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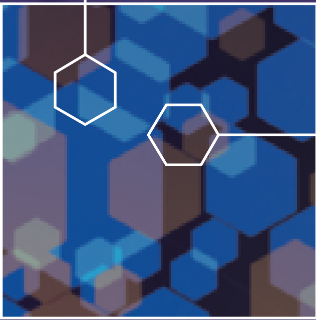
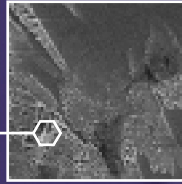

ESEM™

Imaging tissue cultured cells

Conditions for imaging cells in their natural state

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The University of Cambridge has long been at the forefront of electron microscopy due to discoveries including the electron and the neutron. The Cavendish Laboratory is the Department of Physics at the University of Cambridge and has a long history of excellence in Physics research. A decade ago, the Polymers & Colloids Group of the Cavendish laboratory, were among the first establishments in the UK to install an ESEM™. Today, the lab is owner of three ESEM™'s – an Electroscan E3 (LaB_6 filament), a 2010 (tungsten hair-pin filament) and, more recently, an FEI/Philips ESEM™ FEG.

During this time, ESEM has been intensively used by many group members both as a research tool and as the subject of research in its own right. Example applications using ESEM mode include the swelling behavior of textile fibers [1] and mechanical properties of foods as functions of moisture content [2, 3] water contact angle measurements on a variety of substrates [4, 5] phase behavior of opto-electronic polymer blends [6] and water-oil emulsions [7] as well as structure development and film formation in aqueous dispersions of polymer latex particles [8, 9].

Investigating the physics of imaging in low vacuum and ESEM modes has led to the rigorous study of such processes as gaseous signal amplification, [10, 11] intrinsic and dynamic secondary electron contrast mechanisms [7, 12] and the distribution of electric fields in and around dielectric materials, particularly in the presence of positive ions [13, 14, 15].

We have also seen growing numbers of researchers in a wide range of disciplines become aware of the advantages of using ESEM, particularly in the materials & biological sciences. However, there is presently very little literature on the subject of imaging specimens such as tissue cultured cells, in a hydrated state, using this technique.

The very nature of these specimens makes it a challenging task to obtain good quality images of the 'native' state: cells can be very thin in places (around 1 – 5 µm) and their organic composition gives very weak electron signals. The presence of water or physiological secretions can significantly obscure surface features, yet these fluids are essential for maintaining specimen stability.

But, despite the inherent difficulties, it is possible to draw together a set of parameters that satisfy the needs of both the observed and the observer. The aim of this application note is to identify these parameters and outline their importance. Such information should be of interest to those wishing to study the attachment, proliferation and morphology of cells, perhaps in a tissue engineering context, and will also be of relevance in any study where control over specimen stability is paramount.

Figure 1: The Cavendish Laboratory, University of Cambridge

Thermodynamic phase behavior of water

For specimens containing water, consideration must be given to the conditions inside the ESEM chamber, controlled by choosing the appropriate combination of specimen temperature and water vapor pressure. Since the variation in phase behavior of water is so important over the biological temperature range, it is worth reiterating some of the basic principles involved. These ideas will be extended later, when considering the characteristics of mammalian cells.

Water molecules can escape across an air-liquid interface, transported by diffusion and convection, and some proportion of these molecules will inevitably return to the liquid. In a closed system, the exchange of water molecules between liquid and vapor eventually settles down to a thermodynamic equilibrium between the two phases [16]. Hence evaporation and condensation occur at equal rates. Under equilibrium conditions at a given temperature, there is a specific amount of vapor above the liquid, described as the saturation vapor concentration or, equivalently, saturated vapor pressure. Thermodynamic theory, embodied in the Clausius-Clapeyron equation, can be used to plot the phase behavior of a liquid as a function of temperature. The phase diagram for water, or saturated vapor pressure (SVP) curve, is shown in figure 2. Equally, such a plot can be produced from tables of experimental values [17] some of which are tabulated in figure 3 for convenience. Points that lie on the curve represent thermodynamic equilibria – water molecules are evaporating and condensing all the time, but the net liquid-vapor ratio remains constant for a given temperature.

