Investigating Biological Ultrastructure using Environmental Scanning Electron Microscopy (ESEM)

Dr D.J. Stokes

Polymers & Colloids Group, University of Cambridge, Dept of Physics, Cavendish Laboratory, Madingley Road, Cambridge, CB3 0HE, UK.

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Abstract. The ability of environmental scanning electron microscopy (ESEM) to image insulating and/or moist specimens without the need for the removal of volatile components or the application of a conductive coating has significantly increased the potential range of experiments and observations that can be performed at the high resolution of electron microscopy. Such a technological advance has particularly important implications for the study of biological systems, as well as other soft, moist materials and complex fluids. Native-state biological ultrastructures are demonstrated across a range of length scales, from whole organisms to tissues and cells.

Introduction

A fundamental requirement of conventional SEM is the need for high vacuum conditions (10^{-5}-10^{-7} torr) throughout the system, in order to prevent unacceptable scattering of the primary electron beam. Specimens must be in a solid, dry state so that this high vacuum is not compromised. However, many biological specimens contain volatile substances that evaporate under high vacuum. Preparation of such specimens therefore involves treatments such as chemical fixing and dehydration or freezing. These procedures can be very sophisticated or time consuming and may change the very structural features to be examined, particularly if the specimen contains delicate membranes, leading to unwanted artefacts. Furthermore, unless the specimen is electrically conductive, a build up of negative charge can quickly result. Hence insulators must be subjected to further treatment in the form of a metallic coating. Again, the introduction of artefacts is a possibility, along with the risk of obscuring fine structural details under the coating. Dynamic experiments become difficult or impossible under these circumstances. Coated specimens give only topographic contrast, due to the short escape depths of secondary electrons from metals, and therefore valuable compositional contrast from the underlying specimen can be lost.

Development of ESEM was originally driven by a growing need to overcome the limitations of conventional SEM in order to facilitate the research and characterisation of a greater range of specimen types and expand the available experimental methodologies. In the early 1970's, research by Moncrieff *et al.* [1] led to an SEM capable of maintaining a relatively high pressure, further demonstrated by Danilatos & Robinson in 1979 [2]. The term 'environmental scanning electron microscopy' was coined[1] and, by the late 1980's, ESEMs were commercially available.

[1]In the early 1950's, workers were already experimenting with differentially pumped, aperture-limited transmission electron microscopes (others were trying thin windows). These instruments and their relative

merits are discussed in Parsons [3]. In fact, the expression 'environmental chamber' was being used at that time [4].

Modern models of this versatile microscope can be operated at three principal levels: high vacuum, low vacuum and ESEM modes. The latter two modes of operation are discussed in more detail below, but the differences between all three modes can be defined as follows: High vacuum mode is essentially the same as in conventional SEM and is used for the observation of dry, electrically conducting specimens. Hence the traditional specimen preparation constraints apply to the use of this mode. Low vacuum mode involves a small partial pressure of gas in the specimen chamber (nominally around 0.1-2.5 torr) and is used to study dry, electrically *insulating* specimens without a conductive coating. ESEM mode involves the specific use of water vapour in the specimen chamber and operates at higher pressures (around 2.5-10 torr). In conjunction with specimen temperature control, ESEM mode is used for imaging *moist, insulating* specimens without dehydration or coating [5].

ESEM mode is usually the most appropriate means of stabilising and imaging biological specimens in their natural state, since the vapour itself plays an active role in maintaining equilibrium between the chamber environment and the specimen. Even entirely liquid, bulk specimens can be observed in this way [6]. Low vacuum mode can be used for biological specimens that contain small amounts of water, particularly if the specimen has a tough or waxy exterior (for example, many insects and some plants), since moisture loss is not a critical consideration.

