Towards Real Time ESEM Imaging of Biological Processes

Historically, electron microscopy of dynamic biological processes has been impossible to achieve in real time because conventional electron microscopy requires specimen fixation, dehydration and metallic coating. The advent of the Environmental Scanning Electron Microscope (ESEM) removed these restrictions, allowing fully hydrated samples to be imaged in their native state. This raises the possibility of secondary electron imaging of dynamic biological processes.

Well Suited to Biological Imaging

The ESEM differs from a Conventional Scanning Electron Microscope (CSEM) in that a differential pumping system maintains a pressure of gas (typically water vapour) in the specimen chamber whilst the gun remains at high vacuum. Ionizing collisions between electrons and these gas molecules create positive ions which drift down onto the sample tending to neutralize specimen charge. In this way it is possible to image insulating samples without the need for metallic coating. The presence of water vapour in the chamber also means that a high relative humidity can be maintained and therefore samples can be imaged in a hydrated state without the need for dehydration and fixation. These features suggest that ESEM could be well suited to biological imaging, offering high resolution topographic images with a remarkable depth of field, yet without the need for sample preparation or the artefacts it may introduce. Our work focuses on optimizing the ESEM for biological use and most recently [1] has involved exploring the possibility of imaging of biological systems undergoing natural morphological changes.

Visualizing Stomatal Closure

The closure of stomatal pores in leaf epidermal tissue was chosen as a test case as leaf tissue is fairly robust, readily available and stomatal movements occur on a timescale suitable for ESEM imaging. Stomatal pores are present in almost every species of terrestrial plant and control gaseous exchange with the atmosphere. A typical ESEM image of tradescantia epidermis, with widely open stomata pores, is shown in figure 1. Each pore is surrounded by two guard cells, which can swell and change shape, opening or closing the pore depending on their turgor pressure.
In nature, stomatal movements occur in response to environmental cues. In the microscope chamber the temperature and hence relative humidity can be varied with a view to inducing stomatal closure.

By optimizing the microscope to view stomatal movements in living *Tradescantia andersonia* (Spiderwort) leaf tissue, we were able to demonstrate that it is possible to follow a biological system undergoing dynamic morphological changes in real time. Considerations included minimizing beam damage and reconciling the need for an adequate physiological temperature and a low gas pressure favourable for imaging, with the thermodynamic constraints on achieving a high relative humidity. An imaging protocol was developed utilizing a custom pumpdown protocol developed by Cameron and Donald [2], for a specimen held at 7°C. The lower epidermis of *Tradescantia andersonia* was held at a water vapour pressure of 7.3 torr and imaged with a 10 kV, 0.09 nA beam, blanked between imaging scans. Stomatal closure could be triggered and observed in a controlled way by increasing the temperature and thus reducing the relative humidity. Figure 2 shows a series of three images taken over a 20 minute period, following the closure of a single pore in response to a reduction in relative humidity.

**Live Biological Specimens**

This proof of principle work also illustrated the many challenges associated with use of the technique. First amongst these, is the problem of beam damage. Some progress can be made towards lessening the destructive action of the beam by selecting the appropriate working conditions. Reducing the beam voltage, magnification and exposure time all appeared to limit damage. Working with less radiation sensitive samples was also important. This brings us to a more general point concerning ESEM imaging of live biological specimens, namely that working with a suitable specimen is almost as important as optimizing the instrument [3]. Robust leaf tissues with thick waxy cuticles and viscous sap, (of which *Tradescantia* is an excellent example) appear able to withstand the radiation damage and dehydration for tens of minutes [4], sufficient to image stomatal closure. With more delicate specimens such as *Vicia faba* and *Commelina communis*, this was considerably more challenging [5].

The health of the tissue was also observed to be a key determinant of how the cells withstood conditions in the chamber. Towards the end of the season some of the *Vicia faba* plants became infected with *Uromyces vicia faba* or 'broad bean rust', a fungal pathogen. Figure 3 shows some aspects of the diseased tissue. This tissue was not suitable for stomatal pore closure experiments, not least as the ruptures in
the epidermis in areas of necrosis would have increased the susceptibility of the
tissue to dehydration.

Considering the broader applicability of ESEM to the imaging of dynamic processes
it is interesting to note that the timescale of the process is critical. Too fast (under a
minute) and high quality images could not be taken fast enough, too slow (many
hours) and the living specimen could not survive the chamber conditions for long
enough. Outside of these time restrictions CSEM imaging would be just as effective
in following a process by imaging multiple specimens in a range of arrested states,
provided that CSEM specimen preparation does not introduce artefacts.

**Future Applications**

Imaging at longer timescales, such that developmental processes can be followed,
must be one of the long terms goals of dynamic imaging using ESEM. To follow the
development of plants it would be necessary to image attached tissue, or whole
seedlings, install a sufficiently bright light source in the ESEM chamber and modify
the gaseous environment to include carbon dioxide. It is likely that developing
tissues are very beam sensitive. However even with robust tissues, imaging could
not be continuous due to the severe radiation damage that would be incurred. In
mammalian systems imaging tissue development in real time seems a remote
possibility. The high temperatures and culture medium necessary, not least the
outgasing problems described by Kirk et al. [6] are currently insurmountable.
Developmental processes can instead be imaged in the ESEM using fixed but
hydrated tissues exhibiting morphology at a range of developmental stages. CSEM
can also be used to image fixed tissue in this way, but it is important to note that
the material must be dehydrated/cryopreserved and metal coated; steps which can
easily introduce artefacts or damage delicate structures.

**References**


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