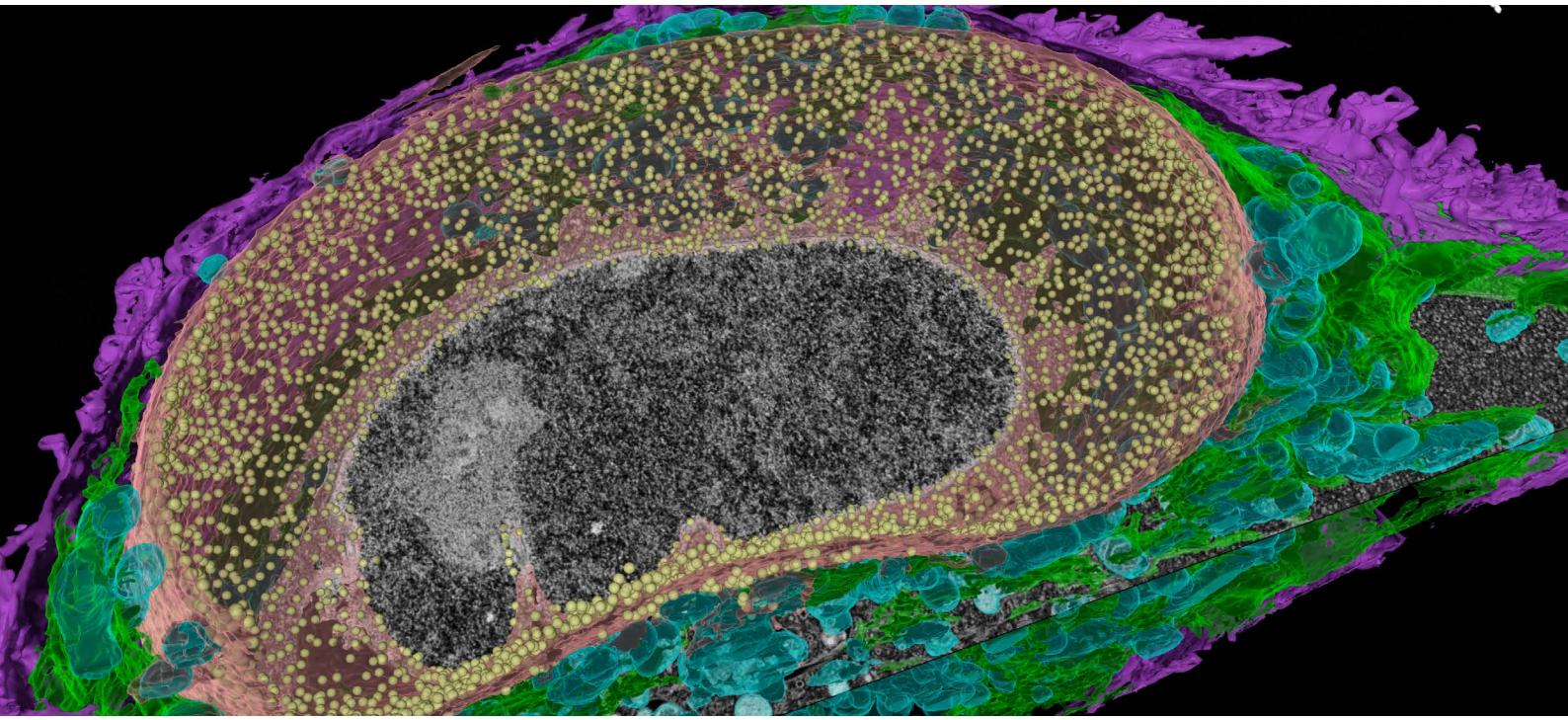
White Paper

Fundamentals of Scanning Electron Microscopy in Life Science Research

An Introduction to SEM and the Versatile SEM Techniques Available for Biological Specimens



Dataset kindly provided by Anna Steyer and Yannick Schwab, EMBL Heidelberg, Germany.

ZEISS

Seeing beyond

Fundamentals of Scanning Electron Microscopy in Life Science Research

Introduction

The scanning electron microscope (SEM) is a powerful tool that uses high energy electrons, instead of visible light, to methodically scan the surface of a specimen to form a magnified, high resolution image. While magnification is a vital part of microscopy, resolution, the smallest distance at which two points can be seen as distinct entities, is of equal importance. Because the SEM relies on electrons to form the image, as opposed to light rays with much longer wavelengths, the resolving power of the electron microscope is approximately 100 times greater than a standard light microscope. Magnifications up to 1,000,000x and 0.3 – 1.0 nanometers (nm) resolution are possible with an SEM. This level of high spatial resolution enables segmentation of ultrastructural information, revealing unrivaled levels of detail in life science specimens.

Many biological applications benefit from this amount of detail, including neuroscience, cell and cancer biology, developmental and structural biology, plant science, and many more. While the SEM provides a wealth of information about biological surface structures (Figure 1), this powerful technology can also be used for high resolution imaging of 2D ultrastructure across large fields of view (Figure 2). 3D structural imaging is possible using an array of sectioning and reconstruction techniques. Serial block face scanning electron microscopy (SBFSEM), array tomography and focused ion beam scanning electron microscopy (FIBSEM) allow you to take your high resolution imaging to a whole new dimension.

