

Sample compatibility with the Hydra Bio Plasma-FIB

3D imaging of mouse brain tissue embedded in acrylic and epoxy resins

In recent years, combined focused ion beam milling and scanning electron microscopy (FIB-SEM) has become a key technology in the life sciences, and is rapidly becoming a powerful tool for 3D volume analysis and ultrastructural imaging of biological samples.

The most common FIB systems used today are liquid metal ion sources (LMIS), typically gallium. Although LMIS tools are already commonly used for biological 3D imaging, gallium FIB-SEM systems are limited by throughput and sample compatibility. The general sample preparation workflow consists of chemical fixation, heavy-metal contrasting, and resin embedding. For conventional FIB-SEM analysis, samples are embedded in high-density epoxy-based resins such as EPON or Durcupan, as these are less prone to milling artifacts.

It is well known that the radiation response of various resins is drastically different. Acrylic-based resins (e.g. LR White) tend to

generate severe milling artifacts, such as curtaining (see **Figure 2B**), even at low doses. Due to this poor stability under the gallium FIB, the use of acrylic resins in 3D FIB-SEM analysis is very limited.

More recently, FIB-SEM systems with inductively coupled plasma (ICP) ion sources have been introduced, capable of delivering an order of magnitude higher currents (up to 2.5 μA). The Thermo Scientific™ Helios Hydra™ Bio Plasma FIB is a plasma FIB (PFIB) SEM with the unique capability to deliver four ion species (Xe, Ar, O, or N) as the ion beam source, enabling researchers to find the optimal ion beam for every sample. The Helios Hydra Bio alternates between ion-beam milling of the sample block and imaging of the exposed surface with SEM. This results in a high-resolution 2D-image stack that can be combined into a 3D representation of the sample, revealing features of interest at nanoscale resolution (**Figure 1**).

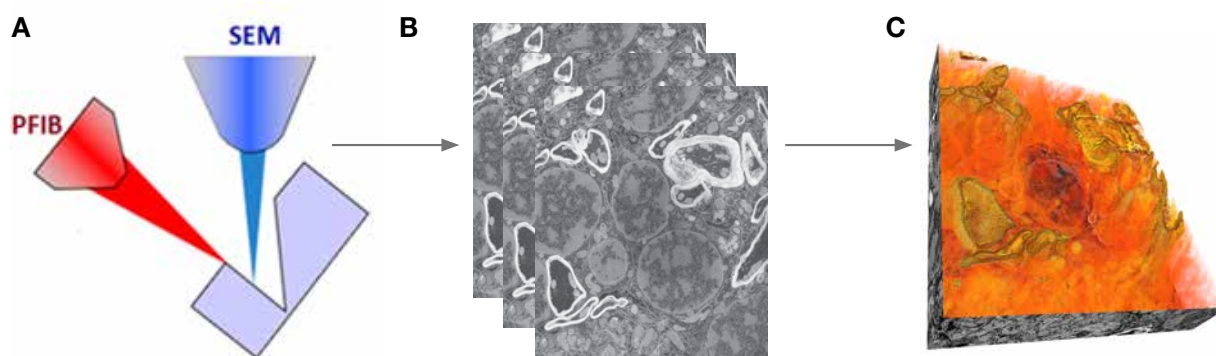


Figure 1. PFIB-SEM workflow for biological experiments. (A) The PFIB-SEM system is used for iterative sample milling. (B) SEM imaging of the revealed cut-faces produces a stack of 2D images. (C) Images are computationally converted into a 3D volume, allowing for segmentation and visualization of features.

Optimizing the ion beam source for biological samples

When preparing biological specimens for analysis, the sample preparation method and resin type determine how well subcellular structures and fluorescent signals are preserved. In this application note, we explored the use of several ion species (Ga, Xe, O, and N) on chemically fixed mouse brain tissue embedded in LR White (acrylic-based) and EPON (epoxy-based) resin.

Mouse brain tissue samples were fixed in glutaraldehyde, paraformaldehyde, and sodium cacodylate buffer and then post-fixed with osmium tetroxide and potassium ferrocyanide. Increased heavy-metal content in the sample improves SEM image contrast, so the fixation step was followed by additional staining by uranyl acetate and lead aspartate. Following fixation and staining, dehydration removed all remaining water in the tissue. The samples were then infiltrated with increasing concentrations of epoxy

(EPON) or acrylic (LR White) resin and polymerized. The stained and embedded samples were trimmed to a volume of ~1 mm³ and coated with a thin gold layer.

