Cryo-scanning electron microscopy of plant samples without metal coating, utilizing bulk conductivity

Hans-Jürgen Ensikat and Maximilian Weigend
Nees Institute for Biodiversity of Plants, University of Bonn, Bonn, Germany

INTRODUCTION
Cryo scanning electron microscopy (cryo-SEM) is, alongside environmental SEM, the most reliable technique to avoid drying artifacts in biological SEM specimens [1-4]. But due to the very low electrical conductivity of deeply frozen samples, metal coating is conventionally used to increase surface conductivity. This automatically precludes the application of compositional contrast imaging on these samples. In recent years, the availability of advanced preparation equipment and cryo-SEM stages has led to an increased utilization of this technique. However, preparation and handling are not trivial. They require some experience and complex equipment.

The standard preparation protocol includes shock freezing, the transfer of the sample into a vacuum chamber, optionally partial sublimation of ice, sputter coating with metal, and a transfer into the SEM. Shock freezing reduces the disruptive growth of ice crystals in the tissue, and metal coating provides the necessary electrical conductivity to avoid charging. Occasional publications [5] show the feasibility of cryo-preparations with simple equipment and the examination of frozen samples without metal coating, but often at the cost of poor image quality.

In our studies, we observed that the electrical conductivity of uncoated frozen hydrated specimens increases with the temperature. Above -90°C conductivity is often sufficiently high to avoid charging, so that an examination is possible with satisfactory imaging quality in both secondary electron (SE) and backscattered electron (BSE) modes [6-7].

In this article we document the possibilities of cryo-SEM without metal coating on several botanical samples, using simplified preparation methods.

MATERIAL AND METHODS

EQUIPMENT
Scanning electron microscopy was carried out using a Cambridge Stereoscan S200 SEM and a LEO 1450 SEM, both equipped with SE and BSE detectors, an energy-dispersive X-ray (EDX) system and with custom-made cooling holders for low-temperature examinations (Figure 1).

Topographical images with the SE signal were recorded preferentially with 5 to 10 kV accelerating voltage and 10 pA (or less) beam intensity, whereas BSE imaging was performed with 15 to 20 kV and at least 20 pA.

CRYO-PREPARATION AND SCANNING ELECTRON MICROSCOPY
The fresh samples were cooled relatively slowly to below -100°C by placing the entire cooling holder in a liqN₂ bath. The initial cooling rate was ca 2 K sec⁻¹ (see diagram Figure 1b). Sample immersion in the coolant was avoided in order to preserve the original specimen surface. Optionally, the frozen samples were freeze-fractured. After closing the sample holder chamber with a cover, the holder was inserted into the SEM. After evacuation of the specimen chamber, the cover of the holder was removed and the sample was examined using very low beam intensities. Due to the thermal insulation, the temperature of the holder rises only slowly (see diagram Figure 1c). Thus, the samples can be examined for several hours at sufficiently low temperatures.

FREEZE DRYING
The cooling holder with the frozen sample was simply left in the SEM overnight, gradually reaching room temperature. In this interval the tissue water sublimated at temperatures far below 0°C. The dried samples were mounted on normal SEM stubs and sputter-coated.

RESULTS
The frozen samples were examined, beginning at temperatures far below -100°C, and continuing while the temperatures rose slowly. Image quality gradually improved with increasing temperature. Figure 2 shows the superhydrophobic surface of a leaf of Xanthosoma robustum (‘elephant ear’), which resembles a lotus leaf in its hierarchical structure. The morphology of the papillose epidermis cells is well preserved on the frozen hydrated samples (Figure 2 a-c). At a temperature of -130°C...
Cryo-SEM of plants

(Figure 2a), insufficient conductivity of the sample causes bad contrast due to charging. At -70°C, the charging effects have vanished and the contrast is good, even without metal coating. Despite the slow freezing process, the surface structure is not influenced by the formation of ice crystals in the tissue. At higher magnifications, resolution and contrast of fine structures such as the tiny wax crystals appear not optimal (Figure 2c). However, for a high-resolution examination, the following simple freeze-drying produced suitable results. Figures 2d,e show the freeze-dried sputter-coated sample with only slight shrinkage artifacts and the fine detail of the wax platelets.

Using adequate test liquids, it is possible to study, for example, wetting phenomena on superhydrophobic plant surfaces in a frozen state by cryo-SEM. Figures 3 and 4 show a drop of a glycerol/water/lead acetate (5:3:2) solution with suitable wetting properties, that does not crystallize during freezing and can be detected by the BSE signal due to the heavy metal content. Figures 3a-c show the decreasing charging effects at increasing temperatures. At -150°C (Figure 3a) strong charging of the drop causes distortion of the image by deflection of the electron beam in the vicinity of the drop. At -120°C (Figure 3b), contrast is still unsatisfactory due to deflection of the secondary electrons. Finally, at -80°C (Figure 3c) charging disappears, so that the sample can be examined in detail.