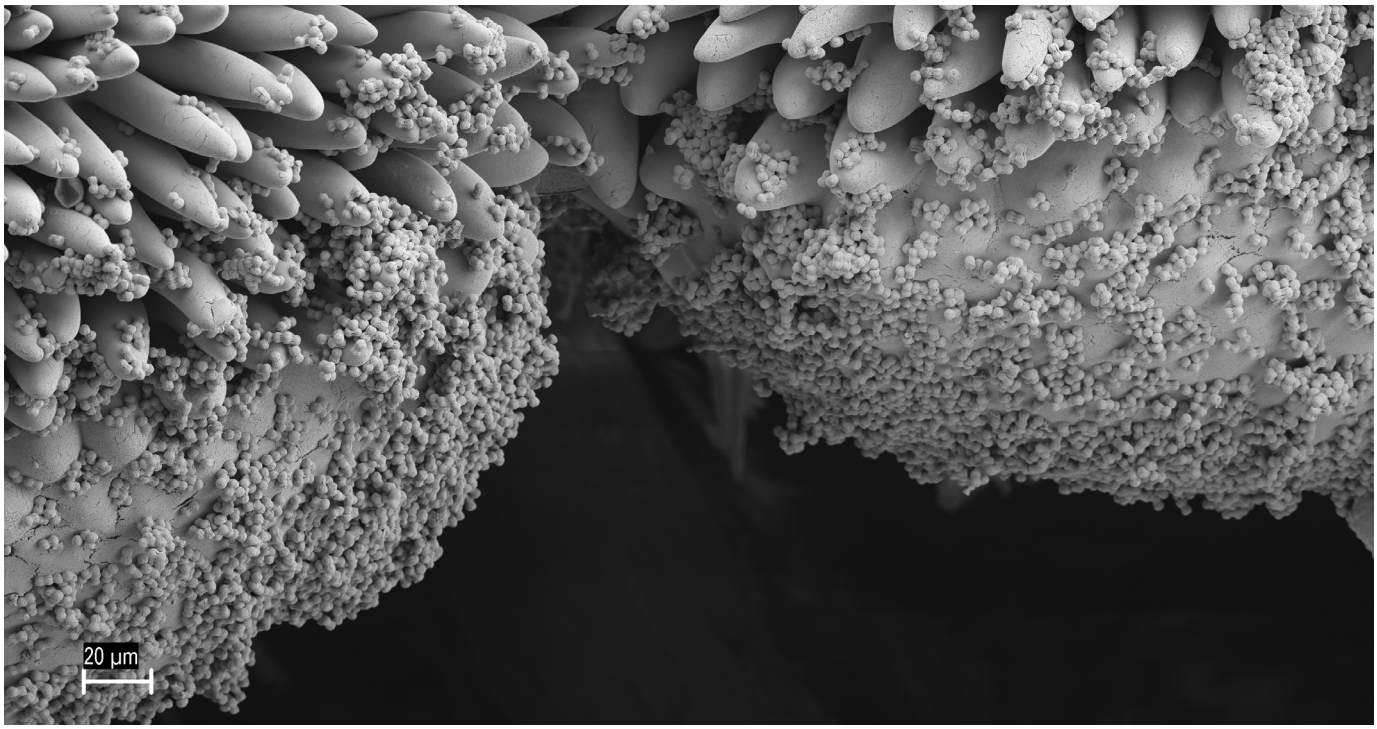


Figure 1: Forget-me-not flower pollen affixed to a petal. Imaged at 5kV accelerating voltage with ZEISS Sigma FESEM.

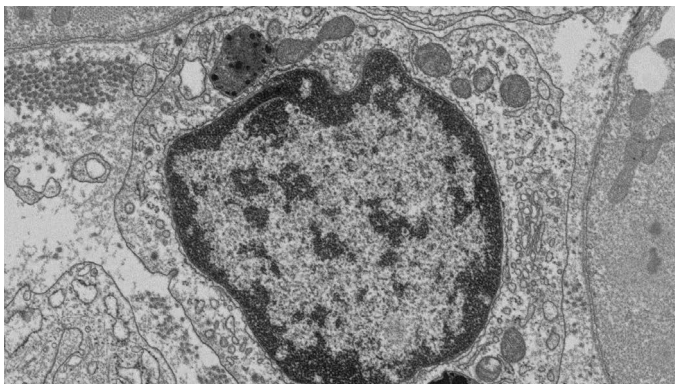


Figure 2 (left): Chemically fixed and stained mouse muscle tissue, imaged with ZEISS GeminiSEM and ZEISS Sense BSD (2 kV, pixel size: 20 nm). Bundles of muscle fibers and individual myofibrils with myofilaments as well as cellular compartments inside the cells are visible.

