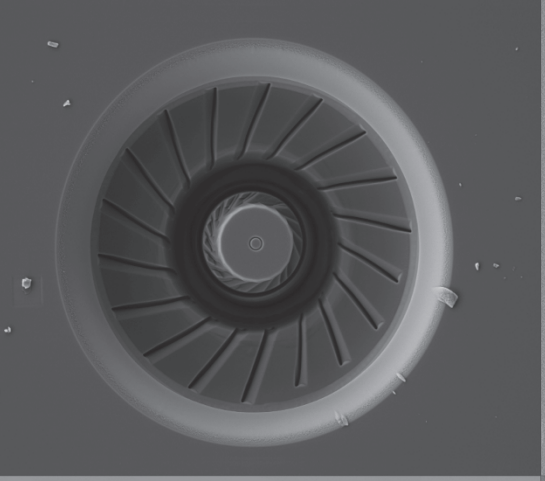
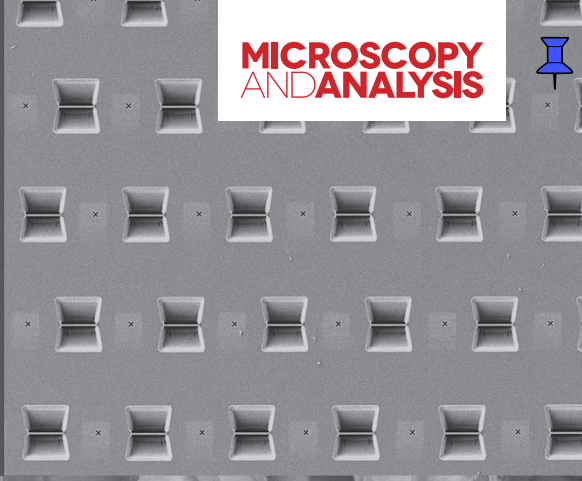
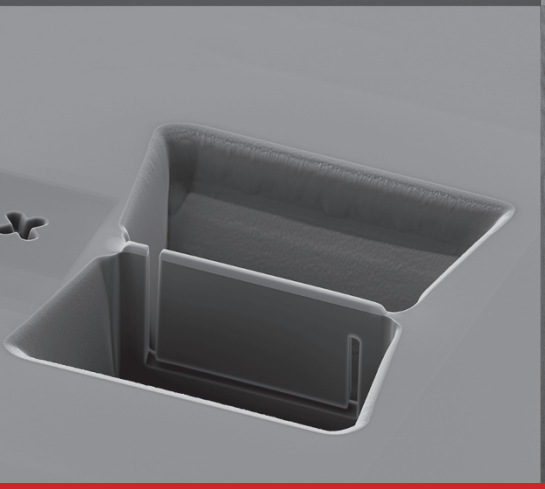


MICROSCOPY AND ANALYSIS

# Focused Ion Beam-Scanning Electron Microscopy

Essential Knowledge Briefings

First Edition, 2017

ZEISS

Front cover image. Top left: top view of a FIB-machined micro-pillar for compression testing. Top right: array of TEM lamellas produced automatically. Bottom left: lamella in silicon. Bottom right: cross section of a material failure in a steel sheet (courtesy of Audi AG).


Microscopy EKB Series Editor: Dr Julian Heath
Spectroscopy and Separations EKB Series Editor: Nick Taylor

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# INTRODUCTION

With any kind of work, it helps to be able to see what you're doing, but that can be a challenge when working at microscopic scales or below. By taking advantage of a system that combines two separate instruments – a focused ion beam (FIB) and a scanning electron microscope (SEM) – researchers are now able to do just that. They can modify a wide range of materials with the FIB while monitoring the process in real time with the SEM.

This modification can include removing material from a sample by dislodging atoms with the ion beam to access its interior, as well as depositing material by reacting gases with the ion beam. The monitoring involves detecting the various signals given off as the electron beam from the SEM is scanned across the sample, including secondary electrons, backscattered electrons and X-rays, to produce a visual image. This modification and monitoring can happen simultaneously, with the FIB and the SEM's electron beam both interacting with the sample, allowing the modification to be visualized in real time.

Since its development in 1989, FIB-SEM has shown its impressive abilities in a wide range of applications, both industrial and academic. These include identifying defects in electronic circuits, analyzing corrosion in metal components, quantifying the porosity of rocks, fabricating atom force microscopy probes, and revealing the detailed structure of a range of biological materials, from teeth to brain regions to pollen.

Over this time, the manufacturers of commercial FIB-SEM systems have continually improved their performance, increasing the precision and speed of material removal by the ion beam, improving the imaging capabilities of the SEM and making the

whole system more automated. As a consequence, FIB-SEM has never been more effective, adaptable or easy to use.

This EKB offers an introduction to FIB-SEM. It begins by describing the two component instruments and how they work, before detailing the advantages of combining them in one system. This is followed by a detailed explanation of the main ways in which researchers utilize FIB-SEM, from preparing samples for analysis by transmission electron microscopy (TEM) to conducting three-dimensional (3D) tomography to fabricating small-scale features.

The EKB also discusses practical issues that need to be considered when working with FIB-SEM, including sample preparation and reducing sample damage. In addition, it details several examples of how FIB-SEM is currently being utilized by scientists in their research, and showcases some of the latest developments, including advanced ion sources and combining FIB-SEM with complementary analytical techniques such as super-resolution light and X-ray microscopy.

