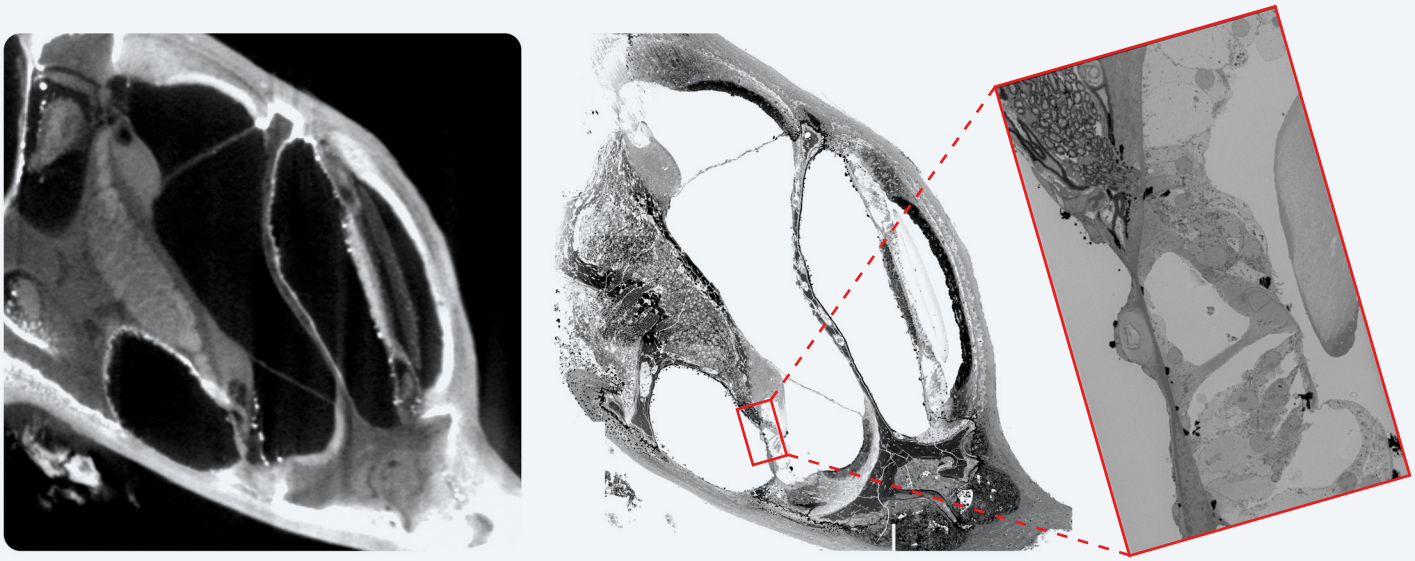
Application Note

X-ray Tomography: The Perfect Partner for High Resolution Volume Electron Microscopy (vEM)

Optimizing Multimodal Workflows in Life Science Research



X-ray Microscopy | Scanning Electron Microscopy | Volume Electron Microscopy

ZEISS

Seeing beyond

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Date: July 2024

In the life sciences field, electron microscopy provides nanometer-level resolution of specimens, with volume electron microscopy (vEM) extending this into three dimensions. Despite its transformative capabilities, challenges remain for acquisition of vEM data, including time-intensive and artifact prone sample preparation, accurate identification of target regions and long imaging times to find the structure of interest. To tackle these challenges, X-ray tomography is emerging as a powerful complementary technology.

X-ray tomography harnesses the penetrative capability of X-rays to generate 3D datasets without physically cutting the specimen. X-ray technologies that are designed to provide both high resolution and high contrast can be used for time-resolved visualization of staining efficacy as well as pre-imaging quality checks of the opaque samples to uncover potential issues that could hinder optimal vEM data acquisition. The inclusive 3D overviews generated by these instruments also provide a strategic roadmap for sample sectioning and streamlined identification of regions to target with the vEM technology of choice.

Combining X-ray tomography with vEM ensures a streamlined workflow to high resolution 3D insights, boosting efficiency, quality and consistency of the results.

Introduction

Electron microscopy is a powerful technology for exploring features in life science specimens with nanometer-level resolution, far surpassing the limits of light microscopy. Volume EM (vEM) further extends this power by expanding the acquired information into three-dimensions. This allows researchers to investigate biological structures in their full spatial context, revealing connections and interactions that may be missed in two-dimensional imaging.

There are different approaches for generating the electron microscope volume including array tomography, serial block-face SEM (SBF-SEM) and focused ion beam SEM (FIB-SEM). A brief summary of some of these technologies is shown in Figure 1 and the details of each can be found here and are also nicely described in [1].

