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Liquid substitution: a versatile procedure for SEM specimen preparation of biological materials without drying or coating

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Key words. SEM preparation, glycerol, uncoated specimens, SEM of liquids, infiltration, cathodoluminescence.

Summary

Certain liquids with a very low vapour pressure, such as glycerol or triethylene glycol, can be used to infiltrate biological specimens so that they may be observed in the scanning electron microscope (SEM) without drying. The conductive properties of the fluids allow specimens to be examined either uncoated or with very thin coatings. The advantages of liquid substitution include the retention of lipids, waxes, loose particles, and surface contaminants. Since the procedure does not require expensive equipment, it offers an alternative to critical point drying or cryo-preparation. For certain types of specimens, liquid substitution may represent the best preparation procedure. In addition, the fluids themselves may be imaged directly in the SEM, or indirectly by cathodoluminescence following labelling with fluorochromes.

1. Introduction

The diverse properties of biological specimens and the variety of problems addressed by scanning electron microscope (SEM) investigations have resulted in a wealth of specimen preparation procedures, each with its particular advantages and shortcomings, dating from the advent of scanning electron microscopy (Parsons *et al.*, 1974; Falk, 1980). The main objectives of these preparation methods are to stabilize the specimen, to prevent shrinkage and other artefacts during dehydration, and to render the sample electrically conductive. Standard methods range from simple air-drying of stable specimens (e.g. chitin, pollen), critical point drying (CPD), and cryo-fixation, to the use of special environmental SEMs which enable the investigation of fresh specimens under saturated water vapour conditions. In the method described here, specimens are not dried, but their water is instead substituted for a liquid (glycerol or triethylene glycol) which evaporates very slowly under high vacuum. The concept of liquid substitution (LS) by infiltration with glycerol or other fluids is by no means new; root surfaces (Dart, 1971) and *Dionaea* leaves (Mozingo *et al.*, 1970) have previously been prepared for SEM using glycerol. Falk *et al.* (1971) used glycerol to infiltrate specimens in their comparative study of fixation methods (as applied to the shoot apex of *Tropaeolum*). Idle (1971) substituted the water in the specimen for polyethylene glycol and observed that the electrical conductivity was sufficient to obviate metal coating.

However, no significant advantages over other methods have previously been reported. Obviously, the method was never optimized and fell into oblivion with the increasing use of CPD and cryo methods (Falk, 1980).

In this report, an optimized LS procedure (using glycerol or triethylene glycol) is described. The advantages of LS are demonstrated for a variety of specimens, and new applications are suggested for SEM investigations of liquids, drying processes, uncoated specimens, and cathodoluminescence.

1.1. Properties of glycerol

Glycerol is miscible with water, has a low vapour pressure at room temperature (*c.* 10^{-4} mbar = 10^{-2} Pa; see Fig. 1), and evaporates very slowly under high vacuum. Glycerol-infiltrated samples can thus be examined in the SEM without further treatment.

Certain other favourable properties of glycerol are advantageous for LS:

1. The electrical conductivity is sufficient to prevent specimen charging in the electron beam. Thus specimens do not require coating. The specific resistivity (at 293 K) of the glycerol sample used in this study ('Glycerin >99%, Merck No. 12011') was *c.* 2×10^7 Ω·cm.
2. The surface tension of glycerol (63·4 mN m^{-1}) is close to that of water (72·75 mN m^{-1}) whereas it is substantially lower in most other organic liquids (e.g. ethanol: 22·75 mN

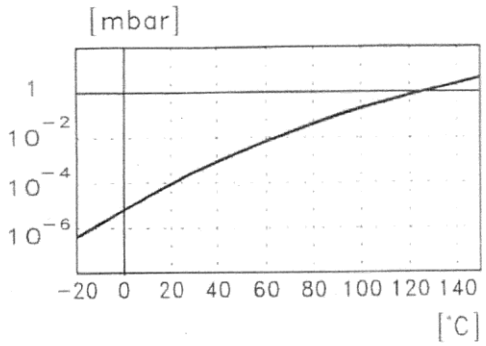

Fig. 1. Vapour pressure versus temperature diagram for glycerol modified from Kirk & Othmer (1951).

m^{-1}) (Weast, 1977). Consequently, hydrophobic surfaces (of leaves for instance) are not wetted and remain dry during preparation.

3. The polar characteristics of glycerol are useful when studying fat-containing samples, since fat extraction and changes in fat morphology are largely avoided.

4. Investigations at low temperature are enabled by the reluctance of glycerol to crystallize at temperatures below its freezing point (293 K).

5. Vapour pressure and electrical conductivity are greatly dependent on temperature. Cooling to 273–253 K reduces the evaporation rate significantly, but below *c.* 253 K conductivity is not sufficient to prevent the charging of uncoated specimens. (At 253 K the specific resistivity of the glycerol utilized amounted to *c.* 2×10^9 Ω·cm.)

