Workflow-Centric Cryo-SEM for Biological Research

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Introduction

Biologists using electron microscopy to understand the form and function of biological systems at the nanometer scale have long been confronted by a number of difficulties related to fundamental characteristics shared by most biological samples. First is the complexity and range of scales at which significant information is present, from the mesoscopic level in tissues, through the microscopic level of cells and organelles, to the nanoscopic level of macromolecules. Next is the hydrated state of most biological materials, in which water is essential to maintaining functional structure, but, at the same time, is incompatible with the vacuum environment of the scanning electron microscope (SEM). Finally, because of their fragile nature, biological structures are vulnerable to damage, by the electron beam itself or by procedures used to prepare them for SEM analysis. A new cryogenic scanning electron microscope (the Magellan cryo-SEM) workflow solution from FEI addresses all of these issues [1–6]. It is an integrated workflow from sample preparation to sub-nanometer imaging that addresses the specific needs of biological research.

Scale and Complexity of the Problem

One of the great challenges posed by biological systems is their complexity and the tremendous range of scales and modalities over which meaningful information exists. At the nanometer scale, atoms are organized into molecules and macromolecules, and these, in turn, into membranes, filaments, liposomes, ribosomes, chromosomes, capsids, and a myriad of other functional structures. At the micrometer scale, these are organized into the classic cellular components and organelles that were first revealed by light microscopy. Above them, at tens or hundreds of micrometers, are cells and tissues and whole organisms. The biologist must interrogate detail at the finest spatial scale, over the widest possible field, in order to correlate information and navigate the complexity across the full range of size scales.

Instrumental Requirements

Resolution. An SEM scans a finely focused beam of electrons over the sample surface and measures the strength of various signals created by interactions between beam electrons and sample atoms at each point in the scanned area. These values are then mapped into an image where the grayscale value at any point represents the signal level at the corresponding point in the sample. Resolution in an SEM is dominated by the size of the interaction volume—the region at, around, and just below the beam spot from which the imaged signal originates at any instant in time (Figure 1). The type of signal, the size of the spot (beam diameter), and the distance and direction beam electrons travel within the sample (beam penetration) determine the achievable image resolution.

Beam penetration generally degrades image resolution, its effects increasing at higher beam energies and in specimens with lower atomic number composition (which includes most biological specimens). In conventional SEMs, decreasing beam energy to reduce penetration is prohibited by the countervailing effect of chromatic aberration, which increases beam diameter. The extent to which chromatic aberration degrades the final spot size depends on the ratio of the energy spread within the beam to the accelerating voltage, becoming worse as the accelerating voltage decreases. The new cryo-SEM uses a field emission source with an inherently low energy spread and then selects a reduced range of electron energies to form the beam, allowing sub-nanometer beam diameters at accelerating voltages as low as 1 kV. The ability to achieve small spot sizes at low accelerating voltages permits dramatic reductions in the size of the interaction volume (Figure 2) and unprecedented image resolution on light element samples.

Beam penetration can be further reduced by decelerating beam electrons with an opposing electric field at the sample. Beam deceleration can achieve landing energies as low as 50 eV. As an added benefit, the deceleration field serves as an electrostatic lens that further reduces spot size by as much as 50%.

Contrast and noise. Resolution is not the only requirement for high-quality imaging. Good contrast and low noise are also important so that the desired image features