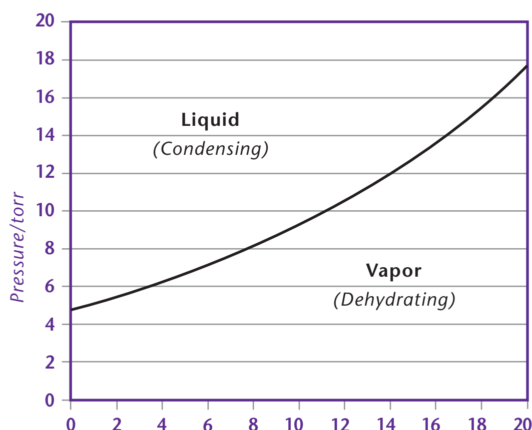

Figure 2: **Thermodynamics**
Part of the phase diagram (SVP curve) for water

T/ºC	P/torr	P/kPa	T/ºC	P/torr	P/kPa
0	4.58	0.61	11	9.85	1.31
1	4.93	0.65	12	10.52	1.40
2	5.30	0.71	13	11.23	1.50
3	5.69	0.76	14	11.99	1.60
4	6.10	0.81	15	12.80	1.71
5	6.55	0.87			
6	7.01	0.94	20	17.54	2.34
7	7.52	1.00	25	23.77	3.17
8	8.05	1.07	30	31.84	4.25
9	8.61	1.15	35	42.20	5.63
10	9.21	1.23	40	55.36	7.38

Figure 3: Water Temperaure/Pressure table

Kinetic considerations

In ESEM, specimen temperatures and chamber pressures can be controlled independently: they can be adjusted in order to attain equilibrium or non-equilibrium conditions, as required. A non-equilibrium state means that the concentration of vapor molecules above the specimen is either higher or lower than that required for a stable state. This will lead to an imbalance in the exchange of molecules between the liquid and the vapor. A higher concentration will lead to an increase in the number of vapor molecules landing on the specimen surface (condensation), while a lower concentration will shift the balance in favor of molecules escaping the surface (evaporation). Appropriate control over this very useful property enables dynamic experiments to be carried out *in situ*.

If the liquid and the vapor have different temperatures, this will also affect escape and landing rates, since the concentration of molecules in the vapor phase increases with temperature. For example, if water vapor is admitted to the ESEM chamber from a source bottle at room temperature and the specimen is on a cooled Peltier stage, it is possible for condensation to occur even if parameters are set for equilibrium conditions (depending on the Peltier calibration for a specific system, see figure 4).

The phase behavior of water is a non-linear function of temperature: by analogy with the Maxwell distribution of speeds in gases, the probability that an individual molecule will have a speed much in excess of the average increases with temperature. Hence, evaporation occurs more readily at higher temperatures. A simple illustration is provided (see figure 4), where the rate of mass loss from a body of free water is plotted as a function of pressure for a range of temperatures. This kinetic behavior has important implications in the case of controlling water in and around specimens in the ESEM chamber. Typical specimen temperatures in ESEM mode tend to be around 2 – 6°C, where the rate of moisture loss is really quite low. It is therefore acceptable to employ pressures somewhat below the equilibrium vapor pressure given by the SVP curve: specimens can usually withstand slowly dehydrating conditions for a finite period of time.

