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SOME PROBLEMS ASSOCIATED WITH LOW TEMPERATURE MICROMANIPULATION IN THE SEM

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ABSTRACT

In order to perform micromanipulation on frozen specimens in the SEM, specimen temperature must be kept sufficiently low that sublimation will be negligible during the required observation period. The micromanipulator needle must also be cooled to prevent local melting and drying during the manipulation. Because the specimen itself is one of the coldest surfaces in the specimen chamber and can act as a 'cold finger,' special attention should be paid to efforts to monitor and reduce condensables in the specimen chamber atmosphere. Results with a test specimen (Pelargonium leaf) indicate that SEM micromanipulation can be performed at low temperatures (-150°C) with minimum sublimation over periods of time that would allow for quite extended maneuvers. Fresh surfaces can be produced by fracture over small areas of the specimen, even down to fractures of a single cell on the plant surface. Particulates can be removed from the surface to allow study of the frozen specimen surface beneath. The separate problem of initial rapid freezing with minimum artifacts is an integral part of low temperature micromanipulation, but has been reviewed extensively in recent reports by others.

KEY WORDS: Biological Specimens, Vacuum, Scanning Electron Microscopy, Pelargonium Leaf

Some Problems Associated with Low Temperature Micromanipulation in the SEM

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Introduction

The combination of a low temperature specimen and micromanipulation in the column of the SEM offers some unique advantages and poses some special problems. The advantage of being able to expose fresh surfaces by fracturing or removing small parts of a sample held in the frozen hydrated state can be very useful in both morphological and chemical analytic SEM studies. The ability of very rapid freezing to hold even small molecule constituents in place and the fact that no fixation, dehydration or drying is necessary can help to reduce chemical extraction.[1] On the other hand, special problems of contamination by condensation, micromanipulator needle temperature, and fracture properties of ice are also associated with low temperature micromanipulation. The proper rapid freezing of the specimen is essential to this technique, but has been discussed by others,[2,3] and will not be covered here. Suffice to say that the very strict freezing requirements set up for freeze-drying apply equally to samples being prepared for low temperature SEM micromanipulation.

Several of the problem areas of particular importance to the combined operation of micromanipulation and low temperature specimen methods will be considered below.

Condensation onto the low temperature specimen

When fresh surfaces of the cold sample are created by micromanipulation, the problem of condensation from the specimen chamber atmosphere is particularly acute. These contamination problems are reduced by careful control of the chamber atmosphere, particularly in the immediate area of the sample. The first step toward such control is to assure that the vacuum system is leak tight. Because rather large pressure gradients may exist between the pressure transducer and the specimen, a small leak close to the specimen may result in serious specimen contamination and be essentially undetected by the base pressure measurement of vacuum. Residual gas analysis by mass spectrometry is important to the control of specimen chamber pressure and atmosphere composition. The reduction of condensables when the system is fitted with a liquid nitrogen cold trap can be seen by comparing Figures 1 and 2 and a liquid nitrogen cooled anti-contamination cold plate near the specimen is also effective. Backfilling with dry nitrogen and a continuous nitrogen bleed while the chamber is at atmosphere reduces the condensable load on these anti-contamination devices.

Specimen temperature and stage temperature

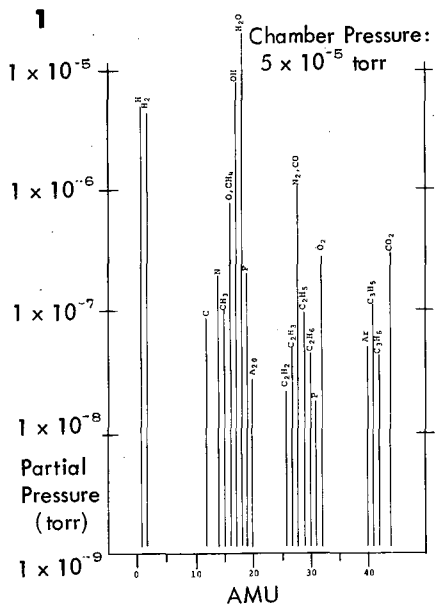
Micromanipulation takes place on the sample surface and the temperature of the sample may be somewhat different from the temperature of the stage as indicated by the cold stage monitor. A test system to establish the amount of this differential helps to predict true specimen temperature as a function of the monitored stage temperature. A fine (0.1mm diameter) thermocouple imbedded in a drop of ice on the specimen mount can indicate actual specimen temperature. (A similar thermocouple arrangement can be used to monitor specimen cooling rates during the initial freezing step, Figure 3.) The specimen temperature in this test system can be compared to the temperature indicated by the cold stage monitor during warming and cooling cycles. When the specimen mount is being cooled through contact with the liquid nitrogen reservoir, specimen temperature is higher than the indicated temperature, and when the specimen mount is being warmed by the small heater in the stage, the specimen temperature is slightly below that indicated by the stage monitor. Warming and cooling curves for the specimen and the stage monitor are shown in Figure 4.

