted as both microscopes are imaging the same area of tissue but due to the destructive nature, this is not possible.
2. Include why using the 3view for targeting is beneficial over other methods, such as taking sections at the microtome and checking them.
3. In the discussion the authors mention the differences in fixation techniques (chemical vs HPF) and mention that other parts of the processing also need to be optimized for

**Commented [cg41]:** We thank the reviewer and have changed the title which we think is more accurate.

**Commented [cg42]:** We have added the rationale for our approach to the discussion.



different samples. It may be beneficial for readers to mentions some more specific examples or suggest a good reference/review for EM staining

4. The major point of this article is outline the use of 3view to find the ROI in a sample however this is one line in the method (line 197, step 25). Provide details on how it was monitored. Eg. Cutting thickness, imaging resolution - are you acquiring images quickly while it is monitored?

5. I suggest including images of the block/ sample at each stage of the process (initial embedded/ trimmed/ on pin for imaging). The authors will show this during the video however for those who read the manuscript only it will be very useful.

#### Minor Concerns:

1. Provide reference for staining procedure used if it was modified from a previously published method

2. Line 165 and table 1: do rinse steps include any initial solution exchange on the bench prior to microwave?

3. Line 180: how are samples embedded? Molds used? (include on materials list)

4. Line 185: during trimming are any parts of the tissue exposed?

5. Line 186: removing samples from resin blocks with a razor blade can be difficult, are any steps taken to ensure sample is not lost at this step?

6. Line 195: clarify why block face needs to be smoothed again

7. Line 200: face of sample was trimmed off in 3view including pt coating. Does this need to be coated again prior to being placed in the FIB? The deposition in the FIB will only cover a small area not the entire block face

**Commented [cg43]:** Both references 3 and 9 have further helpful information on diverse tissue types and we have indicated this in the text.

**Commented [cg44]:** We have rewritten the detailed protocol and now include this information

**Commented [cg45]:** We thank the reviewer for this comment and have now correctly acknowledged the NCMIR protocol (ref. 8)

**Commented [cg46]:** This is made clear in the revised protocol.

**Commented [cg47]:** This is made clear in the revised protocol.

**Commented [cg48]:** This is made clear in the revised protocol.

**Commented [cg49]:** Excellent observation. We have included a reference to Anne Weston's parafilm trick as shown in the Russel et al J Cell Sci article.

**Commented [cg50]:** This has been clarified in protocol step 2.4.

**Commented [cg51]:** This is made clear in the revised protocol.

8. Line 201: is an overview image used for relocating area of interest? Or does the lack of pt coating allow features to be seen? It may be worth noting somewhere that those using this technique need to have an idea of the area they wish to image including depth, as this is important when setting up the acquisition in the FIB

**Commented [cg52]:** We use an accelerating voltage of 15kV to image through the Pt coat, this is now clarified in the protocol step 5.3.

9. Figure 2 and 3: more detailed description of screenshots. Maybe include some annotation of images to explain what the reader is looking at, particularly for the FIB where you can see images which including the trench and tracking marks. This will be particularly important for those who are not familiar with the cutting angle change between the 3view and FIB (this is worth mentioning somewhere in the procedure, may be described nicely using a schematic)

**Commented [cg53]:** We have clarified this with image annotations as well as a note in the protocol step 5.4. Additionally a schematic is now included as Figure 3A.

10. Line 224: SB-EM and SBFi seem to be used interchangeably. For clarity this should be consistent throughout.

**Commented [cg54]:** Corrected, thank you. We now use SBFSEM as was introduced in the Denk and Horstmann paper.

11. Paragraph starting line 224: Wording suggests the same part of the tissue can be re-imaged in the FIB following 3view acquisition, however this is not the case.

**Commented [cg55]:** We think the new protocol makes this clear

12. Line 239: on the effect mentioned caused by anisotropic voxels, can this be eliminated by using image analysis software that takes into account the z slice thickness?