These techniques have found utility across numerous disciplines. For example, in cell biology, vEM can be used to understand organelle structure and interaction [2], while in neuroscience, it can help in mapping 3D neural circuits to a level of detail unachievable by any other technique [3].

Despite these remarkable capabilities, acquiring high-resolution images using vEM presents unique challenges, which can impact on the quality and usability of the resulting data.

Reaching Optimal Results using vEM

The imaging process for any vEM technique is relatively slow. Acquisitions can take many hours, days or even weeks. It is therefore vital that steps are taken to ensure the best imaging outcome.

The sample preparation process for vEM can be time-consuming and requires expertise and optimization. Specimens must be prepared in several steps including fixation, staining, dehydration, embedding and trimming. Each of these stages can introduce artifacts or damage to the sample and without a reliable means of identifying these problems and checking staining success, sub-optimal samples can progress to the imaging stage, which wastes valuable time.

Cover image *This mouse cochlea was imaged with the ZEISS Xradia X-ray microscope to locate the region of the sample for capture at higher resolution. The sample was trimmed to the right dimensions based on the X-ray microscope data. The surface of the trimmed block was imaged with SEM and aligned with the digital slice of the XRM data. Targeted acquisition of the precise region of interest was then performed using Serial Blockface SEM to generate a volume of high resolution data. Data Courtesy of Haoyu Wang and Yunfeng Hua, Shanghai Institute of Precision Medicine, Shanghai Ninth People's Hospital, China. For full experimental details please see [7]*