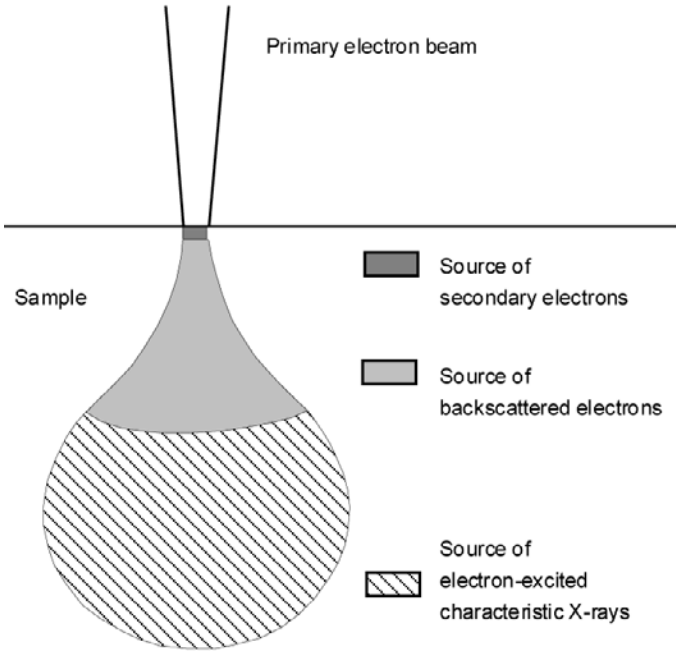

Figure 1: The size and shape of the interaction volume, within which imaging signals are generated by interactions between beam electrons and sample atoms, determines the resolution achievable in the image. The size and shape of the volume depends on many factors, including the signal type, beam diameter, accelerating voltage, sample composition, and more.

https://doi.org/10.1017/S1551929411001743X Published online by Cambridge University Press

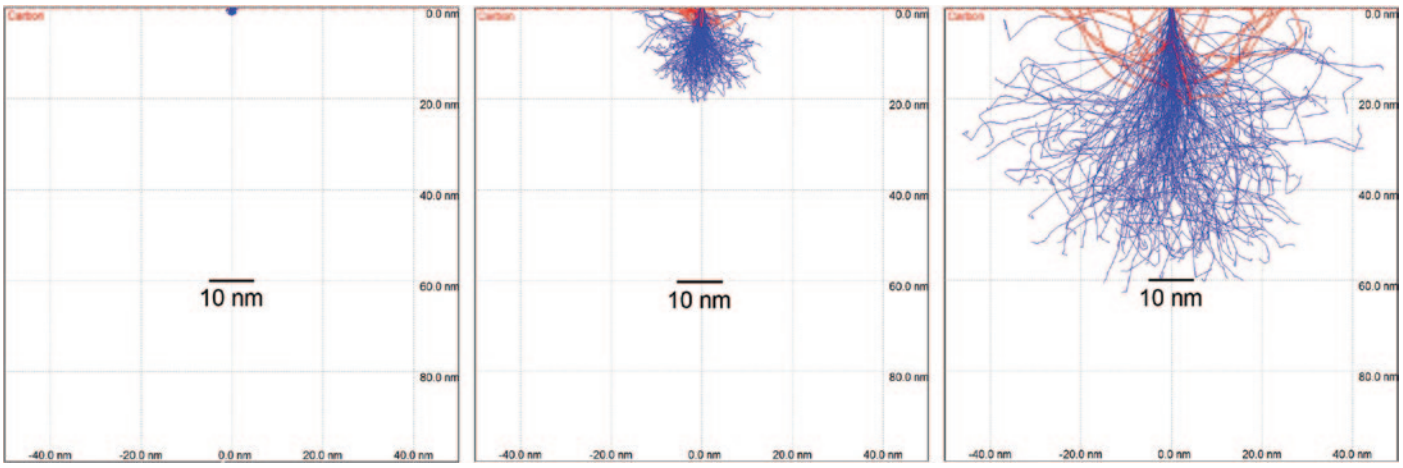

Figure 2: Monte Carlo simulations showing beam penetration in carbon at (left to right) 200 eV, 1 keV, and 2 keV. The red tracks are backscattered electrons, which may also create type II secondary electrons as they exit the surface at locations quite distant from the beam. Simulations were performed using Casino v2.42, Université de Sherbrooke, Québec, Canada.