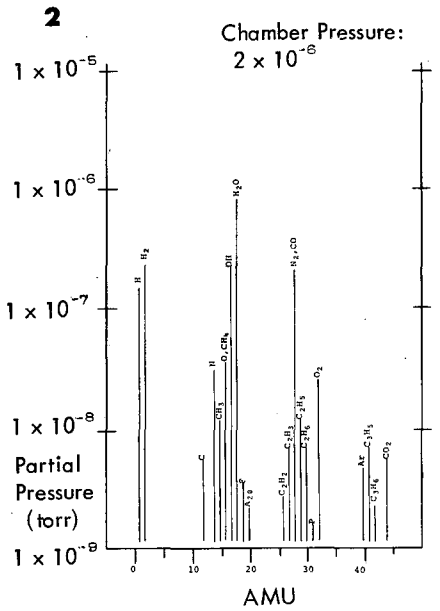
Heat flow paths and schematics of the heat flow during cooling and heating help to explain these data with respect to a typical stage design.

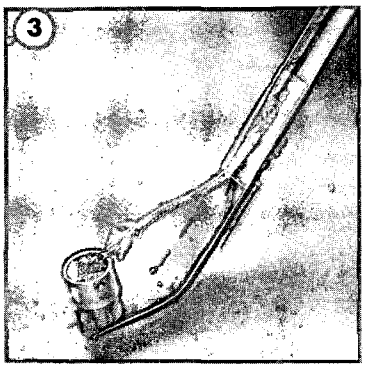
Sample temperature may rise during transfer (under low frost conditions) from the rapid freezing apparatus to the low temperature stage, and during metal evaporation procedures for the reduction of charging of freshly fractured surfaces. The low temperature module must have sufficient thermal mass to ensure reasonably constant low temperature during the period of time that it is disconnected from the cold stage. It is important to know the warming rate of the specimen module under conditions of vacuum (in airlock for metal evaporation) and the atmosphere (in introducing the specimen into the cold stage.) Typically we use such temperature monitoring results over a 10 minute interval. Specimen introduction can be accomplished in time that maintains specimen temperature below -120°C, and metal evaporation in the airlock can be carried out in a time such that the temperature rise from cold stage temperature (-150°C) is 10°C or less, to about -140°C.

Specimen stability at low temperature

In order that the micromanipulation procedures can be carried out on the frozen sample, it is necessary that changes due to sublimation and contamination be small







Figures 1 and 2
Mass spectrometry of SEM chamber atmosphere without (Figure 1) and with (Figure 2) LN cold trap.

Figure 3.
Sample mock-up used to monitor specimen temperature.

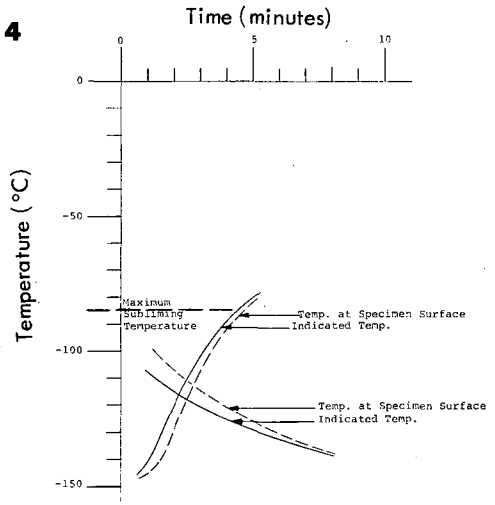


Figure 4. Warming and cooling curves showing temperature differential.

over quite long periods of time. Micromanipulation, even with the fairly simple deFonbrune type in the SEM[4], requires many minutes of observation and the more intricate maneuvers possible with the dual needle piezoelectric micromanipulator [5] may require hours. A test specimen that has several levels of structural detail extending to the smaller sized particles visible in the SEM can be helpful in assessing the stability of the sample at low temperatures, and when compared to critical point dried material, can help to indicate changes produced in the initial rapid freezing step. The leaf of the common plant Pelargonium can be useful as such a test specimen.