Figure 2 shows comparative results after Ga-FIB and PFIB milling for the LR White resin sample. Use of both the gallium and xenon ion source led to curtaining, while oxygen and nitrogen resulted in smoother block faces. Additionally, oxygen demonstrated better milling efficiency compared to nitrogen.

3D volume imaging

An overview of the sample preparation workflow for 3D volume imaging is provided in Figure 3. Initially, a 105 x 90 µm region was exposed in 40 seconds using the oxygen beam (30 kV, 45 nA), opening a block-face of the sample. Once the region of interest (ROI) was defined, trenches were milled around it using the oxygen beam. Subsequently, a 1-µm-thick protective layer of tungsten was deposited on the ROI using xenon PFIB (8 kV, 10 nA) for 3 minutes (note: platinum or carbon deposition can also be used). A fiducial marker (cross-milled with xenon) was

used as a reference point during automated milling and data acquisition, which were performed with Thermo Scientific Auto Slice & View™ Software.

An oxygen ion source (30 kV, 0.23 nA) was used to mill the EPON-embedded sample, with a slice thickness of 4 nm (22 s milling time per frame). For the LR-White-embedded sample, O⁺ (30 kV, 0.61 nA) was used to mill 10-nm-thick slices (20 s milling time per frame). It is important to note that higher, more common beam currents (up to 45 nA) can be used; these will further accelerate the milling process. After each slice was milled, a backscattered electron image was acquired using either the in-column (ICD) or through-lens detector (TLD) in ultra-high-resolution mode (2 kV, 200 pA).

The stacks of 2D images (721 slices for the EPON-embedded sample, 2,000 slices for the LR-White-embedded sample) were then used to computationally reconstruct the 3D volumes (Figure 4, 5). These results demonstrate that high-quality, high-throughput PFIB-SEM imaging is possible for biological samples, without the typical challenges associated with sample preparation.

Conclusions

When preparing biological specimens for analysis, the sample preparation method and resin type determine how well subcellular structures and fluorescent signals are preserved. In this application note, we compared the sample compatibility of different ions (Xe, Ga, O, and N), with specimens of mouse brain tissue embedded in epoxy- and acrylic-based resins. We demonstrated that oxygen milling resulted in curtain-free surfaces, even for acrylic-embedded sample, which are typically challenging for FIB milling.

In combination with automated data collection, oxygen milling resulted in FIB-SEM tomography with excellent quality images for both EPON and LR-White embedded samples. Oxygen was found to be a versatile ion beam, especially suited for ultrastructural studies of biological samples, regardless of preparation method.