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The SEM can be tailored depending on your unique research goals and requirements. Used in combination with a variety of detector types, various types of imaging and analysis are possible. The SEM can be combined with an energy dispersive spectrometer (EDS) to reveal compositional information, with a backscattered electron detector (BSD) for compositional contrast, or with a secondary electron detector for topographical imaging. SEM data can even be correlated with other types of microscopy modalities, such as a laser scanning microscope (LSM) (Figure 3), in order to link cellular structure and function. Even the most delicate biological specimens can

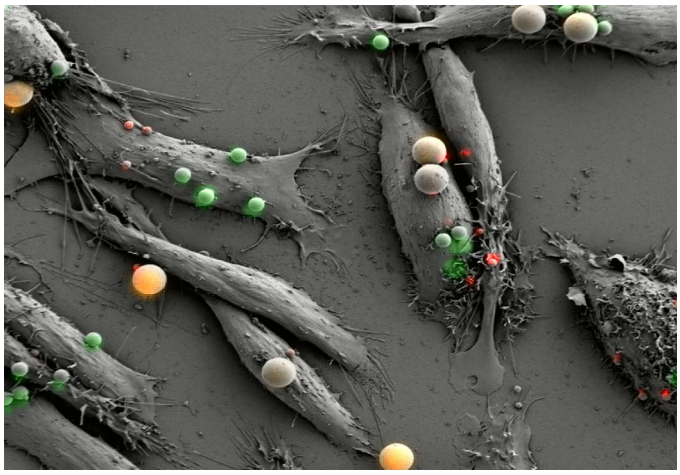

Figure 3: Confocal correlative microscopy with LSM 710 and AURIGA 60 FIBSEM; Fluorescence signals of the beads (2, 3, 5µm) in confocal LSM detection is overlaid with SEM imaging. Imaging by ZEISS Microscopy Labs, Munich, Germany. Sample courtesy of Jeffrey L. Caplan and Kirk J. Czymmek, Bioimaging Center, Delaware Biotechnology Institute.

be imaged with an SEM through the use of variable pressure (VP) vacuum modes or cryogenic imaging.

How does an SEM Work?

The SEM works by scanning a focused beam of high energy electrons, produced by the electron gun, across the surface of a sample, which is positioned on the specimen stage directly below the final lens. Electromagnetic lenses and apertures focus the electron beam from the gun, through the column and onto the sample. Scanning coils, controlled by a scan generator, deflect the electron beam systematically across a given area of the specimen. As the electron beam interacts with the surface of the specimen, various types of signals are produced as a result of this interaction, e.g., secondary and backscattered electrons, X-rays, heat and light. Some of these signals are collected by various types of detectors, processed, and translated to a series of pixels on a display monitor which is scanned in accordance with the specimen. The specimen is scanned in a raster pattern, point to point and line by line, synchronized with the scanning pattern of the display monitor. Each point where the beam strikes the specimen and generates a signal, a corresponding pixel is displayed on the monitor, thereby building the image line by line (reference 1).

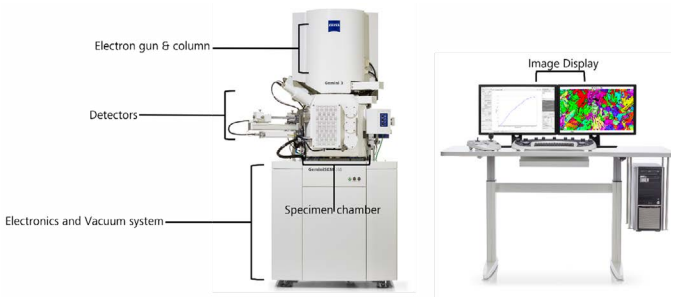

Figure 4: The main components of a standard scanning electron microscope consist of the electron gun, electromagnetic lenses and apertures within the column, electronics and vacuum system, the specimen chamber, the image display and the control panel.

Main components of a standard SEM

The electron gun and column

The electron gun is located at the top of the SEM column and is responsible for producing a stable beam of electrons of adjustable energy. There are two main types of electron guns: thermionic emission guns and field emission guns.

Thermionic emission guns are used in conventional scanning electron microscopes. Electron generation in thermionic emission guns is heat-induced and the gun has three main components: a filament, Wehnelt cap and anode. The filament tip is the source of the electrons, it is composed of tungsten or Lanthanum Hexaboride (LaB_6). By heating the filament, the outer orbital electrons gain sufficient energy to overcome the work function barrier and escape. The emission is localized by surrounding the filament with a negatively biased Wehnelt cap. The voltage potential between the filament and the anode plate accelerates the electrons down the column and is known as the accelerating voltage.

Field emission guns are used in field emission scanning electron microscopes (FESEM). Electron generation is induced by an electrostatic field which yields higher brightness and lower energy spread of the beam compared to thermionic emission guns, making it ideal for very high resolution imaging (reference 2). There are two types of field emission guns: thermally assisted or Schottky field emitters and cold field emitters.

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Lenses and apertures

A series of electromagnetic lenses and apertures are located in the column, directly below the electron gun. They are used to reduce the diameter of the beam produced by the gun and to place a small, focused beam of electrons (spot) onto the specimen. Small spot sizes are essential for high resolution imaging. The lens system consists of condenser and objective lenses, apertures and scanning coils. Optical focusing in light microscopes occurs with a glass lens when the air-glass interface causes refraction of light rays and the spherical shape of the lens leads to focusing. In an electromagnetic lens, however, current passes through a wound copper coil, producing a magnetic field inside the lens. This forces electrons closer together. The magnetic field bends electron paths in a similar way that solid glass lenses bend light rays.

