The application of ESEM to biological samples

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The application of ESEM to biological samples

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Abstract. The Environmental Scanning Electron Microscope (ESEM) differs from a conventional SEM in that a differential pumping system maintains a pressure of gas (typically H_2O) in thespecimen 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 neutralising specimen charge. It is therefore 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 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 imaging biological samples undergoing natural biological processes. We present a proof of principle study on the closure of stomatal pores in *Tradescantia andersonia* leaf tissue. An imaging protocol is developed and the advantages and limitations of this technique are discussed. Images of *Vicia faba* leaf tissue are also presented. Challenges include minimising 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.

1. Introduction

ESEM was first proposed by Danilatos in 1979 [1] and offers the ability to image insulating samples at high relative humidity, features which are not available with conventional SEM. These abilities suggest it may have substantial capacity for high resolution imaging of biological specimens. Donald [2] discusses the ESEM technique and its advantages in more detail. Our work focuses on optimizing the ESEM for biological use and exploring its applications in that field. Specifically, we demonstrate the possibility of using ESEM to image live cells undergoing dynamic morphological changes.

The system under study here is the closure of stomatal pores in leaf epidermal tissue. Each pore is formed by a pair of guard cells which can swell, change shape and open the pore - thereby controlling gas exchange with the atmosphere. A more detailed discussion of stomatal biology can be found in Taiz & Zeiger [3]. In nature stomatal movements occur in response to a range of environmental cues including changes in atmospheric humidity. Varying the temperature of the sample in the microscope chamber allows control of relative humidity with a view to inducing stomatal closure.

ESEM imaging of plant tissue in it's native state was first explored by Danilatos [4] in the early days of the instrument. By optimizing the microscope to view stomatal movements in living *Tradescantia andersonia* (Spiderwort) leaf tissue, we explore the possibility of using ESEM to image dynamic natural processes in living tissue

2. Materials and methods

2.1 Plant Material

Young *Tradescantia andersonia* and *Vicia faba* (broad bean) plants were grown in commercial potting compost (John Innes no. 2) in 25cm plastic pots. A light intensity of 100-300 $\mu molm^{-2}s^{-1}$, dependent on leaf height, (measured using an Apogee quantum sensor) was generated by a 600W Sunmaster metal halide growth lamp. The plants were entrained to a 16h photoperiod and were well watered. Temperatures ranged between approximately 15ºC and 25ºC with relative humidities in the range 35-55%. Plants were transferred to a perspex box with the same temperature range but higher humidity several hours before imaging to ensure the stomatal pores were open wide. The relative humidity levels in this box varied between 40 and 65% during the day and a maximum of 90% during the night. Experiments took place within a 10am to 4pm time window. Leaf tips were cut from the plants using a scalpel and transferred to the microscope chamber within a minute and a half.

2.2 ESEM setup and protocol

The sample was placed, lower epidermis up, on an adhesive carbon tab fixed to a 3.5 cm x 3 cm copper plate in thermal contact with a water cooled Peltier chip in the sample chamber. This allowed temperature control of the whole sample and meant that complete leaves or leaf tips could be accommodated, minimising the number of potentially dehydrating cut edges. All experiments were conducted using an FEI XL30 FEG ESEM with the Secondary electron signal collected using a needle detector, developed by Toth and Baker [6]. The accelerating voltage was 10kV, 7kV or 5kV. A final water vapour pressure of 7.3 Torr (unless otherwise stated) was achieved using a custom pumpdown procedure developed by Cameron and Donald [7] minimizing sample evaporation. Samples were typically maintained at 7 ºC throughout pumpdown unless otherwise stated. The relative humidity at the sample surface was estimated by expressing the actual pressure of water vapour in the sample chamber as a percentage of the theoretical saturated vapour pressure of water at the temperature of the Peltier stage. Saturated vapour pressure increases exponentially with increasing sample temperature, thus it is possible to lower the relative humidity by raising the sample temperature but leaving the gas pressure unchanged. The time elapsing between cutting the leaf tissue and capturing the first image was minimized such that the stomatal pores were maximally open and the tissue was as fresh as possible; this preparation and pumpdown time typically ranged from four and a half to ten minutes. After an initial image was taken, the temperature of the Peltier stage was raised to lower the relative humidity and trigger the closure. Images were taken at five or ten minute intervals, between which the beam was blanked.

