Progress in the specimens using



study of Biological ESEM

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In all areas of electron and optical microscopy, the challenges posed by biological specimens have long provided the stimulus for new and inventive means for their handling, processing and imaging. This paper discusses various recently developed approaches to the study of uncoated hydrated biological specimens using scanning electron microscopy (SEM), environmental SEM (ESEM) and scanning transmission electron microscopy in ESEM (ESEM-STEM). Specifically, a method is described for observing unfixed, even live organic specimens at higher temperatures and pressures than is normally associated with ESEM. It is shown that specimens can be successfully maintained in a hydrated state and imaged under relatively ambient conditions, e.g. room temperature and water vapour pressures up to 20 torr (~2.7 kPa), suggesting that it should be possible to provide organic specimens with a near-physiological environment in this type of instrument.

Introduction

Environmental scanning electron microscopy (ESEM), along with other types of variable pressure SEM, is becoming a well-established technique for the study of uncoated electrically insulating specimens. The ESEM utilises ionising collisions of emitted secondary electrons (SE) with chamber gas molecules to both amplify the signal and provide a source of dynamic charge control. Recent reviews of these principles can be found in, for example, Thiel (2004) and Stokes (2003). The advantages of such systems are already well appreciated across many disciplines, and the range of applications continues to grow. For example, the nature of gas-electron-specimen interactions has recently been shown to be of great importance for contamination-free imaging (Toth *et al*, 2005) and ultra high-resolution nano-metrology of insulators (Toth, Knowles & Thiel, 2006).

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Another aspect of ESEM and similar instruments is the ability to maintain or otherwise influence the physical state of moist or liquid specimens. The use of water vapour as a chamber gas, in conjunction with specimen cooling, means that both the thermodynamics and kinetics of water can be controlled. Typically, temperatures and pressures in ESEM are in the range 2 – 4°C and 3 – 6 torr, respectively. For ease-of-reference, Table 1 lists the

torr	kPa	mbar
3.00	0.40	4.00
5.00	0.67	6.67
5.40	0.72	7.20
6.00	0.80	8.00
7.50	1.00	10.00
9.80	1.31	13.10
10.00	1.33	13.30
13.00	1.73	17.33
14.50	1.93	19.30
20.00	2.67	26.66

Table 1. Water vapour partial pressures and their equivalent values in various units of measurement.

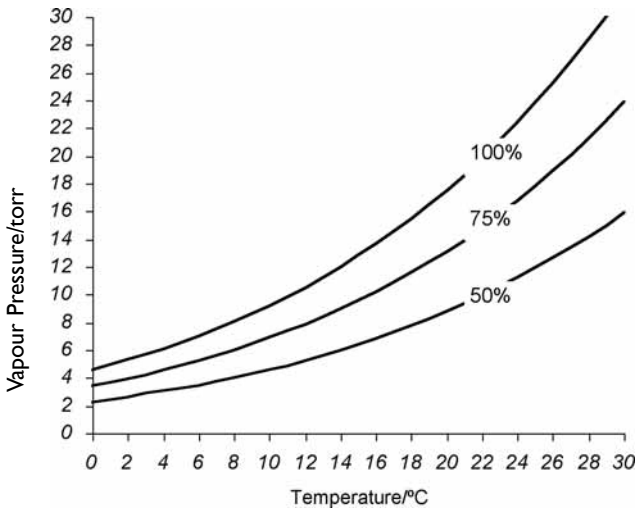

Fig 1. ESEM phase diagram, showing the saturated vapour pressure curve for water (i.e. 100% RH). Also shown are curves for 50% and 75% RH.

values of pressures used throughout this work, in units of torr with conversions to kilopascal (kPa) and millibar (mbar). The conditions of temperature and pressure noted above have been used to observe various tissues and cells: see, for example, Tai & Tang (2001), Donald *et al* (2003), Muscariello *et al* (2005) and Stokes *et al* (2003). Part of the phase diagram for water is shown in Figure 1, where the curves represent 100%, 75% and 50% relative humidity (RH). This diagram is very useful when choosing a chamber pressure for a given specimen temperature. Note that, when dealing with hydrated specimens, it is important to consider the *equilibrium* vapour pressure p_{H_2O} of the aqueous phase and hence select conditions accordingly (Stokes, 2003). For example, in many cases, a chamber environment of about 75% RH is perfectly adequate for maintaining thermodynamic stability (Equation 1). In addition, the kinetics of water loss are quite slow at low temperatures, making it possible to go to even lower pressures for short times.

Equation 1

$$p_{H_2O} \approx 0.75 p_{SVP}$$

where p_{svp} represents 100% RH at a given temperature and pressure.