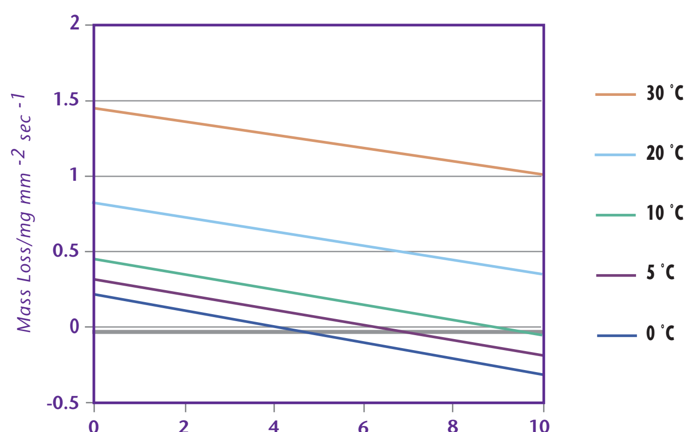

Figure 4: **Kinetics**
Water loss occurs more slowly at lower temperatures

Peltier stage calibration
Using a plain metal stub, condense stable water droplets onto the stub at fixed temperatures to find pressures corresponding to 100% RH for your system. Ensure that the proper purge-flood procedure has been followed prior to commencing, as air in the chamber will contribute to the total pressure readout, affecting the accuracy of the results.

Physiological vapor pressure

Many hydrated specimens consist not of pure water, but of aqueous phases containing dissolved solutes. We should therefore consider what influence this may have when selecting ESEM chamber conditions.

According to Raoult's law, the vapor pressure of a solution is proportional to the mole fraction of solute. An important consequence of this statement is that: Macromolecules in the interior of a mammalian cell substantially reduce the equilibrium vapor pressure of the aqueous phase, relative to pure water. The cell is thermodynamically stable at pressures lower than shown on the vapor pressure curve for pure water.

Figure 5 depicts two vessels of water, one of which contains solute molecules, depicted by white circles. In isolation, the processes of evaporation and condensation occur in each vessel such that the vapor of each is in equilibrium with its liquid. However, the concentration of vapor molecules above pure water is higher than for the solution. Now, if these containers are placed together in a sealed box, there will be a net evaporation from the pure water vessel and condensation into the solution vessel in an osmosis-like flow [18].

This driving force (osmotic pressure) for a solvent to enter a solution, thermodynamically described by Van't Hoff's equation, essentially depends upon the number of solute molecules contained in the solution. Physiological solutions tend to have large osmotic pressures, the magnitudes of which are not always adequately predicted by theory alone [19]. This is because it is assumed that solutions are dilute (solutes occupy negligible volume) and that their behavior is ideal (solute molecules do not interact with each other or with solvent molecules). Real physiological aqueous phases, such as those found in the interiors of mammalian cells are neither dilute nor ideal: macro-molecules such as proteins and polysaccharides take up a large volume (around 30% of the available space) and interact strongly with water molecules (protein folding, for example, is dependent on such inter-actions) [20].

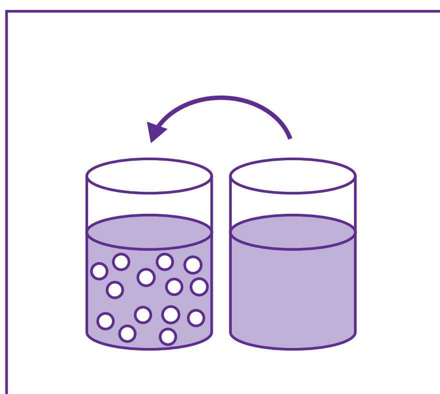

Figure 5: **Osmotic pressure**
The vapor pressure of the solution on the left is lower than that of the pure solvent on the right. Solvent molecules will condense into the solution vessel.

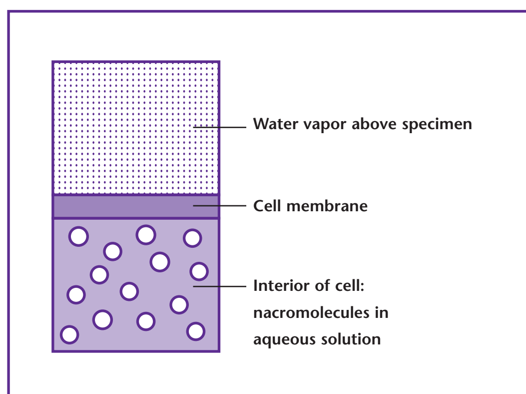

Figure 6: **Cell Model**
The lower compartment represents the interior of a mammalian cell, containing macromolecules such as proteins, enclosed by a semi-permeable membrane. Above the cell is a partial pressure of water vapor in the ESEM chamber. If the vapor pressure above the cell is higher than the equilibrium vapor pressure of the cell contents, vapor will condense out of the environment.