## HISTORY AND BACKGROUND

For decades, scientists valued FIB and SEM as separate instruments, before discovering the benefits of combining them in a single system.

SEM provides information on the morphology (texture), chemical composition and crystalline structure of a sample, down to the nanoscale, with the first commercial SEM introduced by the Cambridge Scientific Instrument Company in 1965.

In a typical SEM, an electron gun fires an electron beam at a sample. This beam is focused by electromagnetic lenses to a spot 0.4–5nm in diameter, which is scanned across the surface, causing electrons in the beam to interact non-destructively with atoms at or near the surface of the sample. This interaction generates various different signals, comprising secondary electrons, back-scattered electrons, X-rays and cathodoluminescence (CL), all of which can be collected by detectors to reveal information about the sample.

Secondary electrons are released when the beam interacts with weakly bonded electrons at the sample surface, and reveal information about the topography (shape) and electrical properties of the sample. In the resultant image, bright areas correspond to areas emitting large quantities of secondary electrons, *eg* edges.

Backscattered electrons are electrons that have been reflected directly back from the sample. They tend to come from slightly deeper regions than secondary electrons and reveal information about a sample's elemental make-up, because different atoms backscatter electrons to different extents. Usually, heavy atoms (with a high atomic number) backscatter electrons more strongly

than light elements and so appear brighter in the resultant SEM images, but it depends on the voltage of the electron beam.

The detectors used for both secondary and backscattered electrons are synchronized with the electron beam, so that as the beam is scanned across the sample the electrons released at each scan point produce a single pixel in the resultant image.

The emission of secondary electrons from the electron shell of the atoms leaves behind positively charged 'holes', which quickly fill with electrons from an atom's outer shells. As outer-shell electrons move to a lower position, their excess energy is discharged in the form of X-rays, which are emitted at an energy that is characteristic of the parent element. Energy dispersive X-ray spectroscopy (EDS) can be used to detect and measure this energy, revealing the identity of the parent element. In this way, EDS can provide rapid qualitative and sometimes quantitative information about a sample's elemental composition.

CL is produced when electrons hit luminescent materials, such as certain minerals. A CL detector picks up the light emitted by these materials, producing an image in which different materials produce distinct levels of contrast. CL is primarily used for studying geological materials, but can also be used in biology to image luminescent proteins.

FIB works in a similar manner to SEM, but uses a beam of positively charged ions rather than negative electrons. Because ions are much heavier than electrons, an ion beam can be used for physically modifying a sample as well as analyzing it.

The beginnings of FIB can be traced back to 1975, when VE Krohn and G Roy Ringo at the Argonne National Laboratory in Illinois, USA, first produced an ion beam with high brightness

(intensity) using liquid gallium as the ion source. Following on from this, Robert Seliger and his team at the Hughes Research Laboratories in Malibu, USA, used gallium as the ion source in the first scanning ion microscope used for patterning.

Most FIB systems now use liquid metal ion sources (LMIS). These are mainly gallium, although some use gold or iridium; gallium is favoured for its low melting point, volatility and vapor pressure. The gallium is housed in a capillary tube with a tungsten needle running through the middle. When heated, liquid gallium flows along the needle to the tip. There, an extraction electrode produces a strong electrostatic force that causes the liquid meniscus to form a cone with a tip radius of 2nm. An electric field ionizes the gallium in this cone, while electrostatic lenses accelerate and focus the gallium ions to form a finely focused beam.

At high ion doses, the FIB can dislodge atoms from the surface of a sample via a process known as sputtering, allowing it to modify, or mill, the sample surface with nanometer precision (Figure 1a). It can also generate secondary electrons and ions, which can both be used to produce images, in the same way as an SEM (Figure 1b). In fact, the FIB and SEM share the same secondary electron detection system, while the secondary ions are detected by their own specific detectors. In order to preserve the sample, FIB imaging is typically conducted at lower ion doses than milling.

The secondary ions can reveal information about sample composition; for example, they can highlight corrosion in metal structures because the raised oxygen levels significantly increase secondary ion yields. In addition, the secondary ions can be

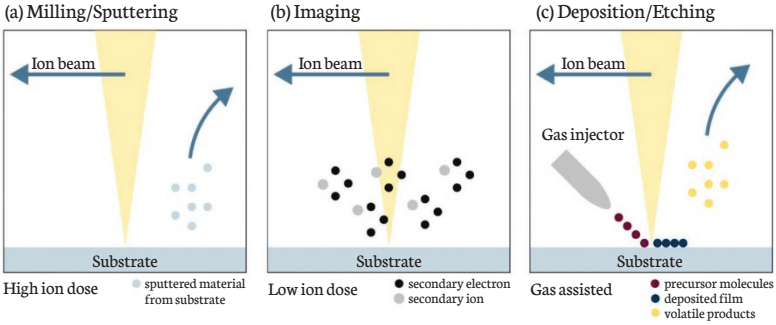



**Figure 1. Schematics showing the interactions of an ion beam with a substrate. The ion beam (a) sputters material, (b) creates secondary electrons and ions for imaging, and (c) induces material deposition in the presence of a precursor gas**

collected and analyzed by a mass spectrometer to conduct secondary ion mass spectrometry (SIMS). This is a very sensitive technique that can detect dopants in a semiconductor down to the parts-per-million level.