rise above the noise. As data for an image accumulates over time, contrast (meaningful changes in signal strength related to point-to-point differences in the sample) grows, while noise (meaningless random signal variations) averages out. High signal levels and high signal-to-noise ratios are difficult to achieve in many biological specimens. Perhaps the most important instrumental capability in this regard is a high, stable beam current. The amount of current that can be focused into a spot of a given size is determined by the brightness of the source (current density per solid angle). Field emission sources are the brightest. They come in two flavors, cold field emission and Schottky field emission. Until now, cold field emission sources have offered the highest brightness and lowest energy spread, but they have suffered from instabilities related to contamination and require periodic cleaning procedures (flashing) to restore performance. The FEI cryo-SEM uses a Schottky source that achieves high brightness, low-energy spread, and stable current without flashing.

Wide field of view. As mentioned previously, one of the difficulties facing biologists is the tremendous range of spatial scales at which important information exists in their specimens: from millimeters to nanometers, a range of a million times. It is now possible to automatically combine data from multiple images into a single large-scale image that still preserves the full resolution detail of the original single images. Figure 3 is a 7 × 7 composite image (23,598 × 18,299 pixels covering 23.6 × 18.3 micrometers). If expanded to make individual pixels just visible to the unaided eye (0.2 mm/pixel, typical computer display resolution), the overall dimensions of the image would be 4.7 m × 3.6 m, and the viewer would be able to see the sub-5-nm bilayer structure of membranes throughout the image. Of course, handling a physical image of that size would be cumbersome, so new acquisition and visualization software (MAPS) facilitates navigation through the image, presenting smaller sections at full resolution.

Correlative navigation. Another consideration related to the range of spatial scales is the difficulty in locating specific

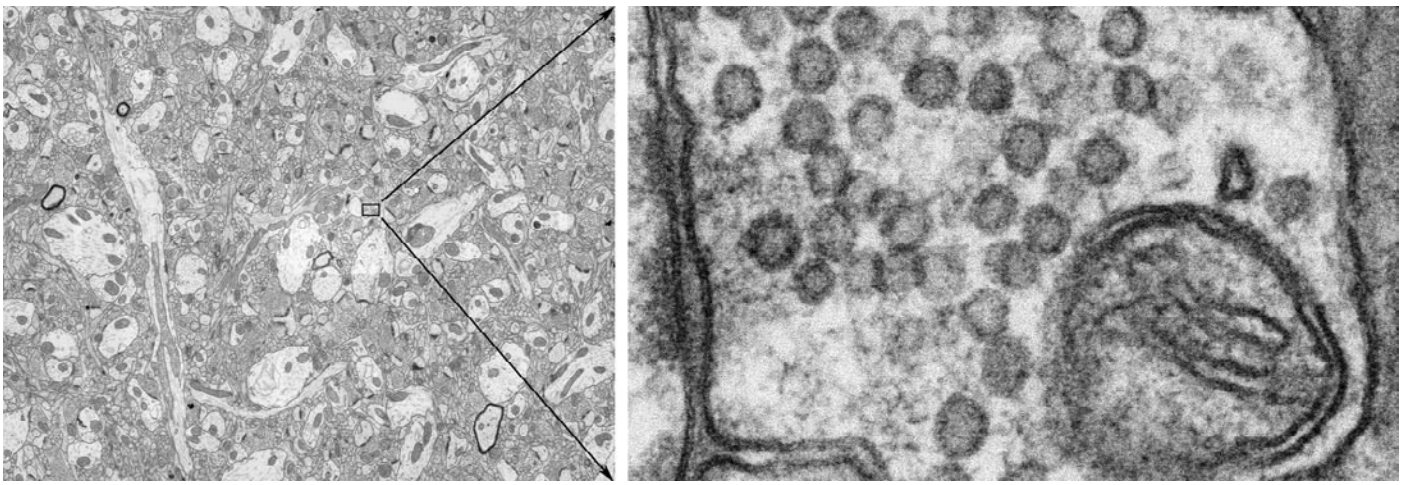

Figure 3: (left) Large-field high-resolution image of adult mouse cerebellum created by stitching together 49 images (7 × 7) resulting in a stitched image with HFW of 23.6 micrometers. (right) Area of interest is shown at higher magnification where the 5 nm bilayer structure of the membranes is resolved. The image was acquired at 1 nm per pixel. Sample courtesy of Graham Knott, EPFL, Lausanne, Switzerland.

