

# A New Approach to Studying Biological and Soft Materials Using Focused Ion Beam Scanning Electron Microscopy (FIB SEM)

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**Abstract.** Over the last decade techniques such as confocal light microscopy, in combination with fluorescent labelling, have helped biologists and life scientists to study biological architectures at tissue and cell level in great detail. Meanwhile, obtaining information at very small length scales is possible with the combination of sample preparation techniques and transmission electron microscopy (TEM) or scanning transmission electron microscopy (STEM). Scanning electron microscopy (SEM) is well known for the determination of surface characteristics and morphology. However, the desire to understand the three dimensional relationships of meso-scale hierarchies has led to the development of advanced microscopy techniques, to give a further complementary approach. A focused ion beam (FIB) can be used as a nano-scalpel and hence allows us to reveal internal microstructure in a site-specific manner. Whilst FIB instruments have been used to study and verify the three-dimensional architecture of man made materials, SEM and FIB technologies have now been brought together in a single instrument representing a powerful combination for the study of biological specimens and soft materials. We demonstrate the use of FIB SEM to study three-dimensional relationships for a range of length scales and materials, from small-scale cellular structures to the larger scale interactions between biomedical materials and tissues. FIB cutting of heterogeneous mixtures of hard and soft materials, resulting in a uniform cross-section, has proved to be of particular value since classical preparation methods tend to introduce artefacts. Furthermore, by appropriate selection, we can sequentially cross-section to create a series of ‘slices’ at specific intervals. 3D reconstruction software can then be used to volume-render information from the 2D slices, enabling us to immediately see the spatial relationships between microstructural components.

## 1. Outline of focused ion beam scanning electron microscopy (FIB SEM)

### 1.1 Introduction

When a beam of focused ions (e.g. gallium) interacts with a specimen, both secondary electrons and ions are emitted and can be used to form an image. Due to the large size of primary ions, the penetration depth of the ion beam is very small (a few tens of nanometers) and so can provide high-resolution imaging, as well as surface-sensitive electron channelling contrast from polycrystalline

materials. An important aspect of the FIB is the high efficiency with which atoms of the specimen can be removed (sputtered). This gives us the possibility to selectively mill material to make cross-sections or patterns. In addition, new materials (metals and insulators) can be added by vapour deposition from a suitable gas. In fact, this type of well-controlled *in situ* chemical vapour deposition is becoming increasingly important in the field of nano-fabrication [1].

FIB technology has been known in the semi-conductor industry for more than a decade, where it is used for circuit edit and mask repair. We now wish to explore the possibilities for applying FIB technology in the rather different context of biological specimens and soft materials.

## 1.2 FIB SEM

There are yet further benefits to having a combined FIB SEM system. For example, with primary ion and electron beams focused to the same point on a specimen surface, it is possible to mill a specimen with the ion beam whilst visualizing or monitoring the results with the electron beam (without having to move the specimen, as with a FIB-only instrument). Figure 1 depicts a specimen, tilted normal to the ion beam, with a wedge-shaped volume of material removed. The final vertical face, parallel to the ion beam, is at a sufficient angle to enable electron imaging.

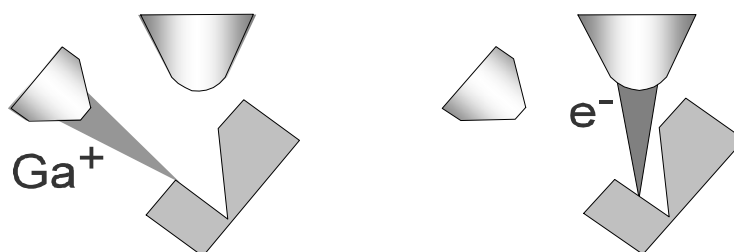


Figure 1. With the specimen tilted towards the ion beam (left), a cross-section is made that can be imaged with the electron beam (right).

The ability to FIB mill into the bulk of a specimen means that we can open up the third dimension and explore hidden internal microstructure. Milling and imaging can be done in a sequential manner: the cross-section can be milled by a given amount, then imaged before the next step. Thus a series of 'slices' can be collected at well-defined distances. The entire process can be automated, enabling data to be collected in the absence of a microscope operator and maximizing the useful work performed by the instrument (e.g. overnight running). For a truly three-dimensional view, the final step is to apply volume rendering to the two-dimensional images, using appropriate software (e.g. Amira).