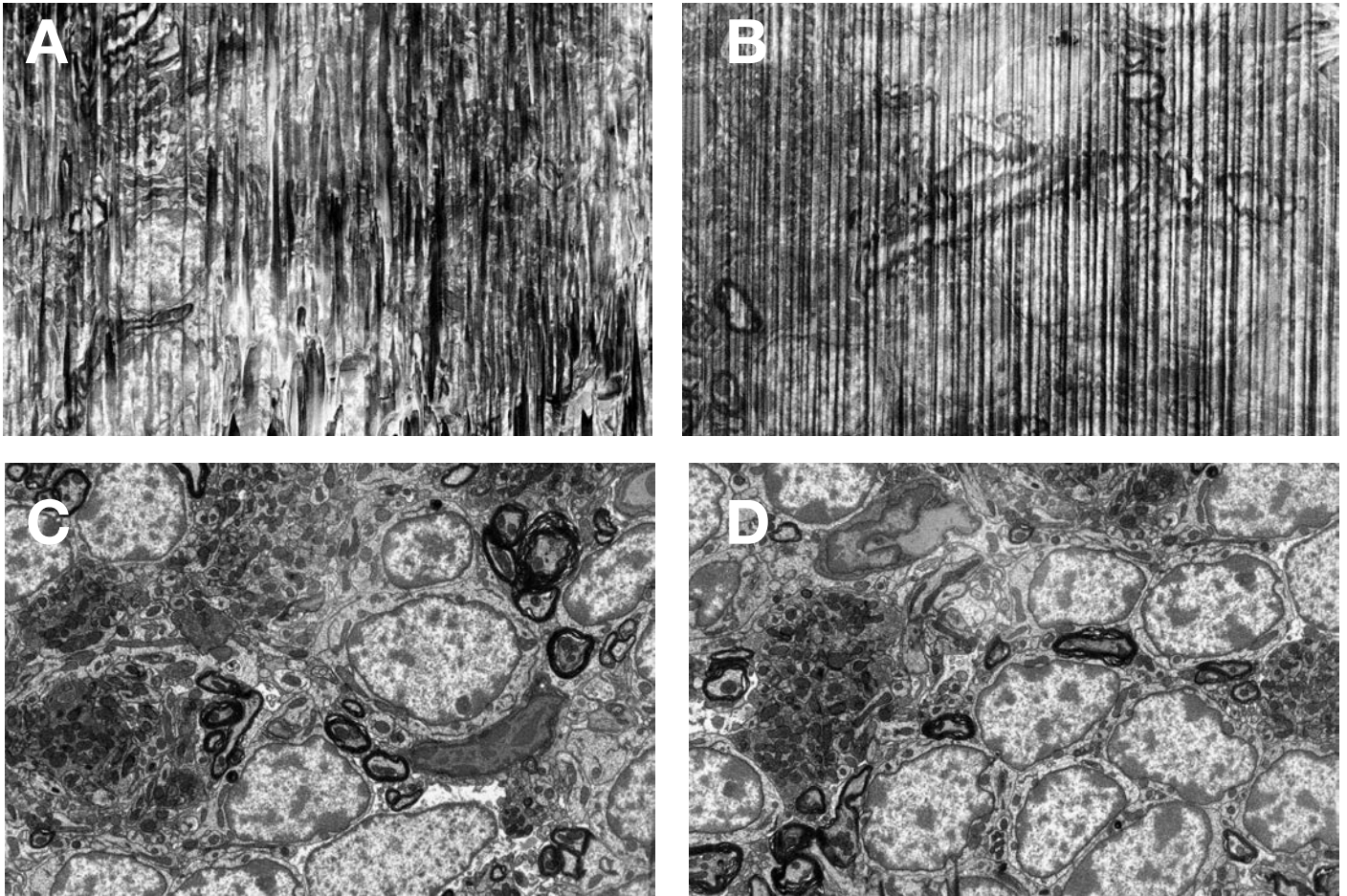


Figure 2: Chemically fixed mouse brain tissue embedded in LR White resin. (A) Xenon PFIB milling at 30 kV, 60 nA. (B) Gallium FIB milling at 30 kV, 65 nA. (C) Oxygen PFIB milling at 30 kV, 45 nA. (D) Nitrogen PFIB milling at 30 kV, 23 nA. An ICD detector was used at 2 kV and 200 pA. Pixel width = 9 nm. Horizontal field width = 28 μm .

