

Methodologies for the preparation of soft materials using CryoFIB SEM

Debbie J. Stokes* & Michael F. Hayles
FEI Company, PO Box 80066, 5600 KA Eindhoven, The Netherlands

ABSTRACT

The recent advent of focused ion beam (FIB) technology in combination with the more familiar scanning electron microscope (SEM) is bringing new insights to the characterization of a range of bulk materials. Furthermore, the FIB SEM can be augmented by a cryo-preparation/transfer system, enabling both frozen and frozen-hydrated soft materials to be FIB-milled at low temperature. This provides an opportunity to perform *in situ* site-specific cross-sectioning, and hence study the interior of a bulk material in two and three dimensions, and serves as an alternative to the freeze-fracturing techniques associated with conventional cryo-SEM. For soft materials in particular, the quality of FIB SEM results is dependent on correct preparation of the specimen's top surface, which is rather challenging for specimens at low temperature. We therefore demonstrate methods for 'cold deposition' of a protective, planarising surface layer on a cryo-prepared sample, enabling high-quality cross-sectioning and investigation of structures at the nano-scale.

Keywords: focused ion beam, FIB SEM, polymers, milling, ice etching, cold deposition, soft materials, biological samples

1. INTRODUCTION TO FIB SEM

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. 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' or 'milled'). This gives us the possibility to selectively mill material to make cross-sections, lamellae and patterns. In addition, materials with various electrical properties can be added by vapor deposition from a suitable gas. In fact, this type of well-controlled *in situ* chemical vapor deposition is becoming increasingly important in the field of nanofabrication and nanoprototyping [1]. We now wish to explore the possibilities for applying FIB technology in the rather different context of soft materials, where we extend cryoSEM techniques to enable cryoFIB SEM.

1.1 Focused ion beam scanning electron microscopy (FIB SEM)

There are 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). The diagram in Figure 1 depicts a specimen tilted normal to the ion beam, with a wedge-shaped volume of material removed by FIB milling. The quantity of material removed is a function of ion flux and material-dependent sputter rate. The final vertical face, parallel to the ion beam, is at a sufficient angle to enable electron imaging. The ability to FIB mill into the bulk of a specimen means that we can open up the third dimension and explore the otherwise hidden internal microstructure.

* Debbie.Stokes@fei.com

Milling and imaging can be done in a sequential manner: the cross-section can be milled by a given amount and 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, maximizing the useful work performed by the instrument (e.g. overnight running). An example is shown in Figure 2, where a frozen polymeric sphere has been successively FIB-milled throughout its bulk. For a truly three-dimensional view, the final step is to apply volume rendering to the two-dimensional images, using appropriate software (see, for example, [2]) to yield a 3D reconstruction.

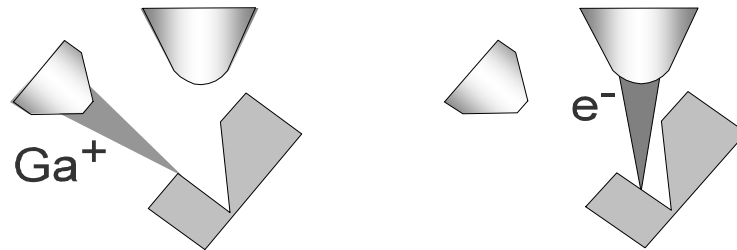


Fig. 1. With the specimen surface tilted normal to the ion beam (left), a perpendicular cross-section is made that can be imaged with the electron beam (right).

