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INCREASING RESOLUTION AND VERSATILITY IN LOW TEMPERATURE CONVENTIONAL AND FIELD EMISSION SCANNING ELECTRON MICROSCOPY

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Abstract

Studies were undertaken to expand the versatility and the resolution of low temperature conventional and field emission scanning electron microscopy (SEM). The results indicated that simple modified specimen holders, which could be used in conjunction with the commercial cryosystems, allowed one to store specimens for several weeks in liquid nitrogen, either before or after observation in a conventional SEM, without incurring degradation of the surface features. Other modified holders permitted one to move the specimen closer to the final lens or to use the upper secondary electron detector, which is available with some SEMs. Both of these procedures increased the resolution that was attainable with the standard holders. In conventional SEM (CSEM) and field emission SEM (FESEM), holders were also modified to allow one to obtain complementary images of fractured specimens. When a conventional vacuum evaporator equipped with a freeze-etch module was used in conjunction with these holders, specimens could be fractured, etched, shadowed with platinum and coated with carbon before the sample was transferred to the cryostage in the SEM. This procedure increased resolution beyond that obtained with the sputter units in two commercial cryosystems that were used on a CSEM and a FESEM, provided membrane particle resolution in the FESEM and produced a coating or replica that could be recovered and examined in a TEM. These results, which demonstrated how resolution of cryospecimens can be enhanced in CSEM and FESEM, indicated that coating specimens in a high vacuum evaporator provided an alternative procedure that could be used to obtain high resolution images in a FESEM.

Key Words: Scanning electron microscopy (SEM); Field emission SEM; Freeze-fracture; Freeze-etching; Low temperature SEM; Platinum shadowing; Replicas; Sputter coating; Vacuum evaporation.

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Introduction

Scanning electron microscopy (SEM) of biological specimens generally requires chemical fixation, dehydration, critical point drying and coating. Each of these procedures is associated with potential artifacts or changes in the tissue (Beckett and Read, 1986; Read and Jeffree, 1988; Wergin and Erbe, 1989; Jeffree and Read, 1991; Read, 1991; Read and Jeffree, 1991). For example, chemical fixation, which is relatively long (5-60 min), has been associated with the extrusion of materials; solvent dehydration may extract cellular constituents; critical point drying can shrink and distort tissues; and sputter coating with 20-30 nm of gold-palladium may obliterate fine structural details. To avoid these problems, it is highly desirable to examine hydrated tissues. However, because samples examined with the SEM must be placed in a vacuum, evaporation of water, which frequently constitutes as much as 90% of the total tissue weight, would quickly result in collapse of the specimens.

To overcome these problems Echlin (Echlin et al., 1970; Echlin, 1978) introduced a procedure to examine fresh, frozen, fully hydrated specimens at low temperature (LT) in a conventional scanning electron microscope (CSEM). Refinements in the procedure, which were made by Pawley and Norton (1978) and Robards and Crosby (1979), led to the introduction of commercial low temperature systems designed for use with a CSEM. Low temperature SEM had two obvious advantages. First, the time required for cryofixation was short. Although the freezing rates for biological tissues may vary depending on the size and nature of the specimen, the time necessary to quench freeze-fix a biological sample was shorter, by several orders of magnitude, than that required for chemical fixation (Bachmann and Talmon, 1984). Second, the specimen would not collapse. When the frozen specimen was observed on a cryostage operating at temperatures below 143° K, the vapor pressure of water was not significant. Therefore, sublimation did not occur at a detectible rate. Furthermore, at temperatures, below 143° K, recrystallization of pure water did not occur (Beckett and Read, 1986). Consequently, as long as the stage remained cold, stable frozen samples could be observed for several hours (Wergin et al., 1988; Wergin

and Erbe, 1989).

In spite of these advantages LTSEM had several disadvantages. Namely, manipulation of the specimens at low temperature was more difficult, the resolution of the technique was generally limited to magnifications below 10,000X and frozen samples could not be readily stored. Introduction of the field emission (FE) SEM (Nagatani and Saito, 1986), which has a resolution of less than 1 nm, and the new generation of cryo-units (Müller et al., 1986; 1990; 1991), which incorporate planar magnetron sputter coating, have enabled biologists to increase resolution considerably. However, the expense of this instrumentation limits its accessibility to many biologists.

The present study describes some relatively inexpensive modifications that can be applied to other commercial cryosystems and discusses alternative preparation procedures that enable biologists to increase the flexibility and resolution of the low temperature applications pursued with CSEM as well as with FESEM.

Materials and Methods

Conventional SEM

Bulk biological samples were prepared for examination by plunging tissues into an EMscope SP2000A freezing chamber that contained liquid nitrogen slush. The sample was then removed from the freezing chamber using the EMscope transfer device and transferred to the cryochamber where it could be fractured, etched and sputter coated with gold. Following the coating procedure, the sample was retrieved with the transfer device and inserted into a Hitachi S-570 SEM equipped with the EMscope low temperature stage.

An alternative coating procedure was carried out in a Denton DV-503 high vacuum evaporator with a modified DFE-2 freeze-etch module (Steere, 1981). In this unit, frozen yeast cells were fractured, etched, shadowed with platinum (Pt) and coated with carbon (C). High resolution images of the fractured membranes of these cells were achieved by mounting the specimen on a modified holder that was used in conjunction with the upper detector on the Hitachi S-570 SEM equipped with the EMscope low temperature stage. After the frozen sample was photographed in the SEM, the cells were dissolved from the Pt/C replica, which was then mounted on a grid for observation in a Hitachi H-500H TEM. Identical cells were located and photographed in the TEM.

