Examination of Hydrated Bacteria in An Environmental Scanning Electron Microscope (ESEM)

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An ESEM has recently become the tool of choice for many laboratories. Biologists have taken advantage of its capability to examine uncoated fresh specimens or certain types of specimens from insect collections that should not be gold-coated for examination in a conventional scanning electron microscope. However, some fresh specimens are more difficult to examine than others when using an ESEM—specimens consisting of bacteria are particularly difficult. Recently, a question on this subject was posted on the Microscopy Listserver, where it brought many responses. They are summarized below.

It should be noted that pathogens in the specimen chamber can contaminate the whole instrument and be aerosolized into the room. Therefore, unknown bacteria and pathogens must be fixed prior to ESEM examination.

General procedures

1. Pre-cool the cold stage to a temperature (T) of 2°C, place the untreated bacteria onto it directly or mounted on a pre-cooled cold-stage stub with excess water surrounding the specimen.
2. Select the "Wet Specimen" mode, "Target Vapor Pressure" (VP) 5.3 Torr, "Flood Cycle" (4-6X) above the target VP (5.5 - 10 Torr). The T and VP may be varied as desired as long as it translates to 100% relative humidity.
3. Place double-distilled water in the corner wells of the cold stage or place 1-2 ml in a tube positioned in the chamber. Pump out the chamber.
4. Work at a low working distance, e.g. 7 mm in the ESEM; reduce the VP very slowly while monitoring the excess water being evaporated. Focus quickly and capture images before the specimen is damaged by the electron beam.

Bacteria on tissue or inorganic matter may be viewed *in situ* by gluing the substrate onto the cold stage stub with colloidal graphite or a double-sided carbon tab. Salts and other organic matter in the culture must be washed away. Therefore, bacteria in liquid media must be centrifuged gently and washed with water. The suspension may be placed on the cold stage directly or filtered with a carbon-coated membrane filter (polycarbonate), which is then glued onto a cold stage stub. For bacteria on agar, it is advisable to cut a small plug, remove excess agar and place it on the cold stage. A TEM grid on the surface of the agar can be used as a focusing aid and for locating the bacteria. However, bacteria usually have a protective polysaccharide layer on the top of their colonies that makes viewing the bacteria difficult or even impossible.

Images of fresh bacteria by ESEM are not as crisp as critical point dried (CPD) and subsequently gold-coated specimens examined by conventional SEM for several reasons:

1) Many bacteria are covered with a layer of polysaccharide. This layer is retained in an ESEM where it obscures the underlying cells, in contrast to conventional SEM, where this layer is removed during specimen preparation.
2) Gold on CPD specimens yields a stronger secondary electrons (SE) signal than a hydrated specimen can generate in ESEM (using a tungsten gun), and the ESEM signal is from a greater depth resulting in softer images.

Although some biologists fail to get high-quality images of fresh bacteria using an ESEM, there are others who recognize the constraints of the technique and are satisfied with the less crisp ESEM images obtained promptly, if they provide the information at the level anticipated. Field emission ESEM should produce better images than microscopes equipped with W or LaB_6 filaments. The satisfaction with image quality depends, to a great extent, upon the research objective.

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Dye is Money

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In our laboratory, we often use fluorescent dyes in aqueous solution, for example calcium indicators that are iontophoretically injected into neurons through a microelectrode. Usually, the dyes are dissolved in water or electrophysiological recording solution as a stock solution, and aliquots are kept frozen until used. This treatment, however, does not prevent the degradation of the dye over a longer period of time. The potassium salt of Fura Red (Molecular Probes), for instance, looses its sensitivity to calcium after approximately three months of storage in aqueous solution at –20°C. The dry salt, in contrast, can be stored for years when kept dark and frozen. Hence, it is advantageous to store aliquots of the dry salt. The amount of dye, which usually is in the range of 1 mg, is too small to be directly divided into smaller portions. Therefore, we dilute the dye in a small volume of methanol, subdivide the solution into aliquots in 1-ml reaction tubes, and then let the methanol evaporate for 1 hour in darkness. For example, 1 mg of Alexa Fluor 488 hydrazide sodium salt is diluted in 100 µl of methanol, and aliquots of 5 µl are made. After evaporation, the aliquots are kept frozen in darkness and are stable for one or more years. We have successfully tried this method for different Alexa Fluor hydrazide