structures, particularly when they may have been identified by other imaging techniques. For example, fluorescent tags can be used in light microscopy to immunologically identify and locate specific molecular structures. Correlative microscopy software permits the use of image data from complementary techniques to navigate to a specific location on the specimen for high-resolution imaging in the electron microscope (Figure 4). Correlation of light microscopy and electron microscopy accelerates the workflow, allowing the researcher to focus on analysis of the data rather than the tedious task of navigation and looking for the biological region of interest.

Fully hydrated samples. As mentioned above, water is a major component of most biological specimens but, in its liquid state, it is not compatible with the vacuum conditions of a typical electron microscope. However, techniques used to remove water for conventional SEM often distort delicate biological structures where the water itself plays a crucial structural role. Cryo-microscopy addresses this issue by freezing the water in place. Depending on the size of the specimen and the structures of interest, it may be possible to freeze the sample so rapidly that the water solidifies without crystallizing. This process, known as vitrification, avoids the microscopic damage caused by volume expansion of ice crystals and preserves all of the structural detail present in the fully hydrated natural state. The new cryo-SEM system includes fully integrated cryo-handling capabilities. Figure 5 shows a yeast cell prepared by freeze fracture after high-pressure freezing.

Maximizing Information from Delicate Biological Materials

Most biological materials are vulnerable to damage by high-energy electron beams. Conventionally prepared samples (dehydrated, stained, and embedded) tend to be more robust under the beam, and staining provides stronger contrast with lower doses. However, such preparation procedures introduce perturbations to cell morphology and composition. Cryo-prepared samples avoid many of these preparation artifacts but are more vulnerable to electron beam damage. In either case, the biological microscopist is often confronted with a race to extract meaningful information from the sample before significant damage occurs. In this race, the efficiency with which signals are detected may be the difference between success and failure. The term "dose" quantifies the total number of beam electrons incident on the sample per unit area. Low-dose techniques seek to extract maximum information with minimum exposure to the beam. Doses below 2,000 electrons per square nanometer are generally considered to be in the low-dose range. The higher the detector efficiency, the lower the dose required to achieve the same image contrast and signal-to-noise ratio.

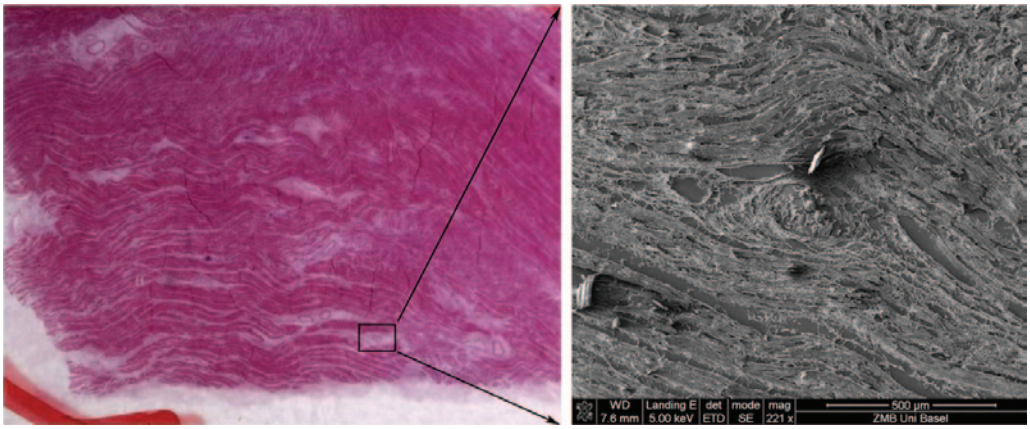

Figure 4: (left) Low-magnification external image of a histology sample taken with a light microscope and used to facilitate navigation within the SEM, and (right) corresponding region of interest under higher magnification in the SEM. Courtesy of Markus Dürrenberger, Center of Microscopy of the University of Basel, Switzerland.

