Electron microscopy in the life sciences

From sample preparation to recent breakthroughs

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Electron microscopy (EM)

Room-temperature EM (RT-EM)

3D reconstruction and volume EM (vEM)

Focused ion beam SEM (FIB-SEM)

Serial block-face SEM (SBF-SEM)

Array tomography

Correlative light and electron microscopy (CLEM)

Biological sample preparation

Ultrastructure preservation

Quantitative ultrastructural analysis

Accurate electron microscopy (EM) is highly dependent on the quality of the sample preparation. Regardless of the modality, optimal fixation, dehydration, staining, and embedding are essential to preserve ultrastructure, minimize artifacts, and achieve sufficient contrast and conductivity. The choice of protocol should be guided by the intended imaging strategy: conventional transmission electron microscopy (TEM) typically requires less heavy-metal staining, whereas volume scanning electron microscopy (SEM) necessitates thorough heavy-metal application to support stable serial imaging and 3D reconstruction. Factors such as sample size, tissue composition, desired resolution, and imaging environment determine compatibility with downstream analysis. These preparation strategies underpin both 2D and 3D room-temperature EM and the included examples highlight how 3D room-temperature EM is helpful for relating ultrastructure to function.

Preparation of biological samples for electron microscopy imaging

Sample preparation for conventional TEM

Conventional TEM uses ultra-thin resin sections to visualize cellular ultrastructure at nanometer resolution. Biological samples are chemically fixed (to crosslink proteins and preserve morphology), followed by post-fixation with osmium and optional *en bloc* staining with aqueous uranyl acetate, and finally dehydration with graded ethanol or acetone. The tissue is then infiltrated and embedded in an epoxy resin (e.g., Epon or Durcupan).

Once the sample is preserved, ultrathin sections (50–90 nm) are cut using an ultramicrotome; thicker sections (200–1000 nm) can also be made for tomography or scanning transmission electron microscopy (STEM). If no *en bloc* stain was applied, sections are post-stained sequentially with uranyl acetate and lead citrate to enhance contrast. For superior structural preservation, chemical fixation can be replaced by high-pressure freezing (HPF) followed by freeze substitution (FS). Samples are vitrified at ~2,100 bar (which prevents crystalline ice formation) followed by substitution in acetone or methanol at –90°C to –50°C with fixatives such as osmium tetroxide or uranyl acetate. The samples are then gradually warmed and resin-embedded using conventional protocols.

Common chemicals and associated purpose for TEM sample preparation

Chemical	Purpose
Glutaraldehyde and paraformaldehyde in cacodylate or phosphate buffer	Tissue fixation that crosslinks protein amines and stabilizes cellular architecture
Osmium tetroxide, often with potassium ferricyanide	Reacts with unsaturated membrane lipids to deposit electron-dense osmium, preserving bilayer contrast
En bloc uranyl acetate (optional)	Chelates phosphate- and carboxylate-rich targets (e.g., nucleic acids, ribosomes)
Tannic acid	Mordant
Graded ethanol or acetone	Dehydration
Epoxy resin (Epon or Durcupan)	Resin embedding
Uranyl acetate and lead citrate	Post-staining to increase mass-thickness and anionic-site contrast

Sample preparation for volume SEM

Volume SEM and related multimodal techniques, such as serial block-face imaging (SBFI) and focused ion beam SEM (FIB-SEM), enable 3D imaging of biological tissues at nanometer scale. Optimal results require uniform heavy-metal contrast, structural integrity, and conductivity. Sample preparation begins with fixation, as in TEM, but with a different initial contrasting step, where osmium-potassium ferrocyanide is reduced with subsequent thiocarbohydrazide application along with a second osmium step. This rOTO protocol achieves higher contrast and conductivity, while also stabilizing sample lipids and reducing charging artifacts (rOTO is a standard protocol in volume electron microscopy, first established by **Deerinck *et al***.[1]). *En bloc* uranyl acetate, together with lead-aspartate staining, further enhance contrast and signal uniformity. The specimen is then dehydrated, embedded in resin, and finally, trimmed down to the required size and glued to a stub.