The extent to which these factors lower the vapor pressure of a physiological solution is estimated to be quite significant [21]. The phase diagram below shows the equilibrium vapor pressures for both pure water and that inside a mammalian cell.

Using this data as a guide, the equilibrium vapor pressure for a cell at 3˚C is approximately 4.3 torr (some 25% lower than for pure water).

These are the conditions that maintain the specimen in a fully hydrated state.

Further lowering of the chamber pressure can be accommodated, due to the kinetic factors previously discussed. In addition, the semi-permeable membrane surrounding cells confers further short-term stability against water loss. The system as a whole is depicted in figure 6.

It is possible to work at just 50% RH (relative to pure water) for about 20 – 30 minutes per specimen.
At 3˚C, this means pressures as low as 2.8 torr which, when using the gaseous secondary electron detector (GSED), turns out to be an optimal pressure for imaging purposes, as explained overleaf.

Despite deviating to conditions well below 100% relative humidity, the specimen itself is not under dehydrating conditions.

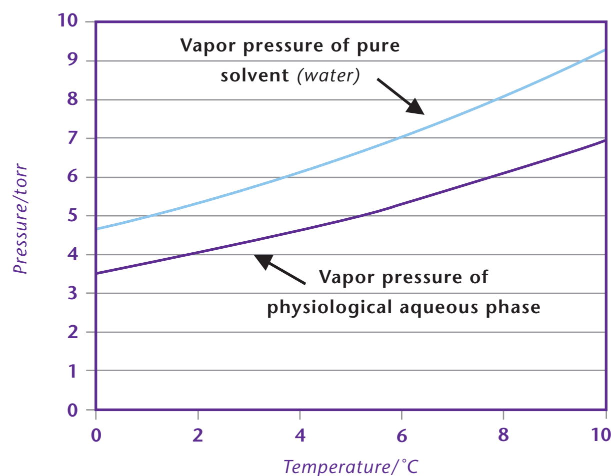

Figure 7: Equilibrium vapor pressures for pure water and that inside a mammalian cell.

Cell cultures

For this work, human osteoblasts (bone cells) were seeded onto 5 mm diameter glass cover slips and cultured for periods ranging from 1 to 5 days. Some specimens were then fixed using 4% para-formaldehyde-0.01% glutaralde-hyde, while others were left completely unfixed.

Once removed from culture, the seeded specimens can be stored in wells containing a buffered salt solution, for up to several hours, before use in the ESEM.

The seeded cover slips should be mounted on a specimen stub (using double-sided adhesive carbon tape, for example) and placed on a pre-cooled Peltier stage (e.g. 3ºC), as shown diagrammatically below.

Finally, before pumping down the chamber, cells must be rinsed to remove the buffer solution, which will otherwise cause salt precipitation as shown in figure 8. Distilled water can be used for rinsing osteoblasts.

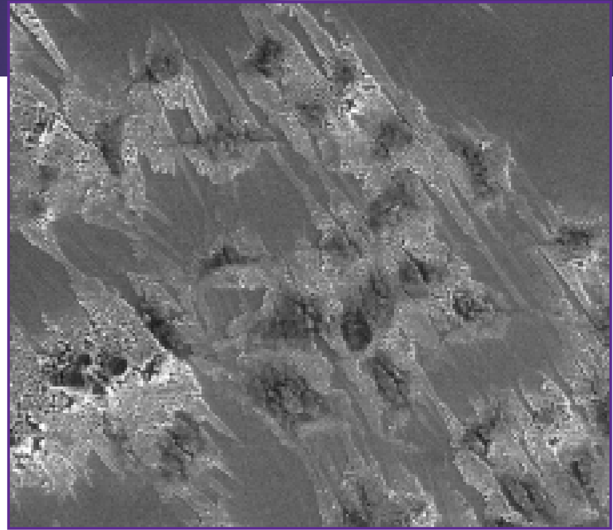

Figure 8: **Salt precipitation**
Precipitates from the culture medium or buffer solution nucleate around cells. Rinsing of the specimen before use helps to minimize this.