3. Results and discussions

3.1 Imaging the closure of stomatal pores

Following the general protocol outlined in section 2.2, stomatal closure could be observed reproducibly occurring in the ESEM chamber. The sequence presented in figure 1 is closure in response to a reduction in humidity from 97 to 91%. In this case the process occurred on a time scale of 20 minutes. Single imaging scans were taken at approximately ten minute intervals with a 10kV beam, spotsize 3. The beam was blanked between scans to minimize beam damage. Tripling the magnitude of humidity decrease halved the closure time, suggesting that we are observing a natural living process rather than the consequences of apoptosis. If, as a control, no step in humidity was introduced closure occurred on a time scale of 45-50 minutes, consistent with the expected response of an excised leaf tip in dark, cold conditions. If the sample had been introduced into the high vacuum conditions of a conventional SEM, the absence of any water water vapour in the air would also have triggered closure however the rapid dehydration of the tissue would severely and irreversibly damage the guard cells; impairing normal function. One would also anticipate imaging difficulties due to charging in high high vacuum conditions.

3.2 Limitations

The images of *vicia faba* presented in figure 2 illustrate the consequences of imaging using unsuitable environmental conditions. In this case the relative humidity was 89% and should therefore have triggered a rapid closure response. Instead the stomata in image B appear open yet the epidermal cells appear flaccid. This appearance was not limited to the irradiated region indicating that it was not a beam damage effect. We suggest that this unusual behaviour was an unexpected consequence of the tissue growth conditions which resulted in tissue adapted to high humidities. As a consequence, the tissue did not respond well to the conditions of the microscope chamber. Non closing stomata have previously been reported in tissue grown at high humidity [5].

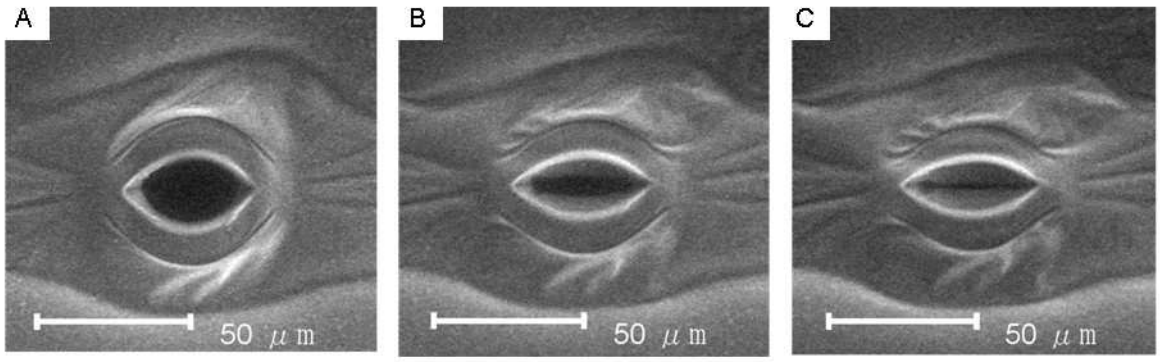

Figure 1: A sequence of three ESEM images following the closure of a single stomatal pore in response to the reduction in chamber relative humidity from 97% to 91%. **(A)** 12 minutes, **(B)** 25 and **(C)** 32 minutes after cutting. Accelerating voltage 10kV, beam current 0.09nA. Water vapour pressure 7.3 Torr, temperature 7°C m in image (A), subsequent pressure 7.2 Torr, temperature 8 °C , in (B) and (C).