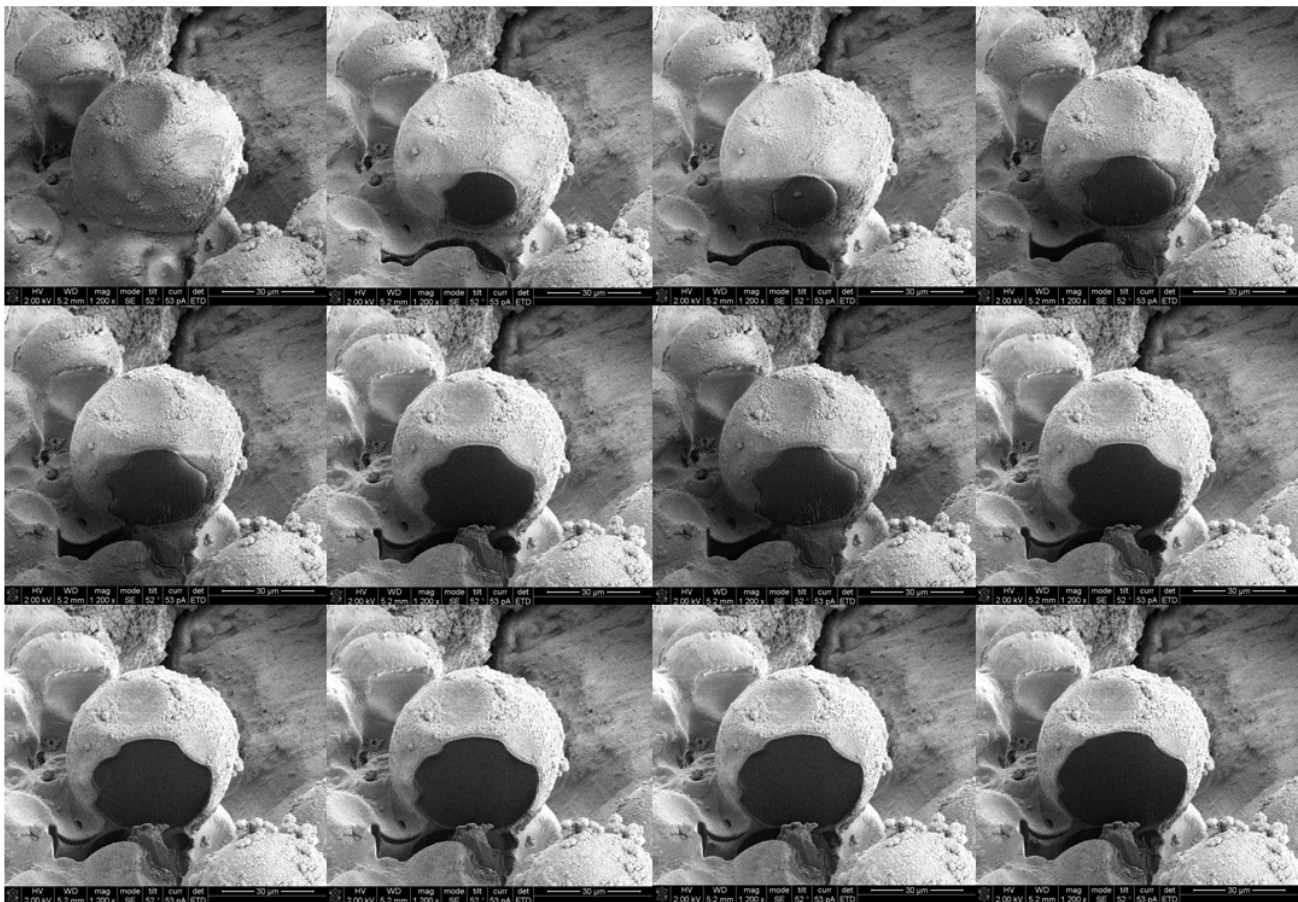


Fig. 2. A series of 'slices' can be milled with the ion beam and sequentially imaged between each step using the electron beam. This is demonstrated on a sample of frozen polymeric spheres. Scale bar = 30 µm.

1.2 Gas injection and chemical vapor deposition

Before FIB milling of specimens, it is advantageous to carry out localised *in situ* chemical vapour deposition (CVD) of material, commonly a metal such as platinum or tungsten, via a gas injection system (GIS). The GIS is specifically designed to deposit or etch materials at or near room temperature. The GIS incorporates a crucible containing an organic precursor material with metal ligands (platinum, tungsten, etc). Under normal operation, the compound is heated in the crucible to produce a desired vapor pressure. The gas is then released by a valve mechanism and delivered to the sample by means of a narrow hypodermic-style needle that is positioned over the sample target area. Low energy excited or secondary electrons or ions, generated by either the primary ion or electron beam, then collide with and decompose the precursor gas into metallic and (volatile) organic components, leaving a metallic layer on the sample surface (see, for example, Orloff *et al* 2003 [3]).

This process helps to protect top surfaces from unwanted ion beam erosion, preserves topographical features against re-deposition of material and minimizes ‘curtaining’ artefacts (uneven vertical striations) on cross-sectioned faces. We will see the results of some of these effects in Section 3.2.

2. CRYO-PREPARATION TECHNIQUES FOR SOFT MATERIALS

2.1 Conventional cryo-preparation techniques

Cryo-transfer systems have been available for electron optical instruments for a few decades, and cryo-scanning electron microscopy (cryoSEM) has established itself as a technique of major importance, especially for specimens with high water content. In general, the advantages of cryo-immobilisation in conjunction with SEM are widely accepted for applications in the life sciences [4] and for soft materials such as polymers, gels and emulsions [5-10].

Frozen-hydrated samples examined by cryo-SEM demonstrate superior preservation compared with chemically-fixed and dried specimens because they retain all or most of their water. Structures such as cells appear fully turgid, showing little distortion or shrinkage, and extracellular secretions are well preserved (it is well-known that chemical fixation and dehydration of living cells can introduce many preparation artefacts). There are numerous reports in the literature of cryo-SEM of botanical specimens, used as a routine technique since the 1980's (see, for example, [11-14]). Cryo-immobilisation allows more rapid immobilisation of the sample than chemical fixation and can therefore capture dynamic or transient events, for example spore discharge [15]. Cryo-prepared samples are not exposed to solvent extraction and therefore any fine structures and the spatial relationships of delicate, labile materials are well preserved, such as the conidia-bearing structures of fungi [16] and powdery mildew growing on the surface of leaves [17]. Such samples cannot be as successfully prepared by chemical fixation and critical point drying, and so cryo-preparation has become the method of choice. Figure 3 shows an Arabidopsis leaf infested with virus, cryo-prepared for conventional cryoSEM.

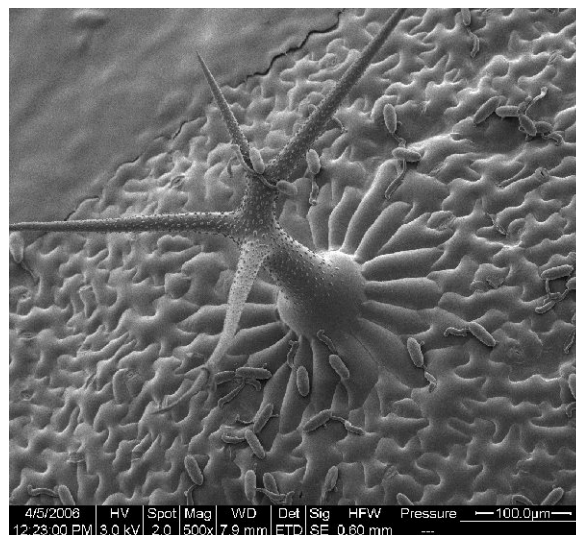


Fig. 3. Cryo-prepared virus-infested Arabidopsis trichome studied by conventional cryoSEM.

Hydrated specimens are generally cryo-immobilised by a process known as plunge-freezing, in sub-cooled ('slushy') liquid N₂ at -210°C (Umrath, 1974), liquid ethane or liquid propane. This method has been well-investigated, both experimentally and theoretically (Elder 1989). Samples can also be prepared by high-pressure freezing, depending on the size and nature of the samples to be studied and the quality and depth of freezing required [18, 19]. Frozen samples are then transferred *in vacuo* to a cryo-preparation chamber where they can be fractured and sputter-coated with a conductive layer. After transfer to the SEM chamber, samples can be examined in high vacuum on the cryo-SEM stage.