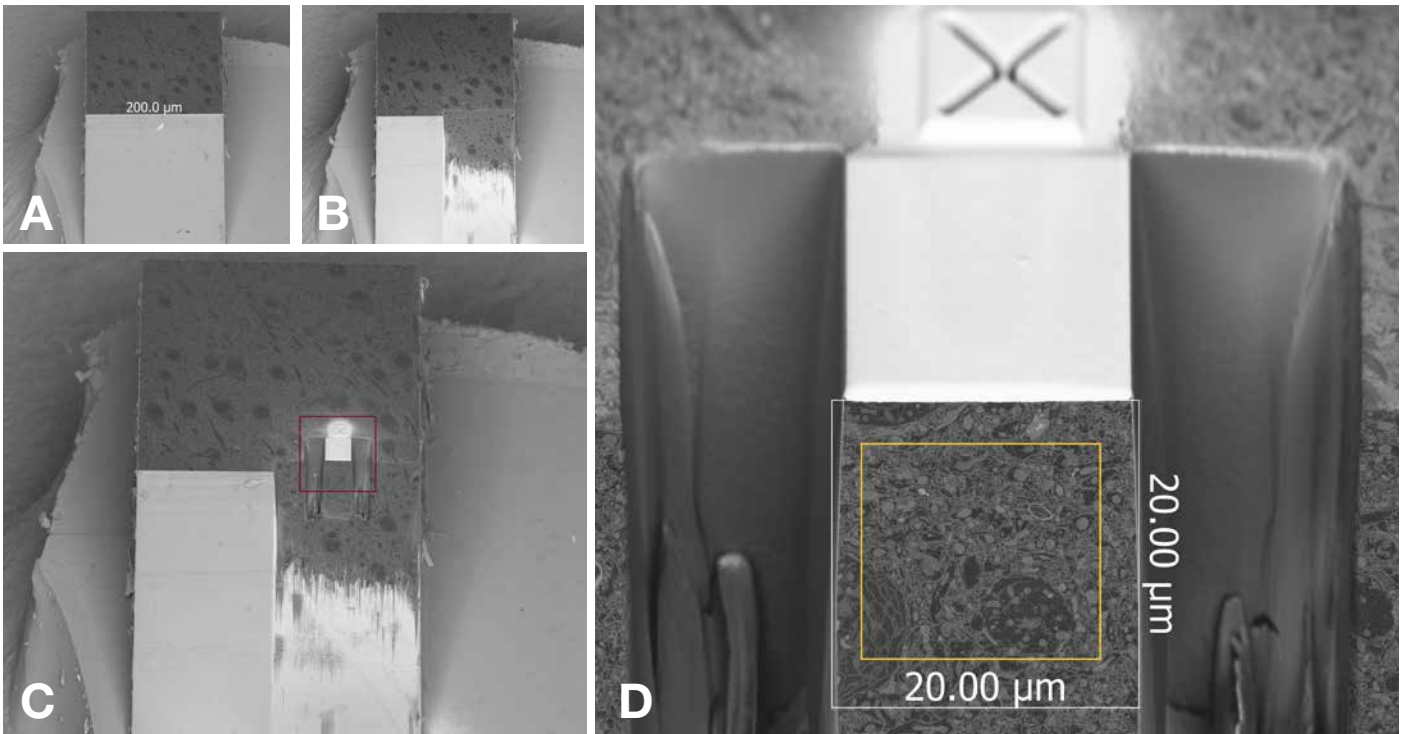


Figure 3: Setup for slice-and-view data acquisition. (A) Overview of the mouse brain tissue sample. (B) Exposure of the region of the interest (105 x 90 μm) using oxygen PFIB. (C) Preparation of the selected area of interest (red rectangle); consists of side trenching, deposition of a protective tungsten layer, and cutting of the X-shaped fiducial. (D) The area of interest (yellow rectangle) is continuously milled and imaged using Auto Slice & View Software.