Since we are dealing with FIB technology in the relatively unexplored realms of biological and soft materials, with their inherently insulating properties, we must consider the effects of charging. Just as non-conductive materials charge negatively under electron irradiation in high vacuum, so they charge positively with ion beam irradiation, and there are times when a conductive coating is not suitable. Negative charging of uncoated insulators can, of course, be controlled by using a primary electron beam voltage low enough to limit the depth from which electrons are emitted, so that electrons leave at roughly the same rate as they arrive.

Electrons emitted as a result of ion irradiation, in the absence of a source of negative charge, leave the surface charged positively, further exacerbated by the accumulation of primary positive ions. One option is to supply low energy electrons using an electron flood gun to help redress the charge balance during ion milling and imaging, familiar to those accustomed to single beam FIB, certain types of FIB SEM and other techniques such as secondary ion mass spectrometry (SIMS). Alternatively, we can make use of the primary electron beam to deliver the required negative charge, by putting the beam in spot mode and defocusing to an appropriate degree.

## 2. Biological Specimens

Ultramicrotomy is an approach regularly used to produce thin samples for TEM or to slice through bulk material for SEM. However, there are many types of heterogeneous specimens for which the method is far from successful, particularly where there is a mixture of hard and soft material. Cutting can blur the interface between adjacent materials or tear a soft covering from a hard substrate. Slicing techniques can also result in preferential fracture, where crack propagation tends to go around rather than through certain components. While this is often a useful property, it can make it very difficult to reveal the internal microstructure of the component. FIB SEM offers the capability to perform accurate, homogeneous slicing and, crucially, to choose a specific site of interest.

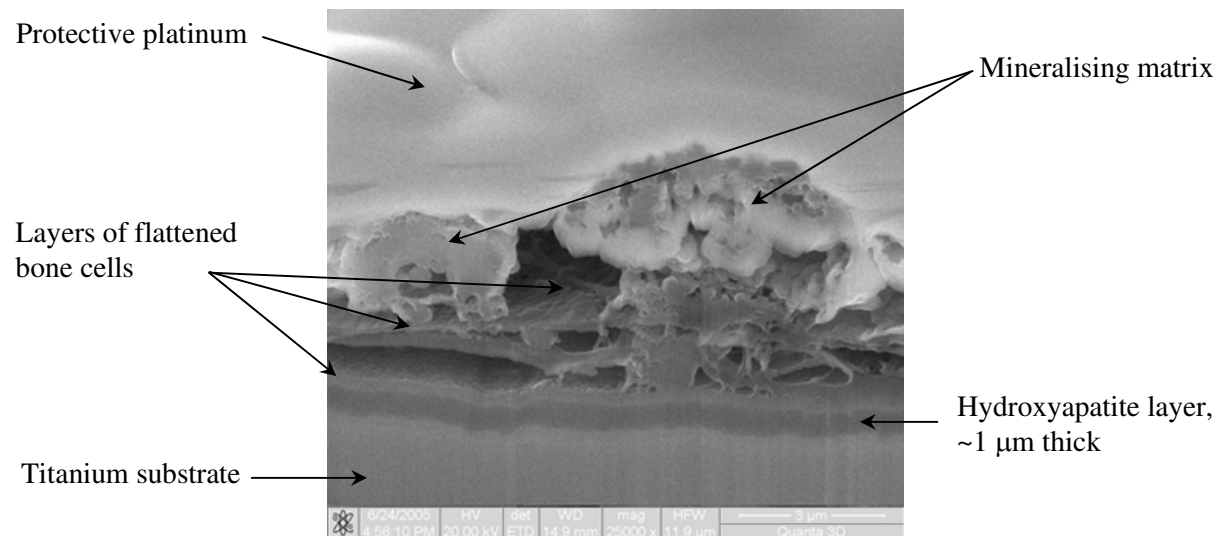


Figure 2. Electron micrograph showing cross-sectional view through bone cells on a biomaterial.

Figure 2 shows a FIB milled cross-section through delicate bone cells that have been cultured for 42 hours on a hydroxyapatite-coated titanium substrate. Such a system is of key interest in the study of orthopaedic applications (such as prosthetic implants). A platinum layer has been vapour deposited *in situ*, in order to protect the top surface of the cells and matrix from the ion beam and ensure that surface topography is preserved during milling. As this example shows, the FIB SEM approach allows us to visualise the interior of the specimen in a very precise manner. In this case, we can measure the thickness of the hydroxyapatite layer deposited on the substrate, check for good adhesion and note any undulations or roughness. We can observe the cells and their interactions with the hydroxyapatite coating, as well as assessing the location and extent of mineralisation as the collagen matrix, secreted by the cells, begins to calcify to form bone. (X-ray microanalysis was also performed on this cross-section, to verify the presence of Ca, P, Ti, C and O). The same approach can be used to observe collections of cells and matrix that form higher order structures such as tissues and organs. Three-dimensional reconstruction of these specimen types has the potential to be immensely instructive, as has recently been shown for osteocytes in bone [2].