As well as removing material from a sample, FIB can also be used to deposit material. This involves using a gas injector to spray a precursor gaseous substance, such as platinum- or carbon-based compounds, onto the surface of the sample, where they react with the ion beam to form a deposit of platinum or carbon (Figure 1c).

At first, FIB was used mainly by the semiconductor industry to remove thin sections, or lamellas, from electronic circuits for analysis by TEM, in order to identify defects. But it could also be used to patch or modify devices, a process known as device editing.

Then, in 1989, Pierre Sudraud's team at Paris-Orsay University in France, became the first to combine FIB and SEM in a single system. FIB and SEM complement each other perfectly, enhancing their respective capabilities. In addition to allowing FIB and SEM images to be collected by the same system, the FIB

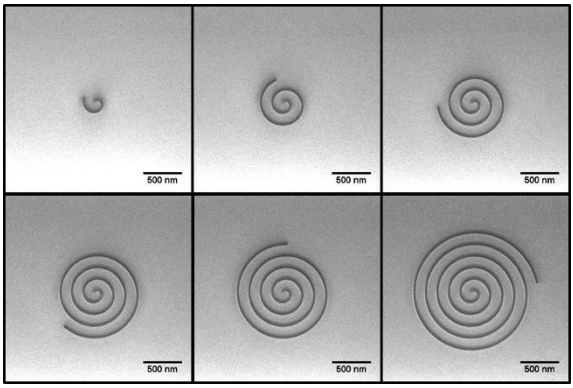



**Figure 2. SEM images acquired during FIB patterning of a spiral in silicon. Spiral pitch and line width are 150nm and 20nm, respectively**

milling process can be monitored by SEM as it happens, offering a much greater degree of control (Figure 2).

Once again, the semiconductor industry was an early adopter, as the addition of SEM made it easier both to identify and study potential defects, and to monitor the milling process. Often the defect could be uncovered by FIB and then analyzed *in situ* by SEM without needing to prepare a lamella and extract it from the sample. Even if a lamella was prepared, it didn't always need to be transferred to a separate TEM, as it could be analyzed by the FIB-SEM in transmission mode, a technique known as scanning transmission electron microscopy (STEM).

Today, FIB-SEM is often used instead of TEM, although only TEM can study samples at atomic resolution. While TEM has to work with thin sections of a sample, because its images are produced by electrons passing through the sample to a detector underneath, the FIB-SEM can work with bulk samples, as its images are generated by secondary and backscattered electrons.

Using FIB-SEM to analyze the nano- and microstructure of samples has subsequently been adopted by scientists to study a wide range of materials, including metals, polymers, rocks, and

even soft biological material, after it has been encased in resin or frozen.

Like FIB on its own, FIB-SEM can also be used to fabricate small-scale structures, by both carving features into material and depositing new material, but with the added advantage that the fabrication process can be monitored in real time (Figure 3 shows some examples of FIB-SEM applications).

For the life sciences, a real game-changer has been the ability to use FIB-SEM to build up highly detailed 3D images of a sample. Known as FIB-SEM tomography or serial section imaging, this involves removing multiple layers of a sample with FIB and then imaging each revealed surface with SEM, before combining all the images together to produce a 3D representation of the original sample.

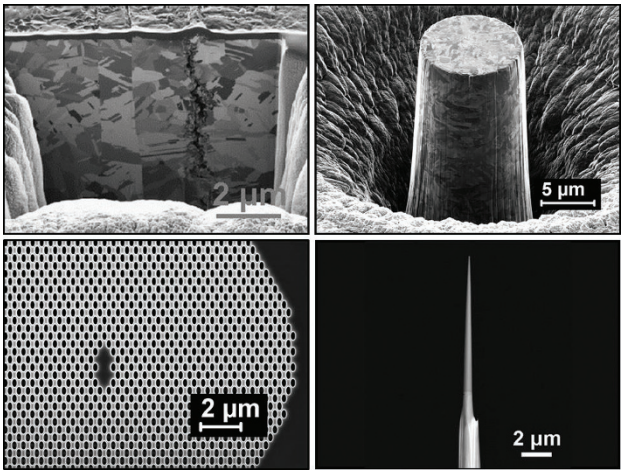



**Figure 3. Some applications of FIB-SEM. (Top left) Cross section for failure analysis of copper contacts (FIB image). (Top right) Pillar machined in steel for micro-indentation experiments (FIB image). (Bottom left) Photonic crystal test structure in silicon (SEM image). (Bottom right) AFM needle for atom probe microscope (SEM image)**

# IN PRACTICE

FIB-SEM systems, often referred to as dual-beam or cross-beam systems, comprise two columns arranged so that the electron and ion beam focal points coincide. Typically, the SEM column is held in a vertical position, above the sample, with the FIB column at an angle of 52° to 56°, depending on the manufacturer (Figure 4).