In a light microscope, glass lenses have a fixed focal length. Focusing is performed by moving the specimen into the proper plane of focus, by moving the stage up and down, for each objective lens. In the electron microscope, however, electromagnetic lenses have variable focal lengths. The focus is adjusted by varying the current running through the lens. The specimen does not move and lenses are not physically changed (reference 3).

Beam-Specimen Interaction

When the electron beam scans the sample surface, signals are created in an interaction volume within the specimen (reference 5). The interaction volume is the volume inside of the sample in which electrons from the primary electron beam spread out and interact with the atoms of the sample (figure 5).

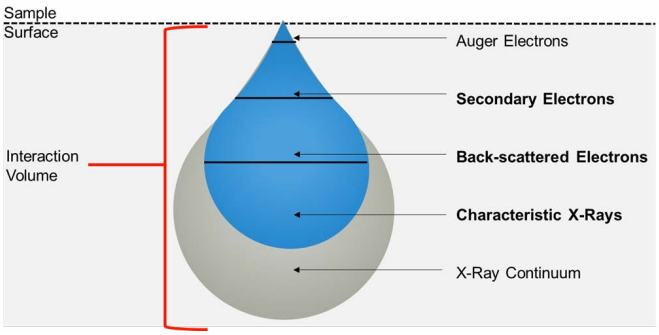

Figure 5: When the primary electron beam scans the specimen surface, signals are created in an interaction volume. These signals are collected and recorded by specific detectors to generate the image of the specimen.

The size of the interaction volume is affected by several factors: the energy of the primary beam, the atomic number of the specimen and the diameter of the primary beam. The dimension of the interaction volume will decrease with higher atomic number elements because higher atomic number materials absorb or stop more electrons and so have a smaller interaction volume. The dimension of the interaction volume will increase with accelerating voltage as higher voltages penetrate deeper into the sample and therefore generate larger interaction volumes (figure 6).

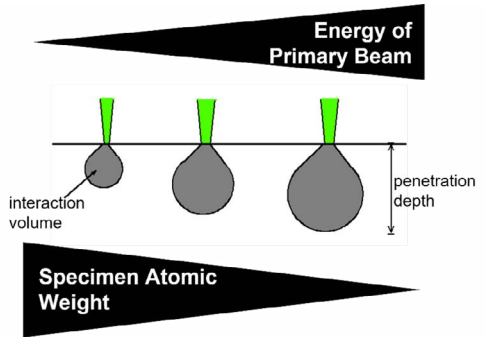

Figure 6: The size of the interaction volume is affected by the energy of the primary beam as well as the specimen atomic weight.

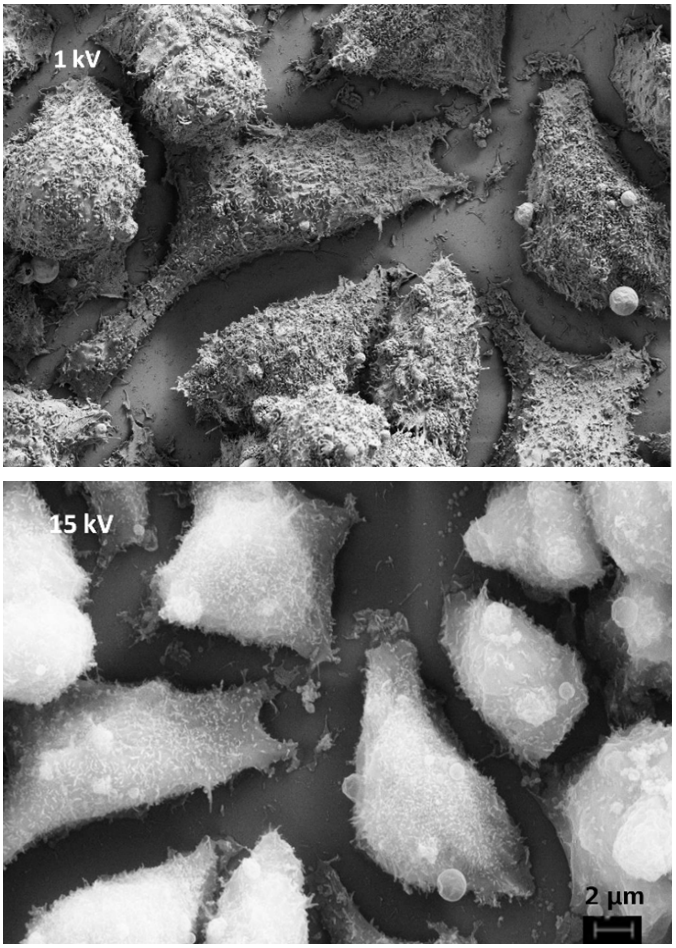

Figure 7: Mouse intestinal cells imaged with a ZEISS FESEM at low accelerating voltage, 1 kV (top), and higher accelerating voltage, 15 kV (bottom). In general, lower kV images of biological samples show better definition of the surface features. At high kV, the interaction volume becomes large and the signal comes from a combination of the surface and the volume beneath the surface. Therefore, the fine surface features are unresolved.