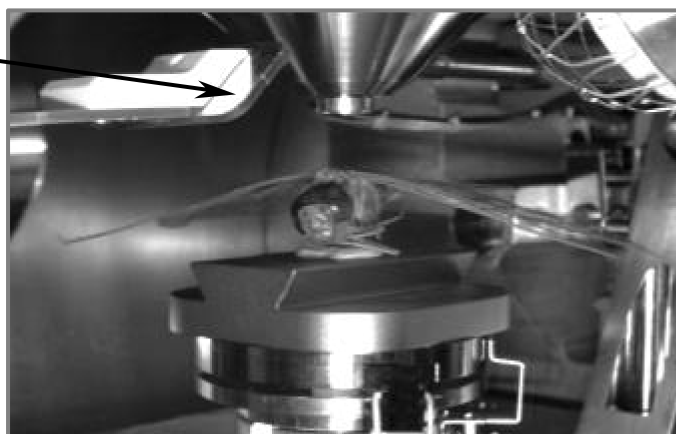

Figure 1. A dragonfly (wingspan ~12 cm) awaits investigation in the ESEM specimen chamber.

Principles of ESEM

ESEM utilises two major innovations in order to operate in ESEM and low vacuum modes: differential pumping of the column and gaseous secondary electron detection.

Differential Pumping. The column of the ESEM is divided into zones of varying pressure using an ion pump, diffusion pumps and rotary pumps. Pressure limiting apertures then allow the electron beam to pass through, but are sufficiently small to maintain the pressure difference between each zone. The filament and upper parts of the column are thus maintained at high vacuum, whilst the sample chamber can be held at a much lower vacuum (up to ~10 torr). The mean free paths of high-energy primary electrons are on the order of several millimeters under these low vacuum conditions. This allows the presence of a gas without destroying the criteria for imaging, provided that the working distance is appropriately short. Placing the specimen around 8mm below the objective lens[2] helps to keep scattering of the primary electron beam at an acceptably low level.

[2]This figure is nearer to 6mm for those using ESEMs from the FEI Quanta™ Series.

Inevitably, some scattering of the primary beam by the gas adds a broad 'skirt' to the beam profile, but does not alter the distribution of the remaining focused beam. Hence the signal-to-background ratio remains sufficiently high and good quality images can be produced without too much interference from the probe skirt. Under optimum conditions, resolution of a few nanometers is possible. However, it is worth mentioning that, due to the large escape depths of secondary electrons (SE) from insulators in general, images of soft, liquid or delicate materials may appear to lack the crisp definition associated with metal-coated specimens. Furthermore, if specimens have a covering of water or physiological secretions, for example, then this will further serve to reduce the sharpness of specimen features. It is therefore important to appreciate that resolution may be limited, not by the microscope's specifications, but by the nature of the specimens themselves.

Gaseous Secondary Electron Detection. The microscope's secondary electron detection system uses the principle of gaseous amplification to achieve a measurable signal. SEs escaping the sample surface are accelerated towards a positively biased electrode (typically 300-500V). The electrode can be an on-axis, annular arrangement (such as the gaseous secondary electron detector, GSED) or an off-axis planar type (the large field detector, LFD, as can be seen in Figure 1).

As SEs traverse the specimen-to-detector gap, they tend to undergo ionising collisions with gas molecules in their path. This has two important consequences: 1) it creates additional SE which, in turn, can undergo ionising collisions, creating a cascade that amplifies the total signal from a given point on the specimen surface and 2) the now-ionized gas molecules (positive ions) drift towards the sample surface, helping to compensate for the build-up of negative charge deposited within the specimen by the primary electron beam, thus obviating the need for a conductive coating on the specimen. The absence of a metallic coating also means that specimen composition-dependent secondary electron contrast can be detected, yielding further information about the material.

The efficiency of signal amplification varies as functions of gas pressure, specimen-to-detector gap, gas type and detector type [7]. Imaging gases may include nitrous oxide, carbon dioxide, helium, nitrogen and water vapour, among others, each exhibiting different amplification properties. Indeed, water vapour has been found to be the most efficient imaging gas so far tested, amplifying the SE signal arriving at the GSED by a factor of up to 10^3 [8].

Environmental Control – ESEM Mode. Maintaining specimens in a moist/liquid state is achieved by choosing appropriate environmental conditions (chamber pressure and specimen temperature) - the specimen should be held in an atmosphere corresponding to its equilibrium state in order to be stabilised against moisture loss or gain over time. Alternatively, pressures and tenperatures can be chosen for non-equilibrium conditions, so that dynamic experiments involving evaporation or condensation of water can be carried out.