Array tomography is a volume SEM method that images dozens to hundreds of serial ultrathin sections mounted on silicon or ITO-coated substrates. With array tomography, the rOTO protocol is unnecessary; the standard sample preparation protocol for TEM imaging can be used. Increased heavy metal would enhance the contrast so much that some biological features would become difficult to spot.

Volume SEM

This table mirrors the rOTO protocol developed by Deerinck *et al*. for volume SEM sample preparation.[1,2]

Chemical	Purpose
Glutaraldehyde and paraformaldehyde in cacodylate or phosphate buffer	Tissue fixation that stabilizes proteins
Osmium tetroxide with potassium ferricyanide	Loads membranes with osmium, boosting electron density and conductivity
Thiocarbohydrazide	Bridges osmium sites, enabling additional metal deposition
Second OsO_4 application	Thickens osmium coat and strengthens the BSE signal
En bloc uranyl acetate	Adds heavy atoms to phosphate/carboxylate groups, further enhancing contrast
Graded ethanol or acetone	Dehydration
Epoxy resin (Epon or Durcupan)	Resin embedding

Types of fixation used to enable high-quality electron microscopy

Immersion fixation is a straightforward and accessible technique in which tissue is submerged directly into a fixative solution. Its simplicity and lack of specialized equipment make it suitable for small specimens like biopsies, or thin samples that generally do not exceed 1 mm^3, e.g., cell monolayers. For these samples, fixatives such as glutaraldehyde and paraformaldehyde are able to efficiently preserve fine structural details. While diffusion is slower in larger tissues, careful preparation of samples through trimming and prompt immersion can still yield good ultrastructural preservation.

Transcardiac perfusion fixation uses an animal's vascular system to deliver a fixative, rapidly replacing blood and distributing the solution throughout the tissue. This approach achieves uniform fixation and preserves ultrastructure with high fidelity. Although more technically demanding, this method provides highly reproducible results when performed correctly. Detailed protocols, such as "Whole Animal Perfusion Fixation for Rodents" by Gage *et al.*, offer established guidelines for its implementation.[3]

High-pressure freezing with freeze substitution (HPF-FS) is an advanced fixation method for samples that require even higher levels of structural preservation, or those sensitive to artifacts from chemical fixation. In this cryogenic approach, specimens are vitrified within milliseconds under high pressure (~2,100 bar), preventing crystalline ice formation, thereby maintaining native ultrastructure. During freeze substitution, water is replaced at low temperatures (–90 °C to –50 °C) with an organic solvent containing fixatives such as osmium tetroxide or uranyl acetate. The sample is then gradually warmed, infiltrated with resin, and polymerized as in conventional workflows. HPF-FS provides near-native preservation of membranes and organelles and due to acrylic resins enables CLEM approaches such as in array tomography. This approach is, however, limited to sample thicknesses ≤200 µm.