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When imaging biological specimens with the SEM, the use of low accelerating voltages is ideal as low kV provides maximum surface sensitivity. At higher accelerating voltages, the interaction volume becomes large and the signal comes from a combination of the surface as well as the volume beneath the surface, resulting in muddied surface features (figure 7). The ZEISS FESEM family features a special Gemini column designed for excellent resolution on any sample, especially at low accelerating voltages.

To visualize a specimen in the SEM, you choose to see an image of the specimen by collecting and displaying one of the signals created by the beam-specimen interaction. These signals are collected by detectors to generate images on the display monitor. The signals generally used for the creation of images are secondary (SE) and backscattered electrons (BSE) (reference 2).

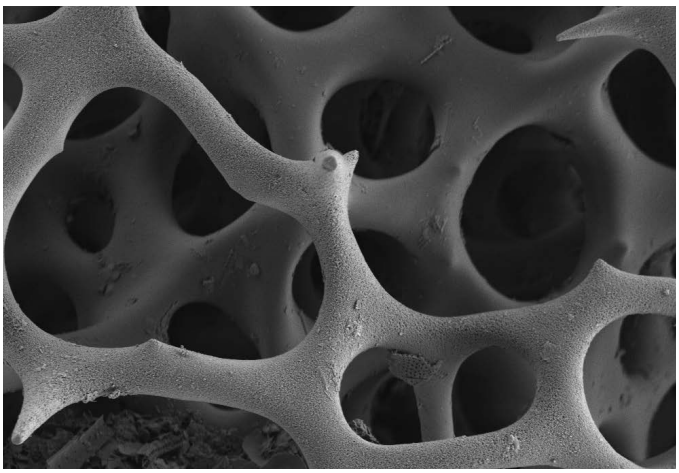

Figure 8: The delicate open structure of a radiolarian is imaged effortlessly by the ETSE detector at 1 kV under high vacuum. Acquired with ZEISS Sigma 500, ETSE detector.

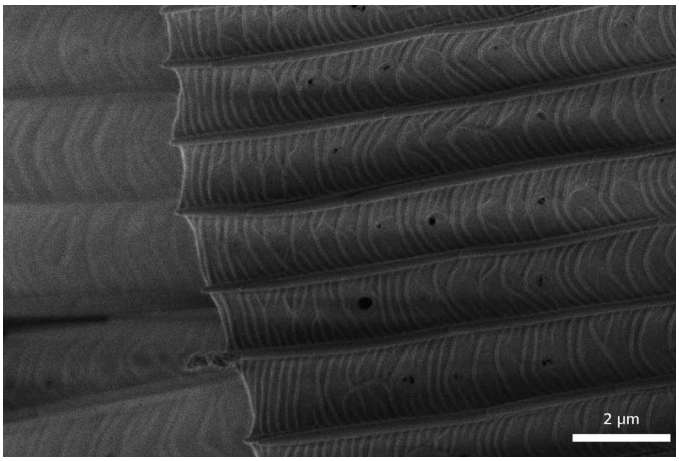

Figure 9: Moth wing, imaged with ZEISS GeminiSEM with the Inlens detector, at 50 V, in high vacuum.

Signal Detection

Secondary electrons

Secondary electrons (SE) are created when a beam electron collides with an electron from the specimen atom and loses significant energy to that atom resulting in a transfer of energy to the specimen atom causing it to ionize. Electrons, called secondary electrons, can be emitted as part of this ionization. Secondary electrons are characterized as having energies less than 50 eV. They are created throughout the interaction volume, but due to their low energy, most of them are absorbed by specimen atoms so only secondary electrons created near the surface of the specimen can escape (reference 4).

Secondary electrons are collected and imaged by secondary electron detectors and show specimen topography. There are many types of secondary electron detectors. The Everhart Thornley detector (ETSE) is an in-chamber detector that provides 3D-like topographical imaging resolution even at long working distances (figure 8).

The Inlens detector (figure 9) is a secondary electron detector located in the SEM column, directly above the final lens. It detects secondary electrons directly in the beam path using Beam Booster technology powered by ZEISS. At acceleration voltages of ≤ 20kV, the electrons of the primary electron beam are additionally accelerated by 8kV inside the electron optics by the Beam Booster. An electrostatic field is generated at the end of the objective lens that decelerates the primary electrons by 8kV to ensure that beam electrons reach the sample surface at the set accelerating voltage. This electrostatic field at the end of the objective lens also acts as an acceleration field to the SE's generated on the sample surface. The SE's are absorbed, re-accelerated and focused through the electromagnetic field of the final lens to the Inlens detector. The Inlens detector is very surface sensitive, making it ideal for low kV imaging of biological specimens.