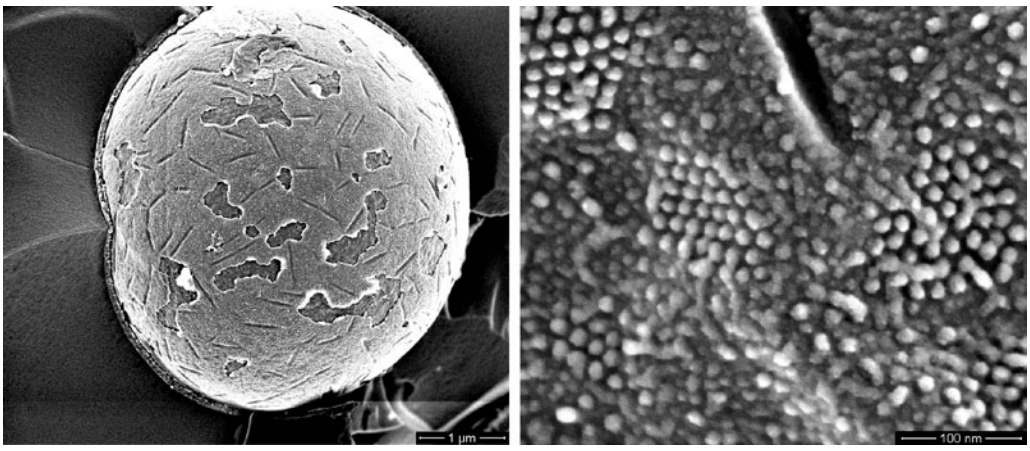

Figure 5: (left) Yeast cell prepared by freeze fracture. (right) Higher magnification of the yeast cell showing the transmembrane particles. Courtesy of Adriaan van Aelst, Wageningen Electron Microscopy Centre, Wageningen University, The Netherlands.

Electron signals. Secondary electrons (SE) and backscattered electrons (BSE) are the two most important SEM imaging signals. Secondary electrons are emitted from sample atoms after interactions with beam electrons. Because they have very low energies—a few eV—SEs can escape from the sample only if they are created very near the surface. Ideally, SEs originating from the first few atomic layers directly beneath the beam spot (Type I SEs) should provide the highest image resolution. However, other SEs generated by backscattered electrons (for example, Type II SEs), collected along with the Type I SEs, carry much poorer resolution

information. Contrast in the SE signal is primarily attributable to topographic features of the sample surface; this has the added advantage of making most SE images easy to interpret.

Backscattered electrons are incident beam electrons that have been scattered out of the sample by the nuclei of sample atoms. They may have energies up to the beam energy and thus can travel relatively large distances through the sample, giving them a large interaction volume. Thus, BSEs produce images of poorer resolution than Type I SE electrons. Backscattered electrons also indirectly degrade the resolution of the SE signal when they create SEs as they exit the sample surface at locations away from the beam spot (Type II SEs).