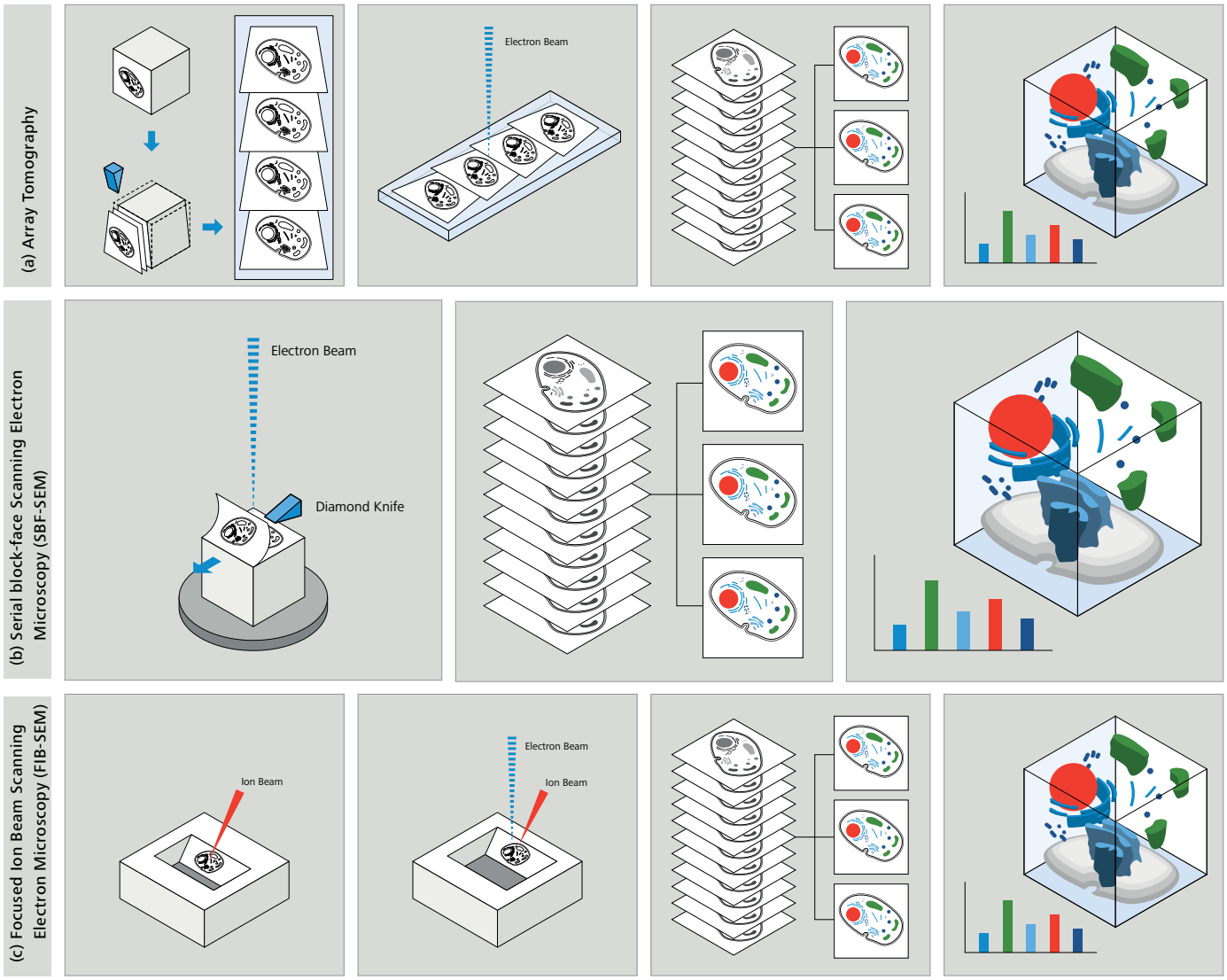

Figure 1 *Common techniques for acquisition of volumes of data using electron microscopy. (a) Array Tomography (AT) describes the process by which thin serial sections are cut and affixed to a sample holder. The sections are then imaged by SEM and digitally reconstructed to create a 3D data set. (b) Serial block-face SEM (SBF-SEM) uses an ultramicrotome located inside the SEM chamber to sequentially cut and then image the surface of a resin-embedded sample. This generates a stack of images that are reconstructed to generate the 3D volume. Typical cutting thickness for each slice is between 25 nm – 50 nm. (c) Focused Ion Beam SEM (FIB-SEM) uses a focused ion beam to mill away the surface of the specimen by as little as 3 – 10nm prior to imaging with the SEM. This process repeats many times resulting in generation of a volume of high-resolution structural information.*

The process of identifying regions of interest (ROIs) for high-resolution imaging in vEM can also be tricky. Conventional methods, such as light microscopy, have limited use since the prepared samples are opaque and visible light cannot penetrate. This means structures of interest must be identified in a rather manual way, which can lead to incorrect sample trimming or the capture of the wrong regions.

These issues point to a need for complementary technologies that can streamline the process, enhance sample quality, provide a means to check sample staining and expedite the ROI identification for vEM acquisitions. This is where X-ray tomography steps in.

High resolution X-ray tomography as a tool to support vEM workflows

X-ray tomography is the ideal partner to overcome some of these vEM workflow challenges. This technique leverages the penetration ability of X-rays to generate 3D datasets without having to physically section the sample (Figure 2) which is transforming the accessibility of these biological workflows.