In addition to the high-quality surface information that can be gained from biological samples using this method, cryo-fracture techniques in the SEM combined with high resolution field emission microscopes, have enabled investigation of internal ultrastructural details, such as intra-membranous particles in yeast [20]. An example of a cryo-preserved, freeze-fractured yeast specimen is shown in Figure 4. Fracturing, however, does not readily show the contact relationship between, for example, membranes, and the extent and complexity of structures continuing within the cell volume. Fracture planes generally propagate through the weakest points of a sample, and are therefore considered to be relatively uncontrolled and random. One might argue that, instead of freeze-fracturing, one should turn to embedding and sectioning to prepare the sample for the transmission electron microscope. However, some samples are not only difficult to chemically fix and embed, but they can also be difficult to physically section using an ultramicrotome, due to density or hardness variations within a sample. In plants, there are often areas, such as the interface between resin, waxy cuticle and thick epidermal cell wall in some leaf samples, which make successful sectioning difficult.

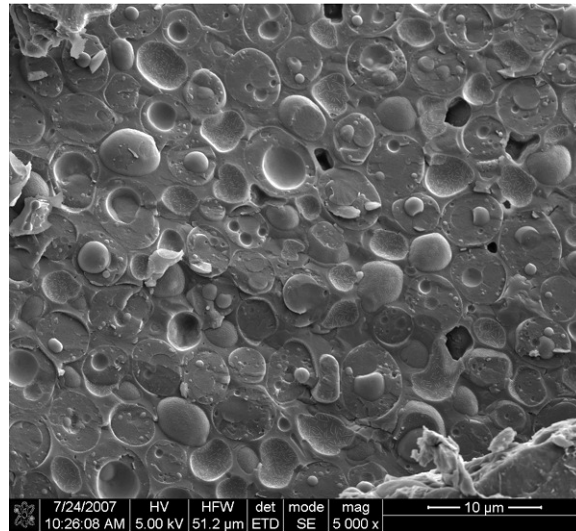


Fig. 4. A frozen-hydrated suspension of yeast cells following freeze-fracture in a cryo-preparation chamber and transfer to the cryo-stage of the SEM.

A further technique, known as cryo-planing, has been used to prepare the surfaces of high-pressure frozen blocks for high resolution cryo-SEM [21-23]. Ultimately, researchers may be interested in specific points within a three-dimensional structure, which are difficult to see or access unless first viewed at electron optical magnification in order to precisely choose a specific feature before cutting. Clearly a method for *in situ* site specific cutting would be advantageous, as we introduce in the next section.

Finally, 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. 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 solar cells, 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.

2.2 Combining cryo-preparation and FIB SEM

For specimens containing soft, polymeric materials, the sectioning methods described in Section 2.1 can give less than optimal results at room temperature, depending on the glass transition temperature T_g of the material. Cryo-immobilisation is therefore sometimes useful for conferring rigidity, to preserve specimen integrity and avoid distortion during the sectioning process. In the case where water is not a significant part of the microstructure, then the freezing process requires few special precautions and the quality of results is not dependent on freezing rate.

Biological specimens, however, present special difficulties for cryogenic treatment; thick cell walls hinder rapid freezing and it is almost impossible to freeze the large, water-filled vacuoles present in many plant cells without the formation of some ice crystals. In practice, freezing methods aim to remove the heat of a hydrated specimen as quickly as possible to keep ice crystals to a sufficiently small size (less than a few nanometres) so that they do not obscure cell ultrastructure. This subject has been reviewed fully by [24]. For a large range of cryo-fractured specimens, plunge freezing in liquid nitrogen slush is perfectly adequate, since this reveals sufficient details at the cellular level to enable, for example, measurements of cell size and cell wall thickness [25]. The simplicity, speed and low cost of simple plunging-freezing methods have led to their widespread and accepted use in many circumstances for cryo-SEM sample preparation. Using no chemical treatments, i.e. chemical fixation or cryoprotection (replacement of water with compounds such as glycerol that freeze amorphously), can be more attractive and often preferable to dealing with the consequences of interpreting chemically induced artefacts. However, this may not prevent all cryo-artefacts, such as eutectic ice ridges.

An alternative means of sectioning is to use a focused ion beam (FIB) to selectively remove material and thereby create a cross-sectional face, as previously discussed. Using FIB, one can decide precisely where one wishes to cut through a sample in a site-specific manner. The ion beam can be successfully used to mill any material, regardless of its hardness, to explore, for example, the interfaces between different materials and the morphology and inter-relationships between phases. As we have already seen, FIB milling capabilities can be combined with SEM imaging (FIB SEM), and some recent examples of the use of FIB SEM for biological materials include cyanobacteria on calcite crystals, [26], gland cells [27], tissue-biomaterial interfaces [28] and bone cells on hydroxyapatite [29]. FIB milling of temperature-sensitive samples such as polymers, foodstuffs, cosmetics and many life science samples can result in damage and/or deformation because of lack of rigidity at room temperature and acute sensitivity to either the ion or electron beam. These samples therefore benefit from cryo-immobilisation to permit successful milling and imaging, although the development of cryo-techniques in conjunction with FIB or FIB SEM is at quite an early stage [2, 30].

Cryo-prepared specimen surfaces should be protected by CVD prior to FIB milling (see Section 3), although the normal practice does not work satisfactorily at low temperatures due to the relatively low vapour pressure of the precursor organometallic gas (such as methylcyclopentadienyl platinum trimethyl, $(CH_3)_3(CH_3C_5H_4)Pt$). Precipitation onto the cold specimen surface occurs before the gas can be degraded. This leaves a thick layer of non-conductive organic compound, with an inhomogeneous morphology that is unsuitable for the purposes of protection and planarisation. We have successfully developed a new approach that has enabled us to perform controlled deposition onto biological samples, polymers, foodstuffs and cosmetics at low temperatures.

3. CRYO-FIB SEM IN PRACTICE

3.1 Materials and methods

Procedures for cryo-preservation and transfer of the various specimens presented here was similar to that described in Hayles *et al* [32]. Experiments were performed using an FEI Nova NanoLab DualBeam™, with a field emission electron source and in-lens electron detectors, and an FEI Quanta 3D DualBeam™ with a tungsten hairpin filament electron source. Both employ similar ion columns with gallium ion sources. Low temperature experiments were carried out using a Quorum PP2000T cryo-transfer system (fitted to a Nova NanoLab DualBeam™) and a Gatan Alto 2500 cryo-transfer system (fitted to a Quanta 3D DualBeam™). For milling of large cross-sectional areas, ion beam current = 20 nA, accelerating voltage = 30 kV. For final polishing, ion beam current = 500 pA. After FIB-milling of biological specimens the sample temperature was raised to -95°C, and the ice allowed to etch briefly, whilst imaging

with the electron beam. For a final, high-quality image, the sample was withdrawn to the cryo-preparation chamber for sputter-coating with a thin layer of platinum (at least 2 nm), before being returned to the cryo-stage in the main chamber of the FIB SEM for electron beam imaging.