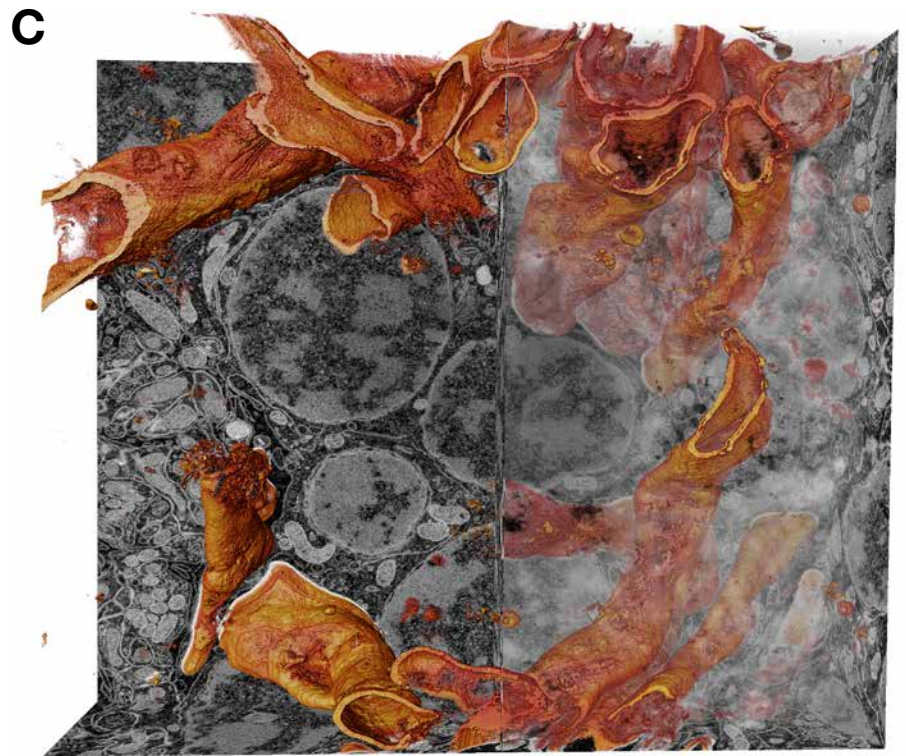
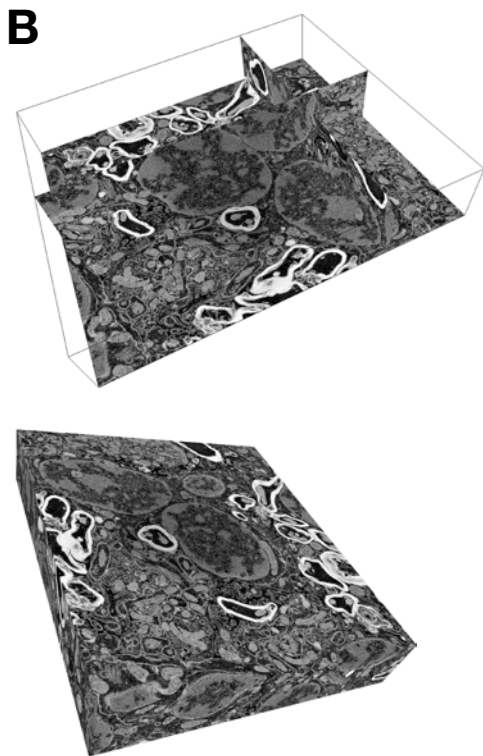