An alternative to ESEM is the concept of a capsule in which moist or liquid specimens are prepared under ambient conditions and then sealed to protect the contents against the vacuum of an SEM (Thiberge *et al*, 2004). Set into the top surface of the capsule is a thin polymer membrane (roughly 100 - 150 nm thickness), supported by a TEM grid. The membrane serves as a 'window', through which primary electrons can reach a specimen adjacent to the underside of the membrane. Backscattered electrons (BSE) and BSE-derived type-II SE can be transmitted back through the top surface of the membrane, thus providing an image. This approach has the advantage that it can be used in a high vacuum SEM, and biological specimens such as cells can be grown on the membrane's lower surface and subsequently held in a suitable buffer solution during imaging. The requirement of a composition-dependent BSE signal (Z-contrast) means that specimens should be suitably fixed and stained or labelled with heavy elements beforehand. Similarly, the specimen must also be in close contact with the membrane.

Meanwhile, one of the most notable recent developments is the ability to perform high resolution scanning transmission electron microscopy in a moist environment: so-called 'wet STEM' or ESEM-STEM. This consists of a Peltier-cooled TEM grid holder and a solid-state detector. The specimen and grid are cooled as described for ESEM above, and likewise maintained in a suitable partial pressure of water vapour. The detector is placed underneath the specimen, the latter of which must be thin enough to allow the transmission of primary electrons (typically around 100 – 200 nm). For a two-segment solid-state detector, one crystal serves to collect minimally scattered electrons (bright field, BF) while the other collects electrons that have been scattered to higher angles (dark field, DF). An alternative detector design allows collection of the high angle annular dark field (HAADF) signal.

ESEM-STEM has recently been used to observe, for example, nano-inclusions in liquid-state suspensions (Bogner *et al*, 2005) and hydrated mouse fibroblasts (Moes *et al*, 2005). This technology is a significant advance in that it allows multi-mode signal detection: in addition to the high-resolution BF, DF and HAADF signals being collected by the STEM detector, SE, BSE and x-ray detectors can all be in place above the specimen to collect surface-sensitive, compositional and chemical data, respectively, giving a very powerful combination of complementary, simultaneous information.

It is clear that a growing number of methods are available for the observation of biological specimens in an SEM, ESEM or similar instrument. However, a common theme is the requirement that the specimen is cooled, except for the sealed capsule method. The main subject of the work presented here concerns imaging hydrated biological specimens under 'ambient' conditions in ESEM, neither constrained within a capsule nor with the need for specimen cooling.

Background

The gaseous SE detector (GSED) and its predecessor the environmental SE detector (ESED) were originally optimised for ESEM chamber pressures in the region of ~ 5 torr. Hence it is traditional to use Peltier cooling in order to maintain water-containing specimens under hydrating conditions, i.e. those corresponding to an appropriate equilibrium vapour pressure relative to the phase diagram for water (discussed in the Introduction). However, there are several reasons why we might wish to work at higher temperatures, and these are outlined below. Crucially, to do this at a sufficiently high vapour pressure to avoid dehydration requires an SE detector capable of producing images at pressures in excess of 10 torr[1]. Now, the efficiency of the gas cascade signal amplification process is drastically comprised at such pressures: the mean free path of SEs becomes severely limited and SEs do not gain the required energy to overcome the ionisation threshold (12.6 eV for water vapour) to initiate the cascade. In addition, the cross section for elastic scattering of the primary electron beam significantly increases, resulting in a large loss of beam current from the focused probe. Existing SE detectors simply weren't designed with the geometries and characteristics to cope with these conditions.

A prototype SE detector that overcomes these limitations was built at the Cavendish Laboratory several years ago (Baker & Toth, 2004). The idea centres around a needle-shaped anode that is highly efficient in amplifying and collecting SE in the adverse imaging environment described above. I was involved in helping to demonstrate applications for the device, and some of those preliminary results are shown here. It should be noted that the properties of the detector were not fully understood at that time and so its operation was not optimised. A recent description of the principles can be found in Toth *et al* (2006).

Imaging at higher pressures, and hence higher temperatures, is an important advance in that it opens up the possibility to study a number of temperature-dependent phenomena that do not occur, or perhaps happen too slowly to be observed, at the low temperatures typically used in ESEM. These phenomena include physiological processes, phase transformations and chemical reactions. Additionally, there is a slight advantage to having the specimen temperature similar to that of the chamber source vapour, since any mismatch can have an effect on thermodynamic equilibria (Cameron & Donald, 1998). Consider a source of vapour held at room temperature (T_r), a specimen cooled to 3°C (T_S) and a chamber pressure p_{H_2O} = 5.4 torr. There will be a slight imbalance between the numbers of vapour molecules arriving at the surface compared to those leaving (greater to

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smaller, respectively), sufficient to cause a small downward shift Δp in the required vapour pressure. In other words, if this shift is not taken into account and the pressure lowered accordingly, water may prematurely condense onto the specimen from the warmer source vapour. The equation below, taken from Cameron & Donald (1998), gives a value of Δp on the order of 0.15 torr for the conditions mentioned[2].