Beam energy: 10 keV, total working distance: 8 mm, pressure: 3 torr, specimen temperature 3ºC, magnification: x 185 (horizontal field width: 490 µm).

Glass cover slips can be useful substrates to practice with: there is good contrast between cells and glass, making cells easier to see.

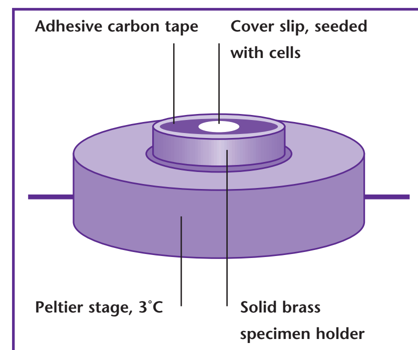

Figure 9: **Sample mounting**

Gas amplification

Water vapor itself, so useful for stabilizing hydrated specimens, plays a key role in amplifying electron signals.

Low energy secondary electrons (SE) emitted by the specimen are accelerated by an electric field between the specimen and gaseous secondary electron detector (GSED). Ionizing collisions with gas molecules generate additional SE and so the signal is amplified before reaching the GSED.

The distance between specimen and detector is a very important parameter: if the gap is too short, the cascade process doesn't really get going, but if it's too long, the signal is degraded by unwanted scattering events. These extremes are strongly dependent on the type and partial pressure of the gas being used, as well as the atomic weight(s) of the specimen.

Clearly, there is a complex interplay between pressure, working distance and specimen composition, but the following result is helpful: for a carbon specimen, the maximum total signal arriving at the GSED is obtained at a partial pressure of around 3 torr of water vapor. Furthermore, the signal-to-background ratio is at its peak, since the signal is dominated by secondary electrons (around 75% of the total) [10].

In practice (see figures 11 and 12), working distances of 8 – 8.5 mm help to increase the strength of the SE signal emitted by mammalian cells. Working distances larger than these begin to introduce significant levels of background noise.

Note that the working distance is defined as the distance between the objective lens and the specimen. For most ESEM models, the GSED is situated around 5 mm below the objective lens and the specimen is then a short distance below the GSED. A working distance of 8.5 mm therefore equates to a specimen-detector gap of 3.5 mm. However, for the Quanta model, the lens-GSED distance is only 3.25 mm, suggesting that, for a specimen-detector gap of 3.5 mm, the working distance will be reduced to 6.75 mm.

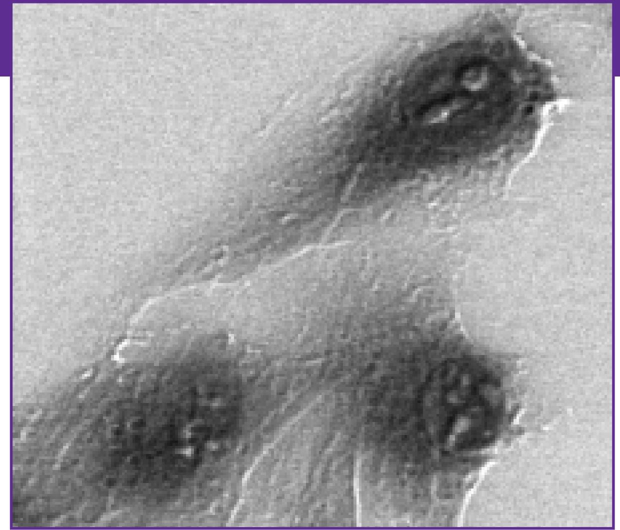

Figure 11: **Chemically fixed cells**
Electroscan 2010, beam energy: 10 keV, total working distance: 8.5 mm, pressure: 2.9 torr, specimen temperature: 3°C, magnification: 750x (horizontal field width: 85 μm)