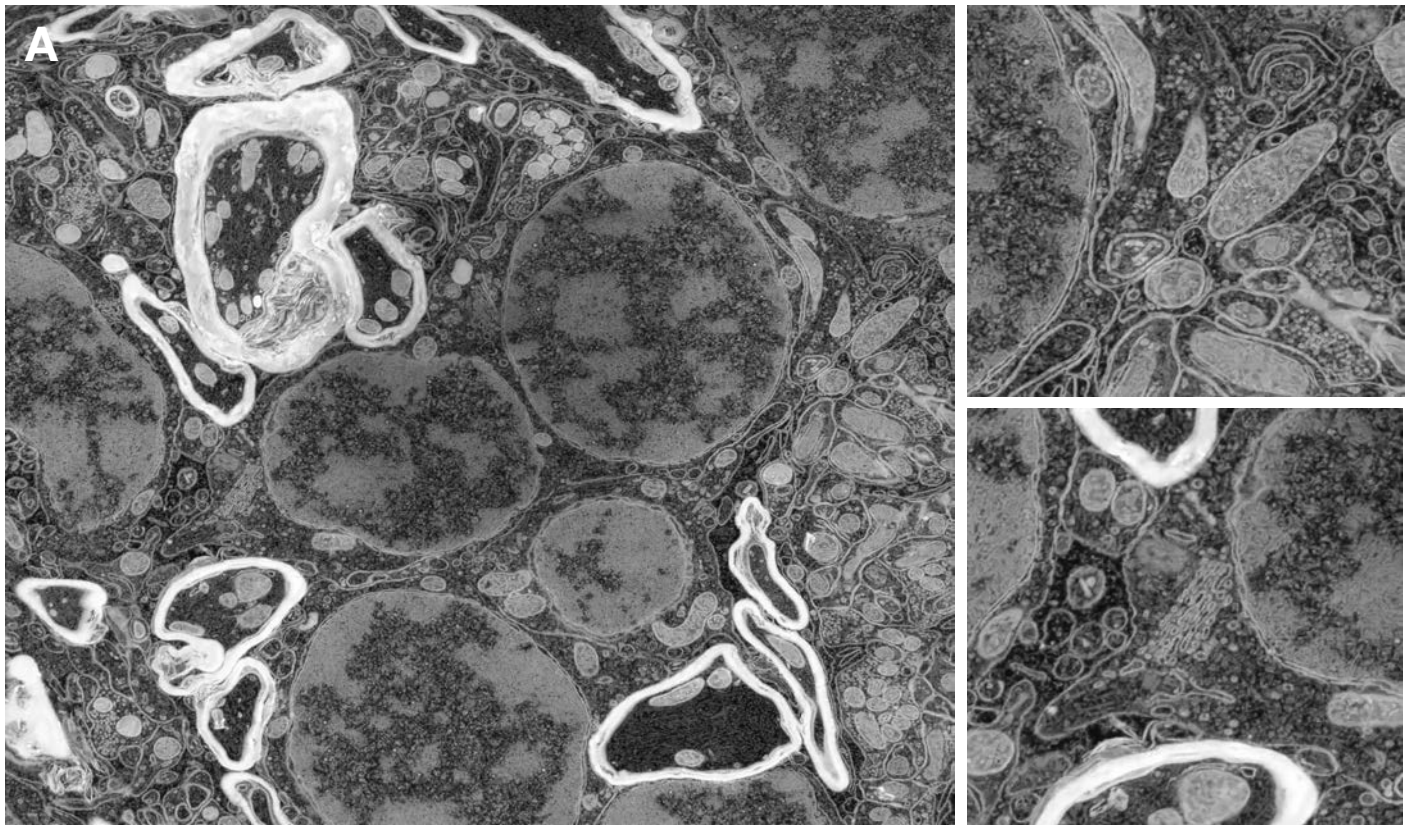