Backscattered electrons

A backscattered electron (BSE) occurs when a primary beam electron comes into close proximity with a specimen atom nucleus or outer shell electron and scatters back out of the specimen atom with minimal energy loss. Backscattered electrons go through single or multiple scattering events in the interaction volume and escape back through the surface of the specimen with energy greater than 50 eV. BSE's are created throughout the interaction volume and escape at greater depths compared to SE's because they are higher energy electrons. Backscattered electrons are collected and imaged by BSE detectors and are useful for highlighting compositional contrast,

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or differences in specimen atomic number (figure 10). As atomic number increases the yield of BSE's increases, therefore higher atomic numbered elements will appear brighter in the BSE image. While the distribution of secondary electrons orientates perpendicularly to topographic structures, backscattered electrons emerge from the bulk material and are therefore less sensitive to surface topography.

BSE detection is essential for low kV, high contrast imaging of heavy metal-stained biological thin sections. The in-column energy selective backscatter (EsB) detector, located above the Inlens detector, is used for this purpose (figure 11). In order to prevent detection of SE's, a filtering grid is installed in front of the EsB Detector. By switching filtering grid voltage on, the SE's are rejected and only BSE's are detected. At low accelerating voltages, below 1.5 kV, the filtering grid has the additional function of selecting the desired energy of the BSE. This equates to enhanced section imaging (figure 12). Instead of lowering the kV to avoid penetrating through the depth of the section, you can simply adjust the filter to reject low energy BSE's that come from deeper depths of the interaction volume.

The ZEISS Sense BSD detector is a BSE detector that is specifically designed for imaging ultrastructure of biological thin sections with a new degree of speed and quality (figure 13). With a new diode design and superior detector sensitivity, Sense BSD can detect very small numbers of backscattered electrons and convert low signals into high-contrast images. Fast image acquisition with low acceleration voltages and low electron doses becomes possible – your biological sample can be imaged without damage, and deterioration of the image quality induced by charging effects is prevented.

Another detector available for BSE detection is the annular backscatter detector (aBSD). It is an in chamber, pneumatic solid-state detector that is inserted into the SEM chamber, in line with the column. It is used to detect backscattered electrons that have been scattered under very low angles. Once inserted, it is located directly below the final lens, and above the specimen. Backscatter electrons exit the specimen, strike the six-segment circular silicon diode which causes ejection of electrons in the silicon and generates a flow of current proportional to the number of BSE's striking it. The current is then amplified and the signal is sent to the display monitor. The six segments can be turned on and off to change image contrast (figure 14).

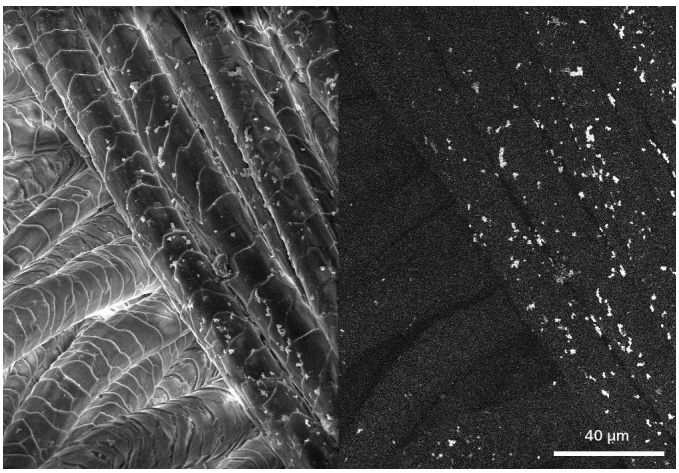

Figure 10: Silver nanoparticle coated natural fibers imaged with NanoVP at 80 Pa, at 10 kV using dual channel Inlens secondary electron detector (left) and EsB backscattered electron detector (right). Secondary electrons emphasize specimen topography and backscattered electrons highlight specimen composition.

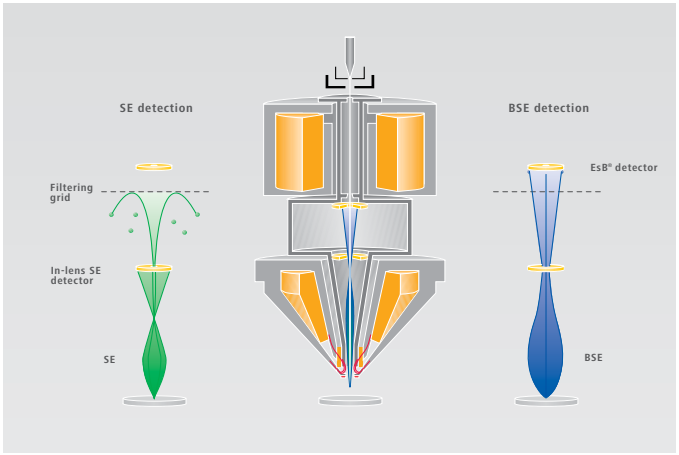

Figure 11: Inlens vs. EsB detectors: Secondary electrons (green) are projected onto the lower Inlens detector and the backscattered electrons (blue) are guided onto the upper EsB detector.

