by Emine Korkmaz

New Approach to 3-D Imaging of Biological Materials Provides Isotropic Resolution Down to a Few Nanometers

Visualizing the three-dimensional (3-D) architecture of cells and tissues is essential for understanding the relationships between structure and function in biological systems. Biologists have primarily used light microscopy (LM) to investigate biological structures and examine living samples. Importantly, many biological samples are nearly transparent to visible light, permitting relatively easy 3-D analysis. However, the resolving power of LM is fundamentally limited by the wavelength of visible light: generally it cannot resolve details smaller than a few hundred nanometers.

Scanning electron microscopy (SEM) can resolve details down to the nanometer scale, sufficient for many of the structures of interest in current biological investigations. Unfortunately, 3-D analysis is challenging because biological materials are generally not transparent to electrons and tissues require heavy metal staining for contrast, necessary for SEM imaging.

Recent developments in a technique known as serial block face imaging (SBFI) allow automated acquisition and reconstruction of high-resolution, large tissue volumes.

Scanning electron microscopy and serial block face imaging

A scanning electron microscope scans a finely focused electron beam in a raster pattern over the surface of a bulk specimen and creates a virtual image by correlating changes in various signals with the position of the beam in the raster. The most useful signal for 3-D imaging is the backscattered electron (BSE) signal, which reflects the average atomic number of the region illuminated by the beam. BSEs are beam electrons that are scattered back out of the sample surface by interactions with nuclei of sample atoms. They originate from a volume of interaction immediately below the beam, and the depth of that volume is determined by the energy of the beam. Biological electron microscopists have developed a number of staining techniques to enhance BSE contrast by selectively staining certain structures with heavy elements.

SEM image acquisition is a serial process, and requirements for resolution and signal-to-noise ratio (SNR) fundamentally determine the time required to acquire an image. Consequently, the strength of the signal and the efficiency are important considerations. The signal strength is determined mainly by the amount of beam current the sample can tolerate before damage occurs; biological samples, even when embedded, can be especially sensitive to beam damage. Therefore, the detection system needs to be optimized to collect low-loss BSE signal with high efficiency; this is best done using an in-lens detection geometry that generates images with strong contrast and high SNR.

Charging artifacts occur in SEM images when charge accumulates on nonconductive samples. Plastic-embedded biological samples can be very difficult to image. In routine imaging applications, conductive coatings may be applied to nonconductive samples to provide a path to ground that prevents charge accumulation. Coatings can be used in SBFI after a fresh surface is exposed with an in situ microtome; however, this increases

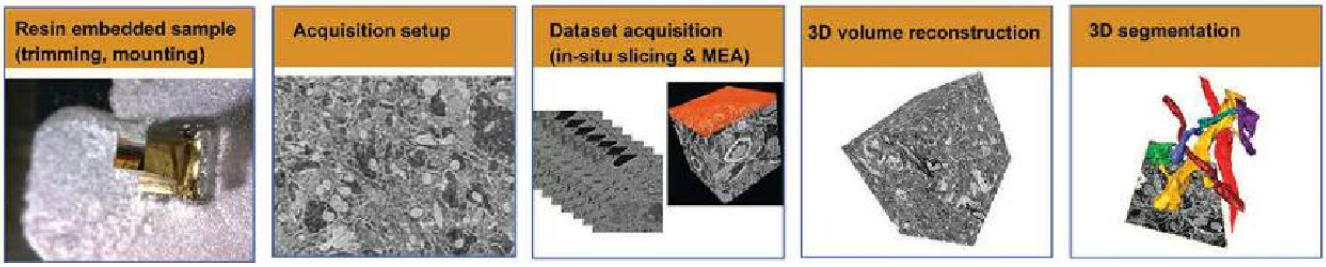


Figure 1 – The serial block face imaging workflow involves chemically fixed, heavy metal stained and resin-embedded biological sample block attached on an Al stub with conductive glue. Several regions of interest can be selected on the block face and imaged at high resolution with either individual fields of view or tile sets stitched together to form a large composite image. A layer of material is removed using a diamond knife, and the multi-energy acquisition (MEA) process is repeated, resulting in a stack of images that can be reconstructed by a computer into a high-resolution representation of the sectioned volume. Segmentation of the data can be used to isolate structures of interest.

the experimental time substantially. Low vacuum (LoVac) operation provides a means to eliminate charging that is compatible with SBFI. In LoVac mode, a gas, in this case water vapor, is injected into the sample chamber and maintained at a pressure low enough to permit the beam to reach the sample without excessive scattering. Some molecules of the gas are ionized by beam electrons or by electrons originating from the sample. These ionized gas molecules are then available to neutralize any charge that accumulates on the sample. LoVac operation is essential to ensure high-quality imaging of difficult samples.