## 3. Soft Materials

Polymers are an important class of materials that fall into this category. For example, there is currently great interest in the development of organic semi-conductors for opto-electronic devices, and hence a need for suitable characterization methods. Often the features of interest are of nano-scale dimensions, and so precise site-selection and high-resolution imaging are required.

Figure 3(a) shows an opto-electronic transistor, consisting of a thin semi-conducting layer over metallic sources and drains, followed by a dielectric layer (polymer) and finally a metallic gate on the surface. The thickness of the entire device is  $\sim 1 \mu\text{m}$ , and has been cast on a glass substrate. This highly

insulating specimen has been left uncoated and imaged in low vacuum mode to minimize charging. Note that we are able to see the buried inter-digitated sources and drains by using a high primary electron beam voltage to give good beam penetration.

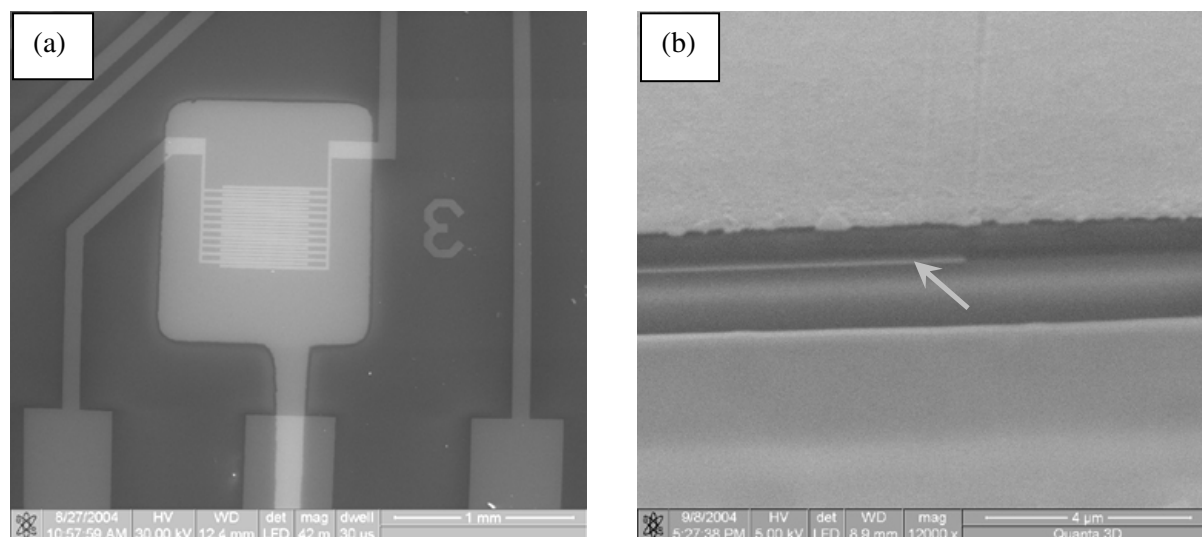


Figure 3. Low vacuum electron micrographs of an opto-electronic transistor, showing the overall structure at low magnification (a) and part of a milled cross-section, at higher magnification (b).

Figure 3(b) shows a FIB milled cross-section through the device, again imaged in low vacuum but this time with a lower primary electron beam voltage to give surface-sensitive information. The feature indicated by the arrow is part of the metallic inter-digitated region located at the base of the device (seen in the centre of figure 3(a)). FIB milling must be carried out in high vacuum, hence the specimen was charge-stabilized using the defocused, low energy primary electron beam method, as mentioned in Section 1.2. Here, FIB SEM allows us to determine the thickness of layers and homogeneity of interfaces, providing valuable feedback about the effects of processing conditions.

### Conclusions

FIB SEM adds new dimensions in the characterization of biological specimens and soft materials, enabling visualization of internal microstructure and three-dimensional representation. At present, the application of this technology is at a very early stage, and methodologies are being developed for optimal results. For example, in addition to charge control, we must quantify the milling rates of organic materials and any inorganic components as a function of ion beam current, and explore the possibilities offered by gas-assisted etching. Another aspect that also promises to make a big impact on the analysis of such materials is FIB milling to yield ultra-thin, flat specimens for TEM and STEM.

### Acknowledgments

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### References

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