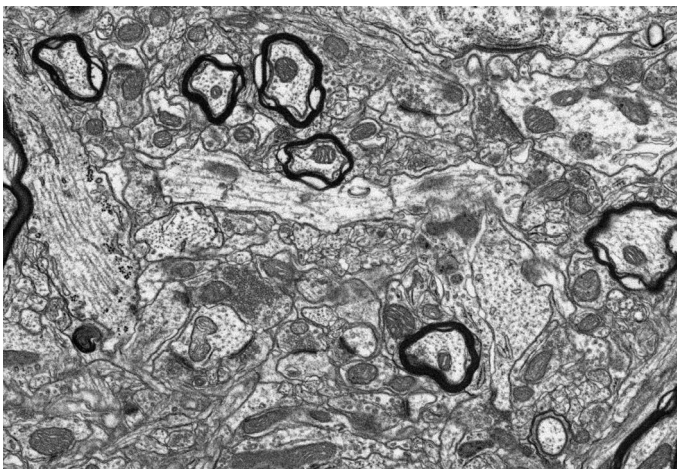

Figure 12: 3D mapping of mouse brain with high z-resolution by serial focused ion beam (FIB) slicing and SEM imaging. The cross section is one of a large image stack with 20 nm slice thickness and a pixel size of 4 nm. The image was taken with the EsB detector at 2 kV. Image courtesy of Javier DeFelipe, Cajal Institute, Madrid, Spain.

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Transmitted Electrons

Thin specimens, such as thin sections prepared by ultramicrotomy or naturally thin specimens placed on a TEM grid, can be placed on a TEM grid holder adapted for the SEM stage. A STEM (short for scanning transmission electron microscope) detector that is mounted on the underside of the specimen, perpendicular to the optical axis, is used to collect the electrons with enough energy to pass through the specimen. These types of collected electrons are called transmitted electrons (reference 1). STEM allows you to benefit from additional information out of your ultra-thin biological specimens without the need to use a dedicated transmission electron microscope (TEM). It functions similarly to a solid-state BSD detector, meaning you can turn segments on and off in order to enhance or change the image contrast (figure 15). The TEM grid holder is well-suited for high throughput studies as it is equipped to hold up to twelve samples at a time.

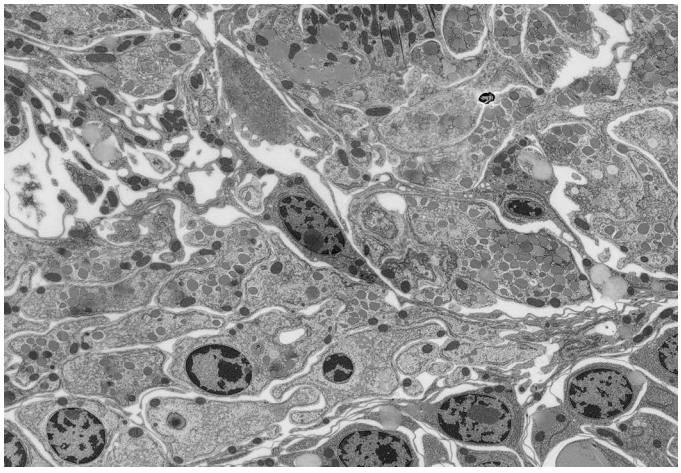

Figure 13: Ultrastructure of Tricellaria inopinata *imaged at 1 kV with ZEISS Sense BSD. Sample courtesy by Anna Seybold & Harald Hausen, Sars Centre for Marine Molecular Biology, University of Bergen, Norway.*

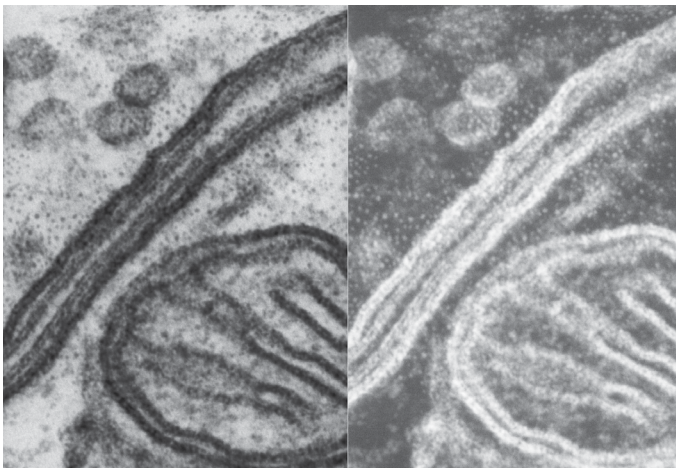

Figure 15: Mouse brain ultrathin section on a TEM grid. Double membrane of mitochondria is resolved down to the lipid bilayer structure. Left: annular STEM (aSTEM) brightfield, Right: aSTEM darkfield. Sample courtesy of EPFL Lausanne, Switzerland.