3.2 The need to protect the specimen surface

The emphasis of the present study is on the successful FIB milling of cryo-prepared soft materials with minimal artefacts. For example, Figure 5 shows how failure to adapt known techniques can lead to sub-optimal results in the FIB SEM. Here, a conventionally cryo-prepared specimen of dandelion leaf exhibits ‘curtaining’ during FIB-milling because the traditional thin conductive sputter coating applied to the top surface has degraded during repeated scans of the ion beam. Sputter coating is a common method for making surfaces electrically conductive for imaging, and the spurious effects of ion beam degradation have been reported for Au/Pd coating on silicon by Kempshall *et al* [31], who also showed that the more continuous nature of sputtered chromium is better in this regard. As a result of ion beam penetration between gaps in the coating, the sample surface becomes damaged. This generates a non-planar texture that is carried perpendicularly down the cross-sectional surface due to differential milling. The solution is to deposit a planarising top layer using CVD. But, as mentioned in the previous section, using a GIS to deposit a (platinum-containing) layer under low temperature conditions calls for a different approach to that for samples at ambient temperatures, as will be described in Section 3.3.

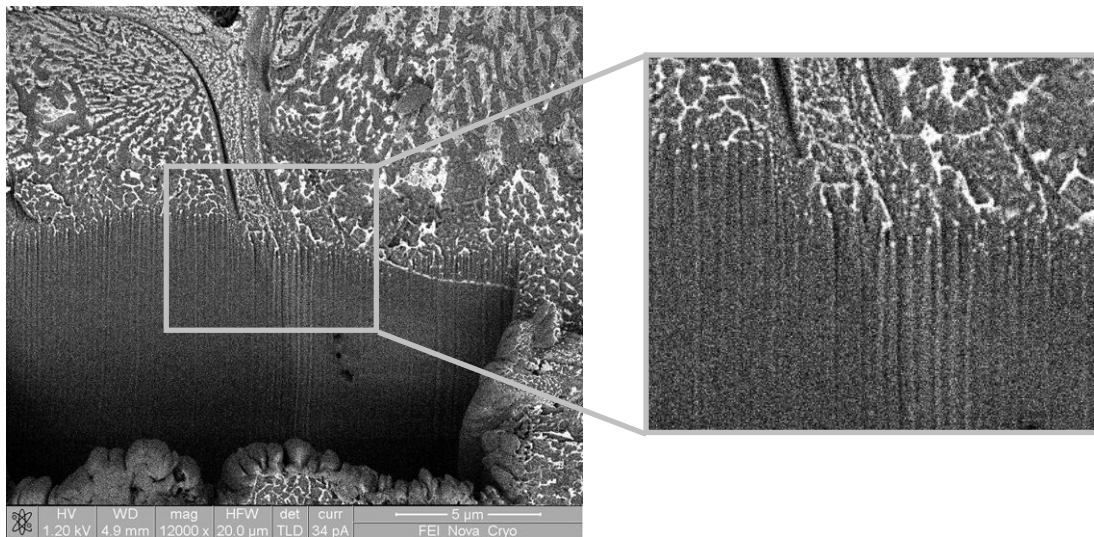


Figure 5. An example of the effects of FIB ‘curtaining’. The specimen has been cryo-preserved, freeze-fractured and coated as for conventional cryoSEM. Scanning of the ion beam normal to the top surface has caused the thin coating to break up into islands, giving this surface a rough texture, resulting in differential milling (curtaining) down the cross-sectional surface, perpendicular to the ion beam. Inset: detail of central features where the curtaining effect is more easily seen.

3.3 Cold deposition to protect the surface

In routine FIB SEM, the precursor gas for CVD is usually heated slightly to increase its vapour pressure. However, by reducing the GIS temperature (to 25°C), the flow of gas being released from the GIS can be slowed to the extent that the deposition process is more easily controlled, and therefore the layer thickness reduced to an appropriate level. Since this is a lower temperature than generally used, together with the fact that the sample is frozen, we term this ‘cold-deposition’.

For standard ambient conditions, a needle-to-sample distance of around 50-100µm is acceptable. But for low temperature conditions, and in particular for highly topographical samples, a much greater distance of between 300 µm - 2 mm is found to be necessary, to disperse the deposit more evenly and also to avoid collision of the needle with any protruding

surface structures. The GIS device can be set so that the needle-to-sample distance at the eucentric position is within these limits and therefore retains coincidence of the ion and electron beams at a specific point on the sample. If the sample topography is of an acute nature then the stage can also be lowered, but eucentricity must be re-established after deposition has taken place. For this work, once the required features of the sample had been located by electron beam imaging, the sample stage was lowered slightly to increase the distance from the inserted needle of the GIS. The sample stage was tilted perpendicular to the GIS needle, for a more even surface coating. Thicknesses of 0.5 to 2 μm of deposition are preferable, particularly for topographic observation of underlying surface features. For 3 seconds deposition time at a GIS temperature of 25°C, approximately 1 - 2 μm thicknesses of Pt were achieved. Electron beam imaging was used to observe the deposition process at low magnification. Note that the ion beam was not in operation during this procedure. Unlike beam-assisted CVD, it is the thermal gradient between the deposition gas and the cold specimen surface that drives the deposition process. Figure 6 shows low magnification secondary electron images depicting the GIS in two positions; the first close to the specimen surface as used for regular beam-assisted CVD, the second a more remote location for cold deposition. The tilt angle of the sample to the GIS is another important factor. Adjusting the tilt angle of the sample to a more favourable position, with the features of interest perpendicular to the GIS needle, can play a significant part in overcoming shadowing effects likely to be caused by other parts of the sample structure. After cold-deposition, the GIS needle is retracted and the sample stage returned to its imaging position.