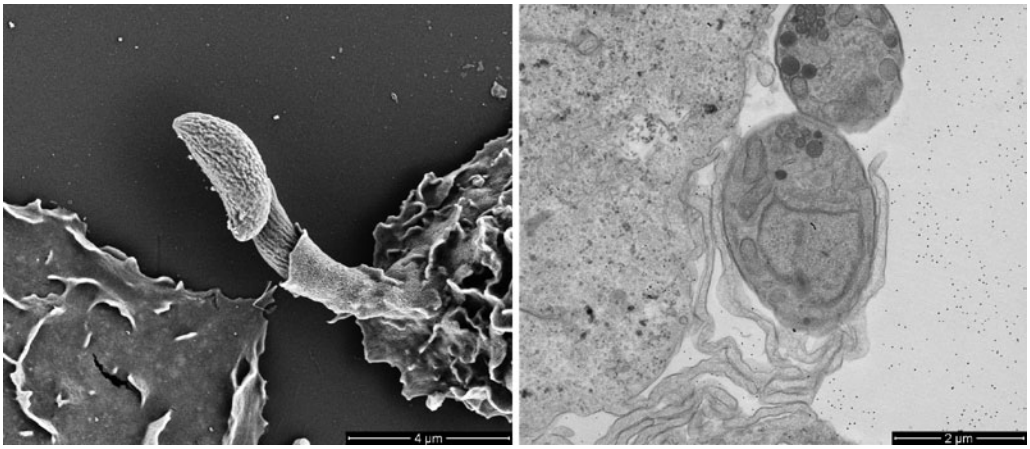

Figure 6: (left) Two tachyzoite forms of the protozoan *Toxoplasma gondii*. One of them is being engulfed by a mammalian cell. (right) Two tachyzoite forms of the protozoan *Toxoplasma gondii* imaged with the STEM detector. Filopodia from a mammalian cell surround one of them, indicating interaction. Sample courtesy of Marcia Attias, Universidade Federal de Rio de Janeiro, Brazil.

Electron signals from surfaces. The Magellan cryo-SEM's through-the-lens (TTL) SE electron detector uses the final lens field to optimize collection of low-energy secondary electrons (Figure 6a). At the very low beam energies used in the Magellan cryo-SEM, the interaction volume approaches the beam size, and therefore, the achievable image resolution using BSEs approaches the resolution obtained from using SEs. At low-beam energies, BSEs also acquire greater sensitivity to topographic changes in the specimen surface. Moreover, by selecting the BSE signal based on backscatter angle, it is possible to vary the relative contribution of topographic and atomic number contrast mechanisms, giving the microscopist an additional tool for enhancing the visualization of specific features. Most conventional BSE detectors were designed to detect high-energy BSEs, and their efficiency drops as the electron energy is decreased. The cryo-SEM system incorporates a specially designed directional backscatter (DBS) detector that maintains detection efficiency for low-energy BSE and provides the capability of selecting the signal based on backscatter angle.

Beam deceleration provides another tool for image acquisition and enhancement. It allows extremely low landing energies that minimize beam penetration. It also influences the trajectories of escaping signal electrons, directing them away from the sample. This capability can be used to enhance BSE detection efficiency by increasing the number of electrons that reach the detector with sufficient energy to be detected and hence lowers the dose required to generate the micrograph.

STEM operation. Another imaging mode useful for biological samples is scanning transmission electron microscopy (STEM), which detects beam electrons transmitted through a thin sample (Figures 6b). An important characteristic of STEM imaging is its ability to maintain small beam sizes and small interaction volumes, allowing good image resolution even at 30 kV accelerating voltages. The small beam size is produced by placing the thin specimen near the final lens where beam aberrations are reduced. This small beam is maintained as the beam traverses the thin specimen because most of the interaction volume has been removed. Although the thin samples used for STEM increase the sample preparation burden, they provide information similar to that obtained with the TEM at modest magnifications. Similar to the backscatter detector, the STEM detector is optimized for contrast with minimum dose.

Conclusion

The Magellan cryo-SEM addresses a number of important challenges posed by the common nature of many biological specimens. It provides high resolution at low beam energies on biological samples. Composite imaging provides large field imaging with full resolution. The correlative workflow approach permits fast accurate location of features identified with other imaging techniques. Cryo sample handling preserves hydrated samples in their natural state. High detector efficiencies extract the maximum information from the sample with minimum exposure to the beam in all imaging modes: SE, BSE, and STEM. Beam deceleration and directional BSE detection provide new mechanisms for enhancing image contrast.

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