The leaf surface exhibits three size levels of structures: numerous large surface hairs (Figure 5), gland structures of about 25μm diameter (Figure 6), and fine particulates of perhaps 0.1μm on the surface of the gland structure (Figure 7). Small pieces (1mm^2) of fresh leaf were quick frozen by plunging them into an LN cooled Freon 22 slush. The samples were then introduced inside a frost shield, into the low temperature stage, and held at -150°C during test periods of viewing and recording. The sequence shown represents many minutes of observation and no sublimation changes could be observed even for viewing times exceeding 1 hour. Long term observations on freshly fractured surfaces (shown later) indicated similar stability at this temperature, and ice recrystallization will be minimal below -130°C[3]. The sublimation rate should be slow enough for extensive micromanipulation at the temperature and vacuum of the available SEM cold stages, but stability testing using a sample of known morphology is advisable before proceeding with the manipulation itself.

Micromanipulator needle temperature

The very fine needle of the micromanipulator has a limited ability for heat capacity and conductance. But when dealing with small structures of the specimen surface, the micromanipulator needle, if not cooled, may cause melting and subsequent air drying and deformation of the sample. One of the gland structures of the test sample is shown in Figure 8 as a room temperature needle pierces it. Note deformation as the needle produces local melting.

It is generally necessary to cool the micromanipulator needle before work on low temperature specimens can be done without unwanted melting. If the needle is cooled by providing contact with the LN reservoir, temperature close to that of the sample can be achieved and

manipulation and fracturing can take place without melting. If the cooled needle is used to contact the gland (Figure 9), ice structure is maintained and the gland can be snapped off without melting. This operation can be repeated over the surface of the leaf to collect a number of glands, which remain frozen to the needle (Figure 10). Adequate cooling of the micromanipulator needle is also essential for cryofracture of the main specimen block as considered in the following section.

Fracture planes in the specimen

Fracture at the base of the gland or hair reveals the interior ice surface (Figure 11), indicating that freeze-drying has not taken place even in the structures projecting from the main surface. The rate of sublimation at -150°C, as mentioned above, is low enough to ensure hydration in the interior of these high surface area structures. The plant material used as the test specimen in these studies may provide special barriers to sublimation, but preliminary work with bone marrow has suggested that the ice will be maintained in animal tissue as well. In both cases, the brittle fracturing of the ice is quite different from the plastic, "cotton candy" response of frozen-dried specimens. The test of mechanical properties is a useful addition to morphological evidence when one is concerned with the question of unwanted freeze-drying of specimens on the low temperature stage.

Fracture of the surface cells of the frozen leaf is also possible with the chilled micromanipulator needle (Figure 12). The fracture proceeds down through the various layers of the cell in a stepwise fashion, exposing surfaces from the outer cuticle to the watery interior (Figure 13). The area of the various intra-cellular surfaces exposed by fracture is quite variable depending on the specimen and is not under the control of the operator, but most surfaces are represented by a planar step that is large enough to permit detailed examination at high magnification. For example, Figure 14 shows a granular layer just beneath the cell wall, and Figure 15 illustrates the texture of a fracture plane through the large, watery, central volume of the Pelargonium leaf cell shown previously in Figures 12 and 13. The ability to fracture selected individual cells of the specimen, and the advantage of being able to repeat the fracturing in the same area, can be useful attributes of fractography by micromanipulation. Fixed tool procedures have also been carried out in the SEM column[6]. The stage movement can be used to maneuver the specimen against a fixed tool and the rigidity and strength of such fixed tools are considerably greater than the micromanipulator needles. Again, proper cooling of the fixed tool would be necessary in order to work with low temperature samples.

Investigation of particulates on the specimen surface

The micromanipulator needle can be used to remove small particles from the surface of the frozen specimen. Figure 16 shows an airborne contaminant particle being picked up from the Pelargonium leaf surface while the specimen is held at -150°C. Structural changes underneath the particle can then be examined without drying artifacts, and chemical analysis of the underlying surface is possible without the chemical extraction associated with critical point drying techniques. The SEM micromanipulator has already been used to dissect out small parts of a critical point dried sample for further study by conventional transmission electron microscopy[4]. In this case, the small samples were transferred directly from the micromanipulator needle to the small embedding capsule while still in the SEM specimen chamber. With the low temperature material, the problem of sampling for further morphological study is complicated by the fresh, non-fixed, non-dried state of the sample and its consequent susceptibility to air drying as the temperature rises. However, it is not impossible to keep the temperature in the range where sublimation can take place, and thereby freeze-dry these small specimens.