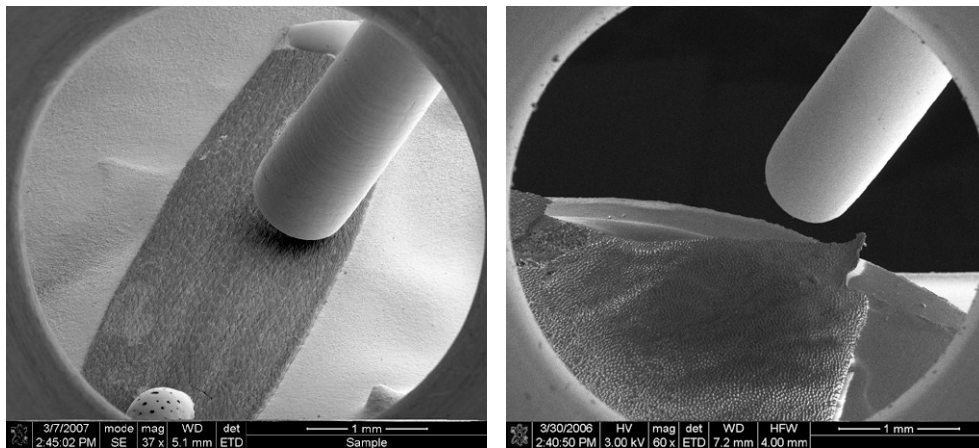


Figure 6. Showing the differences in gas injector (GIS) nozzle proximity to specimen surface for (a) conventional CVD and (b) cold deposition of precursor. The GIS nozzle is typically a few tens of microns above the surface for the situation in (a) whereas it can be hundreds of microns to a couple of millimeters away for the case in (b). Specimens are frozen-hydrated plant material.

Poor conductivity is often an issue when observing soft insulating materials or specimens of high water content which have been cryo-immobilised without the addition of electron dense stains. It should be noted that the platinum-based organic compound deposited in the way described does not aid electrical conductivity, but is used simply to enhance surface protection and provide the necessary planarisation for smooth cross-sectioning. Specimens can be transferred back to the cryo-preparation chamber for sputtering with a thin layer of Pt to make the freshly etched surface conductive before being returned to the sample chamber of the FIB SEM for imaging.

As a next step, we were able to modify the deposited Pt layer to form a very smooth surface and therefore provide an even better cutting platform. This was achieved by exposing an area to a 30kV ion beam at a current of 1 nA for 30 seconds. Figure 7 shows a rectangular area that has been smoothed in this way. When the deposited layer is subsequently milled, it shows a solid homogeneous structure, similar to that expected of material conventionally deposited by beam exposure at room temperature. Provided that the deposition thickness is maintained at around 2 μm or more, no damage to the underlying sample surface has been observed using this technique. Note that it is likely that this compacted material is a result of the driving off of hydrocarbons by the ion beam to give a more pure metallic deposit and so may make for a more conductive surface layer than the unsmoothed deposit referred to in the previous paragraph. This aspect remains to be studied and measured systematically.

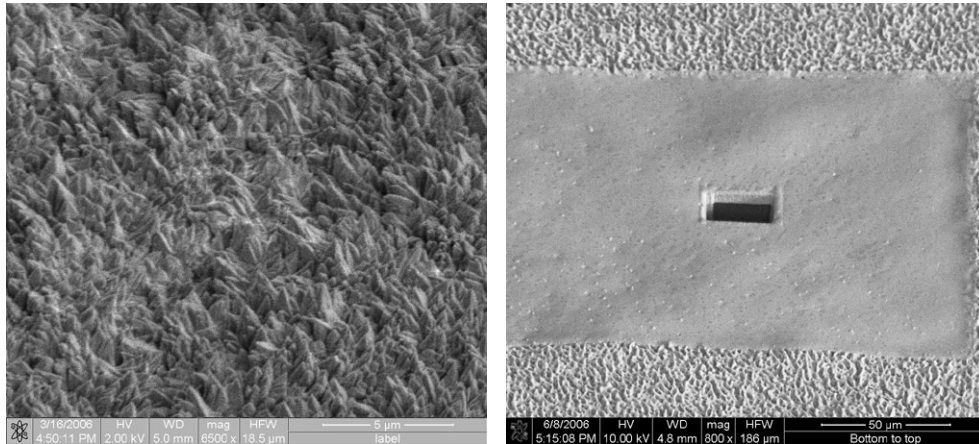


Figure 7 showing (a) a thick, rough deposit that results from the direct deposition of precursor gas as a solid at cryogenic specimen temperatures with the GIS in the position shown in Fig 6(a). (b) A smoother deposit is formed when the precursor is not heated and the GIS held further away from the surface (as per Fig 6(b)). Further planarising of the surface by brief exposure to the ion beam leads to a high-quality surface for FIB milling. Image shows a large smooth area containing a small FIB-milled rectangle.

3.4 Non hydrated specimens

As mentioned in Section 2.2, non hydrated materials such as polymers benefit from cryogenic treatment to maintain structural integrity. Figure 8 shows a relatively large cross-section through a reinforced ‘liquid skin’ patch. Liquid skin patches are made for various injuries from grazed skin to gunshot wounds. The reinforcing is embedded at the back of the patch for patches that are used for severe or large wounds. Bonding of the reinforcement within the liquid skin is important for reliable and durable application.