Selection of suitable chamber pressures and specimen temperatures requires consideration of the phase diagram (or saturated vapour pressure curve) for pure water, as depicted in Figure 2. According to this information, a pressure of ~5.5 torr of water vapour, for example, will give conditions of 100% relative humidity if the specimen temperature is lowered to 3ºC (a water-backed Peltier stage is usually employed to cool the specimen). It is also important to ensure that the specimen's natural moisture is preserved during the initial pumpdown of the chamber. It is therefore usual to perform a sequential pumpdown such that air is purged from the chamber and successively replaced with water vapour (the *purge-flood* cycle). An example regime involves cycling eight times between a set pressure of 5.4 torr and a flood pressure of 9.8 torr [9].

It should be remembered that any aqueous phase in the specimen will contain dissolved solutes, which tend to lower the equilibrium vapour pressure needed, relative to pure water [10]. Thus, for a specimen temperature of 3°C, water vapour pressures in the region of 3 - 4.5 torr (50% – 85% relative humidity)

are preferable [11]. Additionally, working at these slightly lower pressures helps to reduce primary electron scattering, improving the quality of images.

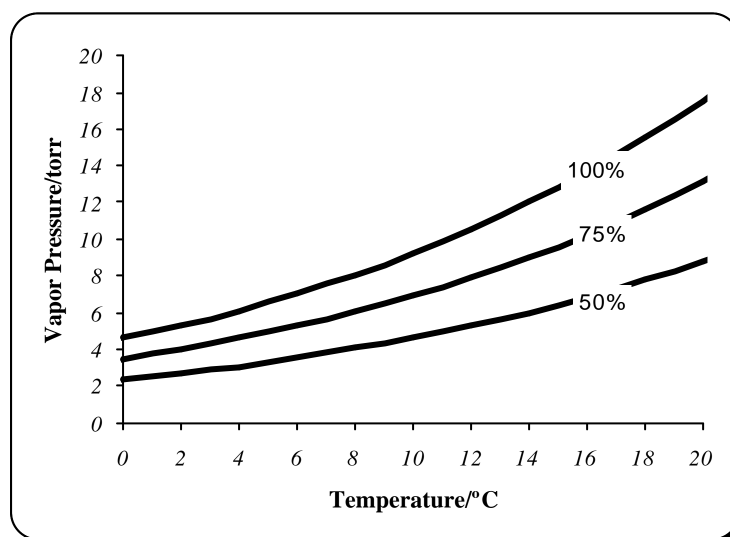

Figure 2. Part of the phase diagram for water showing data for 100%, 75% & 50% relative humidities.

Uncoated Specimens. The absence of a coating means that the native surface can be explored, and this leads to some unique features when using ESEM. Specimen-dependent SE contrast, particularly in the case of organic (low atomic number) specimens, can significantly aid the identification of different phases within the specimen [6]. However, it should be noted that image interpretation of dielectric (insulating) materials can be influenced by a number of factors. For example, localized charging can lead to transient effects on signal intensity: low signals become high signals when the scan speed or magnifications are changed [12]. Moreover, the distribution of electric fields between the specimen and the detector can be affected by positive ions, which can decrease the strength of the detector field [13], or can recombine with the low energy SE emitted by the specimen [14], both of which cause a reduction in the detected signal. Uncoated specimens are also susceptible to radiation damage, particularly in a water vapour environment. For example, a study involving polypropylene [15] showed that the deposition of condensed water layers on the polymer surface significantly increased the likelihood of damage: the highly mobile and reactive nature of radicals in liquid water increases the rate of polymer hydrolysis, compared to the dry state. Care is therefore needed in order to avoid beam-induced artefacts, and a thorough assessment of a specimen's stability under the electron beam should always be carried out prior to engaging in experiments or interpreting results.