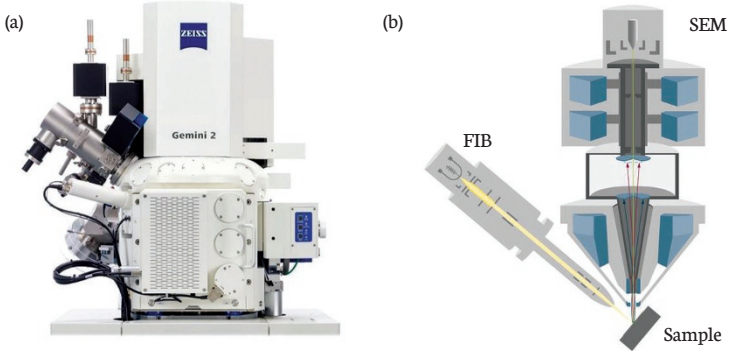



**Figure 4. (a) Photograph of a commercial FIB-SEM instrument. (b) Schematic showing the arrangement of SEM and FIB columns and the sample during FIB-SEM work**

Typical energies for the ion beam range from 1keV to 30keV, with beam currents varying between 1pA to 100nA. Higher beam currents mean higher milling speeds; for silicon, a beam current of 6nA can produce a milling speed of around 100μm$^3$ a minute. The systems can also be adorned with several extra attachments, from cryogenic/heating stages to X-ray detectors to gas injectors for depositing material.

To conduct an analysis with FIB-SEM, the sample is placed at the coincidence point of both beams. The SEM begins by imaging the sample to identify a region of interest (ROI), which might be a grain boundary in a crystalline material or a single cell in biological tissue, and the FIB then uncovers this ROI by cutting

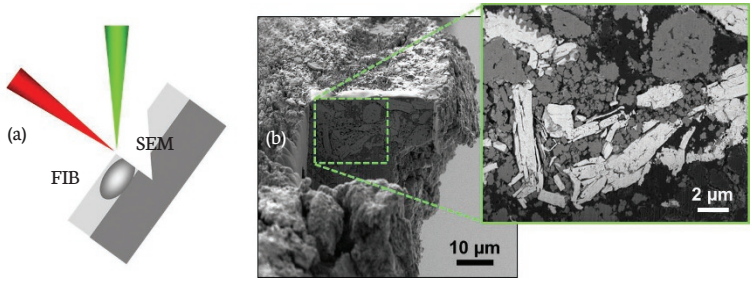



**Figure 5. (a) Schematic showing the arrangement of sample, FIB and SEM beams during cross sectioning. (b) SEM images of a cross section in a lithium ion battery cathode (courtesy of T Bernthaler, University of Aalen)**

a cross section through the sample. If conducting failure analysis or process control, this revealed ROI might simply be studied by SEM (Figure 5).

For more detailed analysis by TEM, the ROI needs to be extracted as a lamella. This is done by using the FIB to remove material in front of and behind the ROI, exposing a flat, vertical lamella (Figure 6). The lamella needs to be thin enough for electrons to pass through, which usually means less than around 100nm thick.

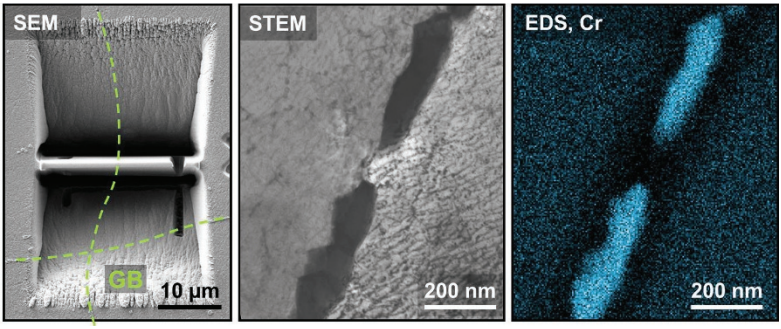



**Figure 6. Site-specific sample preparation of a grain boundary in heat-affected steel. (Left) SEM image showing lamella and grain boundaries (GB, green dashed lines). (Center) STEM image of chromium carbide precipitates at the GB acquired in the FIB-SEM instrument. (Right) Chromium EDS map of the same area. Image courtesy of D Willer, MPA Stuttgart**

Ion beam currents of 10nA or above are often used at the start of the milling process, when lots of material needs to be removed, but the current is then usually reduced to 50–300pA near the end of the process because FIB machining is more precise at lower FIB currents. Finally, the lamella is transferred to a separate TEM or analyzed in the FIB-SEM instrument by STEM; for inorganic materials, the lamella is usually physically extracted for TEM analysis, but for more fragile biological materials it is often left attached to the bulk sample for support.

For FIB-SEM tomography, the ion beam removes successive thin layers of the sample, 3–100nm thick, while the SEM produces an image of the surface of the sample (usually referred to as the 'block face') after each layer has been removed (Figure 7). These images are then combined to create a 3D image of the whole sample.