Figure 4: 3D reconstruction of mouse brain tissue embedded in EPON resin. (A) A selected slice from the FIB-SEM image stack, showing subcellular details, with a horizontal field width = 20.4 μm . (ICD at 2 kV and 200 pA, 4,096 x 3,536 pixel resolution (5 nm per pixel), 5 x 5 x 4 nm voxel size.) (B) Sampled volume with selected slices along the XY, ZX, ZY planes. (C) 3D reconstructed volume, segmented to reveal structural details. Total volume = 20.4 μm x 17.7 μm x 2.9 μm . Total slices = 721. Total acquisition time = ~17 hours. Auto Slice & View Software was used for automated acquisition.

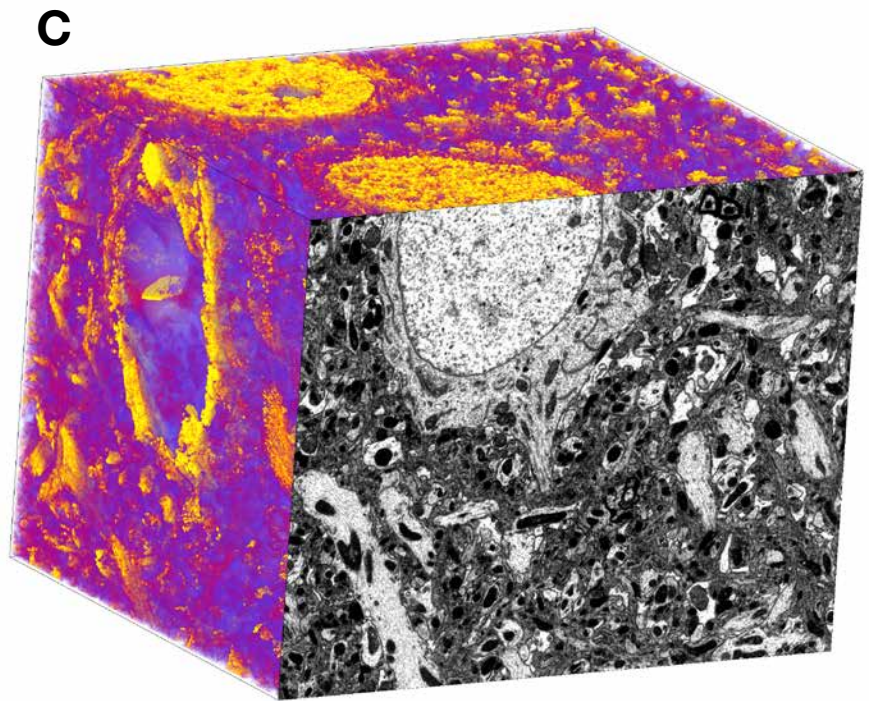
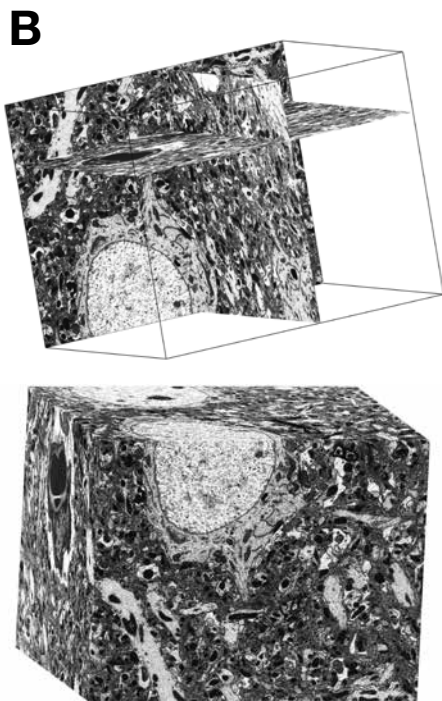
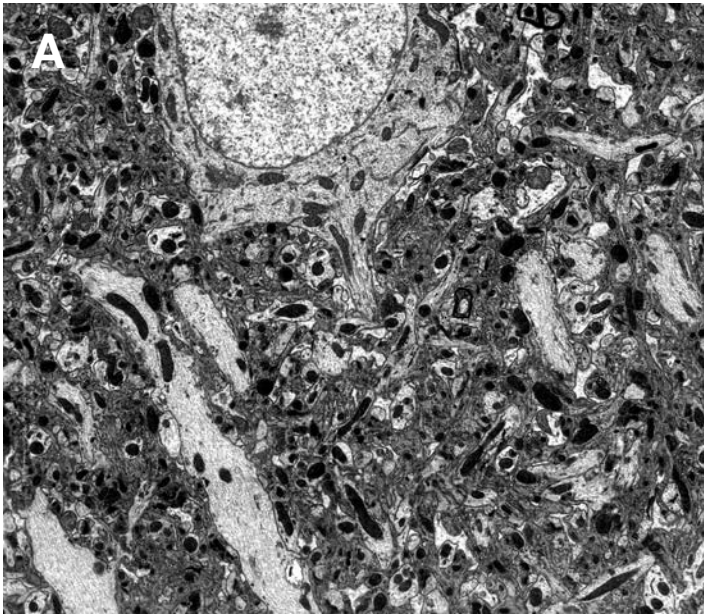


Figure 5: 3D reconstruction of mouse brain tissue embedded in LR White resin. (A) A selected slice from the FIB-SEM image stack with a horizontal field width = $23.4\ \mu\text{m}$. (TLD at 0.2 kV and 200 pA, $3,782 \times 2,929$ pixel resolution (6.2 nm per pixel), $6.2 \times 6.2 \times 10$ nm voxel size.) (B) Sampled volume with selected slices along the XY, ZX, ZY planes. (C) 3D reconstructed volume, segmented to reveal structural details. Total volume = $23.4\ \mu\text{m} \times 18.2\ \mu\text{m} \times 20.0\ \mu\text{m}$.

Thermo Scientific Hydra Bio Cryo-Plasma FIB enables you to:

- Generate smooth, curtain-free surfaces regardless of resin type or sample preparation method
- Perform fast and efficient milling, without compromising quality, in order to access 10× greater volumes than those milled with gallium sources
- Use a 3D data acquisition method similar to serial block-face imaging, but with nanometer-thick slices
- Access multi-modal subsurface and 3D information with precise ROI targeting using Auto Slice & View Software

Further reading

Li, S., Raychaudhuri, S., Lee, S.A. et al. (2021) *Asynchronous release sites align with NMDA receptors in mouse hippocampal synapses*. *Nat Commun* 12:677. doi: 10.1038/s41467-021-21004-x

Gorelick, S., Korneev, D., Handley, A., et al. (2018) *Oxygen plasma focused ion beam scanning electron microscopy for biological samples*. *bioRxiv* 457820. doi: 10.1101/457820

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