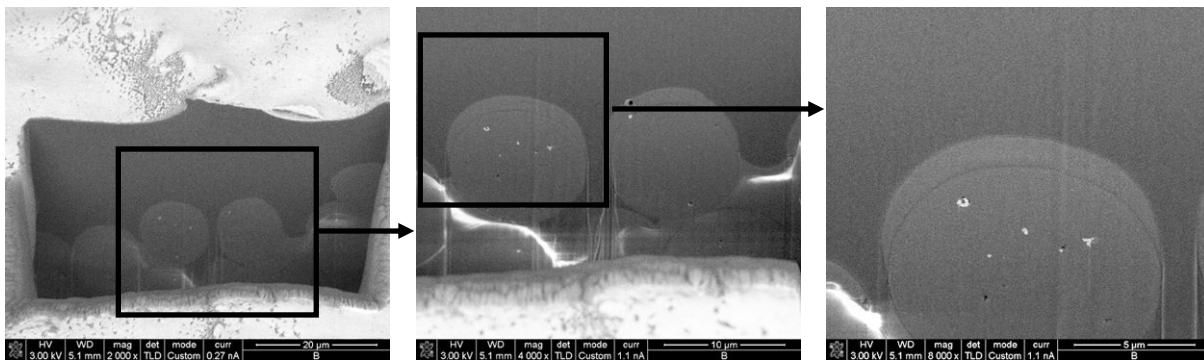


Figure 8. Secondary electron images of a cryo-FIB section through material used as ‘liquid skin’. (a) Shows the thickness of liquid skin before the reinforcement. (b) Shows the well bonded interface of the reinforcing fibres to ‘liquid’ material. (c) Shows the polymer fibre cross-section with coating surround and internal detail.

Figure 9, meanwhile, is an example of backscattered electron imaging to differentiate different components of a specimen based on atomic number (Z) contrast. The specimen is a cosmetic foundation cream in which a lipid component (round particles) appears dark, colour pigment (iron oxide) appears white and protein-rich structures appear as elongated features against an emulsion (oil/water) background.

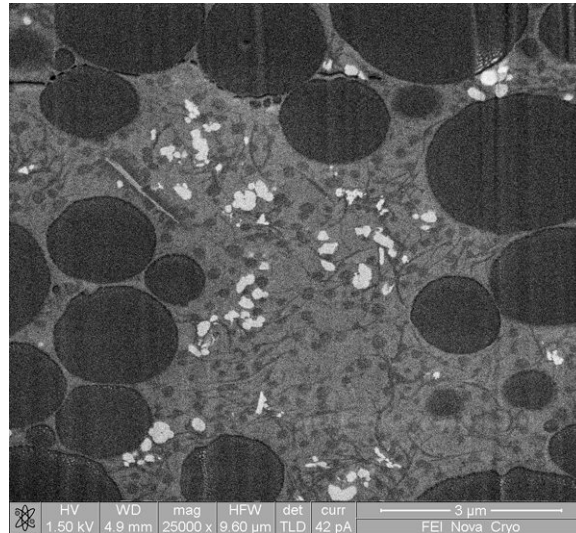


Figure 9. Backscattered electron image (z-contrast) demonstrating the wealth of information available from this cryoFIB-prepared specimen of cosmetic foundation cream. See text for discussion.

3.5 Frozen-hydrated specimens

Although cryo-preservation at the specimen surface will be good, plunge frozen-hydrated specimens will generally be poorly preserved in the centre, due to ice crystal growth during freezing. Only if the sample is extremely small (typically less than 20 μm for plunge-frozen specimens and less than 300 μm if high-pressure frozen), can cryo-immobilisation produce true vitreous ice inside the sample [33]. Therefore, cryo-fracturing can only yield really high-quality information about internal microstructures if the sample is very small and the method of freezing, and the cryogen chosen, provides a fast enough cooling rate to result in a well-frozen sample. Note that, if using high-pressure freezing, it would not be possible to sublimate water without the risk of phase changes that allow re-crystallisation of ice.

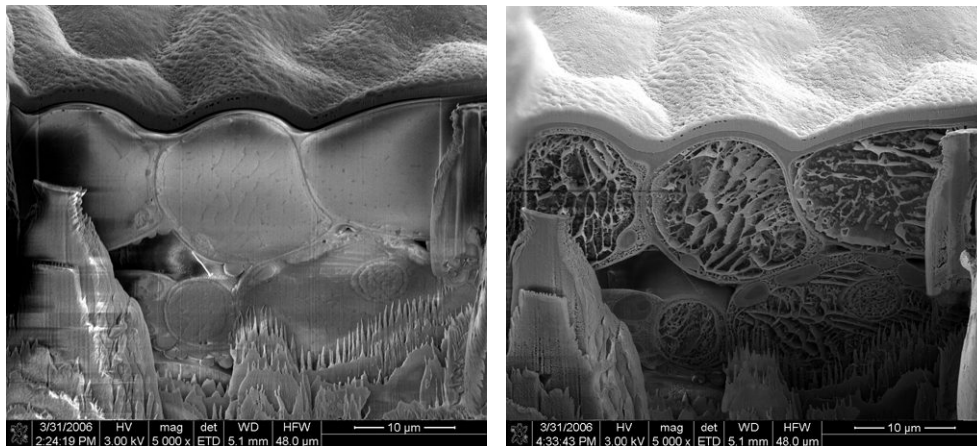


Figure 10. (a) Shows the initially smooth cross-section produced by FIB milling, while (b) demonstrates that a brief thermal etch of the ice helps to bring out structural information. This is similar to the post freeze-fracture step carried out in conventional cryoSEM.

After the milled face of the sample is given a final ‘polish’ with the ion beam, the surface is very smooth and shows little contrast. This is common to cellular material that has been cryo-immobilised but not chemically treated with an electron-

dense stain. A brief temperature-controlled ice-etch is therefore very effective in providing surface relief for topographic imaging, as can be seen in Figure 10. Any devitrification of ice that occurs at the surface does not appear to significantly impair the information being sought. The eutectic ice ridges seen in Figures 10 are within the cells, which are not the areas under investigation in this case, and are virtually impossible to freeze without any ice crystals forming, whatever method of freezing is chosen.

A similar comment applies to the result shown in Figure 11, where an epidermal cell from the petal of a tobacco plant has been cryoFIB-sectioned through its apex. The cell wall, plasma membrane and cytoplasm were sufficiently well frozen to enable cell wall thicknesses to be measured, demonstrating the relationship between the shape of the cell and how the cell wall thickness varies around the contours of the outer epidermal wall. In addition, it is possible to visualise lower-lying structures such as the chloroplasts shown in the inset image to the right. The epidermal cells on these petals vary in shape from flat, slightly-domed to cone shaped, in various positions on the petal. For this sample we aimed to see, in particular, how the cell wall thickness varies at the top of the dome-shaped cells when compared with the base of the same cell. We had been unable to explore this by freeze-fracture methods alone, since the fracture plane almost always goes around the base of the cell and not through the tip.