Although the SEM is the main imaging instrument, both the electron beam and the ion beam can be used for imaging, and differences between FIB and SEM images can provide interesting insights into sample structure. A prime example is when imaging

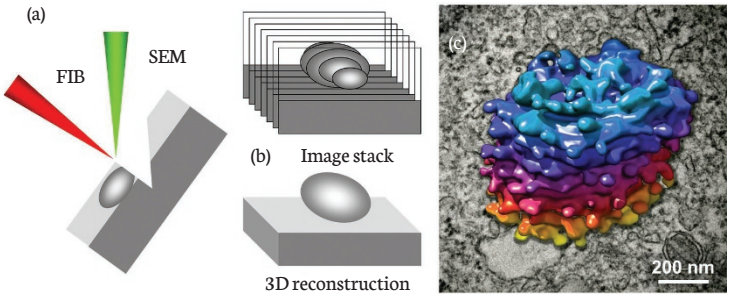



**Figure 7. (a) Schematic showing the arrangement of sample, FIB and SEM beams during FIB-SEM tomography. The FIB produces serial sections of the sample while SEM images are acquired. (b) An image stack results, which can be reconstructed in 3D. (c) Example showing the 3D reconstruction of an algal Golgi body. Image courtesy of Dr. Louise Hughes, Oxford Brookes University**

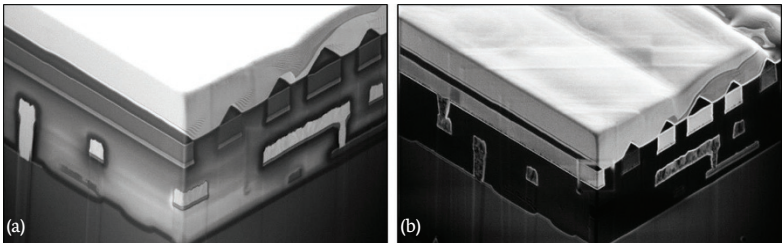



**Figure 8. SEM (a) and FIB (b) images of the same area of an integrated circuit**

computer chips. Insulating layers in integrated circuits appear bright in SEM images, but dark in FIB images (Figure 8). This is because the electron beam used for SEM charges the insulating layer negatively, causing the generated secondary electrons to be actively expelled from the insulating region, making it look bright. In contrast, the positively charged gallium ions making up the ion beam charge the insulator positively, preventing secondary electrons from leaving the insulating region, making it look dark.

FIB-SEM systems contain multiple detectors for collecting the various signals that can be generated by both ion beam and electron beam. The latest FIB-SEM systems can detect several of these signals simultaneously and produce information-rich images by merging them together.

This simultaneous detection works best when all the signals are of a similar intensity. Because the X-ray signal is fairly weak, the electron beam needs to be scanned much more slowly across the sample when conducting EDS than when detecting secondary or backscattered electrons. This means that the sample needs to be scanned twice: first to produce an image from the secondary or backscattered electron signal, and then to obtain EDS chemical information, which is overlaid over the electron image.

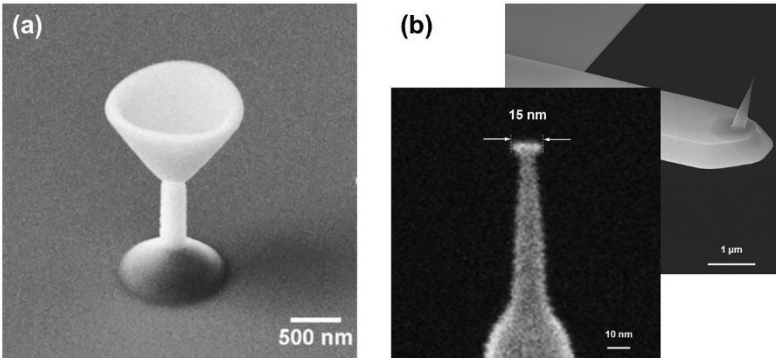



**Figure 9. (a) Carbon nanocup fabricated with IBID. (b) High performance AFM tips produced by EBID. Image (b) courtesy of S Schmidt and B Irmer, nanotools GmbH**

Both FIB and SEM can also be used to deposit material in a process known as ion beam-induced deposition (IBID) or electron beam-induced deposition (EBID). This involves a built-in gas injector releasing a small amount of a gaseous substance near the sample; on interacting with the electron or ion beam, this gaseous substance decomposes to form non-volatile products that deposit on the sample surface. IBID and EBID are often used to apply a protective coating of platinum or carbon onto samples to protect the ROI from ion beam damage and to smooth out rough surfaces. They can also be used to fabricate small-scale structures such as atom force microscopy (AFM) probes (Figure 9).

The other way that FIB-SEM can fabricate small-scale structures is by using the ion beam to carve them out of materials, with the process again monitored by SEM. The exact shape of the fabricated feature depends on the precise milling strategy that is adopted. For example, numerous strategies can be adopted for carving a ring into silicon, including milling

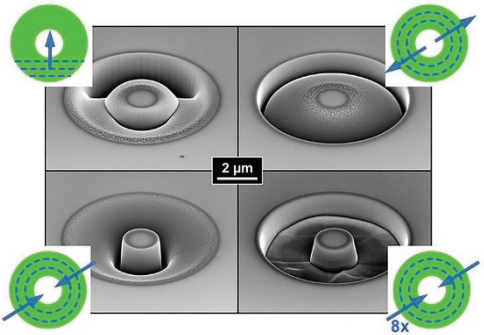



**Figure 10. Pillars milled in silicon with identical dose, but with different milling strategies: line by line upwards, circular milling outwards, inwards, and inwards with eight cycles (clockwise starting from top left image)**

upwards, outwards and inwards, but they all produce dramatically different results (Figure 10).

In modern FIB-SEM systems, all these functions are highly automated, with both milling and imaging controlled by advanced software tools. These tools allow users to set and control a wide range of milling and imaging parameters, including FIB current, milling strategy, pixel spacing and signal mixing.