Figure 4a shows the point of contact between the drop and the tips of the papillae on the ‘hanging’ or receding side of the drop. (The contacts at the advancing side are hidden under the drop and thus more difficult to observe.) Small droplets on the surrounding papillae tips (Figures 4b-d) indicate that they have been in contact with the main drop during sample preparation. The colour images were made by combining a SE image (Figure 4c) and a BSE image (Figure 4d), which shows the droplets as bright spots, by the image processing function ‘Combine HSL’.

Compositional contrast imaging using a BSE detector can be used for several applications, as the samples are not metal coated. Accumulation of silica and calcium compounds in plants (biomineralization) can be examined by compositional contrast imaging and analyzed by EDX. Figures 5a-c show a stinging hair and other small hairs of a stinging nettle (Urtica bienorii) with both silica and calcium incrustations. Micro-organisms such as bacteria and fungi can be recognized well by staining with osmium tetroxide - the leaf epidermis remains unstained (Figures 5d,e). In cryo-fractured samples, calcium oxalate crystals or druses (Figure 5f) can be recognized reliably using the BSE image.

Cryo-fractures are valuable for distinguishing aerial from fluid-filled spaces in plant tissues. The druses in the Opuntia stem (Figure 5f) are clearly visible in the aerial space and are covered with a thin transparent membrane, most likely a residue of a dead cell.

The cryo-fracture images of the Xanthosoma leaf (Figures 6a,b) show the pattern of coarse ice crystals inside the cells, which becomes distinctly visible as the ice begins to sublimate. However, the surface of the inner (parenchymatic) cells appears well preserved, despite their very thin cell wall.
DISCUSSION
It is amazing to observe how the image quality of frozen hydrated samples improves and charging effects vanish, when the temperature rises above approximately -100°C. The reason is the bulk conductivity of ice. Ice is known to be a proton semiconductor [8-9], and its conductivity increases strongly with temperature. Proton conductivity is often found in solids such as ice or acidic salts, in contrast to other ions that provide conductivity only in liquids.

At temperatures above -120°C to -90°C, a sufficient image quality may be achieved for the plant samples, depending on specimen thickness, beam intensity, magnification, and other factors, presuming that the samples are completely hydrated, since dry parts would have insufficient conductivity. At temperatures above ca -100°C ice begins to sublimate. As long as solid surfaces are imaged, the incipient sublimation of ice usually does not impair imaging, apart from a slight drift at higher magnifications. On fractured samples, ice sublimation (etching) reveals details of internal cell structures, particularly on shock-frozen samples.

Omitting the metal coating not only simplifies the preparation. It facilitates several applications that use the detection of material contrast, which would be hidden by a metal coating, or the study of the wetting behaviour of liquids. We thus used the cryo-preparation successfully for the study of biomineralization in plants and drop adhesion on superhydrophobic plant surfaces.

Moreover, cryo-SEM is not limited to botanical or biological specimens (Figure 7). The presented experimental setup without temperature stabilization and without metal coating is not dedicated for high resolution imaging and cannot replace high-performance cryo-workstations with their high resolution and stable temperature control for certain applications. But the study of biological samples needs a versatility of preparation and imaging techniques even for imaging at moderate magnifications.

CONCLUSIONS
Frozen hydrated biological samples without metal coating can be imaged and analysed successfully in a conventional SEM at temperatures above -100°C at magnifications up to at least 2000X, due to the electrical conductivity of ice. This is particularly advantageous for the identification of material contrasts, for example, from mineral accumulations or stained structures, with the cryo-preparation as a reliable technique to avoid dehydration artifacts. Omitting the metal coating simplifies the preparation, which can be performed using a self-developed passively cooled cryo-sample holder. Slow cooling of the samples is sufficient, if...
solid surfaces are to be examined. These methods open up an easy approach to cryo-SEM, at least when high magnifications are not required.

REFERENCES

ABSTRACT
A classical problem in scanning electron microscopy is the alteration of specimen surfaces due to sample preparation. Simplified techniques for sample preparation with minimized surface alteration are therefore desirable. For fresh (hydrated) biological material, cryo-SEM after preceding shock-freezing and metal-coating is favoured when the prevention of drying artifacts has priority. A detailed knowledge of the sample properties and behaviour can facilitate new ways to optimize or simplify preparation and microscopy. Above ca. -100°C, ice has a measurable electrical conductivity due to its proton-conducting properties, which is sufficient to prevent frozen hydrated samples from charging during scanning electron microscopy, at least at low beam intensities. The examination of frozen samples without metal coating enhances the flexibility and versatility of cryo-SEM and simplifies sample preparations. We present some applications for the study of botanical samples, including compositional contrast imaging using backscattered electrons (BSE), and the study of wetting phenomena. The capabilities of simplified procedures are here demonstrated, particularly in combination with slow-freezing and the use of suitable test liquids for certain applications. The techniques can be easily adopted for conventional SEM setups.

CORRESPONDING AUTHOR DETAILS
Hans-Jürgen Ensikat,
Nees Institute for Biodiversity of Plants,
University of Bonn,
Meckenheimer Allee 170,
53115 Bonn, Germany
Tel: +49 (0) 228 733 273
Email: ensikat@uni-bonn.de

Microscopy and Analysis 27(6):7-10 (EU), 2013
©2013 John Wiley & Sons, Ltd