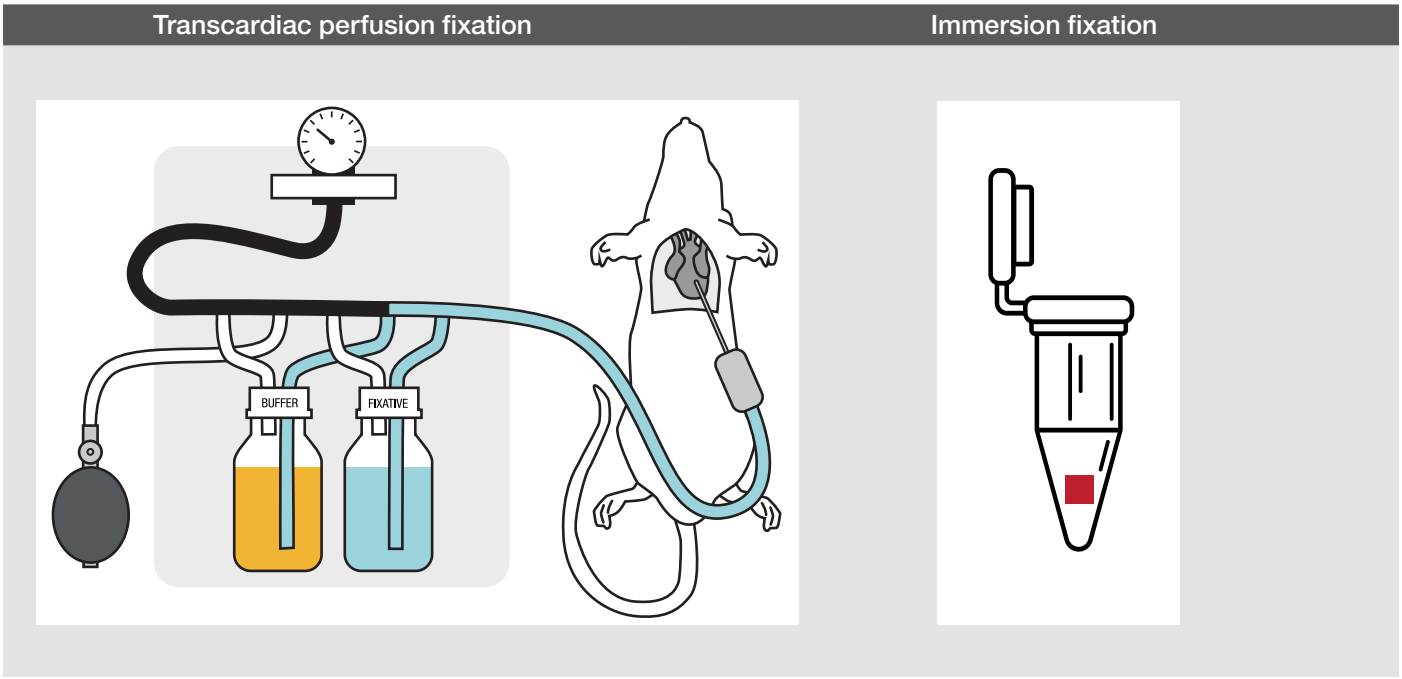

Figure 1. Common sample fixation methods. Transcardiac perfusion and immersion fixation are illustrated. Detailed explanation can be found in Gage *et al.*[3]

Variable sample preparation requirements for TEM and SEM

Conventional TEM uses a high-energy electron beam that must pass through the specimen, and therefore samples are ideally ultrathin sections 50–100 nm thick. For specialized applications such as electron tomography or scanning transmission electron microscopy (STEM), thicker sections ranging from 200–1,000 nm can be used. Image contrast in TEM arises from differences in electron transmission through varying sample densities and atomic compositions. Heavy-metal staining enhances this contrast by increasing electron scattering for specific cellular components, revealing fine ultrastructural details at high spatial resolution.

In SEM, the electron beam interacts with a sample surface to generate two different signals: backscattered electrons (BSE), which are the result of the electron beam interacting with atomic nuclei close to the specimen surface, and secondary electrons (SE), which are produced by inelastic collisions of the electron beam causing the excitation of weakly bound sample electrons.

When SEM systems are used to analyze biological samples at room temperature, BSE are used to generate the images; the intensity of the signal is strongly influenced by the atomic number of the elements present. This is a result of electron–nucleus interactions increasing with higher atomic mass.