## CASE STUDY 1. Brain imaging

*'FIB-SEM is a dream technology because you can semi-automatically do 3D reconstructions that would otherwise be almost impossible,' says Javier DeFelipe from the Laboratorio Cajal de Circuitos Corticales at the Technical University of Madrid in Spain.*

*DeFelipe and his colleague Angel Merchán-Pérez use FIB-SEB to study the minute details of mouse brains. In particular, they use FIB-SEM tomography to drill down into the fine detail of synapses, building up 3D models from the multiple images produced with a serial sectioning approach. By showing the spatial distribution of synapses, these models can be used to determine the number and sizes of synapses in a brain region.*[1]

*Before FIB-SEM, the researchers would carefully prepare tens to hundreds of sample sections for analysis by TEM. 'Today we can make 600 sections in just one session. Now the bottleneck is not the acquisition of images but the analysis in three dimensions of the stacks of sections,' says Merchán-Pérez.*

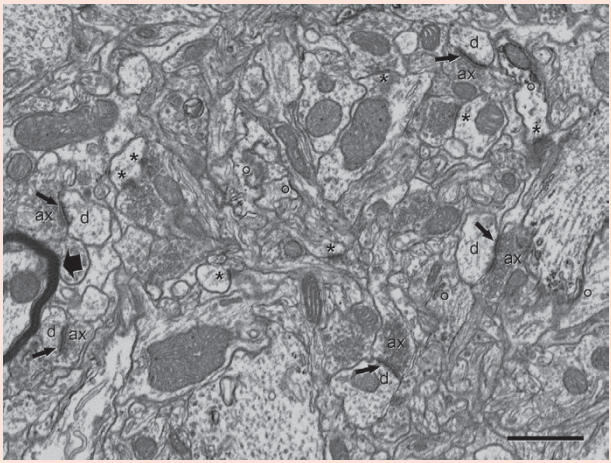
*FIB-SEM can accommodate large samples, so the researchers can put the entire hippocampus region of a mouse's brain on the stage. 'The sample we take with FIB-SEM is tiny, in the order of microns,' explains Merchán-Pérez. 'When you mill a region, you destroy it but you only destroy very small portions of tissue. You can go several microns apart and take another sample. You could have hundreds of samples in one region.'*

*'With this technology, we are getting a much more accurate representation of the synaptic organisation in the brain,' says DeFelipe. 'If you can navigate in three dimensions through your images, you*

*can not only see synapses and dendrites but whether synapses are made on dendritic spines or shafts,' adds Merchán-Pérez.*[2]

*This work has implications for the study of human diseases such as Alzheimer's disease, because the team can measure the distribution of synapses in a healthy brain and compare it with a diseased one. They can also compare healthy and diseased regions of single brains.*

*The data obtained so far has proved particularly useful in improving computer models of brains. 'It's important not only for us to understand the structure and the organization of the brain but also for the modelers to build a model that helps us to understand how the brain works,' says Merchán-Pérez.*




**Panoramic view of the neuropil obtained in backscattered electron imaging mode. Intracellular structures such as filaments, vesicles, cisternae or mitochondria can be identified. Some axon terminals (ax) establish clearly identifiable asymmetric synapses (arrows) with dendritic spines (d). Other membrane densities could only be unambiguously identified as asymmetric or symmetric synaptic densities (asterisks), or non-synaptic densities (circles), when the neighboring serial sections were studied. The myelin sheath to the left of the figure (thick arrow) is very dark and its laminar structure cannot be resolved at this resolution. Scale bar 1µm.**

1. Merchán-Pérez A, Rodriguez J-R, Alonso-Nanclares L, *et al.* Counting synapses using FIB/SEM microscopy: a true revolution for ultrastructural volume reconstruction. *Front Neuroanat* 2009;3:1. (http://dx.doi.org/10.3389/neuro.05.018.2009)

2. Bosch C, Martinez A, Masachs N, *et al.* FIB/SEM technology and high-throughput 3D reconstruction of dendritic spines and synapses in GFP-labeled adult-generated neurons. *Front Neuroanat* 2015;9:1. (http://dx.doi.org/10.3389/fnana.2015.00060)

## CASE STUDY 2. Lithium-ion batteries

*Lithium-ion batteries (LIBs) have taken over much of the rechargeable battery market since their commercial introduction in 1991, thanks largely to their high-energy densities and low discharge rates when not in use.*

*Like all batteries, LIBs consist of an anode and cathode separated by a liquid electrolyte. In this case, the anode is usually made from graphite while the cathode is made from a lithium transition metal oxide such as lithium manganese oxide. During charging and discharging, lithium ions flow back and forth from the anode to the cathode through the liquid electrolyte.*

*With the aim of improving the lifetime of LIBs, Timo Bernthaler and his team from Aalen University in Germany used FIB-SEM to analyze the cathode material from a commercial battery.[1] They cut a small piece of the cathode to create a needle that was free-standing during FIB-SEM analysis.*

*First, they used X-ray microscopy measurements to determine the microscopic structure of the cathode, which revealed some mysterious bright spots below the surface. They then used FIB-SEM to study these bright spots in more detail, collecting secondary electron and backscattered images simultaneously. In the backscattered electron image, the binder material appeared very dark while the particles showed up as two brighter levels of gray.*