Equation 2

$$\Delta p = p_{H_2O}\left(\frac{T_s}{T_r}\right)^{1/2}$$

It has to be said that this refinement is a much less significant factor compared with choosing an appropriate equilibrium vapour pressure to begin with (Equation 1).

Results and discussion

Figure 2 (a) – (d) shows a series of SE images depicting tin balls on carbon over a range of increasing partial pressures of water vapour. A standard GSED[3] was used to obtain the images in Figure 2 (a) – (c) at a typical working distance of 8.5mm. In (a), at p_{H_2O} = 5 torr, the image contains surface-sensitive information, i.e. topographic structure on the surfaces of the balls, as is characteristic of an SE detector. The signal-to-noise ratio is good and the details are clear. In (b), for p_{H_2O} = 7.5 torr, the image is becoming slightly noisy. For p_{H_2O} = 10 torr (Fig 2(c)), an increasing fraction of the primary beam has been scattered *en route* to the specimen (reducing current in the focused probe, discussed earlier) and the signal is quite weak. Notice also that surface details on the tin balls are no longer evident and the image lacks

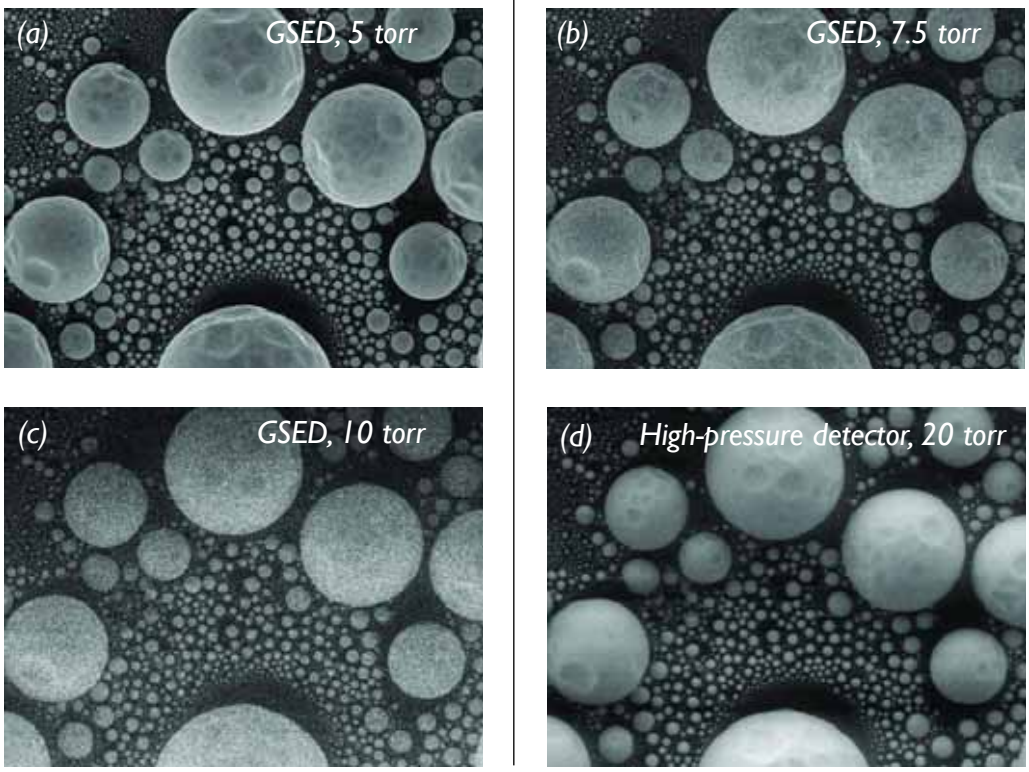

Fig. 2. ESEM micrographs of tin balls on carbon using a standard gaseous SE detector (GSED). The pressure of water vapour is 5 torr, 7.5 torr and 10 torr, respectively. For comparison, the same area imaged with the high-pressure detector is shown in Figure 2 (d), where the water vapour pressure is 20 torr. Imaged using an FEI Quanta 3D DualBeam ESEM (tungsten filament). Horizontal field width = 63 µm.

depth, suggesting that the signal now arriving at the detector is largely made up of BSE, having higher energies than SE.