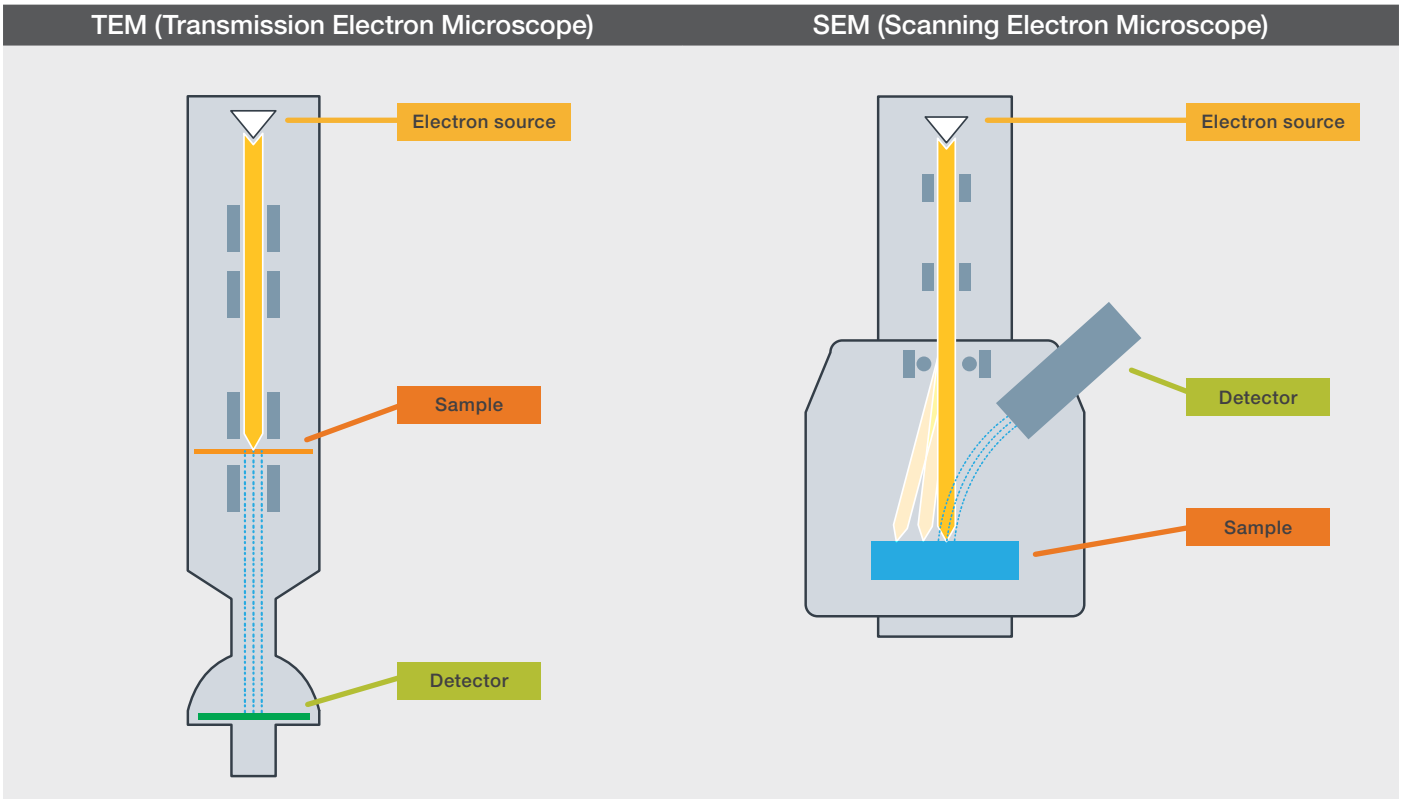

Figure 2. Illustration comparing the layout and signals obtained with TEM and SEM.

Practical considerations for sample preparation

- **Osmium concentration:** Optimal concentrations (typically 1–2%) are critical. Osmium concentrations that are too high precipitate at the tissue surface and hinder deeper penetration, while concentrations that are too low lead to poor membrane contrast and overall under-staining. Final parameters depend on the properties of the sample, particularly thickness.
- **Uranyl acetate:** Applied after osmium treatment, either as *en bloc* staining of resin-embedded samples or as post-section staining in TEM, to ensure effective uranium incorporation without interfering with the osmium crosslinking.
- **Sample-specific adjustments:** The protocol parameters can be fine-tuned for different tissue types and experimental requirements.

Moving from 2D to 3D Room-Temperature EM

The preceding section outlined how different preparation strategies support reliable EM imaging. Building on this foundation, 3D EM (or volume EM) extends conventional 2D approaches by reconstructing volumes from serial images, providing a 3D view of cellular structure. This reveals spatial relationships and depth-dependent features that cannot be captured in single thin sections. Because many processes, including membrane trafficking and organelle remodelling, occur in 3D, volume EM offers a more complete representation of cellular organization and supports quantitative analysis.