Conclusion

Several unique technical problems are associated with the micromanipulation of low temperature specimens in the SEM. In addition, the technique requires considerable instrument time, which may be an important consideration with heavily scheduled SEM installations. With attention to questions of specimen temperature and contamination, micromanipulator needle temperature and control of sublimation, it is possible to fracture and sample the SEM specimen and to have sufficient observation time to monitor these micromanipulation procedures on the low temperature specimen.

All techniques of micromanipulation should benefit markedly from the availability of real time TV speed stereo SEM[6]. The use of stereoscopic vision while micromanipulating helps to overcome the very difficult problem of estimating distances in the direction parallel to the SEM beam.

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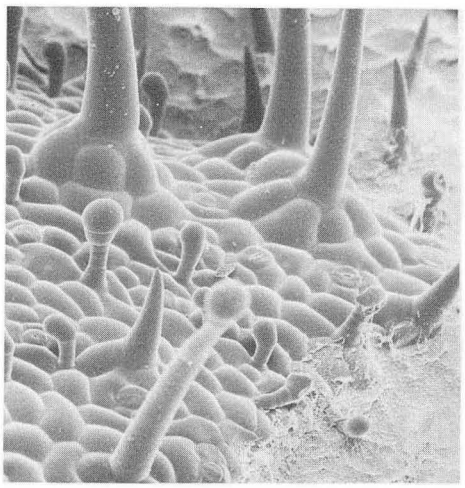


Figure 5. Test specimen at low temperature. Pelargonium leaf, quick frozen in Freon 22 slush. Photo width, 325μm.

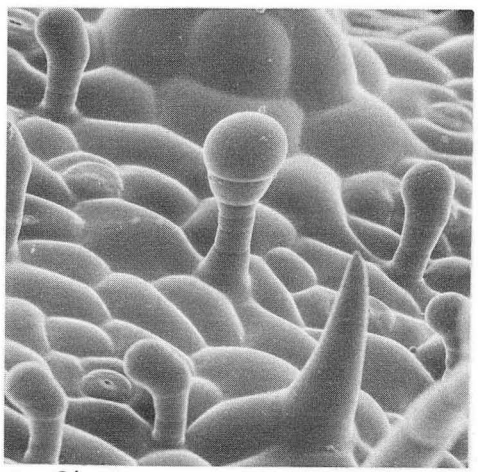


Figure 6. Gland structure, Pelargonium leaf viewed at -150°C. (Width of photograph = 175μm.)

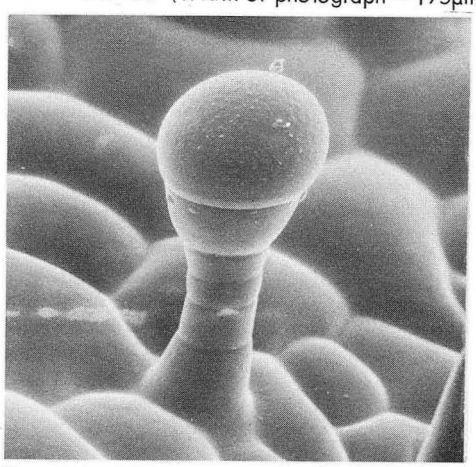


Figure 7. Small particles on surface of gland structure. (Width of photograph = 70μm.)