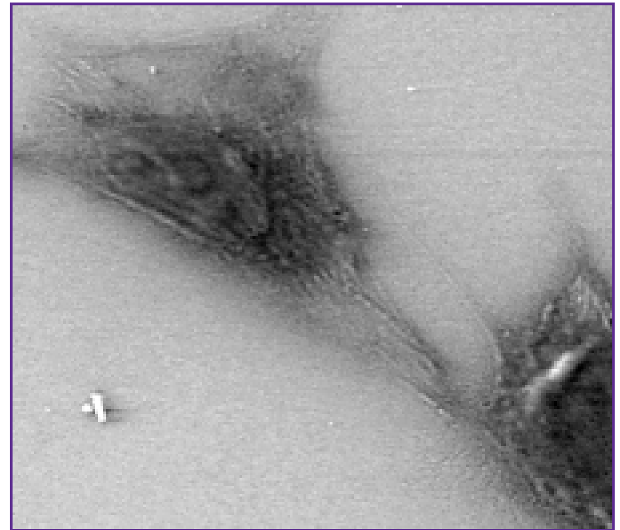

Figure 12: **Unfixed cells**
FEI Philips XL30 FEG, beam energy: 5.5 keV, total working distance: 8.5 mm, pressure: 2.8 torr, specimen temperature: 3°C, magnification: 860x (horizontal field width: 75 μm).

These micrographs show human bone cells, chemically fixed and unfixed. Notice how the cell nuclei are distinct from the cytoplasm, and also that organelles (nucleoli) are visible within nuclei. Such details can be masked if the specimens are conductively coated as they would be for high vacuum SEM.