Field Emission SEM

An Oxford CT 1500 Cryotrans System was mounted on a Hitachi S-4000 FESEM to perform low temperature manipulations and observations. Bulk samples were frozen on an Oxford specimen holder and then transferred to the dedicated cryochamber and cryostage. To obtain complementary images of fractured samples, specimens were frozen in hinged gold specimen holders, which were clamped onto a freeze-etch specimen cap. The cap was mounted on a precooled modified Oxford specimen holder, and transferred, first to the dedicated Oxford cryochamber where the yeast was fractured and then into the microscope where it was etched. The etched specimen was returned to the cryochamber, sputter coated with Pt and then reintroduced to the cryostage in the microscope.

Other samples were mounted in the same type of gold holders, frozen, fractured, etched, shadowed by high vacuum evaporation with platinum and coated with carbon in a Denton freeze-etch module. Next these coated samples were transferred under liquid nitrogen to a modified specimen holder for use with the Oxford cryosystem that was installed on the Hitachi FESEM. The standard Oxford specimen transfer device was used to

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Figure 1. Portion of the surface of a pollen grain from daffodil. Specimen was sputter coated with gold in an EMscope cryosystem and observed using the standard EMscope specimen holder and the lower electron detector in a Hitachi S-570 scanning electron microscope. Bar = 5 μm.

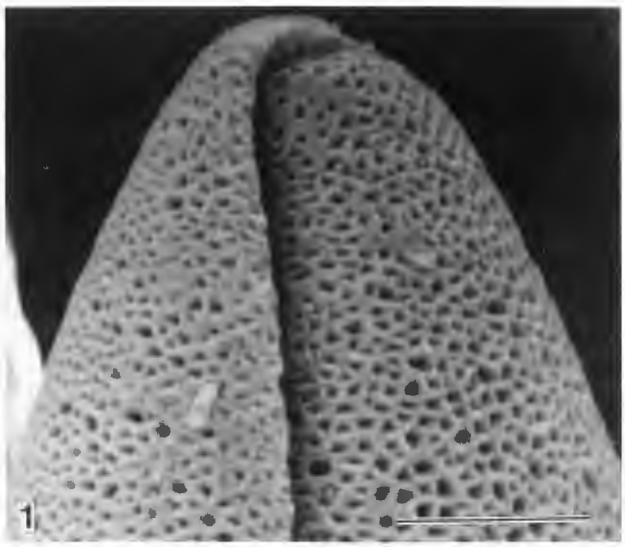
Figure 2. Cross-fractured cell from the radicle of cotton. Fracturing fresh frozen tissue allowed one to observe internal cellular structures such as the nucleus, nucleolus and several other organelles. Bar = 5 μm.

Figure 3. High magnification of the surface of a pollen grain from daffodil. Use of the standard EMscope specimen holder, the gold sputter coating technique and the lower electron detector in the Hitachi S-570 scanning electron microscope did not provide resolution of surface structure beyond magnifications of 15,000X to 20,000X. Bar = 0.5 μm.

Figure 4. EMscope specimen holder that had been modified to retain a small gold secondary holder (inset) on which the specimen was mounted. This combination allowed one to insert the specimen into the final lens and to use the upper detector of the microscope. Scale of holder = 1:1, Bar on inset = 1 mm.

Figure 5. Frozen daffodil pollen grain prepared similar to that shown in **Fig. 3**. However, this sputter coated specimen was mounted on the modified holder shown in **Fig. 4** so that the upper detector of the microscope could be used. As a result, resolution was improved to magnifications greater than 30,000X (compare image with that in **Fig. 3**). Bar = 0.5 μm.