Meanwhile, Figure 2 (d) shows the same field of view imaged with the high-pressure detector at p_{H_2O} = 20 torr. Remarkably, even at this high pressure, the image has depth and contains surface topographical information comparable to that in 2(a), indicating that SEs are being collected. Note that, in this case, the final Pressure Limiting Aperture consisted of a standard cone fitted to the pole piece. This effectively reduces the distance that the primary beam travels through gas, thereby significantly helping to cut down scattering and restore current in the focused probe. Together, these improvements mean that the generation, amplification and collection of SE signals is dramatically increased.

In order to *really* test the potential of the detector, it needed to be tried on a much more challenging specimen: electrically insulating, naturally hydrated, organic (very low SE and BSE emission coefficients), at high pressures (i.e. above 10 torr) and at room temperature.

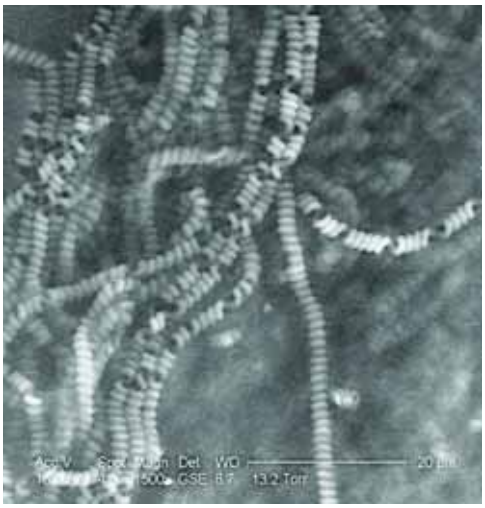

Fig. 3. ESEM micrograph of live cyanobacteria spirulina on agar gel. T = 20°C, p_{H_2O} = 13.2 torr (~ 75 % RH). Reproduced, with permission (Stokes, Baker & Toth, 2004). Imaged using an FEI XL30 FEG ESEM. Horizontal field width = 68 µm.

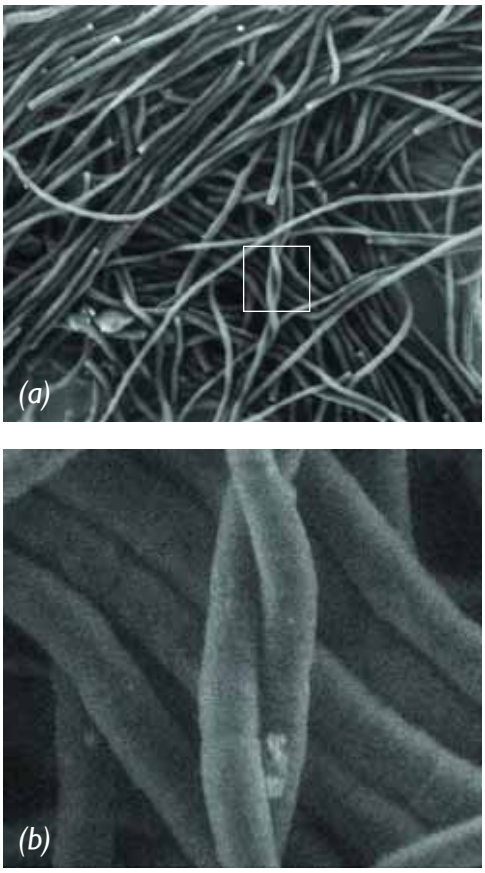

Fig. 4. ESEM micrographs of live cyanobacteria leo. T = 24°C, p_{H_2O} = 14.5 torr (~ 75 % RH).
(a) Low magnification image and, inset,
(b) higher magnification image at very low beam current (~15 pA. See text for discussion). Imaged using a Quanta 3D DualBeam ESEM (tungsten filament). Horizontal field widths (a) = 315 mm (b) = 43 µm.