Figure 10a: 5 keV

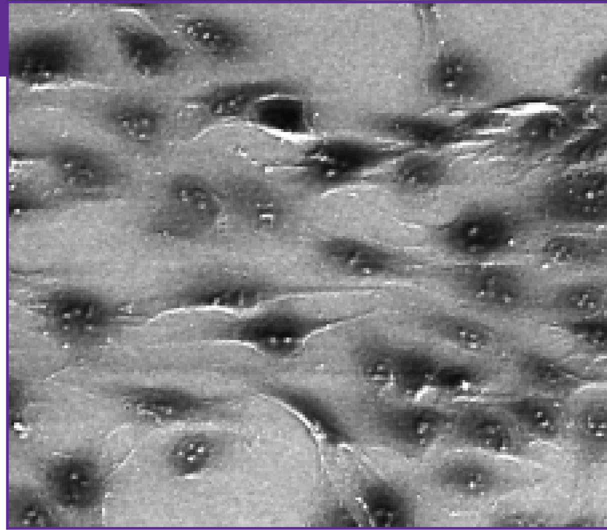

Figure 10c: 15 keV

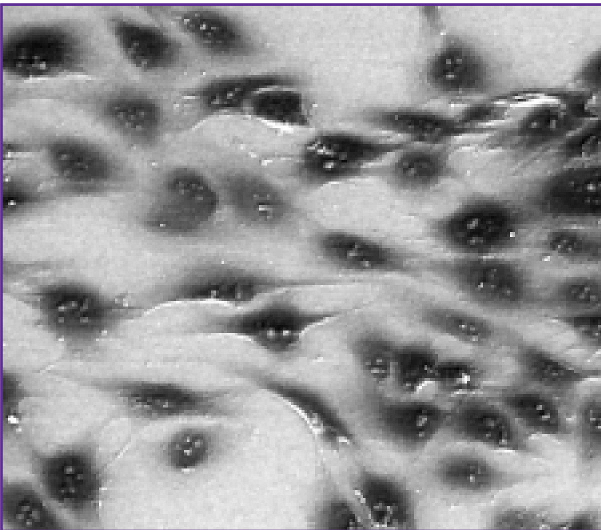

Figure 10b: 10 keV

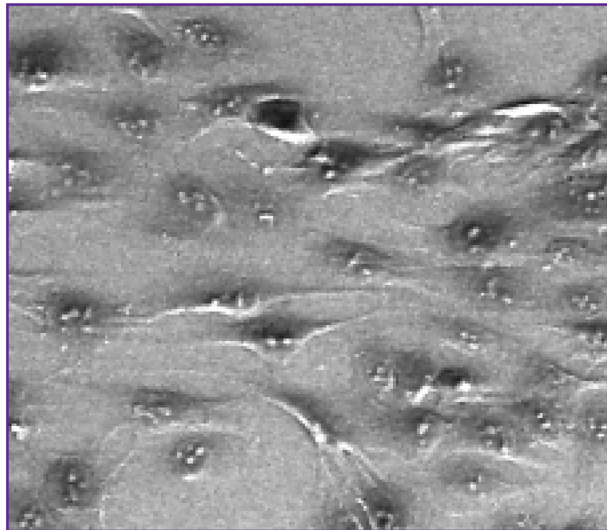

Figure 10d: 20 keV

Figure 10: **Effect of beam energy**
Contrast between cells & substrate decreases as beam energy increases. Total working distance: 8.3 mm, pressure: 2.9 torr, specimen temperature: 3ºC, magnification: 290x (horizontal field width: 350 µm, each image).

Beam energy

Beam energies of 20 keV or so are often used in order to reduce primary electron scattering in the gas. However, organic materials have very low electron stopping powers: primary electrons can travel far into the specimen before becoming thermalized. For thin specimens, a 20 keV beam may penetrate right through the specimen, into the supporting substrate. This is a useful way to deposit energy beyond the specimen and hence reduce the likelihood of charging and radiation damage, but it can reduce surface-sensitive information, depending on the nature of the surface and the information being sought.

This is demonstrated in figure 10 with a series of images of cultured cells, where the beam energy has been increased from 5 keV through 20 keV. The same area of specimen is shown in each image. In a general sense, cells at lower beam energy appear larger, and the contrast between cells and substrate greater. This is because the SE signal is emitted from a smaller depth and thus images features that are very thin (eg.: cytoplasmic membrane). By 20 keV, it is mainly the thicker parts that are seen (nuclei). Perhaps a suitable compromise is to select an 'intermediate' beam energy such as 10 keV.

Pumpdown

Pumpdown in ESEM mode involves several purge-flood cycles. The aim is to remove air from the chamber and replace it with water vapor such that changes in the moisture content of a hydrated specimen are kept to a minimum. The Auto Flood routine built-in to the XL30 software is generally acceptable. An alternative regime is to use eight cycles of 5.4/9.8 torr, finishing at 5.4 torr, for a specimen cooled to 3ºC [22]. Additionally, a small quantity of water (in a 5 mm deep brass specimen cup, for example) placed near to, but not on, the specimen helps to keep the environment reasonably humid during pumpdown. Requirements may vary, as some cell types are more sensitive to moisture changes than others.

Bearing in mind that the equilibrium vapor pressure of mammalian cells is probably around 4.3 torr at 3ºC, the chamber pressure should be adjusted to this value immediately following pump-down. If the partial pressure of water vapor in the chamber is allowed to remain higher than the vapor pressure of the specimen, then water is likely to condense onto the specimen surface. This will impede resolution of surface structure and may also lead to water uptake by the cell, resulting in swelling and perhaps rupture of the cell membrane.

It is good practice to allow a couple of minutes of equilibration time, before reducing the partial pressure further. The choice of final pressure will largely depend on the stability of the specimen against water loss and on signal quality. Pressures below 2.8 torr are not recommended for these specimens, as dehydration occurs too rapidly.

Imaging tissue cultured cells

Summary

It is possible to obtain high magnification images of tissue cultured mammalian cells, without fixing, dehydrating or coating, using ESEM mode. For ease of reference, the main points are summarized in the following suggested protocol:

Mount specimen and place on Peltier stage

- Pre-cool stage to 3°C
- Rinse buffer solution from specimen

Pumpdown

- Auto purge-flood or custom cycle 5.4/9.8 torr x8, finishing at 5.4 torr

Set pressure to give ~ 75% RH

- Specimen temperature 3°C
- Water vapor pressure 4.3 torr
- Equilibrate approx 2 – 5 minutes

Set working distance

- 8 – 8.5 mm

Image at pressures corresponding to 50 - 75% RH

- 2.8 – 4.3 torr water vapor
- 50% RH can be used for periods of up to 20 - 30 minutes

Select appropriate beam energies

- 10 keV for general imaging purposes
- Lower energies for viewing overall cell coverage on substrate
- Higher energies for observing specific details

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