SBFI generates a sequence of images of the sample surface as the sample volume is serially sectioned, each section removing a thin layer of material from the block face (see *Figure 1*). The resulting stack of images can be computationally reconstructed to visualize the original 3-D structure. In an SEM, the lateral resolution of the reconstruction can be as good as a few nanometers; however, the depth resolution is limited by the thickness of the slices removed from the surface. This can be quite thin, since the structural integrity of the removed

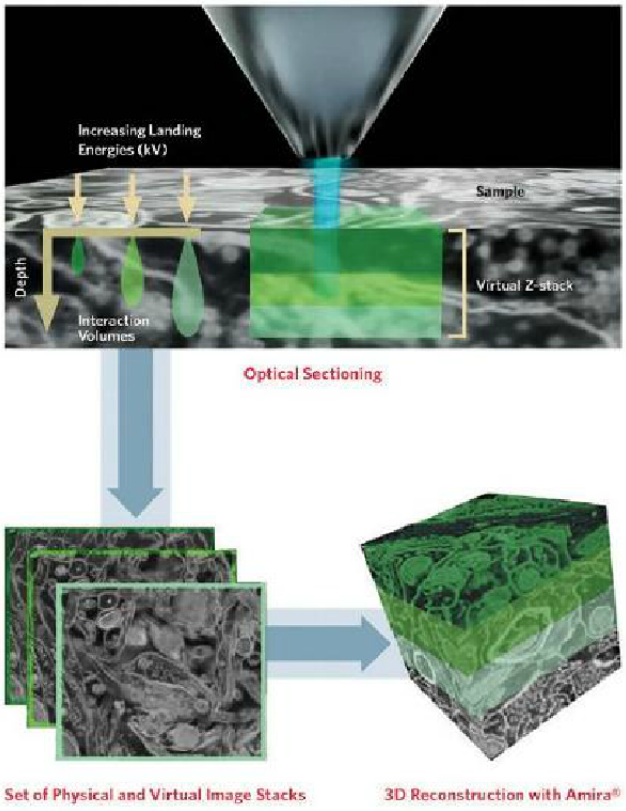


Figure 2 – Multi-energy acquisition captures several images at different beam energies (landing energies). Each image captures information from a different depth below the block face surface, within the next layer of material to be removed. A proprietary software algorithm deconvolutes the data to provide a subset of optical sections for each physical section. The final reconstructed model exhibits isotropic resolution down to a few nanometers.

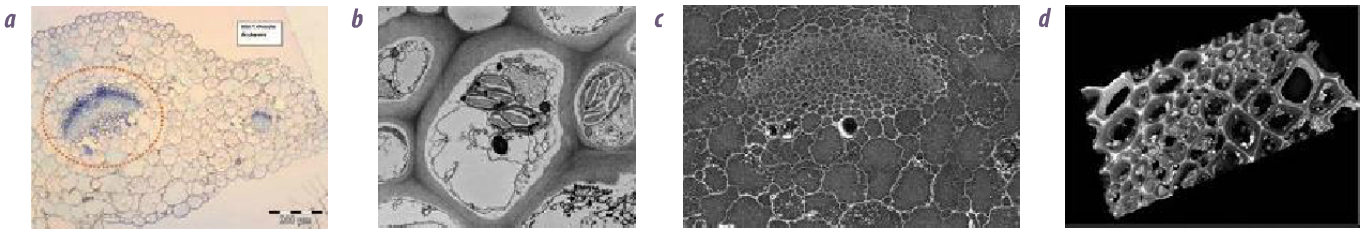


Figure 3 – Arabidopsis leaf. a) LM overview. b) SEM overview. c) SEM of block face. d) 3-D reconstruction.

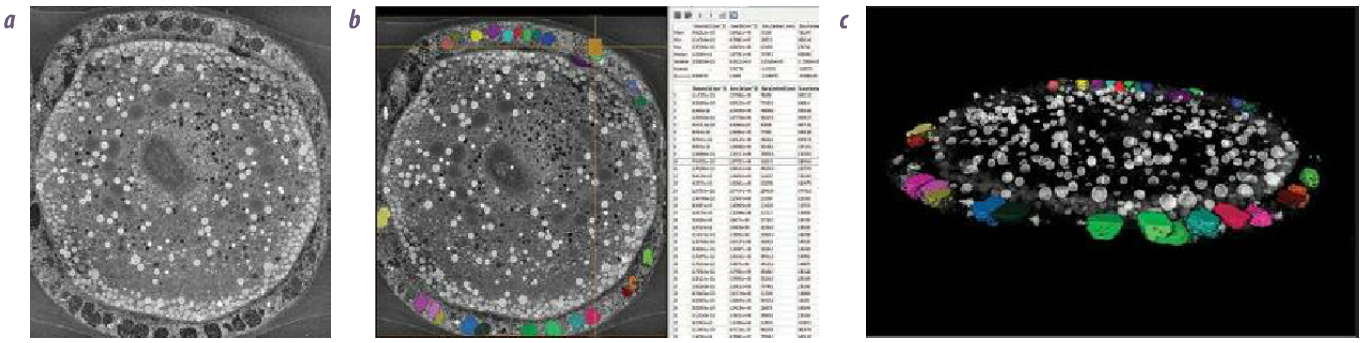


Figure 4 – Oocyte cavity volume measurement. a) A representative slice through the 3-D volume reconstruction. b) The reconstruction has been segmented (colors) to identify cavities and the volume of each cavity is tabulated. c) An oblique view of the 3-D model showing the segmented cavities.

layer need not be preserved, but it is still limited to 25–30 nm for mechanical sectioning techniques.