Figure 6. Portions of cell walls showing glycocalyx filaments from two adjacent yeast cells that were frozen and fractured. The frozen sample was shadowed with platinum/carbon and coated with carbon in a high vacuum evaporator, mounted onto the modified holder shown in **Fig. 3**, transferred to the cold stage and observed with the upper electron detector. This coating procedure further improved resolution of biological specimens. Bar = 0.5 μm.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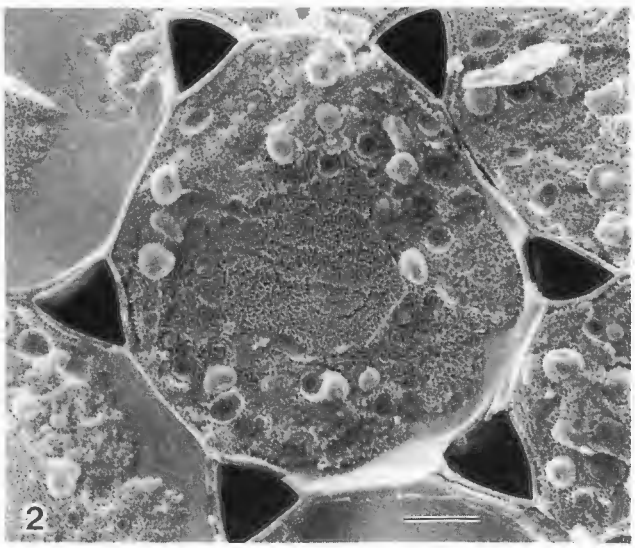
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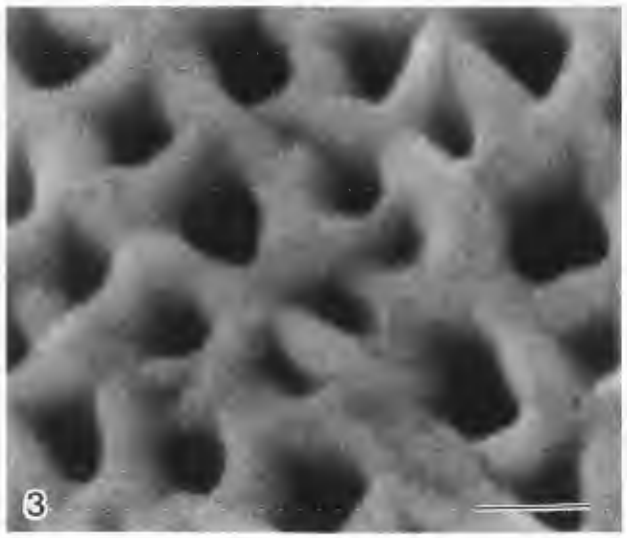
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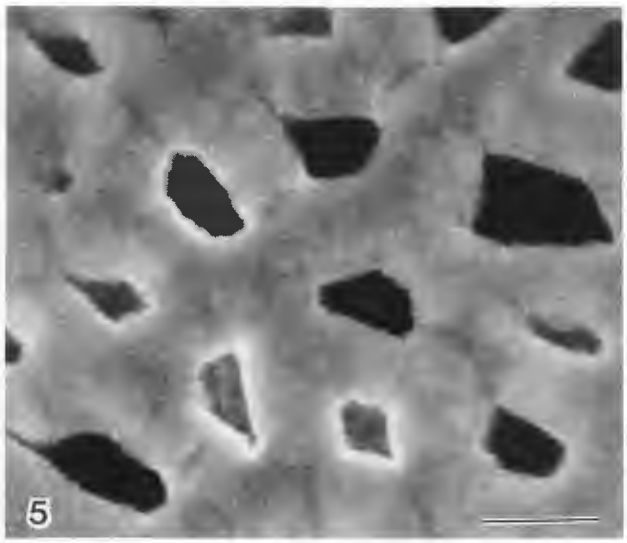
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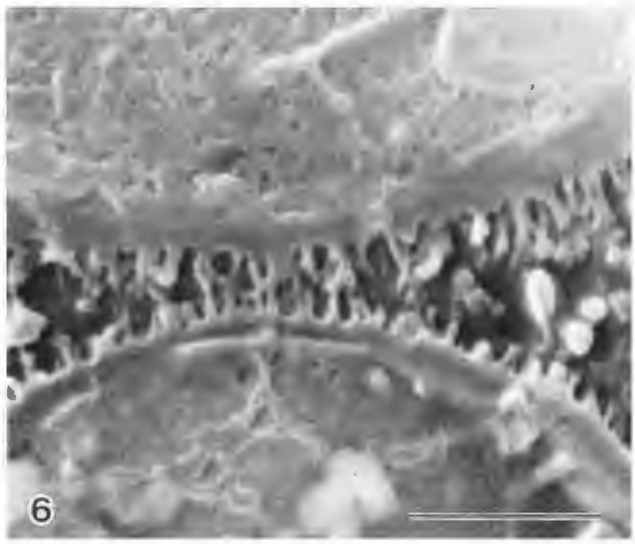
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insert the holder through the cryochamber and onto the cold stage in the microscope where the temperature of the cold stage was temporarily raised to 175° K to remove condensed water vapor so that individual cells, free of water-ice contaminants, could be photographed. Following observation, the frozen sample was removed from the FESEM, the tissue was dissolved from the platinum/carbon replica which was then mounted on a grid for microscopic observation and photographic recording in a TEM.

Low temperature CSEM and FESEM images were obtained with the Hitachi S-570 and S-4000, respectively. Accelerating voltages of 5 kV (**Figs. 2** and **11-13**) or 10 kV (remaining micrographs) were used to observe and record images onto Polaroid Type 55 P/N film. The replicas were observed in a Hitachi H-500H operating at 75 kV and recorded on Kodak Electron Image Plates. TEM images of freeze-etch replicas are presented with black shadows so that these micrographs can be directly compared to those from the SEM without resorting to contrast conversion.

Results

Conventional SEM

The standard EMscope cryosystem used with the recommended preparation procedures provides a quick means of observing surface features of whole (**Fig. 1**) and fractured (**Fig. 2**) biological tissues. However, when magnifications reach about 10,000X, ultimate resolution of structural details appear to have been achieved (**Fig. 3**). Two procedures were employed to improve resolution. First, a modified secondary gold specimen holder (**Fig. 4**, inset) was made to hold the specimen. The secondary gold holder with its specimen could be attached to a modified EMscope specimen holder (**Fig. 4**). These changes allowed insertion of the frozen specimen into the final lens of the microscope and use of its upper secondary electron detector. Use of the upper detector at a working distance of -2 mm improved the resolution of the sample to about 20,000X (**Fig. 5**). At this magnification the granularity of the sputter coating became apparent and prevented further resolution; however, this limitation to resolve further the surface features of the image could be overcome by substituting high vacuum evaporation for sputter coating. Use of high vacuum evaporation to shadow about 2 nm of platinum followed by evaporation of 6-8 nm of carbon allowed us to observe and photograph images up to about 50,000X (**Fig. 6**).