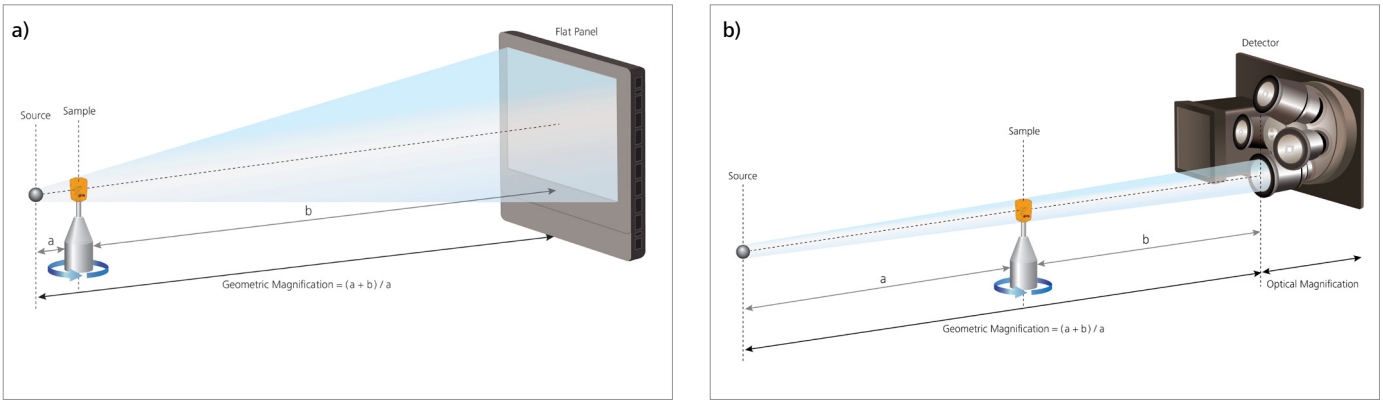

Figure 2 *Graphical representations of X-ray tomography instruments found in the lab. All instruments include an X-ray source, a sample stage and a detection mechanism but the geometry differs since resolution increases are generated in different ways. (a) In the microCT instrument, such as ZEISS Xradia Context, an increase in resolution is achieved using geometric magnification whereby the sample and source are physically moved closer together and the detector is moved further away. This is effective but is ultimately limited by the sample dimensions which limit the achievable resolution in the whole specimen. (b) In the X-ray microscope, such as ZEISS Xradia Versa, a resolution increase is achieved by a range of scintillator coupled objective lenses in combination with geometric magnification. Resolution is increased by selecting higher magnification objectives, in a similar way to a light microscope so sample size does not limit the achievable resolution in the whole specimen. In both cases imaging is non-destructive, so the sample does not require physical sectioning; the intact specimen is mounted and immobilized on the rotating stage. A series of 2D X-ray transmission images is captured at multiple viewing angles by rotating the sample, usually through 360°. These 2D projection images are then reconstructed to create a 3D representation of the specimen based on relative X-ray absorption.*
Both ZEISS Xradia Context microCT and ZEISS Xradia Versa X-ray microscope are optimized to provide the best possible contrast and image quality in low density samples like those used for vEM. This is crucial since once prospective vEM samples have been imaged, regions of interest inside the specimen need to be quickly identified, which is made possible with highly contrasted data. The intact sample can then be trimmed or moved to the vEM instrument of choice for subsequent higher resolution acquisition of these identified regions.

Using X-ray Tomography to Verify Sample Quality

One key advantage of X-ray tomography is its ability to verify sample quality prior to vEM imaging. In traditional workflows, the sample's condition is often only evident after sectioning or imaging, both laborious and often damaging processes. With X-ray tomography, scientists can non-destructively evaluate the entire 3D sample so the internal structure of whole, un-sectioned samples can be evaluated to expose any potential issues (Figure 3).

X-ray tomography datasets that provide high resolution and contrast can detect air bubbles, cracks, or imperfections in the sample, enabling these issues to be addressed before proceeding with vEM [4]. This quality control step is critical for minimizing wasted time and resources on subpar samples.

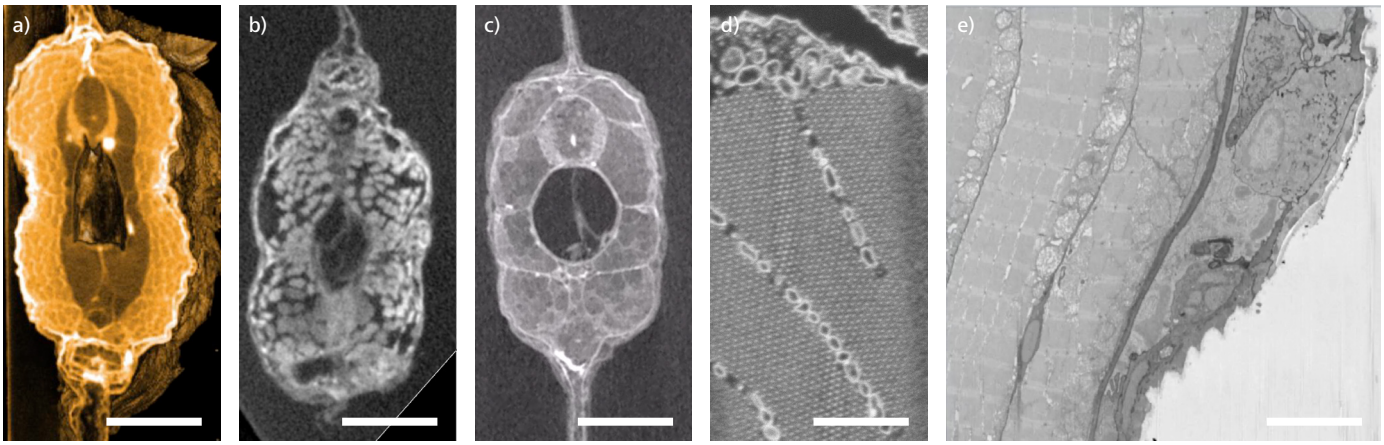

Figure 3 *Zebrafish larva specimens prepared for vEM. (a) 3D render of XRM data viewed longitudinally. Structural information can be attained without the requirement to physically section the specimen. Scalebar 50µm (b) 2D slice taken from 3D XRM dataset. This specimen shows artifacts caused by sub-optimal preservation or bad freezing protocols. These artifacts manifest as condensed punctate structures, assumed to be accumulations of internal components. The original internal structure has been completely disrupted. Since these artifacts are visualized in the X-ray data, this specimen is not selected for further analysis using vEM. Scalebar 50µm (c) 2D slice taken from 3D XRM dataset. The ultrastructure of this specimen seems well represented and the staining is homogeneous. Capturing this non-destructive X-ray tomography dataset provides the necessary confidence that this specimen will produce good results at higher resolution and this sample is therefore selected for vEM. Scalebar 50µm (d) High resolution SEM image of muscle in cross-section of the well-preserved zebrafish larva sample. X-ray imaging determined that staining and sample preparation were good and the resulting SEM data was therefore high quality. Pixel size 1.4 nm; scalebar 500nm (e) 2D slice taken from a 3D FIB-SEM dataset of the same well-prepared sample in longitudinal muscle orientation. Pixel size 20 nm; scalebar 5µm. Specimens courtesy of Prof. Manfred Auer, Department of Biomedical Engineering, Southeast University, Nanjing, China.*