Application examples of 3D room-temperature EM in research

How volume EM builds 3D datasets

In order to build a 3D image, volume EM techniques acquire a series of 2D images through the sample and computationally reconstruct them into a volume. FIB-SEM does this by milling away nanometer-thin layers with an ion beam and imaging each newly exposed surface. SBFI uses an in-chamber ultramicrotome to cut successive slices from a resin block, imaging the block face after each cut. Array tomography collects ribbons of ultrathin serial sections that are individually imaged and digitally aligned into a stack. And in electron tomography, a series of 2D projection images of the same region is recorded at different tilt angles in the electron microscope and then computationally reconstructed into a 3D volume. These acquisition methods provide the basis for the diverse volumetric analyses illustrated in the following examples.

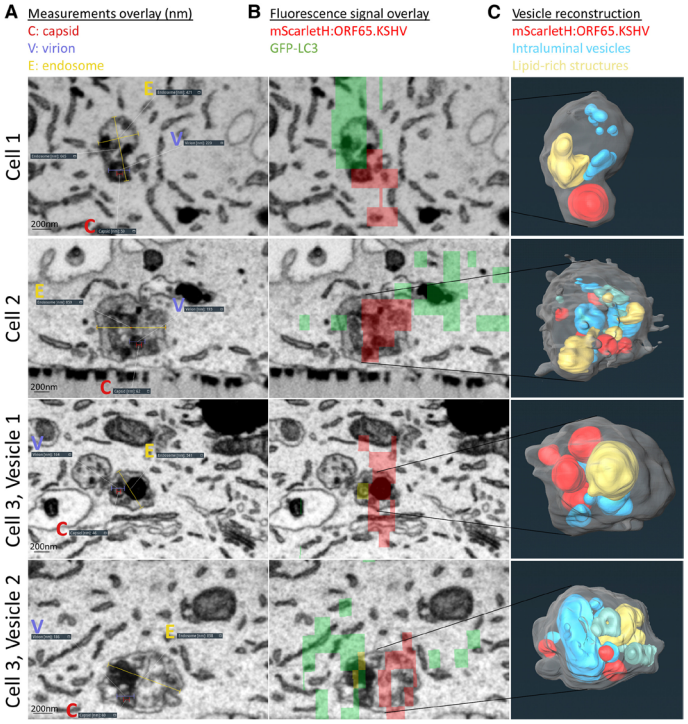

Figure 3. Ultrastructural images of KSHV-containing vesicles, along with the corresponding 3D reconstructions, used to reveal amphisomal structures. Figure reproduced from Schmidt *et al*. under CC BY 4.0.[4]

Correlative volume EM reveals endolysosomal membrane damage and autophagic restriction of KSHV entry

Researchers utilized volumetric FIB-SEM to investigate how entry of the Kaposi sarcoma-associated herpesvirus (KSHV) damages endolysosomal membranes, along with the corresponding physiological response, where the cell utilizes the damage sensor galectin-8, along with selective autophagy machinery, to restrict infection.[4] To visualize these rare entry events *in situ*, the authors used a correlative, volumetric microscopy approach that combines fluorescence microscopy with 3D FIB-SEM (vCLEM). Fluorescence identified virus-positive, galectin-8/LC3-labeled compartments, which were then isolated with FIB milling and analyzed via isotropic 3D SEM imaging. The 3D volumes revealed ultrastructural features of the virus-containing endosomes/amphisomes and their spatial relationships with autophagic and endolysosomal membranes (figure 3). These observations support a model in which membrane damage triggers selective autophagy in order to inhibit viral entry. The vCLEM approach provided the 3D nanometer-scale context needed for quantitative, ultrastructural insights into KSHV entry and inhibition.