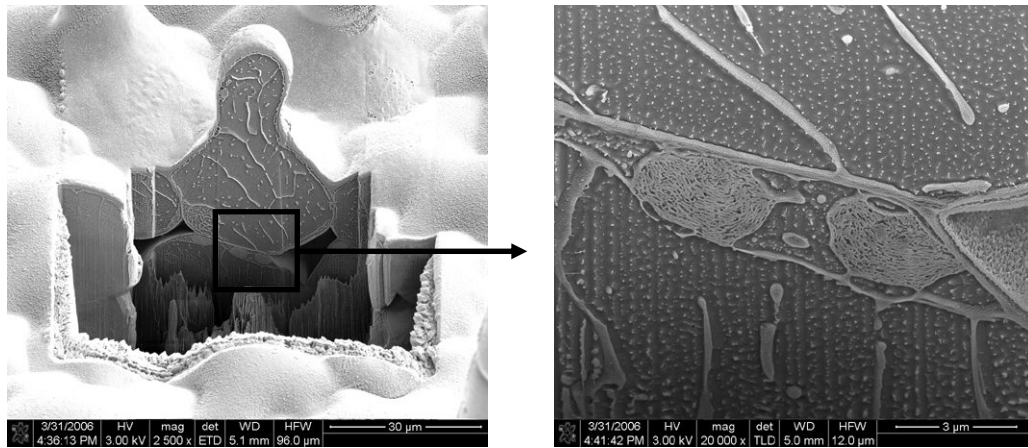


Figure 11. (a) SEM micrograph showing a cryoFIB cross-section of the petal of a tobacco plant flower and (b) a higher magnification image showing details of cell membranes and chloroplasts

4. CONCLUSIONS

FIB-based processing of soft materials is a relatively new technique and holds great potential for site-specific cross-sectioning into the bulk material as well as sequential slicing to produce images for 3D visualisation. We have described a technique that combines cryo-preparation of a sample and gas chemistry to allow milling of frozen, temperature-sensitive samples which, as a result, display little or no damage or degrading effects. This has been achieved by modification of the procedure used for chemical vapour deposition, involving the physical settings of the gas injector system and those of the cryo sample. For high resolution ultrastructural studies at greater depths or to study other types of sample, we intend to progress to high-pressure freezing [34]. In these cases, we shall assess the advantages and disadvantages of aiding contrast by osmication of the material prior to freezing and view the contrast using a backscatter detector method [19], which may mean that the need for any sublimation of frozen-hydrated specimens is avoided. There are many samples from which adequate resolution can be obtained after simple plunge-freezing that would benefit from the cryo-SEM FIB combination. We have tested this process using a variety of samples which would otherwise be difficult, time-consuming or impossible to prepare by other methods. Work on techniques to improve the status of the FIB milled surface of cryo-immobilised samples will also continue, along with more detailed studies of the chemical and physical mechanisms involved.

ACKNOWLEDGEMENTS

The authors would like to thank the following for their valuable contributions to this work: Kim Findlay, John Innes Centre, UK, Steve Reyntjens and Daniel Phifer FEI Company, The Netherlands and Hiroshi Fujitani, FEI Company, Japan.

REFERENCES

- [1] Wilhelmi, O., Reyntjens, S., Mitterbauer, C., Roussel, L., Stokes, D.J., and Hubert, D.H.W., *Rapid prototyping of nanostructured materials with a focused ion beam*. Japanese Journal Of Applied Physics, 2008. 47(6): p. 5010-5014.
- [2] Heymann, J.A.W., Hayles, M., Gestmann, I., Giannuzzi, L.A., Lich, B., and Subramaniam, S., *Site-specific 3D imaging of cells and tissues with a dual beam microscope*. Journal Of Structural Biology, 2006. 155(1): p. 63-73.
- [3] Orloff, J., Utlaut, M and Swanson, L, *High Resolution Focused Ion Beams, FIB and Its Applications*. 2003, New York: Kluwer Academic/Plenum.
- [4] Echlin, P., *Low Temperature Microscopy and Analysis*. 1992, New York: Plenum.
- [5] Goff, H.D., Verespej, E., and Smith, A.K., *A Study of Fat and Air Structures in Ice Cream*. International Dairy Journal, 1999. 9: p. 817-829.
- [6] Kishi, R., Miura, T., Kihara, H., Asano, T., Shibata, M., and Yosomiya, R., *Fast pH-thermo-responsive copolymer hydrogels with micro-porous structures*. Journal Of Applied Polymer Science, 2003. 89(1): p. 75-84.
- [7] Stokes, D.J., Mugnier, J.Y., and Clarke, C.J., *Static and dynamic experiments in cryo-electron microscopy: comparative observations using high-vacuum, low-voltage and low-vacuum SEM*. Journal Of Microscopy-Oxford, 2004. 213: p. 198-204.
- [8] van Duynhoven, J.P.M., Broekmann, I., Sein, A., van Kempen, G.M.P., Goudappel, G.J.W., and Veeman, W.S., *Microstructural investigation of monoglyceride-water coagel systems by NMR and CryoSEM*. Journal Of Colloid And Interface Science, 2005. 285(2): p. 703-710.
- [9] Gonzalez-Meijome, J.M., Lopez-Aleman, A., Almeida, J.B., Parafita, M.A., and Refojo, M.F., *Microscopic observations of superficial ultrastructure of unworn siloxane-hydrogel contact lenses by cryo-scanning electron microscopy*. Journal Of Biomedical Materials Research Part B-Applied Biomaterials, 2006. 76B(2): p. 419-423.
- [10] Schaper, A.K., Yoshioka, T., Ogawa, T., and Tsuji, M., *Electron microscopy and diffraction of radiation-sensitive nanostructured materials*. Journal Of Microscopy-Oxford, 2006. 223: p. 88-95.
- [11] Vartanian, N., Wertheimer, D.S., and Couderc, H., *Scanning electron microscopic aspects of short tuberized roots, with special reference to cell rhizodermis evolution under drought and rehydration*. Plant Cell Environ., 1982. 6(39-46).
- [12] Sargent, J., *Low-temperature scanning electron microscopy - advantages and applications*. Scanning Microscopy, 1988. 2: p. 835-49.
- [13] Read, N. and Jeffree, C., *Low temperature scanning electron microscopy in biology*. J. Microscopy, 1991. 161: p. 59-72.
- [14] Burton, R., Gibeaut, D., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D., and Fincher, G., *Virus-induced silencing of a plant cellulose synthase gene*. The Plant Cell, 2000. 12: p. 691-705.
- [15] McLaughlin, D., Beckett, A., and Yoon, K., *Ultrastructure and evolution of ballistosporic basidiospore*. Bot.J. Linnean Soc, 1985. 91: p. 253-71.
- [16] Sadanandom, A., Findlay, K., Doures, J., Schulze-Lefert, P., and Shirasu, K., *CHPA, a Cysteine- and Histidine-Rich-Domain-Containing Protein, contributes to maintenance of the diploid state in Aspergillus nidulans*. Eukaryotic Cell, 2004. Aug 2004: p. 984-991.
- [17] Xiao, S., Ellwood, S., Findlay, K., Oliver, R., and Turner, J., *Characterization of three loci controlling resistance of Arabidopsis thaliana accession Ms-0 to two powdery mildew diseases*. The Plant Journal, 1997. 12(4): p. 757-768.
- [18] Studer, D., Graber, W., Al-Amoudi, A., and Eggli, P., *A new approach for cryofixation by high-pressure freezing*. Journal Of Microscopy-Oxford, 2001. 203: p. 285-294.
- [19] Walther, P., *Recent progress in freeze-fracturing of high-pressure frozen samples*. Journal Of Microscopy-Oxford, 2003. 212: p. 34-43.
- [20] Walther, P., Hentschel, J., Herter, P., Muller, T., and Zierold, K., *Imaging of Intramembranous Particles in Frozen-Hydrated Cells (Saccharomyces cerevisiae) by High Resolution Cryo-SEM*. Scanning, 1990. 12: p. 300-307.