*'With the FIB-SEM we could see a very clear contrast between a dark phase and a bright additive,' explains Bernthaler (see Figure 5b). This gave the first hint that two different types of particles were present. The team then conducted EDS, which showed that the particles with the brightest contrast in the*

*backscattered electron image were rich in lanthanum rather than the expected manganese.*

*Bernthaler concludes that the cathode material contains far more lanthanum-rich agglomerates or particles than originally assumed: 'The additives are not listed on the data sheet, therefore we were very surprised to find a lot of these particles,' he says.*

*The researchers don't know why the cathode would contain lanthanum, although they think it was added deliberately rather than being an impurity. 'For the function of the battery, it makes no sense to have these additives,' says Bernthaler.*

*Next, the team plans to use FIB-SEM to investigate how lithium-ion active materials age. This should help in understanding the effect of charging and usage conditions, as well as provide an opportunity for improving battery materials.*

*The researchers also plan to study magnetic materials with FIB-SEM, focusing on grain boundaries. 'Typically the grain boundary phase is richer in rare earth element content,' says Bernthaler. The grain boundary phase surrounds larger magnetic regions and is believed to provide magnetic coercivity. 'It is interesting to understand the wetting behavior of the grain boundary phase around the magnetic grains,' he adds.*

1. Weisenberger C, Kopp A, Bernthaler T, *et al.* Multi-scale characterisation of lithium ion battery cathode material by correlative x-ray and FIB-SEM microscopy. *Microsc Anal* 2015;September/October:17. (https://applications.zeiss.com/C125792900358A3F/0/E3943F087E33F98DC1257E6D0031F7A8/$FILE/EN_41_013_103_FIB-SEM_Lithium-Ion-Battery-Cathode-Material.pdf)

## PROBLEMS AND SOLUTIONS

FIB-SEM systems can manipulate and study a wide range of different materials with minimal sample preparation, but users still need to be careful to obtain the best results. The destructive abilities of FIB are essential for removing individual layers of a sample in order to conduct tomography or for carving out a lamella for study by TEM, but they can also induce a range of unwanted effects. These can include physical damage, ion implantation and heating, all of which can affect an analysis and thus need to be understood and, if possible, controlled.

Softer materials are more susceptible to these effects than harder materials, but even hard materials are not immune. For example, at high enough beam intensities, the ions don't just dislodge atoms from the surface of a sample, but can actually be inserted into the material. Known as ion implantation, this can alter the sample's chemical composition and thus its electronic, magnetic, optical and mechanical properties. Further, ion bombardment of crystalline materials can disrupt their regular crystalline structure in a process known as amorphization. The ion beam can also cause radiolysis damage in solid materials, altering their electronic structure.

The most obvious way to control amorphization is by reducing the energy of the ions; this is known as a 'low-kV' FIB polish. Ion implantation can be reduced by lowering the angle of incidence of the ion beam, so that it grazes the surface of the sample, or by using IBID or EBID to cover the sample in a protective coating of platinum or carbon. Such protective coatings can be particularly useful when using FIB to mill a lamella, because the beam always enters the sample at a glancing angle from the same

direction. As a consequence, one side of the sample is continually exposed to the beam, causing that side to become progressively damaged. This damage can be prevented if the outer surface is coated in a protective layer.

A coating can also help when analyzing cross sections or conducting FIB-SEM tomography. In this case, the coating is applied before milling, with the coating helping to protect the revealed surface from physical damage, as well as reducing an unwanted effect of milling known as curtaining. These are stripes that can appear across the surface of the cross section as a result of the sample surface being naturally rough, or the sample being of heterogeneous composition, and thus milled to slightly different depths by the ion beam. The protective coating smooths out these rough surfaces, thereby preventing curtaining.

In polymers, the charged ions can directly sever molecular chains and induce cross-linking in any side chains, both of which can greatly affect the polymer's physical and chemical properties. Radiolysis damage can also alter a polymer's appearance and chemical composition, creating functional organic groups and inducing changes in chemical coordination and oxidation state. Unlike inorganic materials, polymers can also be chemically altered by the heat generated by the ion beam, as they are more sensitive to thermal effects and cannot disperse heat effectively, leading to thermal softening and a reduction in mechanical rigidity.

Once again, the damage caused by the ion beam can be controlled by reducing its intensity and also by applying a protective coating to the polymer sample. The heating can be controlled by conducting FIB at low temperatures, known as cryo-FIB, or by tailoring the scanning strategy so that the beam

doesn't dwell for too long in one place, preventing heat from building up.

Biological materials are particularly prone to the damaging effects of FIB, and unlike crystalline materials and polymers need to undergo sample preparation before manipulation by FIB or analysis by SEM. Understandably, this preparation is basically the same as required for any form of electron microscopy, in order to stabilize the sample and preserve its delicate ultrastructure. Conventionally, this is done chemically, by first fixing the biological material with glutaraldehyde and then embedding it in an epoxy resin.

This produces a solid block that retains all the features of the biological material but is stable enough for FIB-SEM analysis, whether carving out a lamella for TEM or conducting FIB-SEM tomography. In order to ensure the images produced by this analysis have a sufficiently high contrast, extra metal can be added by treating the sample with chemicals such as osmium tetroxide and potassium ferricyanide after fixation. This metal can also help to reduce the build-up of charge and thus radiolysis damage. In addition, the block can also be stained with uranyl acetate.