3D FIB-SEM reveals synaptic nanoarchitecture in human dorsolateral prefrontal cortex

Postmortem, ultrastructural investigation of brain tissue could support a number of neurological studies, but the potential differences between *in-vivo* and *ex-vivo* samples may hamper correlation. A study by Glausier *et al*. utilized 3D FIB-SEM to generate isotropic, ultrafine (~5×5×5 nm) volumes of the human dorsolateral prefrontal cortex, creating a complete reconstruction of glutamatergic synapses and sub-synaptic organelles (i.e., PSD, active zone, spine apparatus, smooth ER, mitochondria) with excellent ultrastructural preservation.[5] The authors were able to compute a number of structure–structure relationships (e.g., bouton–active zone and spine–PSD) across sizeable neuropil. They found that their observations closely match benchmarks from *in-vivo* experimental models, indicating that *ex-vivo* EM provides relevant organizational insights; FIB-SEM offers the isotropic resolution, context, and measurement reliability necessary to substantiate such conclusions (figure 4).

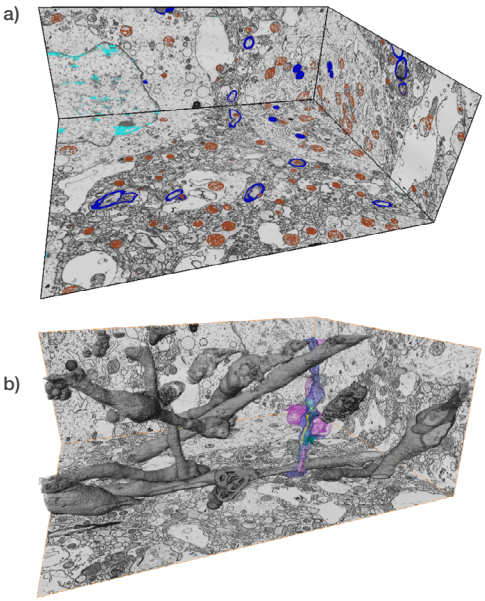

Figure 4. 3D volumetric data from post-mortem human brain tissue obtained with FIB-SEM. a) The data shows excellent ultrastructural preservation of the tissue, with segmentation identifying myelin in blue, mitochondria in red, and the nucleus in turquoise. b) A complex dendritic shaft with filopodia-like structures coursing through the dendritic cytoplasm was identified and segmented. Images courtesy of Dr. Jill Glausier and Dr. Zachary Freyberg, University of Pittsburgh. Full experimental details can be found in Glausier *et al.*[5]

Bone mineralization and osteopontin effects visualized with FIB-SEM

3D EM can provide new insight into the behavior of osteopontin (OPN), and how elevated OPN disrupts bone mineralization. Deering *et al.* used FIB-SEM to show how excess OPN stabilizes amorphous mineral phases and interferes with the normal coalescence of mineral foci.[6]

They were able to show that OPN-rich bone samples have mineral foci that remain small, irregular, and unmerged, preventing the formation of the typical space-filling, tessellated structures seen in healthy bone. The resulting high ratio of surface area to volume slowed mineral maturation and impaired the integration of bone ultrastructure (figure 5). These results underscore how 3D EM can link molecular regulation (OPN accumulation) to altered nanoscale architecture, providing mechanistic insights into bone diseases such as hypophosphatemic osteomalacia.