**Commented [cg56]:** We agree and since that is no longer relevant to the new sample we have omitted this figure.

13. Line 278: remove colon and use a comma.

**Commented [cg57]:** Rewritten.

14. Line 284: suggest changing making to keeping

**Commented [cg58]:** Rewritten.

15. Line 286: the word samples may be missing between for and other

**Commented [cg59]:** Rewritten

16. Line 334: of instead of to

**Commented [cg60]:** Rewritten.

17. Table of materials: lead citrate is mentioned but not included in the protocol

**Commented [cg61]:** This has been fixed in the new protocol.

**Reviewer #3:****Manuscript Summary:**

In this draft, Guerin et al. describe how serial block face and focused ion beam scanning electron microscopy can be used to locate and analyse structures within a single specimen. In this protocol, the SBF SEM is used as a trimming tool (albeit an expensive one!) to locate a specific region of interest, prior to targeted FIB SEM on the preidentified region for high-resolution 3D analysis and isotropic visualisation.

The complex and technical nature of the workflow is well suited to presentation in a video format, and in the text the authors highlight several important considerations that are often ignored such as the crucial nature of optimal sample preparation, imaging resolution (contrasting the differences in scale between the two modalities, which is very often still misunderstood), and more indirectly, the particular benefits of shared resources and expert operators in projects of this scale.

**Major Concerns:**

There are a couple of areas in particular where some refinement of the text would be of benefit:

1) The main message could be more clearly defined in the abstract and introduction (which currently end with sample preparation, and the intercalated disc model system, respectively). Some rearrangement and more emphasis on the use of two imaging techniques to find a specific target area would help the reader.

2) The representative data included for illustration of the protocol does not follow a logical sequence - the introduction finishes on the intercalated disc as a model system, with reference to another very recent paper from the

**Commented [cg62]:** We appreciate this helpful and perceptive comment and have adjusted both the abstract and the introduction accordingly.

authors, but this does not feature again except as a single image in figure 2, and in the discussion, explaining why the increased resolution of FIB SEM is needed. To really illustrate the point of using two microscopes to target a specific area, it would be much clearer if the authors could use a series of linked and annotated images in the figures to illustrate exactly this point. This will presumably be achieved in the video, but the current examples in the text don't flow very well because they don't seem to be related.

**Commented [cg63]:** Again, we appreciate the helpful comment. We have changed both the tissue type and the results section to make this clearer.

Minor Concerns:

Other comments and suggestions:

Main text

40, 52 - SBFI or SB-EM?

**Commented [cg64]:** Corrected, thank you. We now use SBFSEM as was introduced in the Denk and Horstmann paper.

59-61 - "Preparing fixed cells...", the end of this sentence is the wrong way around. Extra contrasting is not needed for en bloc staining as such, but rather is for enhanced signal generation.

**Commented [cg65]:** Corrected, now on line 60.

173-175 - Suitable containers for propylene oxide? i.e. something that won't dissolve.

**Commented [cg66]:** We have added a note to step 1.17 to clarify this.

180 - Hours should read 36-48 hours.

**Commented [cg67]:** Corrected, thanks.

191 - How much platinum?

**Commented [cg68]:** 2-5 nm, added to revised protocol.

200 - Should be carrier not sample.

**Commented [cg69]:** Corrected.

203 - Deposition isn't achieved using the e-beam.

**Commented [cg70]:** Corrected in revised protocol.

228-232 - Check figure referencing as the descriptions don't match the figure panels or the legend.

**Commented [cg71]:** Figures have been revised and legends checked, thank you.

268-271 - Worth mentioning also that samples must be kept small enough for consistent staining throughout, in addition to optimizing the timing, so very large structures are still hard to work with.

**Commented [cg72]:** Clarifying note added to step 1.2.

275 - Impure, not inpure.

**Commented [cg73]:** Corrected.

282-283 - In vivo, not in vitro.

**Commented [cg74]:** Corrected.

## Figures

Figure 2 - The screenshots in A and B should be separated slightly and labelled for clarity; it is currently hard to immediately see which screenshot is which with respect to the legend.

Figure 3 - As mentioned above, the references to the panels in the main text don't match the figure.

Figure 4 - As one of the main points of discussion is the need for appropriate resolution choice/imaging modality, and possible artefacts that may arise in analysis and rendering, this figure would benefit significantly from an additional row of panels to emphasise this point more clearly e.g. zooms of the presynaptic bouton from different angles showing more clearly the difference in segmentation/rendering from each imaging modality. As it stands, it is hard to see the vesicles in 4B in particular.

**Commented [cg75]:** The figures have been revised and all the issues are resolved.