A new technique, multi-energy acquisition (MEA), addresses the depth resolution limitation of SBFI (see *Figure 2*). In an SEM, the depth to which the electron beam penetrates the sample is a function of the beam energy. MEA acquires multiple images of the block face at different energies, and then combines and deconvolutes them computationally to provide additional depth resolution within each of the mechanically sectioned layers, ultimately achieving resolution in all directions as low as a few nanometers, down to a few nanometers.

The Teneo VolumeScope SEM platform (FEI, Hillsboro, Ore.) tightly integrates SBFI and MEA while automating and streamlining the complete 3-D imaging workflow. The system can analyze large area/volume acquisitions by automatically tiling multiple fields of view into a large composite image of each block. It can also import and overlay images from light microscopes to allow direct targeting of regions of particular interest based on fluorescence staining or other LM techniques. An automated, in-chamber microtome can be easily removed to permit use of the instrument for other applications. A newly designed electron column and in-column detection system provide high-efficiency, high-contrast images of biological materials. The system may be operated in high-vacuum mode to achieve the highest resolution or low-vacuum mode with water vapor in the chamber to mitigate sample charging effects. Fully automated column alignments limit the need for operator training and predefined use cases for common conditions boost productivity for everyone. Amira visualization software (FEI) presents the 3-D reconstruction and allows the operator to segment the data to isolate structures of interest.

Application examples

SBFI permits unambiguous visualization of 3-D structures that are difficult or impossible to discern in 2-D images. It is particularly powerful for mapping complex, extended 3-D networks such as neurons in the brain. Once the data are reconstructed, the model can be virtually sectioned in any plane. Animations that show a virtual section is moving through the model provide a powerful and intuitive analytical tool. Segmentation of the data permits the analyst to isolate and highlight features of interest.

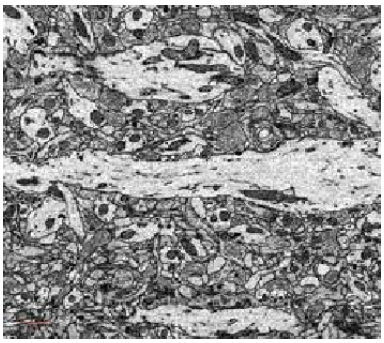
Cell walls in arabidopsis

Arabidopsis is a genus of small flowering plants. It is a popular model in plant biology and genetics. It was the first plant to have its genome sequenced, and is widely used for understanding the molecular biology of many plant traits. Advances in volume imaging permit direct visualization of the cell wall architecture, revealing modifications that occur during growth and development (see *Figure 3*). Understanding the structural and functional relationships of individual cell wall components requires better characterization of a wide range of structural and architectural alterations in cell walls, which could be a consequence of developmental regulation, environmental adaptation or possible genetic modification.

Oocyte cavity volume measurement

An oocyte is an immature egg cell destined to be fertilized and ultimately grow into a fully functioning organism. It must therefore be ready to regulate multiple cellular and developmental processes and supplied with numerous molecules that will direct the growth of the embryo and control cellular activities. *Figure 4* shows representative images from a 3-D analysis that measured the volumes of numerous cavities surrounding an oocyte.

a



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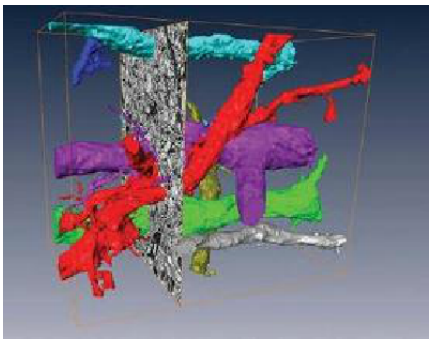

Figure 5 – Volume reconstruction of a mouse brain volume. a) A representative SEM image. b) The segmented 3-D reconstruction has isotropic resolution (x, y and z) of 10 nm. The physical sections were 50 nm thick. A total of 1040 sections, physical and optical, were used to reconstruct the 15.00 µm × 12.9 µm × 10.4 µm volume.

Mitochondrial network

Figure 5 shows the reconstruction of a complex mitochondrial network within neurons of mouse brain. Mitochondria are highly dynamic organelles that have central importance for ATP production and undergo fusion and fission events continuously, leading to a diverse array of mitochondrial morphologies that range from fragmented states to continuous networks. Three-dimensional volume imaging allows reconstruction, visualization and analysis of these highly complex organelles within the context of the biological tissue under investigation.

Conclusion

A new approach to 3-D volume imaging of biological samples combines mechanical sectioning with optical sectioning using multi-energy deconvolution to provide isotropic resolution. Optimized detectors and low-vacuum operation ensure high-quality imaging. Software provides automation of processes ranging from low-level setup and alignment to walkaway acquisition of a complete image series. The approach includes a complete workflow from initial setup to final results, including software solutions for large area/volume analysis, LM image overlay, reconstruction, visualization and segmentation.

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