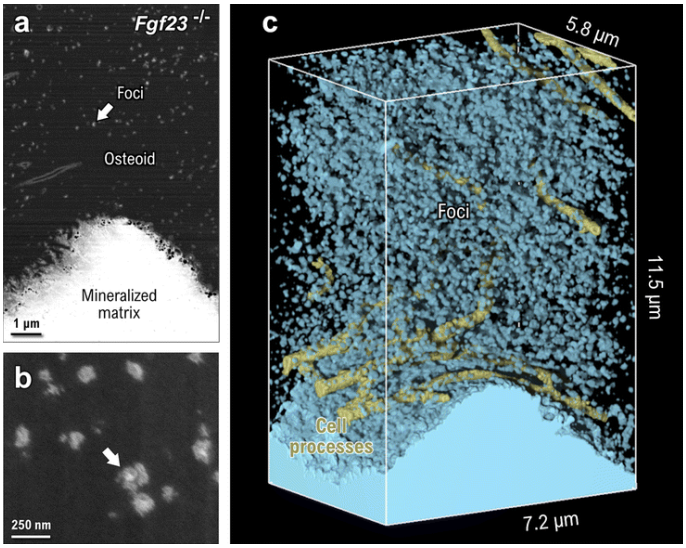

Figure 5. Core-shell mineral foci in FGF23-deficient bone mineralization. a) A single 2D slice from a FIB-SEM series, showing the high abundance of small mineral foci commonly seen with elevated OPN. b) Higher-magnification SEM imaging shows that the foci consist of a bright mineral interior with a surrounding diffuse mineral shell. c) Segmentation of the 3D FIB-SEM dataset shows the 3D distribution of the mineral foci. Figure reproduced from Deering *et al.* under CC BY 4.0.[6]

SBFI reveals cellular remodeling and gene function

Serial block-face imaging can be used to visualize the phenotypic effects of gene knockdown or overexpression, capturing the reshaping of organelles in high detail. Using this approach, Sheikh *et al.* demonstrated that a novel group of dynamin-related proteins, shared by eukaryotes and giant viruses, can remodel mitochondria from within the matrix.[7] This process was only detectable through high-resolution 3D reconstructions (figure 6).

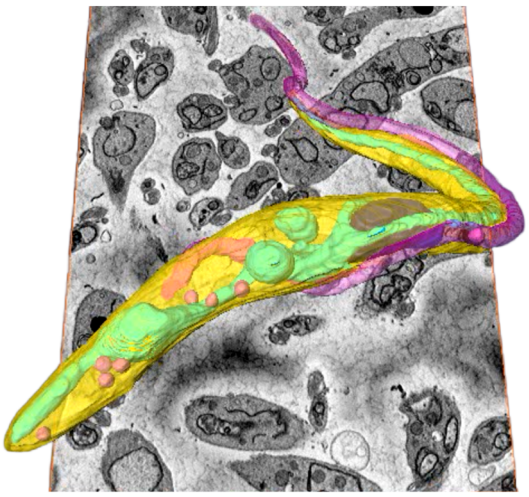

Figure 6. *Trypanosoma brucei* with altered mitochondria, caused by the overexpression of a viral protein. Image courtesy of Jiří Týč, Shaghayegh Sheikh, and Hassan Hashimi, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences. Full experimental details can be found in Sheikh *et al.*[7]

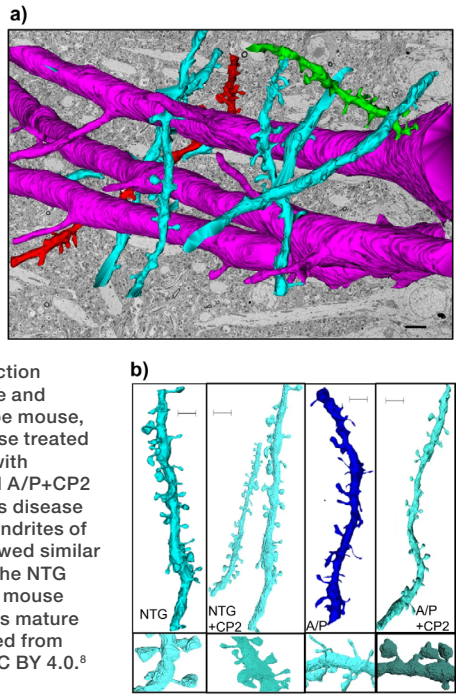

Figure 7. SBFI reconstruction of murine neuronal tissue and dendrites. NTG = wildtype mouse, NTG+CP2 = wiltype mouse treated with CP2, A/P = mouse with Alzheimer's disease, and A/P+CP2 = mouse with Alzheimer's disease treated with CP2. The dendrites of the A/P+CP2 mouse showed similar morphology to those of the NTG mouse, whereas the A/P mouse exhibited thinner and less mature spines. Figure reproduced from Stojakovic *et al.* under CC BY 4.0.[8]

Neuroscience and connectomics: drug effects on neuronal morphology revealed by 3D EM

SBFI can produce comprehensive 3D maps of dendritic spines, synaptic connections, and neuronal networks, enabling precise assessment of drug effects in neurodegenerative disease models.