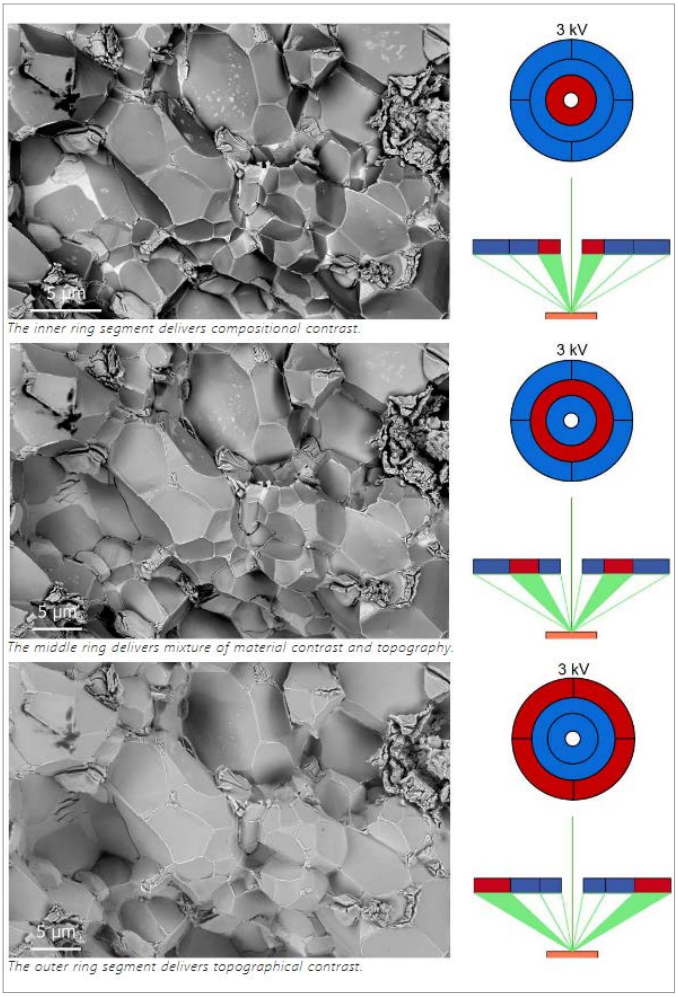

Figure 14: The six segments of the aBSD detector can be turned on and off to change image contrast. The inner ring is especially used to image the material contrast as it has a high take-off angle. The middle ring shows a mixture of material contrast and topography. The outer ring is divided into four segments to explore the topographic contrast with different illumination.

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Variable Pressure Studies

Typically, the SEM is operated at high vacuum (approximately 10^{-6} - 10^{-7} torr), to allow passage of the electron beam through the column without interference of dirt, debris or air molecules (reference 1). This high vacuum state is great for conductive samples; however, non-conductive samples, such as many biological specimens, will experience charging. Charging occurs when there is no conducting path for the electrons to flow from the sample surface to ground, therefore the electrons stick to the sample surface (reference 3). Sample charging is an imaging issue as it causes drift, blur, and low contrast.

Variable Pressure SEM (VPSEM) has emerged as a strategy to deal with charging. The motivation behind the development of VPSEM was the ability to observe non-conductive samples without modifying the surface with the addition of a conductive coating. A gas (air, nitrogen, water vapor) is introduced into the sample chamber, affecting the chamber pressure. Static charge on sample is neutralized by ionization of the gas molecules. The ionized atmosphere necessitates use of special detectors, standard ETSE detectors cannot be used safely in variable pressure mode due to arcing between the sample and ETSE detector. Several types of variable pressure detectors are available, one such detector is the VPSE (variable pressure secondary electron detector). The VPSE is an indirect electron detector based on photon detection. VPSE allows high-quality SEM imaging while protecting fragile, non-conductive samples from the high vacuum environment of the SEM (figure 16).

Discover more information about detector types and applications

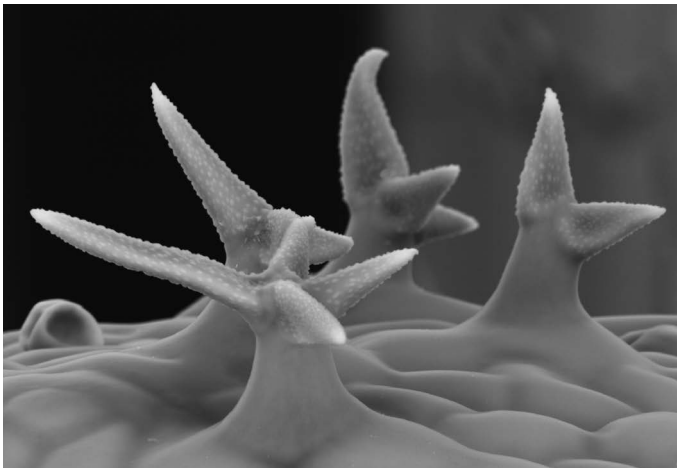

Figure 16: The intricate structure of the surface of a rosemary leaf is visualized by the VPSE G3 detector at 20 kV. The SEM was configured for environmental imaging with water vapor introduction and a coolstage.

Summary

Scanning electron microscopy of life science samples provides a wealth of specimen information. From compositional studies to topographical imaging and 3D reconstruction of ultrastructure, there are countless applications that can benefit from the high resolution, low voltage imaging of the SEM. The SEM is a truly unique imaging solution for the fact that it can be specifically tailored to the needs of the individual researcher or an entire imaging core with the addition of a variety of detectors, correlative microscopy, and room temperature and cryogenic modes available today.

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