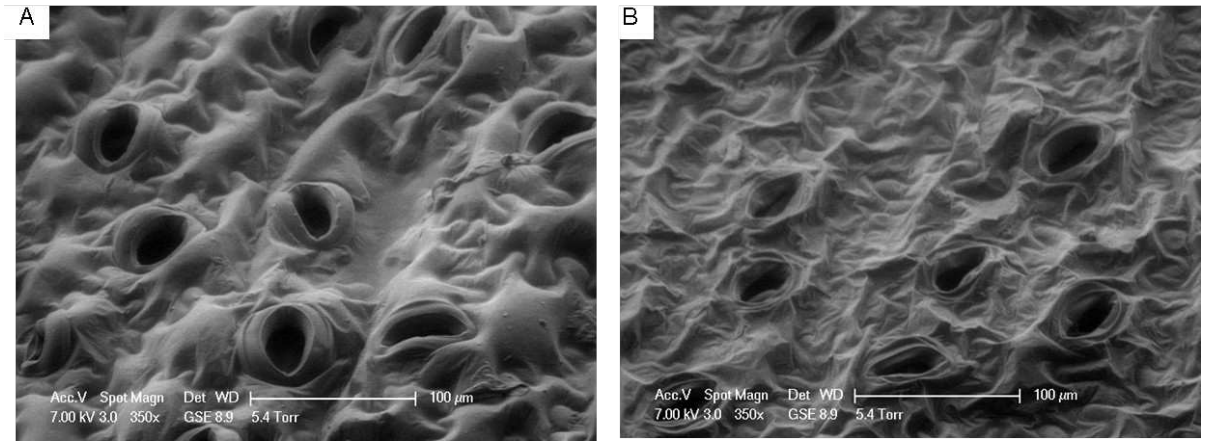

Figure 2: Images of the lower epidermis of *vicia faba* grown at high humidity (described in section 2.1) Imaged using a 7kV beam, FEI spotsize 3. After pumpdown, the sample maintained at 4°C, 5.4 Torr, 89% relative humidity throughout imaging. Image B is taken 5 minutes after image A. The sample is tilted to 45 degrees, revealing more of the three dimensional structure.

Figure 3 shows the changes to tissue character induced by the action of the beam. Beam damage is distinct from environmental damage, being localised to the region being scanned. The tissue in figure 3B has been continuously scanned for 35 minutes with a 10kV beam. This epidermal cells appear deflated and the surface wrinkled. The damage caused by continuous scanning at 10kV necessitated the single imaging scans and beam blanking adopted in the protocol generating the sequence in figure 1, recent work has, however shown that continuous scanning at 5kV is less damaging to the surface structure.

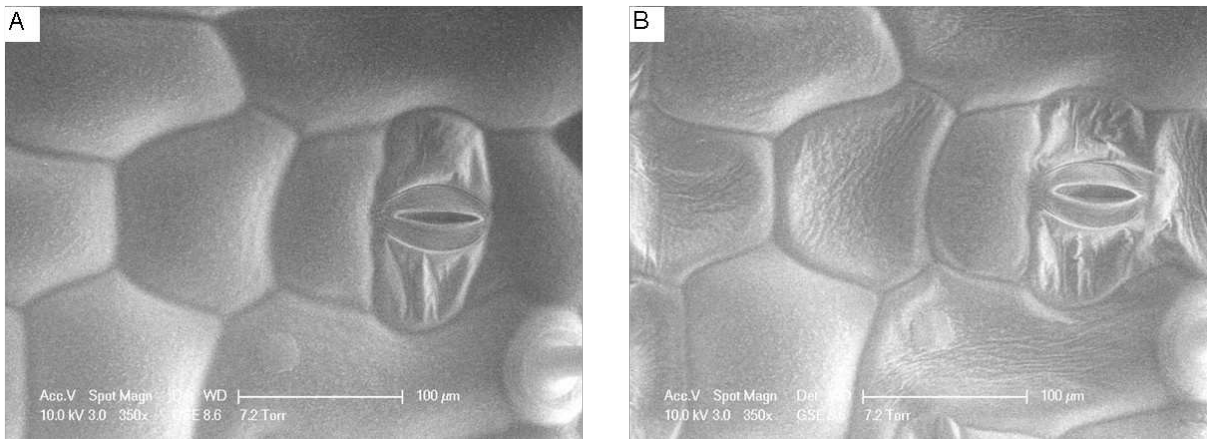

Figure 3: Two images of the epidermis of Tradescantia andersonia imaged at 10kV, FEI spotsize 3, 7.2 Torr, 7°C Image A is the initial image taken mins after excision. Image B is the same region as B but taken following 35 minutes of continuous scanning. The cells in B appear sunken and the surface is wrinkled.

4. Conclusions

This work indicates that it is possible to observe dynamic biological processes occurring in plant tissue. Stomatal closure can be observed inside the ESEM chamber using the protocol presented in section 2. We observe that progress toward continuous imaging may be possible by imaging at 5kV and stress the importance of adapting plant tissue for the imaging environment.

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