Using X-ray Tomography to Optimize Staining Protocols

Staining protocols used in vEM require optimization for each sample type to ensure the best outcome for every experiment. Particularly for larger specimens, variation in stain uptake can be difficult to manage and this can lead to inconsistent results. Using X-ray tomography, the uptake of stain into the specimen can be visualized in real time [5]. Optimal conditions, timings and concentrations can be established in this way to ensure consistent and comprehensive uptake into a specific specimen type (Figure 4).

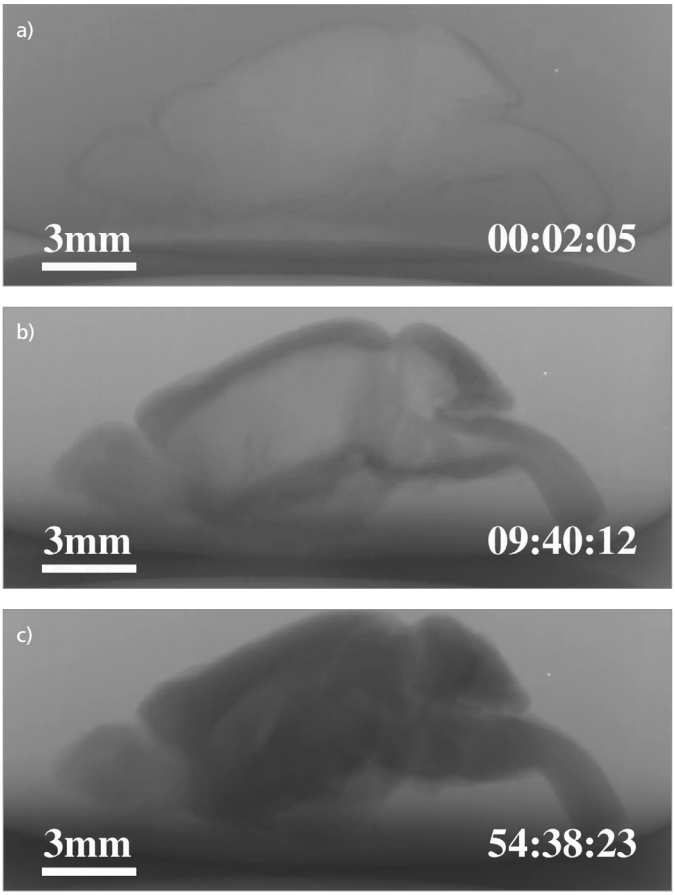

Figure 4 *2D projection images of a whole mouse brain immersed in osmium and sequentially imaged to assess osmium penetration. Representative projection images from 3 different timepoints in the series: (a) 2 minutes and 5 seconds (b) 9 hours, 40 minutes and 12 seconds and (c) 54 hours, 38 minutes and 23 seconds. By capturing sequential images of the specimen immersed in osmium, optimization of the staining protocol becomes much more straightforward. The sample can be removed from the osmium solution once optimal staining has been reached and protocols can be established that are optimized for a particular sample type to generate the best possible result for subsequent imaging using vEM. Images courtesy of Eric Hammerschmith, Princeton Neuroscience Institute, USA. Full experimental details can be found in [5].*

Using X-ray Tomography to Locate Regions for Subsequent High-Resolution Image Acquisition

Locating regions and structures of interest inside the opaque specimens is another challenging aspect of vEM. Traditional workflows rely on manual methods for region of interest (ROI) selection, but this can be time-consuming and prone to error. However, X-ray tomography can quickly and accurately locate ROIs within large samples.

By imaging the entire sample in 3D, X-ray tomography provides an inclusive overview, which is not limited to the sample surface. From this large-scale image, scientists can easily identify areas of interest for high-resolution vEM. Targeted acquisition using the vEM technology of choice can take place based on the 3D X-ray tomography map [6]. Tools are available to assist with the automation of navigating to the identified location from the X-ray tomography dataset. For example, if the region of interest is near the surface of the specimen, data from the ZEISS Xradia Versa X-ray microscope or ZEISS Xradia Context microCT can be used to navigate to precisely the right location in the FIB-SEM once the sample is mounted in the ZEISS Crossbeam.
The Atlas 5 software is used to power this navigation which ensures efficient and precise capture of the region of particular interest (Figure 5). If the region of interest is located more deeply into the specimen, the sample may first need to be trimmed using an ultramicrotome prior to this navigation step (see next section for details).

