

# The Use of a SEM/FIB DualBeam Applied to Biological Samples

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A DualBeam instrument using both a focusing electron and ion column, can effectively be applied to biological samples using cryogenic temperatures to ensure compatibility with the vacuum conditions of the instrument. A cryo stage as commonly used for SEM can be made compatible with a DualBeam geometry taking into account the requirements for both techniques. Experiments on biological samples have shown that milling into the frozen substrate to create site-specific cross-sections is fast and easy and that the obtained cross-sections show very good information that is comparable or better than obtained with non-site-specific cryo fracturing. This opens the way to three-dimensional local analysis of the sample.

## Introduction

In the world of Scanning Electron Microscopy (SEM) the use of a cryo stage is a very common way to accommodate wet samples and colloidal solutions or mixtures of water and fat, such as present in many food and cosmetic products. By freezing the sample to a low temperature, the vapor pressure becomes so low that it is well below the operating pressure of the instrument and thus the sample is stable. For example, at a temperature of  $-140^{\circ}\text{C}$  the vapor pressure is in the range of  $10^{-10}$  mBar, whereas the working chamber pressure is between  $10^{-4}$  and  $10^{-6}$  mBar. In regular use the practical operating temperature of the cryo system is between an upper limit and a lower limit of liquid nitrogen temperature (77 K) and for this specific range the evaporation of ice can be neglected. The very low temperature can be used to prevent local heating of the sample by electron beam irradiation to a temperature well above the upper limit where the evaporation of ice starts to be noticeable. Note that within the chamber it is very common to have at least one surface at an even lower temperature than the sample and this surface will act as a collector: it is generally referred to as anti-contaminator.

## System Components

A DualBeam Strata 235M makes use of the following geometry for the two columns (Fig. 1). The two beams have an

intersection point at 5 mm below the electron column, which coincides with the eucentric point of the stage (at this point the tilt axis is perpendicular to the electron optical axis and hence the image does not move when tilting the sample). It can be seen from this set-up that it is necessary to be able to mill into the sample when the stage is tilted to 52 degree and as a consequence the cryo stage that is mounted on top of the standard stage will be tilted as well.

A Gatan Alto 2500 cryo stage was used with a facility for cryo fracture and thin film coating. The cryo stage and the anti-contaminator and its supplies take up a considerable space in the DualBeam chamber. Following detailed engineering discussions a configuration was found where all components fit well and uncompromised operation was guaranteed. It was particularly important to ensure that the required tilt of 52 degree can be obtained and that the anti-contaminator could be sited to ensure optimal cryo performance with minimal geometric disturbance. For loading of samples into the chamber use is made of the transfer valve of the Alto 2500 cryo system and standard procedures for cooling of the sample from room temperature to cryogenic temperature are followed. It is important that during the freezing of the sample, care is taken to avoid ice formation on the top surface, as this will obstruct selection of the proper sample area and decent imaging of the sample.

In the standard operation of the DualBeam it is common practice to apply a

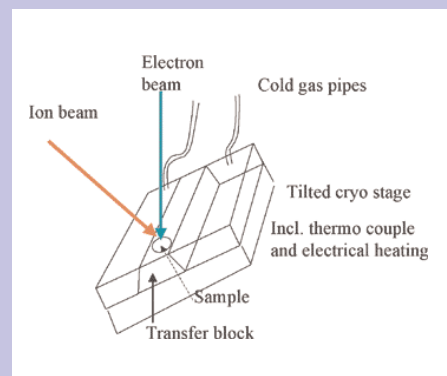


Fig. 1: Schematic set-up of the cryo stage on the standard stage and the position of the two columns

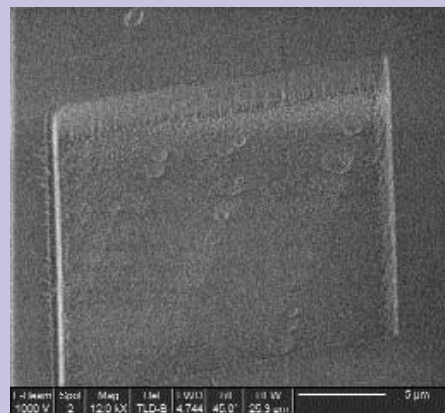


Fig. 2: Milling a square hole into pure, frozen water helps to understand parameters such as dwell time, overlap, beam current and allows to quantify the milling rate.