Use of the small gold secondary specimens holders also allowed us to store numerous specimens that resulted from large experiments. During storage in liquid nitrogen two types of changes occurred on the specimen surface. Occasionally, fragments of material or debris, which were initially observed on the surface of freshly prepared tissue (**Fig. 7**), were not present when the stored specimens were reexamined. Their absence apparently resulted from the turbulence that occurred during storage in liquid nitrogen. Alternatively, other contaminants, which were not present during the initial observation, appeared on the specimen surface after storage (**Fig. 8**). Because these contaminants could be removed by raising the temperature of the stage to 175° K (**Fig. 9**), they were believed to be water-ice that attached during the storage period or formed during the transfers - either when the specimen was moved from the microscope to the storage vessel or when it was subsequently returned to the cold stage.

Field Emission SEM

Similar hinged gold specimen holders were used in conjunction with a complementary freeze-etch specimen cap (**Fig. 10**). This combination was used in an Oxford CT 1500 cryochamber to fracture and sputter coat samples. Use of these hinged gold holders provided complementary images of fractured specimens. The degree of etching that was exhibited between the two halves varied (**Figs. 11** and **12**). This resulted from differential thermal conduction, which provided different etching temperatures, to the two halves of the holder. The half that was firmly clamped to the specimen cap had good thermal contact with the stage; therefore it received a lower etching temperature than its complementary half, which was only loosely attached via the hinge. Because this half was considerably less efficient at conducting heat away from the sample, it was exposed to a warmer etching temperature.

Platinum sputter coatings in the Oxford cryochamber provided ample resolutions to about 30,000X, a point at which granularity representing either the coating material or surface contaminants began to obscure further meaningful resolution (**Fig. 13**). Further increases in the resolution of the SEM image could be obtained with the

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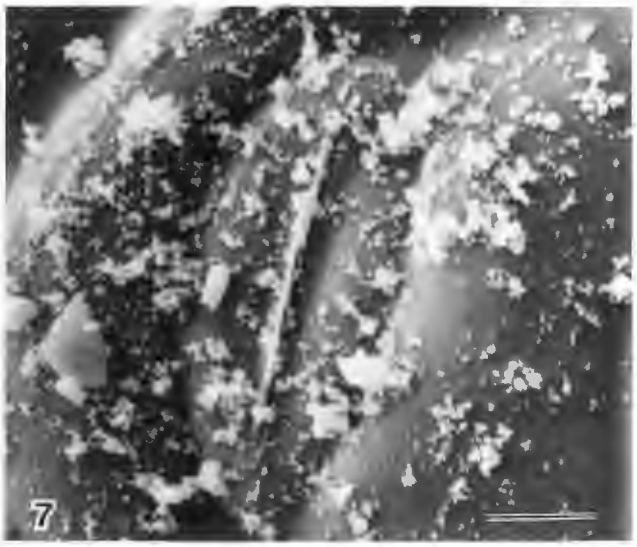
Figures 7 to 9. Upper surface of a soybean leaf illustrating the surfaces of a closed pair of guard cells and portions of several adjacent epidermal cells. This specimen was initially observed and photographed in the microscope (**Fig. 7**) and then stored in liquid nitrogen. Observation of samples after storage (**Fig. 8**) frequently revealed the presence of contaminants (arrows) but less surface debris. The contaminants could be sublimed by raising the temperature of the stage to 175° K (**Fig. 9**). Bars = 5 μm.

Figure 10. Oxford specimen holder that had been modified to accept a complementary freeze-etch specimen cap which holds six hinged gold specimen holders (inset). Scale of holder = 1:1, Bar on inset = 1 mm.