- [21] Nijssse, J. and van Aelst, A.C., *Cryo-planing for cryo-scanning electron microscopy*. Scanning, 1999. 21(6): p. 372-378.
- [22] Walther, P. and Muller, M., *Biological ultrastructure as revealed by high resolution cryo-SEM of block faces after cryo-sectioning*. Journal Of Microscopy-Oxford, 1999. 196: p. 279-287.
- [23] Refshauge, S., Watt, M., McCully, M.E., and Huang, C.X., *Frozen in time: a new method using cryo-scanning electron microscopy to visualize root-fungal interactions*. New Phytologist, 2006. 172(2): p. 369-374.
- [24] Sitte, H., Edelmann, L., and Neumann, K., *Cryo fixation without pretreatment at ambient pressure*, in *Cryo Techniques in Biological Electron Microscopy*, R.A.S.a.K. Zierold, Editor. 1987, Springer-Verlag: Heidelberg, Berlin. p. 87-113.
- [25] Ryden, P., Sugimoto Shirasu, K., Smith, A., Findlay, K., Dieter Rieter, W. and McCann, M., *Tensile properties of Arabidopsis cell walls depend on both a Xyloglucan cross-linked microfibrillar network and Rhamnogalacturonan II-borate complexes*. Plant Physiology, 2003. 132(June): p. 1033-1040.
- [26] Obst, M., Gasser, P., Mavrocordatos, D., and Dittrich, M., *TEM-specimen preparation of cell/mineral interfaces by Focused Ion Beam milling*. American Mineralogist, 2005. 90(8-9): p. 1270-1277.
- [27] Drobne, D., Milani, M., Zrimec, A., Leser, V., and Berden Zrimec, M., *Electron and ion imaging of gland cells using the FIB/SEM system*. Journal Of Microscopy-Oxford, 2005. 219: p. 29-35.
- [28] Burkhardt, C. and Nisch, W., *Electron Microscopy on FIB prepared interfaces of biological and technical materials: First results*. Praktische Metallographie-Practical Metallography, 2005. 42(4): p. 161-171.
- [29] Stokes, D., Morrissey, F and Lich, B. *A New Approach to Studying Biological and Soft Materials using Focused Ion Beam Scanning Electron Microscopy (FIB SEM)*. in *EMAG-Nano 2005: Imaging, Analysis and Fabrication on the Nanoscale*. 2006. Leeds, UK: Institute of Physics.
- [30] Marko, M., Hsieh, C., Moberlychan, W., Mannella, C.A., and Frank, J., *Focused ion beam milling of vitreous water: prospects for an alternative to cryo-ultramicrotomy of frozen-hydrated biological samples*. Journal Of Microscopy-Oxford, 2006. 222: p. 42-47.
- [31] Kempshall, B.W., Giannuzzi, L.A., Prenitzer, B.I., Stevie, F.A., and Da, S.X., *Comparative evaluation of protective coatings and focused ion beam chemical vapor deposition processes*. Journal Of Vacuum Science & Technology B, 2002. 20(1): p. 286-290.
- [32] Hayles, M., Stokes, D.J., Phifer, D., and Findlay, K.C., *A technique for improved focused ion beam milling of cryo-prepared life science specimens*. Journal of Microscopy, 2007. 226(Pt. 3): p. 263-269.
- [33] Dubochet, J., Adrian, M., Chang, J.-J., Homo, J.-C., Lepault, J., McDowell, A.W., and Schultz, P., *Cryo-electron microscopy of vitrified specimens*. Q. Rev. Biophys, 1988. 21: p. 129-228.
- [34] Osumi, M., Konomi, M, Sugawara, T, Takagi T & Baba, M, *High-pressure freezing is a powerful tool for visualisation of Schizosaccharomyces pombe cells: ultra-low temperature and low-voltage scanning electron microscopy and immunoelectron microscopy*. Journal of Electron Microscopy, 2006. 55(2): p. 75-88.

Proc. of SPIE Vol. 7378 73780G-12

See Beyond at FEI.com

World Headquarters
Phone: +1.503.726.7500

FEI Europe
Phone: +31.40.23.56000

FEI Japan
Phone: +81.3.3740.0970

FEI Asia
Phone: +65.6272.0050