For instance, Stojakovic *et al.* used SBFI to investigate how partial inhibition of mitochondrial complex I (using the drug CP2) influences neuronal morphology in a mouse model of Alzheimer's disease (AD).[8] The 3D reconstructions revealed that AD mice displayed thinner and less mature dendritic spines compared to the wildtype control. Remarkably, treatment with CP2 restored dendritic spine morphology in AD mice, making it comparable to that of the untreated wildtype mice. These findings illustrate how 3D EM can capture subtle morphological changes in neuronal structures (figure 7). When combined with light microscopy, histology, and behavioral experiments, SBFI can offer valuable insights into the efficacy of therapeutics against neurodegeneration.

Impact of hemodynamic load on the embryonic myocardium, revealed with FIBSEM

FIB-SEM can be leveraged to study structural changes that occur during embryonic development. Midgett *et al.* investigated how increased hemodynamic load during early embryonic development influences myocardial ultrastructure.[9] They demonstrated that elevated load leads to subtle but significant 3D rearrangements of myofibrils and mitochondria (figure 8). These ultrastructural alterations, undetectable in traditional 2D imaging, were quantified and directly correlated with molecular findings using 3D EM, ultimately linking biomechanical stress to developmental remodeling at the organelle level.

Summary

The sample preparation approaches outlined in this paper summarize the key factors that influence the suitability of biological specimens for room-temperature EM and how these considerations support both 2D and 3D imaging. These methods, as demonstrated in published studies, enable the acquisition of structurally preserved, high-contrast datasets for ultrastructural visualization and quantitative analysis combined with multimodal experiments, helping to correlate ultrastructure with function. The application examples illustrate how room-temperature EM can be used across different specimen types to examine cellular organization and morphology, providing a practical overview of its capabilities in biological research.

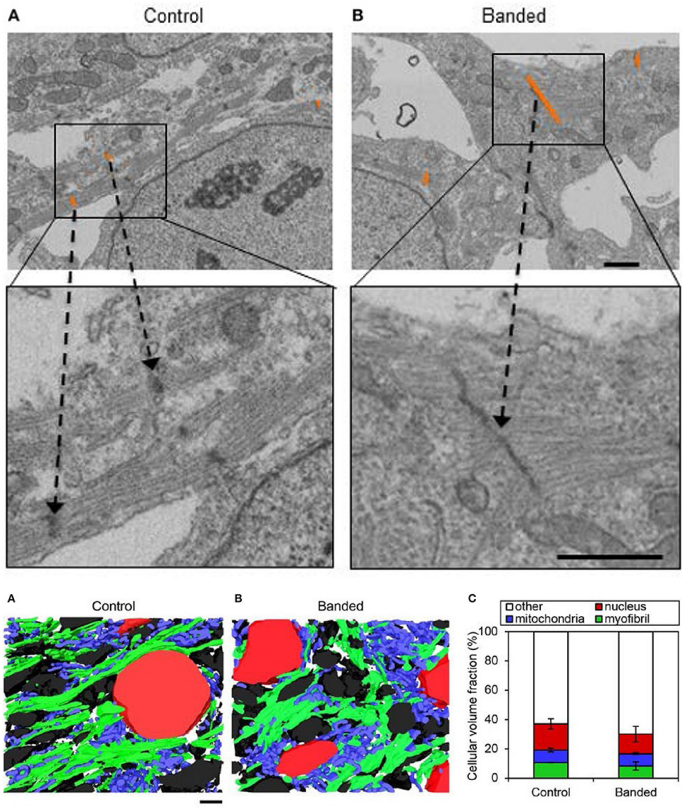

Figure 8. 3D FIB-SEM analysis shows how ultrastructural organization in myofibrils is affected by increased hemodynamic load in the embryonic myocardium. Reproduced from Midgett *et al.* under CC BY 4.0.[9]

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