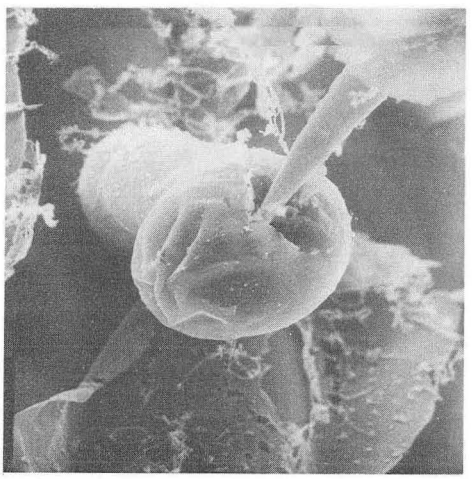
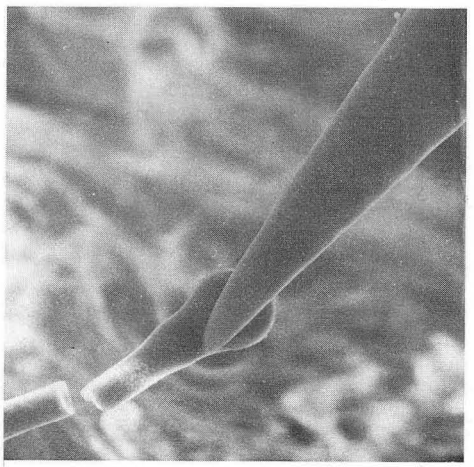


Figure 8. Non-cooled micromanipulator produces local melting and distortion. (Photo width, 70μm.)



Fiaure 9. Cooled micromanipulator needle fractures gland structure without melting. (Width of photograph = 140μm.)

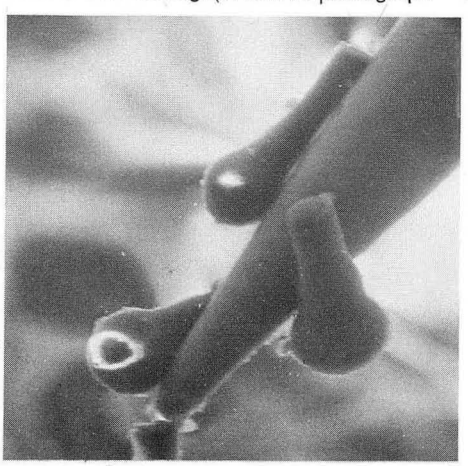


Figure 10. Gland structures are collected from various parts of the leaf surface. (Width of photograph = 140μm.)

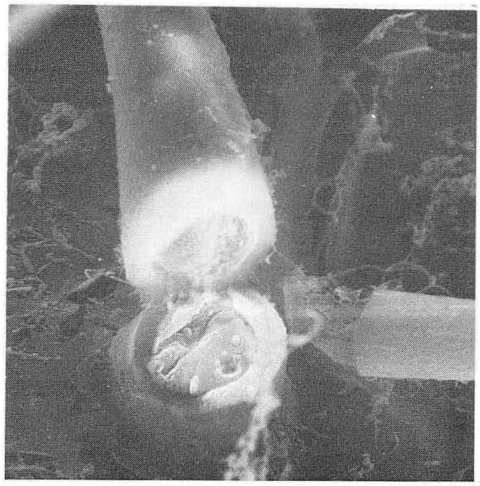


Figure 11. Fractured shaft showing solid ice interior. (Width of photograph = 70μm.)

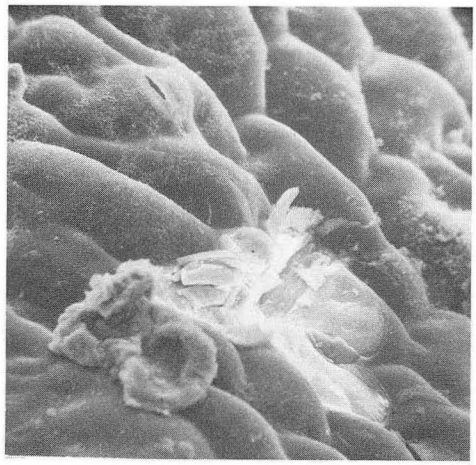


Figure 12. Fracture of selected group of surface cells. (Width of photograph = 160μm.)

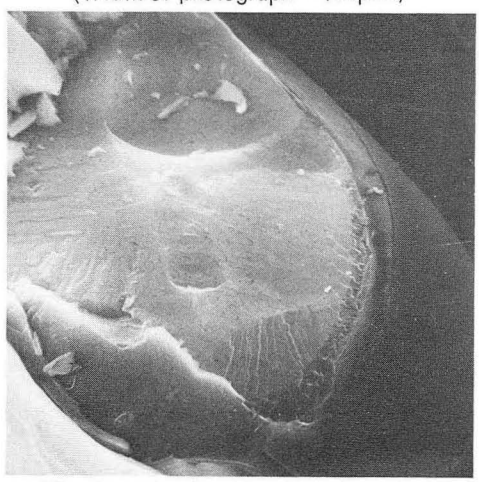


Figure 13. Stepwise fracture through surface cell. (Width of photograph = 40μm.)