Figures 11 and 12. Fractured cells of yeast from one of the complementary hinged gold holders shown in the inset, **Fig. 10**. The degree of etching in **Fig. 12** was greater than that exhibited in **Fig. 11** due to differential thermal conduction in the two halves during etching. The specimen was frozen, fractured and sputter coated with platinum in the Oxford cryosystem and then observed in the Hitachi S-4000 FESEM. Bars = 5 μm.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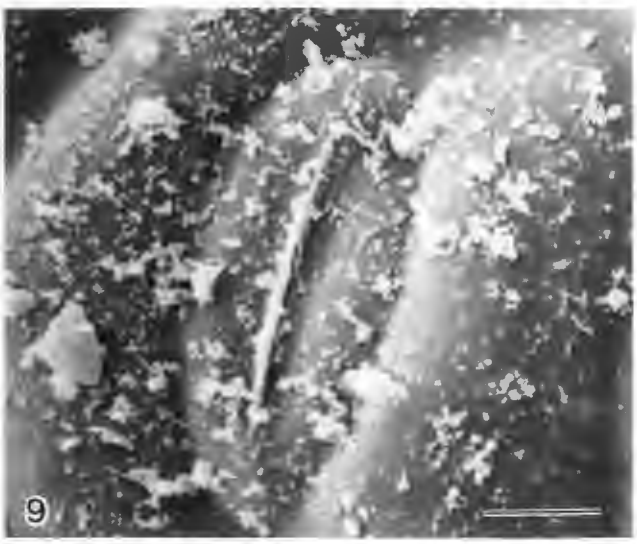
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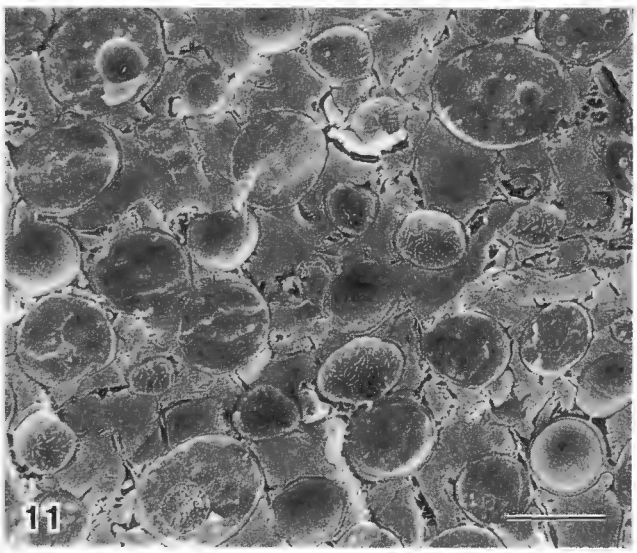
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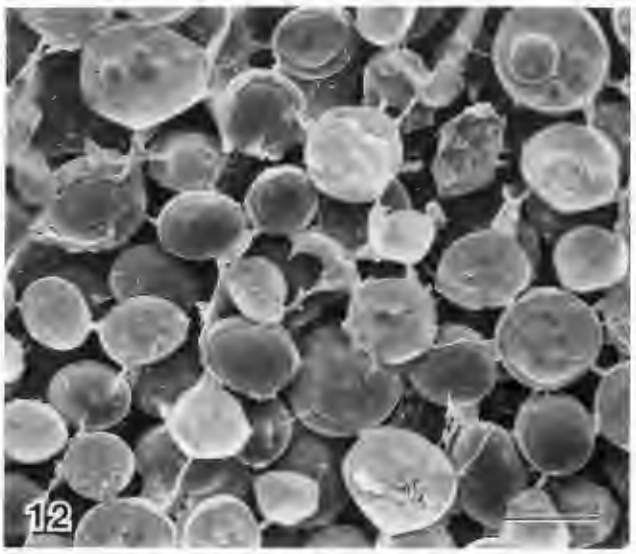
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complementary holders by substituting high vacuum evaporation for sputter coating (**Fig. 14**). However, after the sample was coated in the evaporator, a "through air" transfer was necessary to move the specimen into the microscope. As a result, contamination in the form of water-ice condensed on the surface of the sample (**Fig. 15**) and had to be removed by raising the temperature of the stage to 175° K to etch or sublime the contaminants (**Fig. 16**). This process did not appear to degrade the quality of the final image. Use of high vacuum evaporation to shadow about 2 nm of Pt followed by evaporation of 6-8 nm of C and transfer to the cryostage allowed us to observe and photograph images up to about 100,000X. At this magnification, contamination, thermal drift and vibration prevented further resolution. However, the magnification was adequate to resolve particles, which were reported to have a diameter of about 10 nm in TEM replicas (Gross et al., 1978), on the P-face of the yeast plasma membranes (**Fig. 14**).

Coating that was performed in the high vacuum evaporator provided an advantage not possible with sputter coating. Namely, the unidirectional Pt/C coating was analogous to the procedure that has been classically used to produce freeze-etch replicas which have been examined in the TEM for many years. Therefore, after the frozen specimen containing the Pt/C coating was observed and photographed in the SEM, it was removed from the instrument and placed in acid to dissolve away the tissue. The replica was then recovered, mounted on a grid and observed in a TEM. This procedure allowed us to use low temperature FESEM to observe the surfaces of frozen, fully hydrated specimens (**Fig. 17**), and then to recover replicas of those surfaces and use TEM to observe and photograph the same cell or surface areas (**Fig. 18**).

Discussion

One of the biggest disappointments of low temperature SEM is that most of the studies that successfully illustrate fresh frozen biological tissues, have been performed at relatively low magnifications i. e., less than 8,000X. This is generally the case with CSEM because the sizes of the cryostages and the specimen holders usually require long working distances, which greatly reduce resolution (Beckett and Read, 1986). Our study indicates that the resolution in a CSEM can be enhanced merely by modifying the specimen holder so that one effectively shortens the working distance. In addition, if a CSEM is equipped with an upper secondary electron detector and the holder is modified to allow use of this feature, a 3 to 5 fold gain in resolution is achievable.

CSEM specimens, which are viewed at ambient temperature, are viewed and usually stored in a desiccator for future reference; this procedure is not possible with frozen specimens. Our results, which support those by Van Gardingen et al. (1989), indicate that specimens can be easily stored in liquid nitrogen before or after observation. The main disadvantage is the appearance of contaminants, which seem to consist of water-ice, on the surface of the specimen. However, most of these contaminants can be sublimed by raising the temperature of the stage. An unexpected advantage of storing frozen specimens was the occasional disappearance of surface debris which was thought to arise during the fracturing process. The turbulence of the liquid nitrogen during storage apparently dislodged this material.