Materials & Methods

Specimens were imaged using an Electroscan E3 ESEM equipped with a LaB_6 filament, an Electroscan 2010 ESEM equipped with a tungsten hairpin filament and a Philips XL30 ESEM equipped with a field emission gun. The beam energy was 10 keV and signals were collected using a GSED. Water vapour was used as the imaging gas in all cases, with pressures ranging from 0.9 torr to 4.5 torr, depending on specimen type. Specimens containing water were maintained at a temperature of 3°C during pumpdown

and imaging, and a purge-flood cycle was performed at pumpdown. Specimens were mounted on double-sided carbon adhesive tape.

Results and Discussion

Three categories of specimen were selected on the basis of demonstrating the broad applicability of the technique. Examples using both ESEM and low vacuum modes serve to illustrate biological specimens of varying properties and lengthscales.

The first example demonstrates the application of ESEM mode to a whole (live) organism. Fig. 3 shows part of the eye of a fruit fly (*Drosophila*): the specimen was held at a temperature of 3ºC in a water vapour environment at a pressure of 3.5 torr. These conditions were used to successfully preserve the fly in a living state throughout and beyond the imaging process. For a creature such as this in a non-living state, low vacuum conditions would suffice (c.f. the chamber set-up shown in Fig 1, for example). Note that a cool thermal environment tends to slow down living processes, and so very little movement of the fly was observed during imaging. The compound sections of the eye are clear of the detritus associated with organisms after death, and the spiny protrusions are erect, indicative of the living state.

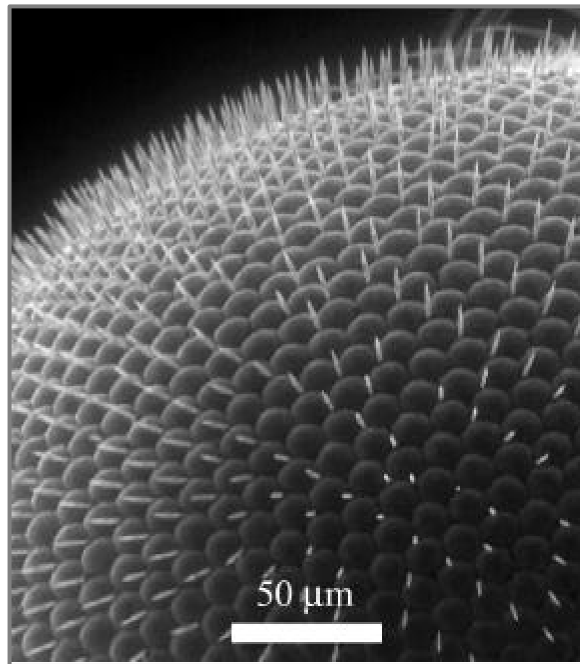

Figure 3. ESEM micrograph showing part of the eye of a live fruit fly.

Low vacuum mode (e.g. room temperature, water vapour pressure: 2.5 torr) can be applied to the study of hard tissue such as bone. The role of water vapour in this case is to provide mechanisms for signal amplification and charge compensation, rather than conferring moisture stability. Fig 4 shows a ground and polished section of human cortical bone (very dense material that occurs at the edges of bones). Vascular channels are surrounded by lamellar osteons, with evidence of older systems that have been remodelled over time. Also visible are osteocyte lacunae – regions where bone cells have become incorporated into the bone matrix. These osteocytes communicate with each other via tiny, interconnected channels (canaliculi). The ability to image such tissues uncoated means that the specimen is not altered in any way, making it available for further analysis, for example, using other techniques. Additionally, simultaneous x-

ray microanalysis can be carried out in ESEM, enabling light elements, especially, to be detected without the hindrance of the coating.

Returning to ESEM mode, and at an even smaller length scale, ESEM can be used to image individual mammalian cells, as is demonstrated in Fig 5 (temperature: 3ºC, water vapour pressure: 3 torr). The human bone tumour cells have in this case been chemically fixed, although recent work has shown that this is not actually necessary [11], i.e. tissue cultured mammalian cells can be placed in the chamber directly from culture, with no preparation other than rinsing with distilled water. With or without fixing, cells remain in a hydrated state using ESEM mode, and intracellular components are clearly visible - application of a coating could mask these features.