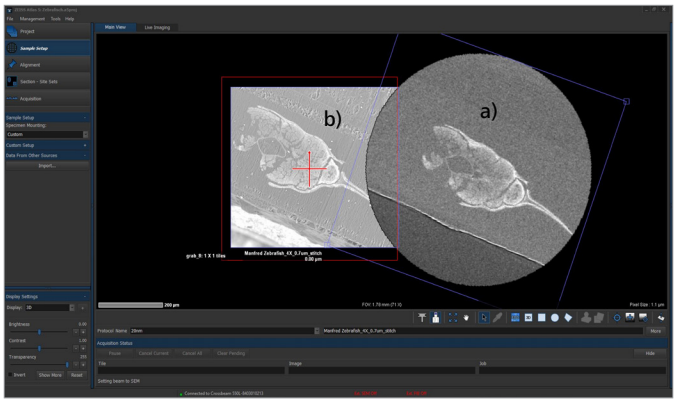

Figure 5 *Figure 5: A zebrafish larva was prepared for imaging with vEM. The specimen was first imaged with the ZEISS Xradia Versa X-ray microscope (a). The same specimen was then mounted in ZEISS Crossbeam 550 (b). Using the Atlas 5 software, the X-ray microscopy dataset from ZEISS Xradia Versa is aligned with the live image of the specimen from ZEISS Crossbeam. This allows quick and easy navigation to the region of interest for high resolution image capture. This streamlined workflow saves hours of time that would be otherwise wasted trying to locate and navigate to the region for higher resolution acquisition. Specimen courtesy of Prof. Manfred Auer, Department of Biomedical Engineering, Southeast University, Nanjing, China.*

Using X-ray Tomography to generate a roadmap for directed sample trimming

As discussed, locating regions of interest using X-ray tomography is very valuable, but for subsequent imaging, samples often require additional trimming to reach a more appropriate size for EM. X-ray tomography can be used to help plan these sample trimming or sectioning processes by providing a "roadmap" of the sample's internal structure (for example [7]). Navigation points on the sample (which could be fiducial markers, unique features of the sample itself, or strategic markings made for navigational purposes by etching) are used to define the required measurements. The sample can then be strategically cut in ways that optimize data acquisition while minimizing damage to

crucial regions. Trimming and sectioning can be performed manually based on measurements from the 3D map [4], as shown in figure 6. More automated approaches have also been developed to further streamline this process [8].

Save Time, Improve Quality, and Streamline Your Workflows using X-ray Tomography

Incorporating X-ray tomography into multimodal workflows is a strategic choice that offers significant benefits for vEM imaging.

The first benefit is operational efficiency. X-ray tomography provides rapid, non-destructive sample screening and comprehensive ROI identification, speeding up the overall workflow.

Secondly, the technique offers improved data quality.

By enabling upfront quality control, staining optimization, and targeted sample sectioning, it increases the chances of acquiring high-quality vEM data.
Finally, X-ray tomography can enhance the value of vEM data. A seamless correlation between whole-sample and high-resolution views is provided by the X-ray scan. This adds context to the smaller volume of high-resolution information revealed by vEM which enriches the interpretability of the data.
Based on these advantages, any core facility or lab using vEM that invests in high quality X-ray tomography technology gains a competitive edge; Researchers and technical staff benefit from optimized and streamlined workflows for vEM acquisitions that generate high-quality, context-rich data. The complementary nature of the high resolution and contrast images from the ZEISS Xradia Context microCT or ZEISS Xradia Versa X-ray microscope ensures that these technologies are not just valuable scientific tools, but also strategic assets that can help to drive growth and innovation for core facilities.