Recently resolution of frozen, hydrated biological specimens to less than 10 nm (Müller, et al., 1990; Walther et al., 1990) has been made possible through the use of FESEM and the Balzers SCU 020 - a dedicated cryochamber that uses planar magnetron sputtering. We have achieved similar results that allow us to resolve membrane particles on frozen, fully hydrated cells, by using high vacuum evaporation to coat samples for low temperature FESEM (Wergin and Erbe, 1991). This procedure, which can be carried out in an existing freeze-etch unit in a vacuum evaporator, required transfer of the frozen, fractured, coated specimen to the cryostage. Although this procedure involved a through air transfer, which resulted in water-ice contamination, direct vacuum transfer would largely eliminate this problem.

Figure 13. Portion of the E-face of the plasma membrane from a single fractured yeast cell. This specimen was prepared and observed similar to that described for **Fig. 12.** At magnifications greater than about 30,000X, granularity, possibly representing the platinum coating or condensed contaminants, could be observed on the surface of the specimen. Bar = 0.5 μm.

Figure 14. Portion of the P-face of the plasma membrane from a fractured yeast cell. This specimen was fractured, shadowed with platinum/carbon and coated with carbon in a high vacuum evaporator and then transferred through the Oxford chamber to the cold stage on the Hitachi S-4000 FESEM. This procedure considerably improved resolution so that 10 nm intramembrane particles could be observed on the surface of the fractured plasmalemma. Bar = 0.5 μm.

Figures 15 and 16. Yeast cells that were prepared as described in **Fig. 14.** When the frozen sample was transferred through the air from the evaporator to the cryosystem, contaminants condensed on its surface (**Fig. 15**). However, these contaminants, which consisted largely of water-ice, could be removed by sublimation by raising the temperature of the stage to 175° K (**Fig. 16**). Bars = 5 μm.

Figures 17 and 18. Portion of the E-face of the plasma membrane of yeast that was prepared similar to that shown in **Fig. 14.** This procedure not only allowed observation of the frozen, fully hydrated cell in the FESEM as shown in **Fig. 17**, but the identical cell could be observed in a TEM after retrieving the frozen sample from the FESEM, removing the Pt/C coating or replica and mounting it on a grid (**Fig. 18**). Bars = 0.5 μm.


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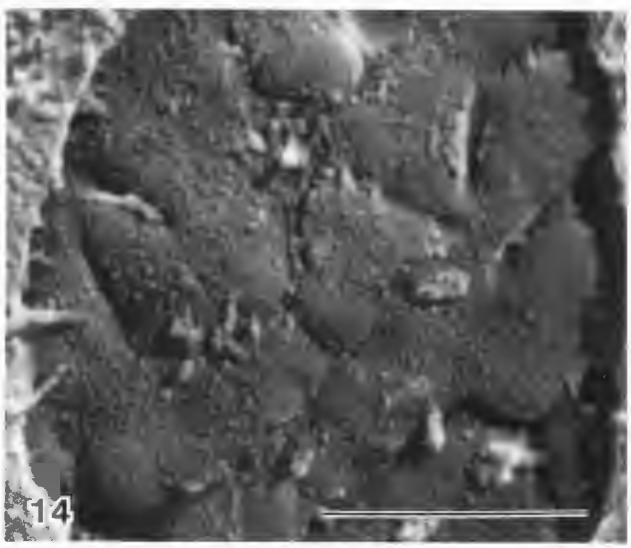
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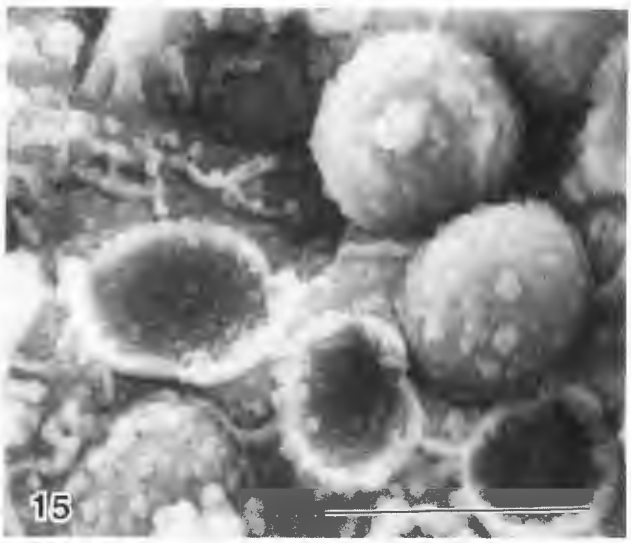
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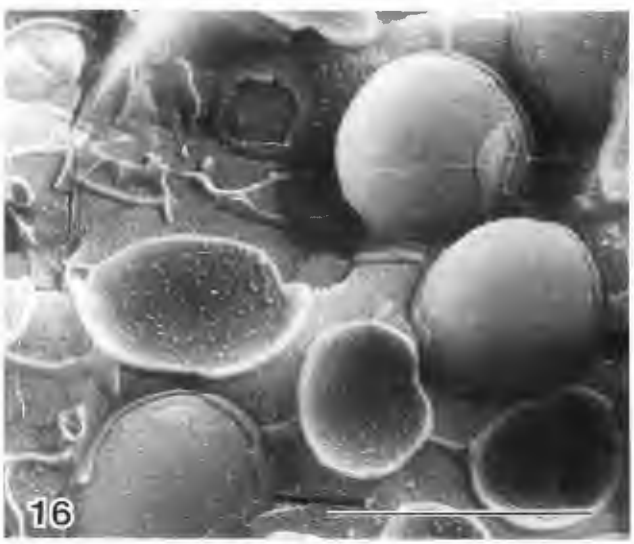
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The high vacuum evaporation method for coating combined with the use of the hinged gold holders had two additional advantages. First, the procedure used to produce the Pt/C coating was identical to that employed to make replicas for observation in a TEM. Therefore, after the frozen sample was observed in the SEM, the specimen could be removed from the instrument, the tissue dissolved in acid and the replica recovered for observation in a TEM. At the present time, resolution of the replicas is greater in the TEM than that obtained by viewing a replica at ambient temperatures in a FESEM (Wergin and Erbe, 1989; 1990; Wergin et al, 1989). However, our current results indicate that further resolution of structural detail at low temperature appears to be limited by specimen contamination and stage vibration rather than by the grain size resulting from the evaporated Pt/C coating. Reducing contamination and eliminating stage vibration may further increase the resolution obtainable with low temperature FESEM. A second advantage of our procedure results from the use of the hinged gold holders. These holders allow complementary images of the frozen, fractured specimens to be observed not only in the FESEM, but if the replicas are recovered, complementary images can also be observed and photographed with the TEM (Wergin et al., 1991).