Figure 3 shows one of the earliest results of the high-pressure detector on such a specimen (Stokes, Baker & Toth, 2004). Unfixed, live cyanobacteria *Spirulina* on agar gel were mounted directly onto a 1 cm circular aluminium Peltier stage stub and allowed to cool to 3°C before closing the chamber door. After a controlled purge-flood cycle (p_{H_2O} = 9.8 to 5.4 torr, x 8), the temperature and pressure were increased to 20°C and 13.2 torr, respectively, giving an RH in the region of 75%. In Figure 4 (a) and (b), unfixed, live cyanobacteria *Leo* were imaged at T = 24°C, p_{H_2O} = 14.5 torr, again corresponding to RH ~ 75%. For

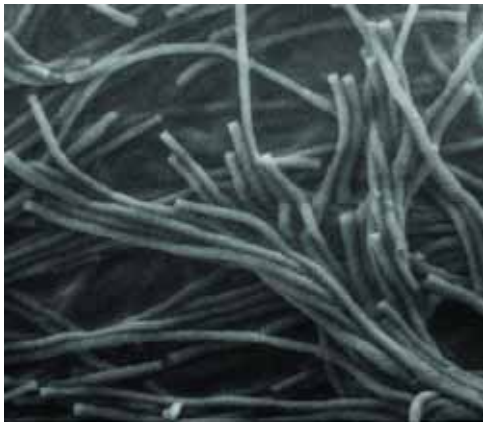

Fig. 5. ESEM micrograph of live cyanobacteria leo for T = 24°C, p_{H_2O} = 20 torr (RH ~ 90%). Imaged using an FEI Quanta 3D DualBeam ESEM (tungsten filament). Horizontal field width = 216 μm.

the higher magnification image (Figure 4 (b)), a very small beam current was used (~15 pA) in order to minimise the risk of radiation damage. Unfortunately this is likely to make the resolution more sensitive to aberrations in the electron optics. Also, some fraction of this current will inevitably have been scattered out of the probe, hence the actual current would have been somewhat lower. These points, together with the fact that the detector configuration was not optimal at this time, make it all the more remarkable that an image is formed at all.



The latest addition to the Cavendish Laboratory's EM facility: a Quanta 3D DualBeam ESEM. Debbie Stokes puts this unique microscope through its paces during a press launch, accompanied by Tony Edwards of FEI Company and Prof Athene Donald, Deputy Head of the Cavendish Lab.

At the maximum chamber pressure of 20 torr, we find that the detector continues to perform quite well, even for these organic specimens, as is demonstrated in Figure 5. The conditions used here, T = 24°C, p_{H_2O} = 20 torr, give ~ 90% RH, which is rather high. Since it is acceptable to operate at lower RH (discussed earlier), we can afford to increase the temperature. For example, aiming for 65% RH at p_{H_2O} = 20 torr would enable the temperature to be increased to roughly 30°C, only a few degrees below body temperature. With a little further development and an appropriately subtle mixture of gases, we can extend the range of conditions attainable in the chamber, enabling us to realise the goal of working to biologically relevant criteria. Meanwhile, the issue of whether live cells and organisms can tolerate and survive electron beam irradiation becomes rather important, and is the subject of ongoing research by workers at the Cavendish Laboratory.

Conclusion

The microscopical study of biological specimens is by no means a static discipline, and a brief summary has been given regarding aspects of progress in SEM, ESEM and ESEM-STEM. Of course, each approach has its strengths, suitability and limitations, and often no one technique will provide all the necessary answers. However, the range of possibilities has certainly increased and will no doubt continue to develop and improve to make tools available so that, when we specify a set of criteria, we can choose a combination of techniques most appropriate to the questions being asked. The ability to detect SE signals at high pressures and removal of the limitation on specimen temperatures extends our parameter space and hence the range of experiments and is likely to impact on those wishing to study dynamic biological processes and chemical reactions or observe temperature-dependent microstructures.



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Debbie Stokes has been carrying out ESEM research at the Cavendish Laboratory, Cambridge, for the past decade. She obtained her PhD there in 1999 and went on to become a Royal Society Research Fellow & Senior Research Fellow, Newnham College, Cambridge. Debbie has worked on the physics of secondary electron emission from organic and aqueous materials, as well as effects on image interpretation due to transient charge-related phenomena. She has devised protocols for stabilising and imaging hydrated/uncoated biological and biomedical materials and demonstrated the use of low temperature, low vacuum SEM to study sub-zero phase transitions and crystallisation.

More recently, Debbie has been helping to develop new applications in focused ion beam scanning electron microscopy (FIB SEM) for advanced materials characterisation, preparation and fabrication. She continues to be a long-term academic visitor at the Cavendish and has just taken up a new appointment with FEI Company. Debbie is the RMS Honorary Secretary (Physical Sciences) and joint Chair of **MICRO**SCIENCE 2006.

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[1]For example, an equilibrium vapour pressure of 75% RH at T = 20°C requires p_{H_2O} ~ 13 torr.

[2]T is measured in Kelvin.

[3]The GSED is an annular electrode placed under the final lens on the axis of the primary beam, several millimetres above the specimen. The aperture of the GSED serves as a pressure-limiting aperture (PLA) helping to maintain the pressure difference between the specimen chamber and the rest of the differentially pumped column.