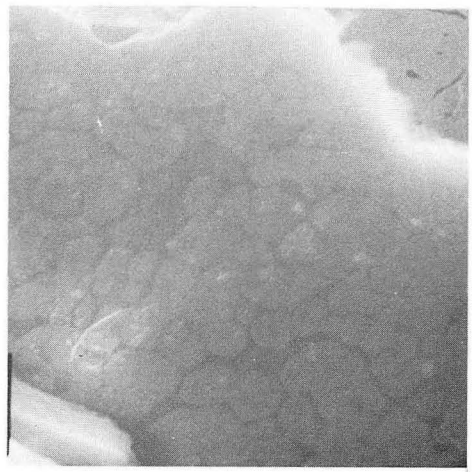


Figure 14. Fracture step through granular layer near cell wall. (Width of photograph = 13μm.)

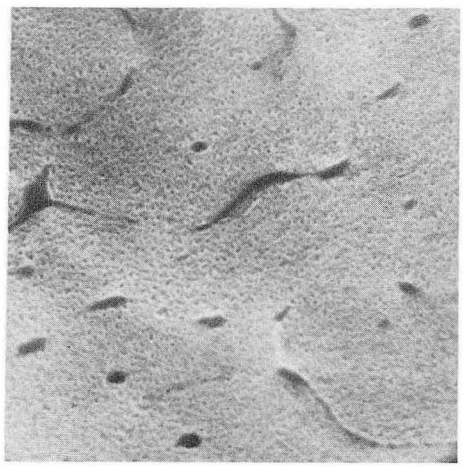


Figure 15. Fracture step across cell watery interior. (Width of photograph = 5μm.)

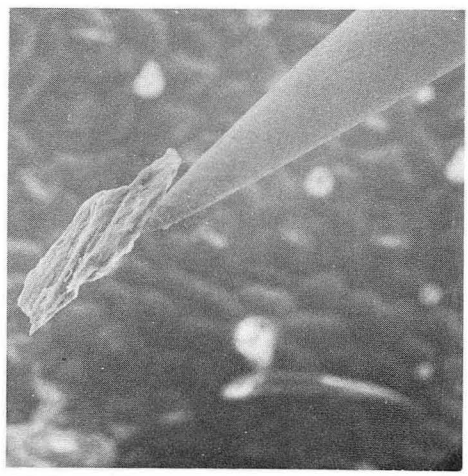


Figure 16. Particulate contaminant removed from Pelar. surface with cooled manipulator needle. Width, 140μm.

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DISCUSSION WITH REVIEWERS

Reviewers II & III: Please give details of the technique for transfer of the specimen into the SEM.

Authors: The specimen is frozen to a copper mount which is held in a collet-type transporting device. A shallow depression in the copper specimen mount permits the entrapment of a small amount of liquid nitrogen around the specimen during loading into the airlock. In addition, the transporting device contains a shield which surrounds the specimen mount and reduces atmospheric contamination.

Once the specimen is in the airlock chamber in an atmosphere of dry nitrogen, the transporting device is removed and the trapped liquid nitrogen in the specimen mount evaporates during evacuation.

Reviewer II: What is the pressure inside the microscope, particularly at the level of the specimen? Wouldn't a liquid nitrogen cold plate near the specimen at -150°C tend to increase the sublimation rate?

Authors: We are unable to measure directly the pressure at the level of the specimen. Pressure is measured by a conventional ionization gauge. There may be some cryo-pumping at the level of the specimen that would provide for somewhat better vacuum conditions in that area.

The column pressure was read as 5×10^{-7} Torr, and as the cold plate and cold stage are acting as cryo-pumps the pressure in the specimen area might be somewhat lower. The liquid nitrogen cold plate does increase the sublimation rate, but the observed rate still appears to be acceptable for these experiments.

Reviewer I: Could you give details of the liquid nitrogen cold plate?

Authors: The liquid nitrogen cold plate was a rectangle of approximately 12 square inches. Measurement of temperature at various points on the surface using thermocouples indicated the warmest point on the surface was at about -170°C. We did not experiment with different designs.

Reviewer II: How do you judge that no sublimation has occurred?

Authors: Repeated photographs of the hydrated frozen specimen showed no visible changes. When the stage temperature was raised to above -100°C, sublimation was observed.