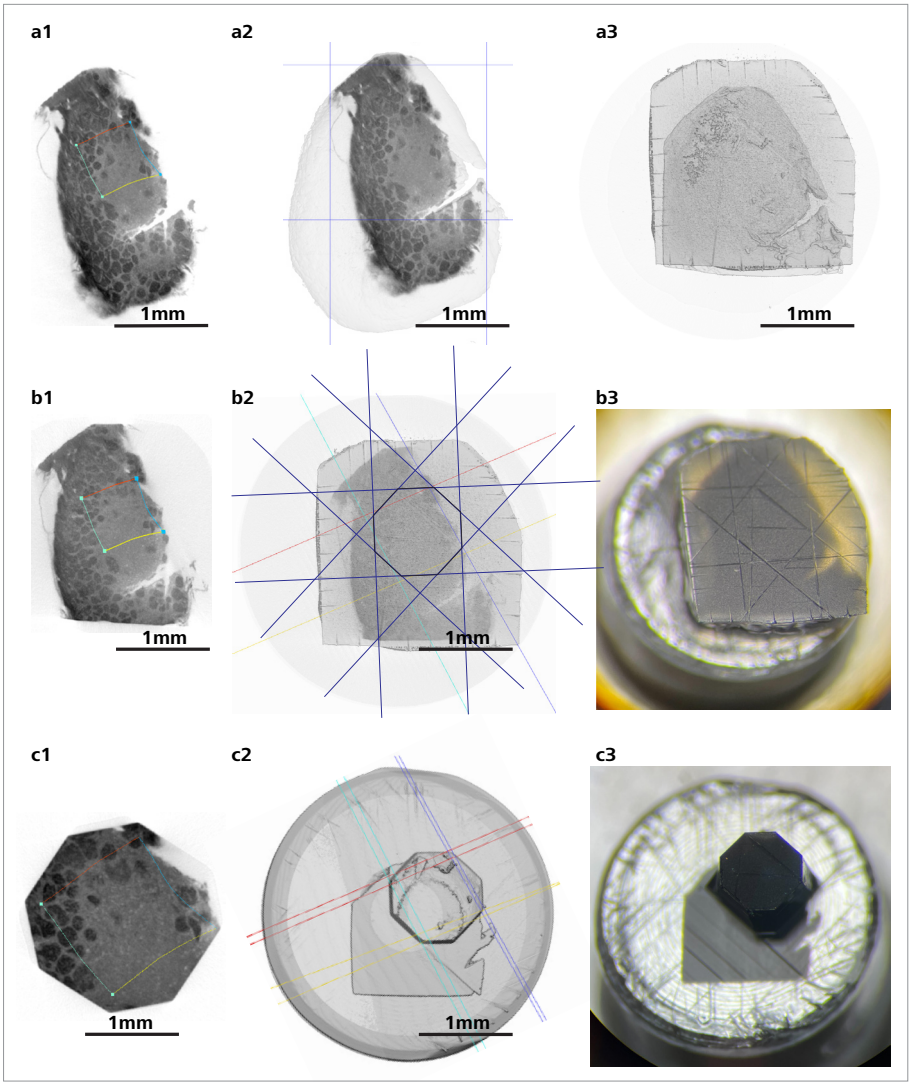

Figure 6 *Prior to higher resolution acquisition this resin embedded mouse olfactory bulb sample was trimmed to the right dimensions. The trimming pattern was very specific since it needed to include the region that had been functionally imaged in the live animal to allow correlation of cell function and structure. The sample was imaged with the ZEISS Versa X-ray microscope to generate a 3D representation of the whole specimen. Marks made on the surface of the resin can be seen in the X-ray microscope data and also when the specimen is transferred to the ultramicrotome. Dimensions taken from the X-ray dataset can be used to direct cutting of the specimen by using these navigational points to generate a sample size optimal for subsequent higher resolution acquisition. In this way, the region of interest is preserved in the resulting smaller block, and this can be imaged using synchrotron X-ray methods or vEM. Specimen and images courtesy of Yuxin Zhang, the Francis Crick Institute, London*

(a1) Single virtual slice from a 3D dataset taken with the ZEISS Xradia Versa. The region that was functionally imaged previously (ROI) is overlaid onto the sample before it is trimmed.

(a2) Trim lines are planned based on the location of the ROI box, using the sample shape as a reference. The sample is trimmed using a razor blade and glued on top of an aluminum sample holder pin.

(a3) Overlay of two 3D renderings after the sample is trimmed for the first time; one showing the resin block and the other the sample itself inside the resin block. Note that marks are etched on the surface of the resin block around the tissue, to aid navigation during the second round of trim planning.

(b1) Single virtual slice from a 3D dataset of the specimen taken with the ZEISS Xradia Versa after the first trim. The ROI is shown overlaid onto the trimmed sample.

(b2) Eight trim lines are planned based on the location of ROI box, using the etched marks as references.

(b3) Photograph of the specimen with the 8 trim lines carved on top of the sample surface. Note that the location of these lines relative to the etched marks is precisely as planned. The sample is then trimmed according to this pattern using the ultramicrotome.

(c1) Single virtual slice from a 3D dataset of the specimen taken with the ZEISS Xradia Versa after the second trim. The ROI is shown overlaid onto the trimmed sample. Note that the ROI is entirely preserved, and the sample is just big enough to capture the entire ROI.

(c2) 3D rendering of the final sample shape, with lines indicating the ROI box overlaid; the 2 sets of lines take into account the tilt of the 3D ROI box in z.

(c3) Photograph of the final sample, after 2 rounds of trimming.