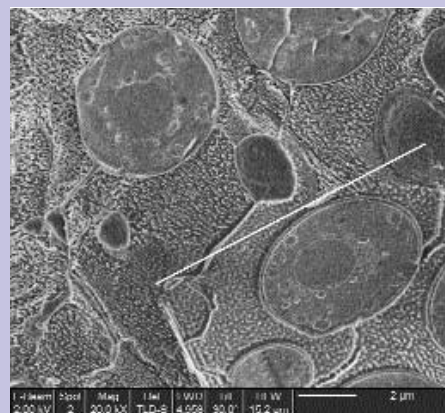


Fig. 3: Bakers yeast showing both a cryo fracture part and a FIB created cross-section part (below the drawn line). Image is slightly scan rotated.

coating on the sample prior to milling, especially for non-conductive samples. This very local coating is created by ion beam or electron beam induced deposition from a local gas supply of an organo-metallic vapor. This commonly used system, referred to as Gas Injector System or GIS, however cannot be applied to a very cold sample: gas molecules from the GIS system will immediately condense on the cold surface and form a (thick) film of frozen precursor gas rather than a thin film of decomposed organo-metallic material. As a consequence all operation of the DualBeam with cold samples has been realized without use of any GIS system. Note that the use of the coating equipment of the cryo system is primarily applied for the purpose of high-resolution imaging and not for enhancing milling properties: as such this kind of coating is too thin (nm range instead of  $\mu\text{m}$  range).

## Experiment

Prior to using real samples, some experiments were done with milling into pure water. A simple square was created by top down milling as shown in Fig. 2. With a simple experiment like this it was confirmed that the milling rate in ice actually is very high and around  $10 \text{ um}^3/\text{nC}$ . This is a big advantage as making relatively large holes and cross-sections or multiple sections in the third dimension is now possible in an acceptable time frame. Another consequence is that an ion beam, when used for imaging, will rapidly remove sample layers even in low current mode: as a consequence a DualBeam, where the electron column can be used for non-destructive imaging is far more ideal for this application than a single beam FIB. As many biological systems have a very high water content, the milling rate measured here can be used as a good reference value for setting up automated milling jobs.

Another characteristic of the milling process is the smoothness of the cross-section. Using pure ice as a reference sample helps to define the overlap and the milling strategy for optimal smoothness of the cross-section.

## Results

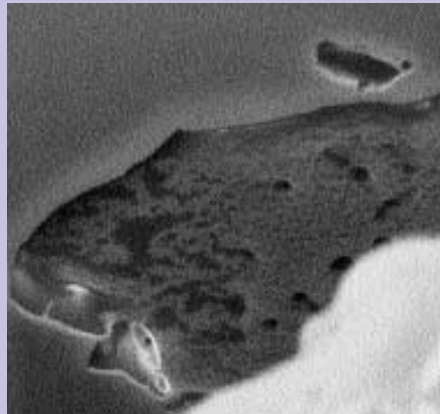
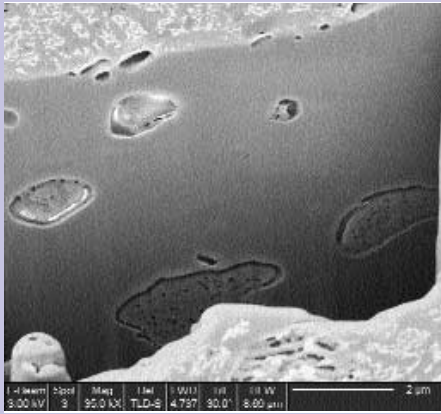
One of the first tests was done using bakers yeast as a reference sample. This sample is commonly used as a test sample. In this case it was cryo fractured in the preparation chamber, passed through the transfer valve and loaded onto the stage. A cross-section was then made with the ion beam, followed by imaging with the electron beam. The result is

shown in Fig. 3. It should be noted that in an image such as this, the actual start of the cross-section does not show up as a straight transition line from top surface to vertical section. This is due to the original uneven fracture surface. This would easily be seen if the sample top surface was flat. The image in Fig. 3 has a slight scan rotation and the actual milling direction is perpendicular to the solid line drawn in the image. The cell in the upper left corner is opened by the cryo-fracture, whereas the cell in the lower right section is opened up by the FIB cut. It is clear that the information revealed by the FIB cut is very good and that the internal bodies in the cell are not damaged, but nicely sectioned in a straight plane. Details like cell membrane and nucleus are very visible. It would be possible to make another section using an image like this, by cutting just a little deeper in the material and so revealing the third dimension.

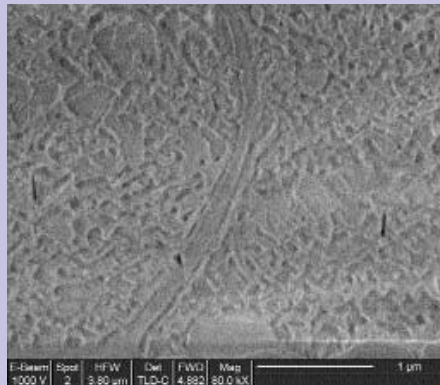
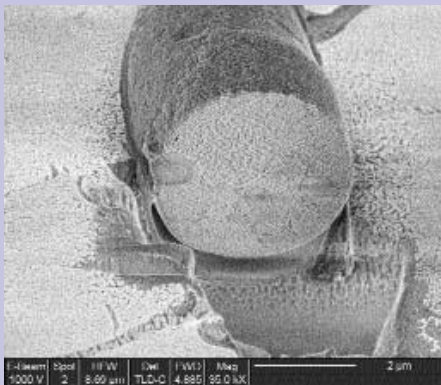
Another example is a food product such as liquid margarine: this basically is an emulsion of water droplets in oil that is stabilized by emulsifiers and proteins. In material combinations such as these, interest in the mechanisms at the interface between water droplets and oil lead to a better understanding of the structuring mechanism of emulsions.