Reviewer II: What evidence is there that the specimen remains frozen-hydrated, and that the contamination rates are low?

Authors: When the structure is touched with the room temperature micro-manipulator needle, one can observe melting and drying. Also, the fracturing process is typical of the breaking of ice and quite different from the mechanical manipulation properties of the frozen, dried material. Again, sublimation can be produced by elevating the stage temperatures.

Reviewer I: Is ice condensation more important than hydrocarbon condensation? For complete removal of hydrocarbons, it has been shown that liquid helium temperatures are required.

Authors: Because of beam-induced interactions, hydrocarbons tend to form permanent layers of contamination. Ice condensation is more common and of greater mass, but is susceptible to removal by sublimation. At stage temperatures between -100°C and -80°C, condensed ice will be sublimated along with inherent specimen ice. For present work, it is desirable to keep the stage temperature always below -130°C, and sublimation is so slow that condensed ice also is a permanent contaminant. All our work was with a liquid nitrogen cold plate only, and no work at liquid helium temperatures was attempted.

Reviewer II: In Fig. 7, small particles on the gland look very much like ice contamination!

Authors: This is a possibility for the very small particles, although they are not found on all specimen surfaces. Critical point drying procedures subject the specimen surface to multiple washings with fixatives, dehydrating agents, etc., which may remove some surface material; thus, direct comparison is difficult.

Reviewer III: You referred to minimum ice crystal damage. What is the smallest structure that is visible?

Authors: We have made no attempt to measure resolution during these experiments, but suggest that particles of 200Å diameter could readily be seen.

Reviewer II: What is the temperature of the specimen? What calculations were used to get the graphs shown in Fig. 4? How much warmer is the specimen than the stage? The curves in Fig. 4 are misleading, as the specimen surface temperature has not been measured, and ice has very poor thermal conductivity.

Authors: The temperature was measured inside of the specimen, not at the surface. The specimen interior temperature was felt to be more useful than relying solely on the measured stage temperature, but is not as useful as the surface temperature.

The graphs shown in Fig. 4 were not calculated, but represent the measured temperature inside of a mock specimen. The heat flow charts are a schematic representation of a flow pattern suggested by these measured values. It would be very useful to be able to measure the specimen surface temperature directly.

Reviewer II: What sort of thermocouples were used? The thermal mass of 100μm thermocouples is probably too large to accurately measure the temperature. Thin film thermocouples are probably the only answer.

Authors: Iron-constantan thermocouples were used to measure the static temperature inside of the mock specimen. Thin film thermocouples offer an exciting advance in techniques for measuring specimen surface temperatures, and will undoubtedly result in more accurate information with respect to surface-beam interaction.

Reviewer II: What are the vacuum and thermal conditions of the specimen and environment during coating?

Authors: The coating chamber is connected to the microscope column and remains essentially at column pressure during coating. Temperature of the specimen is maintained by the thermal mass of the specimen mount, and the rise in temperature during a typical coating procedure is less than 10°C.

Reviewer IV: Several objects were collected from the leaf surface using the micro-manipulator needle. Why did the objects stick to the needle, and how cold is the needle?

Authors: We feel that as the needle touches the object to be collected, pressure causes a very localized melting and refreezing that serves to attach the object to the needle. The temperature of the needle tip is difficult to know, but we estimate it to be somewhat below -150°C.

Reviewer III: It is easy to see how the gland can be broken off, but how are the cells on the surface separated into layers? The fine micro-manipulator needles must be susceptible to bending and breaking against the ice. How were the needles made?

Authors: Needles with a very fine point are not strong enough to be used for micro-manipulation of the frozen structures. Slightly more blunt needles were chosen for fracturing the gland stems. In order to fracture the surface cells, the rigid shaft of the needle was placed against the base of the gland stem and then displaced rapidly. The resulting fracture passed through several of the surrounding surface cells. The needles were made by Etec Corporation using a fairly standard electropolishing technique.

Reviewer IV: Was there any observable change in the secondary electron image of the frozen, uncoated material as the temperature was changed?

Authors: We observed no marked change in the secondary electron image as the stage temperature was warmed even as high as -60°C.

Reviewer II: Have you made any attempt to measure the heat input into the specimen from the beam and the environment?

Authors: We have made no measurements, but have made some preliminary calculations (Pease and Hayes, Nature, 210, 1049, 1966).

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