An alternative option for sample preparation is cryo-fixation, in which the biological material is rapidly frozen, and then manipulated and imaged while maintained in this frozen state. The advantage of cryo-fixation over chemical fixation is that it allows biological material to be imaged under conditions that are closer to its natural environment.

Cryo-fixed samples can be modified by FIB in exactly the same ways as those embedded in resin, but need to be maintained

at the necessary low temperatures during all stages of the FIB-SEM analysis, including a possible transfer to a TEM. The resolution of FIB-SEM tomography is also lower for cryo-fixed samples than for chemically fixed samples, because the layers etched away by the ion beam are typically much thicker, usually 100–500nm thick.

Even after sample preparation, whether chemical or cryo, biological samples are still highly prone to the damaging effects of the ion beam, and so require the same mitigating measures as other materials, including reducing the ion beam intensity, lowering the temperature and applying a protective coating. In addition to the ion beam causing physical damage to a sample, another potential problem is the redeposition of volatile material that has been etched away. This material obviously needs to go somewhere and if not dealt with can simply condense back onto the sample surface. The easiest way to prevent this happening is by introducing a surface cooled to a temperature below that of the sample and placed close to it, in which case the volatile material will condense onto the cooled surface rather than the sample.

## WHAT'S NEXT?

Despite the undoubted abilities of FIB-SEM, there is still scope for improving both of its component instruments. For the FIB, manufacturers are faced with two opposing aims: to increase the resolution of the instrument, so that it can mill smaller features; and to increase the rate at which large volumes of material can be removed. Fortunately, both of these aims can be met by adopting novel ion sources, although different ion sources are required to meet each aim.

High-resolution milling requires an ion beam with a small focal spot, while large-volume milling requires an ion beam with a large current and a high current density. The latest generation of gallium LMIS offer an effective compromise, able to produce an ion beam with a focal spot of around 5nm in diameter at small currents of 1pA, while also producing a beam at a current of 100nA for large-volume milling. Two ion sources that can produce ion beams with even smaller focal spots are a gas field ionization source (GFIS) and a low temperature ion source (LoTIS).

In a GFIS, which is just beginning to appear in commercial FIB systems, the ion beam is generated by exposing a neutral noble gas atom, usually helium or neon, to a large electric field established between a pointed needle and an electrode. This causes electrons to be stripped away from the gas atom, transforming it into a positively charged ion that is then accelerated away from the needle by the electric field, joining with others to form the ion beam.

Because the GFIS source is the size of a single atom, the resultant ion beam is very narrow and thus can be focused to a very small spot. Helium GFIS can produce a beam with a focal

spot just 0.35nm in diameter, whereas neon GFIS produces a slightly wider beam, with a focal spot of 1.9nm. This comes at the expense of the ion current, which is typically less than 10pA, and so GFIS is not suitable for milling large volumes of material.

In LoTIS, gaseous caesium ions are cooled and photoionized by one or more laser beams, and then accelerated by an applied electric field to form an ion beam, which can be focused onto a sample. Caesium is often used in LoTIS because it is the heaviest atom amenable to laser cooling and heavier atoms tend to generate narrower beams. Caesium beams are able to produce focal spots below 1nm in diameter at currents of 1pA, and thus have a similar resolution to the helium beams produced by GFIS but with higher removal rates. Up to now, however, LoTIS has not been integrated into a commercial FIB system.

In contrast to GFIS and LoTIS, inductively coupled plasma (ICP) ion sources, which are also just beginning to appear in FIB-SEM systems, produce thicker, more powerful ion beams, allowing higher removal rates and faster milling speeds. The ion beam is produced from a cloud of charged ions and electrons known as a plasma, which itself is generated by exposing inert gases such as helium, neon, argon and xenon to radio frequency waves. For FIB, a xenon plasma is usually used to produce ion beams with currents up to a few micro-amps, which are ideal for milling large volumes. Another option being explored for milling larger volumes is to combine FIB-SEM with laser ablation.

For SEM, the main scope for improvement comes from enhancing the efficiencies of the various detectors rather than modifying the electron beam. Although modern FIB-SEM systems possess multiple detectors, they can currently only

actually capture a fraction of the signal that is generated, whereas ideally they should detect all the signal, especially when that signal is inherently rather weak, as is the case for X-rays.

While manufacturers are continually trying to improve FIB-SEM systems, users are exploring the benefits of combining them with other analytical and microscopy techniques. Obviously, FIB-SEM systems have always been designed to work with TEM, but now users are also combining it with techniques such as X-ray microscopy, confocal light microscopy and super-resolution microscopy to conduct multi-scale, 3D characterization of materials, devices and biological tissue. Often, this involves using X-ray microscopy or confocal light microscopy to identify interesting features in a sample, which are then exposed and studied in more detail using FIB-SEM.

Super-resolution microscopy, a collection of techniques that push the resolution of light microscopy beyond previous physical limits, can be combined directly with the information generated by electron microscopy, as both can reveal features at similar scales. Already, scientists have successfully combined super-resolution microscopy with FIB-SEM to study cellular organelles such as mitochondria.

## FURTHER INFORMATION

AAAS-Zeiss poster on SEM. (http://poster.sciencemag.org/sem)

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