Use of high vacuum evaporation in combination with low temperature SEM provides direct comparison of many of the same structural features by two distinct electron imaging techniques. A comparison of the fractured membranes indicates that the gross three-dimensional structure of a sample is observed better when a Pt/C replica, which remains attached to the tissue and functions as the conductive coating, is examined with low temperature CSEM or FESEM. Although resolution of less than 10 nm can be obtained on frozen, fully hydrated tissue in the FESEM, the resolution of the fine structural details is more clearly discerned on the replica in a CTEM. However, use of evaporative coatings provides a means for many EM labs to pursue high resolution cryotechniques by making use of existing freeze-etch modules in combination with a modestly priced cryostage.

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Discussion with Reviewers

N. D. Read: Could the authors explain in more detail how and for how long the frozen-hydrated samples were stored under liquid nitrogen.

Authors: To store samples under liquid nitrogen after they had been observed in the SEM, the specimen holder containing the mounted sample was removed from the SEM and inserted into a plastic tube filled with liquid nitrogen. The tube, which was attached to a cord, was then lowered into a dewar of liquid nitrogen for storage. The soybean leaf surfaces illustrated in **Figures 8** and **9** were stored in liquid nitrogen for 2 days after the initial observations and micrographs (e.g., **Figure 7**) were recorded. These identical areas and other biological samples have been observed and photographed after six weeks of storage in liquid nitrogen (unpublished results).

P. Walther: On the sample in **Fig. 16** you removed the water contaminants on the membranes by raising the temperature to 175°K. Did this not produce shrinking artifacts, changing the structure of the membranes at high resolution?

Authors: This sample had been etched and coated in the vacuum evaporator and then transferred through air to the cryostage in the FESEM. The temperature of the stage was then raised to remove the condensed water from the surface of the Pt/C coating without affecting the frozen water that is present in the sample (below the coating). The presence of the coating beneath the surface contamination prevented charging; therefore, the sample could be observed in the FESEM while sublimation of the water-ice contaminants was performed. During this process, sublimation occurring from **below** the coating would definitely result in shrinking artifacts. However, when this happened it disrupted the coating and charging occurred. Consequently surface contaminants were carefully monitored during sublimation and the sample was rapidly recooled before the coating was ruptured and shrinking artifacts of underlying membranes could occur. This etching step will no longer be necessary when our samples can be transferred under high vacuum from the evaporator to the microscope.

P. Walther: **Figs. 17** and **18** show a comparison of the frozen-hydrated sample in the cryo-SEM (**Fig.17**) with the same portion after replication in the TEM (**Fig. 18**). The resolution is better in the TEM image (**Fig. 18**). In our experience this is not only an effect of the poorer resolving power of the SEM, but also of the coating method used in this study. The samples were coated first with 2 nm of platinum and then with an additional 6-8 nm carbon coat. When the sample is imaged with secondary electrons (**Fig. 17**) it is mainly the carbon coat that is visualized. This carbon layer is relatively thick and hides the small surface features. To overcome this limitation, we used the mass dependent backscattered electron imaging to visualize the underlying platinum coat (Walther P, Hentschel J [1989]. Improved representation of cell surface structures by freeze substitution and backscattered electron imaging. Scanning Microsc. **3** [Suppl], 201-211). The platinum coat is thin and in close contact to the biological surface and therefore provides better surface information. Our studies were, however, done with air dried samples. Did you try backscattered electron imaging of your frozen-hydrated samples?

T. Müller: I can agree with your statement, that the granularity of the Pt-C does not seem to limit the resolution in your micrographs. Platinum deposited by planar magnetron sputter coating in our system did not show any granularity in the LTSEM either. For the moment we don't feel any need to choose another coating material or to use evaporative techniques. But if so, the choice of the material would be chromium by planar magnetron sputter coating (Apkarian RP, Gutekunst MD, Joy DC [1990]. High resolution SE-I SEM study of enamel crystal morphology. J. Electron Microsc. Technique **14**, 70-78) or electron beam evaporation of chromium (Hermann R, Müller M [1991]. High resolution biological scanning electron microscopy: a comparative study of low temperature metal coating techniques. J. Electron Microsc. Technique **18**, 440-449) or platinum-iridium-carbon (Wepf R, Amrein M, Bürkli U, Gross H [1991]. Platinum/iridium/carbon: a high-

resolution shadowing material for TEM, STM and SEM of biological macromolecular structures. J. Microsc. **163**, 51-64). To start with the simple techniques first: did you ever check the application of planar magnetron sputtered chromium?