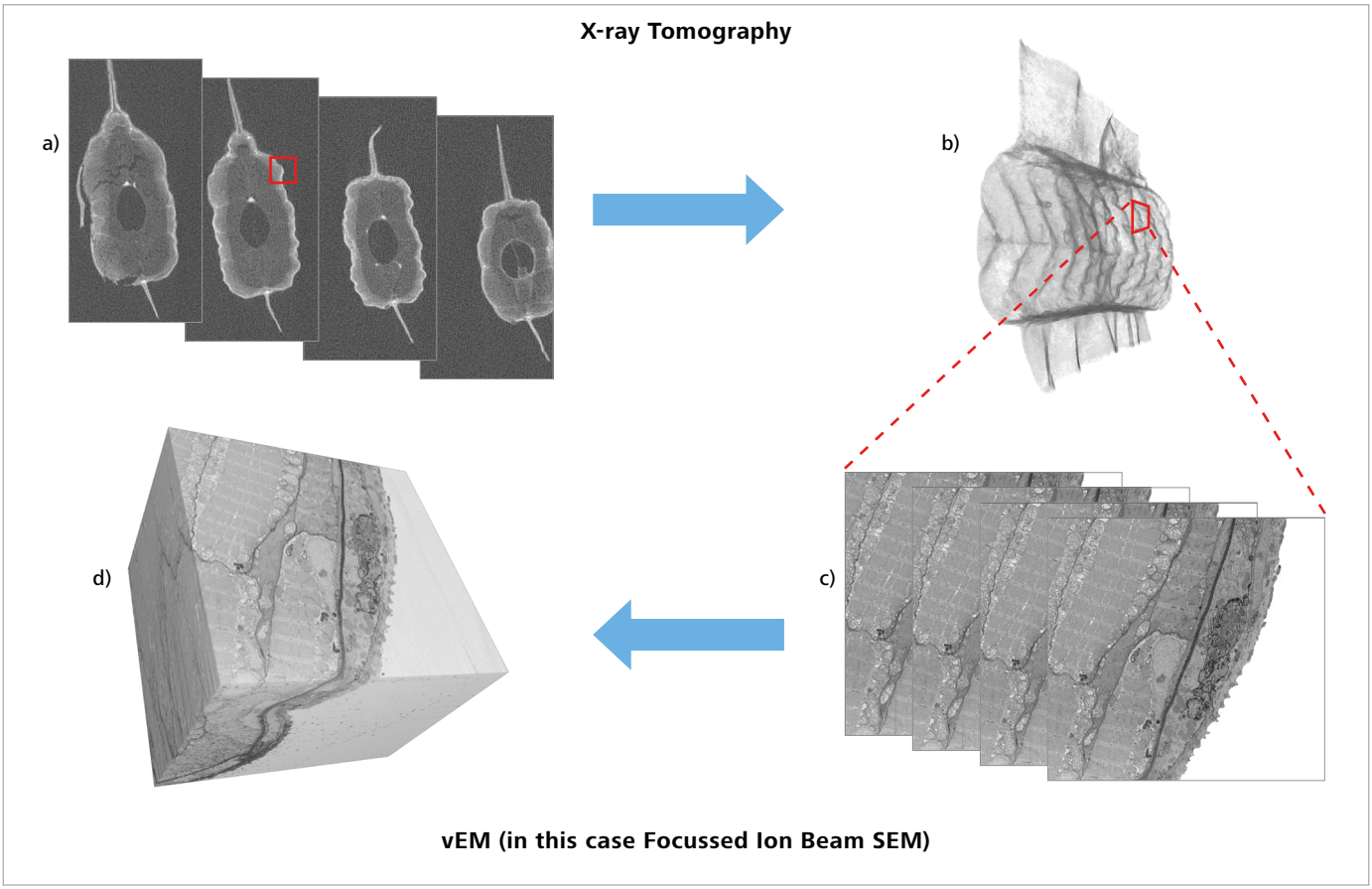

Figure 7 *Common workflow to ensure optimal results using vEM. The zebrafish larva is non-destructively imaged in 3D using ZEISS Xradia Versa X-ray microscope. Using both the 2D slices (a) and the 3D reconstruction (b) (both imaged with 0.7nm voxel size), the quality of the sample preparation and staining can be assessed and the region(s) of interest for higher resolution acquisition (red box) can be visualized. Once identified as a high-quality specimen, the sample is transferred to the FIB-SEM for higher resolution acquisition using ZEISS Crossbeam. 2D slices (c) and 3D renderings (d) of the FIB-SEM data can provide ultrastructural insights of small regions whilst retaining the context of the entire specimen from the X-ray tomography scan. Specimen courtesy of Prof. Manfred Auer, Department of Biomedical Engineering, Southeast University, Nanjing, China.*

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