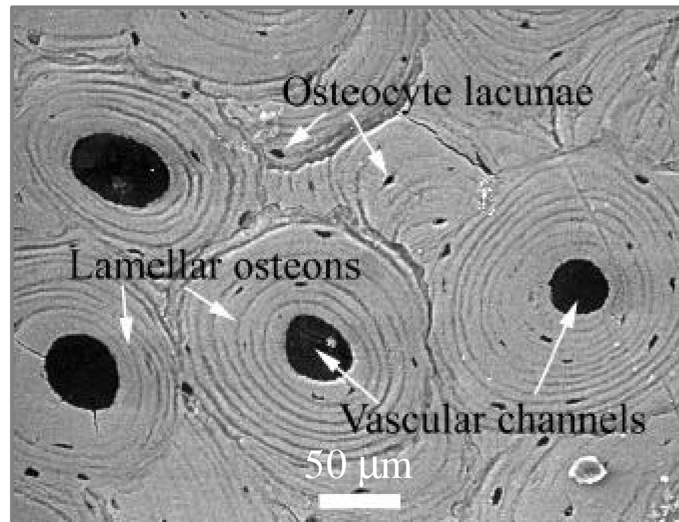

Figure 4. ESEM micrograph of a section of human cortical bone.

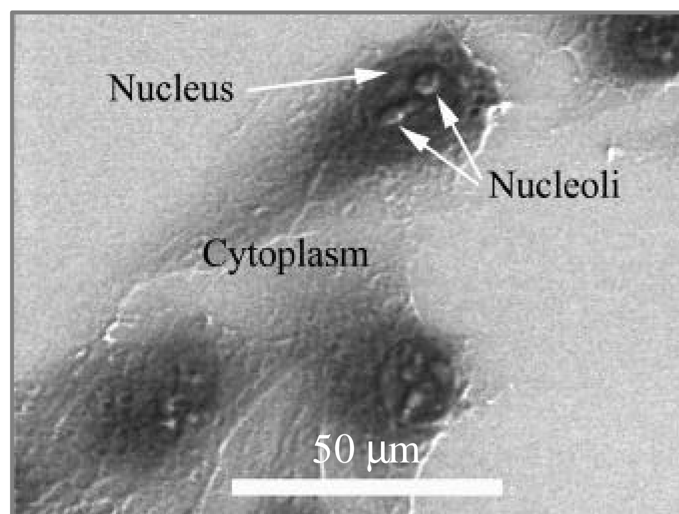

Figure 5. ESEM micrograph of tissue cultured human bone tumour cells, seeded onto a glass cover slip. The cells have been chemically fixed but are still hydrated, and imaged in the absence of a conductive coating. The central nucleus, containing nucleoli, is clearly distinguishable from the surrounding cytoplasm.

A potential application of ESEM, in terms of imaging cells and tissues, is in the very active field of tissue engineering. Many biomimetic materials are being developed, including those that mimic bone tissues, and

microscopy can play an active part towards elucidating the mechanisms underlying the bioactivity of these materials. In particular, ESEM is a promising candidate for the *in vitro* study of cellular morphology, attachment and proliferation on biomaterial substrates and also for observing bone-implant interfaces and quantifying bone ingrowth around implanted materials following *in vivo* experiments.

Summary

Environmental scanning electron microscopy (ESEM) represents a significant advance in the field of electron microscopy. Aside from its conventional high vacuum capabilities, ESEM can be used in low vacuum and ESEM modes, depending on specimen type. For insulating specimens containing little or no volatile components, low vacuum mode offers a means of controlling specimen charging, obviating the need for a conductive coating. For specimens containing high levels of moisture, ESEM mode specifically utilizes water vapour in a dual role, combining charge compensation with specimen stability, enabling hydrated & biological matter to be viewed both uncoated & without the need for dehydration or freezing. In ESEM mode, particularly, consideration should be given to the criteria for obtaining high quality images under conditions that are favourable to maintaining a specimen in a given state.

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