T. Müller: On your micrographs the invaginations ("mountains") on the EF are broadened by the additional carbon layer when imaged by SE (**Fig. 17**) as compared to these structures when imaged by TEM (**Fig. 18**). Therefore, important consequences of making replicas for SEM and TEM observation must be considered. For example, the resolution in the SE mode is reduced by the C layer as shown by Walther and Hentschel (1989), with a conventional FESEM (Hitachi S-800) and by Hermann and Müller (1991) with an in-lens FESEM (Hitachi S-900). As a result the resolution of the microscope is not fully utilized in the SE mode. This loss of resolution can be partially corrected with the help of an Autrata BSE detector (YAG type). But according to Hermann and Müller (1991), the SE signal can be expected to be superior to the BSE signal. For these reasons you might improve the resolution shown in **Fig. 14** by doing electron beam evaporation of the Pt-C without the additional C layer. Then of course, observation in the TEM would not be possible. If you compare different coatings, use similar test specimens because the degree of hydration among samples can be highly variable. Our experiments indicate that the structural resolution of the SEM is affected by electron diffusion inside the hydrated specimen (Walther P, Hentschel J, Herter P, Müller T, Zierold K [1990]. Imaging of intramembranous particles in frozen-hydrated cells [*Saccharomyces cerevisiae*] by high resolution cryo SEM. Scanning **12**, 300-7). Furthermore, hydration within the specimens themselves can be variable. On the EF of the yeast plasmalemma (**Fig. 13**) small ring-like depressions are present which correspond to the volcano-like transmembrane particles on the PF (Gross H, Müller T, Wildhaber I, Winkler H [1985]. High resolution metal replication, quantified by image processing of periodic test specimens. Ultramicroscopy **16**, 287-304). On the PF in **Fig. 14**, the particles are just visible; however debris, which may consist of ice-contamination, covers some of the areas of interest. One would have to be careful in etching this sample because the cytoplasm and the PF are much more delicate than the EF attached to the wall. My specific suggestion would be to look exclusively at the PF of yeast cells and compare the results of the following experiments:

- planar magnetron sputter coating of platinum, chromium and LTSEM;
- evaporation coating of Pt-C and LTSEM;
- evaporation coating of Pt-C and C and LTSEM and TEM of the replicas.

Direct comparison of planar magnetron sputtering with Pt-C electron beam evaporation or with Pt-C/C replicas will then be possible.

Authors: We do not have a backscattering detector, planar magnetron sputter coater or electron beam evaporation capabilities at this time; therefore these experiments have not been performed. We do plan to perform a modification of the experiment suggested by Müller, namely to shadow with Pt/C in the high vacuum evaporator but not coat with carbon. If the Pt/C shadowing were adequate to prevent charging, perhaps the resolution could be enhanced in the SE images. After observation in the FESEM, if the frozen sample can be transferred from the cryostage back into the vacuum evaporator, we could then coat with carbon to provide a replica for TEM comparisons. We would like to reiterate that the intent of our study was to "discuss some relatively inexpensive modifications" that could be used to explore high resolution SEM, complementary imaging of frozen, hydrated freeze-fractured samples in the FESEM and to compare the results to those that have been reported for replicas viewed in the TEM. We used Pt/C shadowing and C coating because this procedure provided a coating sufficient for imaging samples in the SEM as well as a replica that could be recovered for observation in the TEM. Furthermore, this procedure could be carried out with an existing high vacuum evaporator and a relatively simple cryostage. During our experiments (and in spite of the fact that we were not equipped to make a high vacuum transfer from our evaporator to the microscope), we did obtain excellent complementary images in the SEM and the identical halves of fractured membranes could be imaged and compared to the same cells in the TEM replicas (Wergin WP, Erbe EF [1992] Techniques for obtaining and observing complementary images with a low temperature field emission SEM and subsequent comparison of the identical cells in freeze-etch replicas viewed with a TEM. Scanning **14**, No.1, In Press). Furthermore, this coating procedure provided sufficient resolution to resolve membrane particles without having to resort to other more expensive techniques such as planar magnetron sputter coating or electron beam evaporation. In the future we believe that our resolution can be further increased and we would indeed like to make comparisons with other coating procedures.

T. Müller: You write in Materials and Methods that all the water contamination that occurred during the transfer was removed by etching at 175°K. **Fig. 13** and more clearly **Fig. 14** show many ice crystals. Can you explain the source of these contamination products?

Authors: **Fig. 13**, which was sputter coated with gold, exhibits the type of grain and contamination that limits this coating procedure. In our system, the prechamber, where sputter coating occurred, was not a high vacuum chamber; therefore water contamination from numerous sources undoubtedly condensed onto the frozen, newly fractured specimen. Converting the prechamber to high vacuum will help to alleviate this problem. **Fig. 14** was Pt/C shadowed and C coated in a high vacuum evaporator. At the present time, a through air transfer and submersion into liquid nitrogen is required when moving the sample from the evaporator to the cryostage. As one can see from **Figs. 7-9** and **Fig. 15**, both of these procedures are associated with contaminants - some of which apparently are resistant to sublimation in the microscope.