A complete milled section is presented in Figs. 4a and 4b, showing individual water droplets in the cross-section and a higher magnification image showing more fine- detail. This example shows again that milling into biological samples such as emulsions of fat, oil and water can be done easily, and that interface studies at localized positions can be performed very well. Most important in this respect are the absence of milling artifacts (due to different material behavior) and good control over the final milling steps and the geometric stability of the system.

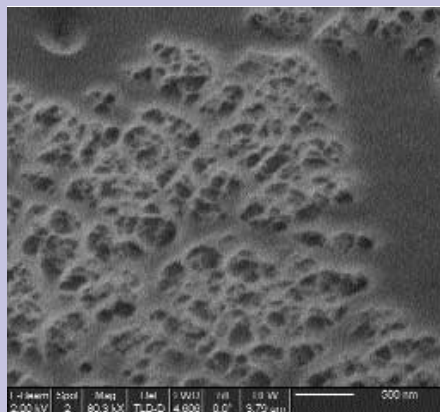
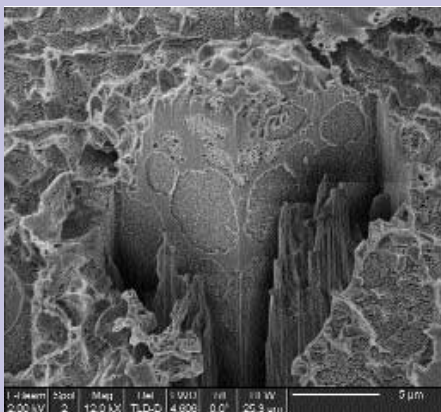
Another biological system is presented by a bacteria culture or cell culture in a rich nutrition and friendly environment. If the dilution of the cells / bacteria is low enough and the growth conditions are favorable, then it will be possible to create a sample with individual and separated organisms, in a thin water film on a substrate. Such cells can be presented as monolayers on a flat substrate such as a microscope cover glass (eucaryotic cells) and covered in a thin water film only. Bacterial cells can be presented as isolated colonies from petri dish culture or dispersed within cryopreservative storage gel as here. In this way, once the sample is frozen, individual cells / bacteria can be selected with the electron beam when looking top/down and cut at specific sites with the FIB to reveal the internal struc-



Figs. 4 a, b: Cross-section into liquid margarine, showing individual water droplets (left) and higher detail about the interface (right) at 100 kx.



Figs. 5a, b: Single bacterium completely cross-sectioned by FIB after the wall has been stripped away. In Fig. 5a a loop of mesosomal membrane can be seen to penetrate the cell at the approximate 9 o'clock position. Attached to the mesosome is the bacterial circular chromosome. Much of the bacterial cytoplasm is occupied by densely packed ribosomes (Fig. 5b).



Figs. 6a, b: 90 degree „double FIB-cut“ on a single gut epithelial cell. The edge on the right of Fig. 6a has not been cleaned up. Higher magnification on the right (Fig. 6b) shows a greater detail of what is thought to be supercoils of DNA exposed by ice sublimation.

tures. So it is possible to directly use living material to work from and examine it without staining or chemical fixation. Its internal structure can be investigated in a cross-section at a very local site, created by the ion beam. As an example a *Ractobacillus* bacterium has been cut perpendicular to its long axis, similar to macro analogue situation of a sausage being cut with a knife. The FIB really acts now as a micro-surgical knife, that creates a very smooth and straight cut.

Vertebrate gut epithelial cells were chosen as model systems from more complex higher organisms where the cell not only contains complexly coiled chromosomes within a distinct nucleus but also many sub cellular cytoplasmic organelles. In the figures shown the cell has been milled on two faces at right angles to each other (Fig. 6a). This double cut was employed to follow the projection of different features into the bulk of the sample. The area framed within the two

cut faces contains several profiles through the eucaryote cell nucleus. Within this nucleoplasm the DNA exhibits molecular coiling to differing complexities. Many such supercoils can be seen in Fig. 6b. In some occasions a thin metal coating deposited on the sample, after it has been cut with FIB, is very helpful to increase the image quality: this coating is created in the preparation chamber of the cryo system so it means re-loading and repositioning of the sample. In practice, using stage automation of the instrument, this can be done very rapidly. The coating not only helps to reduce charging of the sample, but also enhances the edges in the image. For any further milling the coating is not a problem as it will be milled away easily.

### Conclusion

For samples that are commonly used in cryo SEM observation (such as biological and cosmetic / food samples) an added FIB capability for cutting into the sample is a very valuable extension. The FIB can be used as a micro-surgery tool, that can open up the third dimension. Milling is very fast and precise and most important, structures that show up are sharply cut without noticeable damage. A Dual-Beam is ideal for such type of investigation as the electron column is used for imaging without cutting and the ion beam for cutting without imaging: the two beams are fully complementary to each other.

### Acknowledgements

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