6. A drawback of glycerol is its high viscosity (Table 1) which, at least in some specimens, may result in shrinkage during dehydration.

1.2. Properties of triethylene glycol

The viscosity of triethylene glycol (TEG), 20·9 mPa s at 293 K, is considerably lower than that of glycerol (1499 mPa s). TEG has also a lower surface tension and is less polar and thus slightly more lipophilic than glycerol. Hydrophobic surfaces are therefore more readily wetted.

TEG resembles glycerol in its miscibility with water, very low vapour pressure, electrical conductivity, and reluctance to crystallize at temperatures below its melting point (266 K).

Table 1. Viscosity (mPa s) of aqueous glycerol solutions dependent on the concentration (at 293 K)

Glycerol concentration	Viscosity
10%	1·311
20%	1·769
30%	2·501
40%	3·75
50%	6·05
60%	10·96
70%	22·94
80%	62·0
90%	234·6
100%	1499·000

2. Description of the LS procedure

The description that follows is based on the application of glycerol. TEG may be used in the same way: its lower viscosity results in easier and faster infiltration.

2.1. Appropriate specimens and their fixation

Glycerol may be substituted for water in biological (e.g. plant surfaces, animal organs, single cells) as well as in many other water-containing specimens (e.g. food technology, soils, etc.). Some types of biological specimens give unsatisfactory results with LS. Soft objects like flowers, water plants, etc., become too flaccid to maintain their shape. Hairs, leaves, and other flexible structures may adhere to one another when the specimens are taken out of the glycerol in which they have been treated. Specimens with poor permeability (caused by barriers such as cuticular membranes or long diffusion pathways, e.g. in hairs) may shrink during the substitution process due to the high viscosity of glycerol.

Fresh samples may be fixed with glutaraldehyde using standard procedures (e.g. Sabatini *et al.*, 1963), or with OsO_4, under conditions adjusted for the kind of specimen. Some objects (e.g. many leaves) give good results even without fixation.

2.2. Infiltration with glycerol

Depending on the properties of the specimens one of the three following infiltration procedures was chosen.

1. Standard procedure: for specimens that were submerged in liquids.

2. Procedure for small specimens: used for single cells and other small items that were easily handled on a membrane filter.

3. Procedure for specimens with hydrophobic surfaces: for objects with a surface that was required to remain dry during processing.

2.2.1. Standard procedure. Pre-fixed specimens (as above) were dehydrated in an ascending series of aqueous solutions of glycerol in 10% steps to a final concentration of 80 to 100%. They should remain in each solution for at least 1 to 4 h. The specimens were subsequently mounted on SEM stubs and stored in a desiccator (with a desiccating agent)

for a longer period to evaporate all remaining water and to achieve complete substitution. (Residual water can cause specimen movement in the electron beam in the SEM.)

2.2.2. Procedure for preparation of small specimens. Single-celled specimens (pollen etc.) are easily handled on a membrane filter. A piece of fabric soaked with fixative (e.g. glutaraldehyde) was placed in a Petri dish and was covered by the membrane filter. The specimens were then mounted on the membrane filter, either fresh or suspended in fixative. Excess liquid was drained away, leaving the specimens attached to the substrate.

To change solutions, excess fluid was removed from the tilted Petri dish with a pipette, and the new solution applied to the fabric. This avoided washing away the specimens. The procedure can readily be adjusted to object size and permeability.

2.2.3. Procedure for specimens with hydrophobic surfaces. In samples with hydrophobic surfaces (many leaves and fruits) there is no need to moisten the surface, which thus remains intact and unaltered. The specimens were mounted onto a piece of fabric soaked with a fixing agent or with the glycerol solution. The liquid thus infiltrated the objects from below and the upper surfaces remained dry. For large organs (e.g. fruits, stems, succulent leaves), it is recommended that a superficial portion of the plant should be excised and the cut surface placed onto the fabric. Thin cuticularized specimens (e.g. leaves) should be scarified from below to improve infiltration (using a razor blade or sandpaper). The solutions are replaced as described in the preceding section.

After following these procedures, infiltration with glycerol still proved to be insufficient in some specimens, and shrinkage may occur, particularly when infiltrating from below. There are several ways to overcome this problem by varying the preparation methods: continuous (as opposed to stepped) increase in glycerol concentration, infiltration at elevated temperatures, immersion of specimens, and the use of the less viscous liquid TEG may all improve infiltration; optimized conditions (temperature, time, etc.) during fixation can improve specimen stability. More aggressive fixatives (e.g. OsO_4) may increase permeability.

2.3. Mounting, coating, and SEM examination

Excess glycerol must first be blotted from the specimen using filter paper. Larger specimens may be mounted on the SEM stub with a conductive glue. As the filter membrane bearing small specimens contains very little glycerol and therefore dries up too fast, we usually mount it on a glycerol reservoir such as a small piece of filter paper or gelatine soaked in glycerol.

For comparison with LS, air-dried and critical-point-dried (CPD) samples were also investigated. After fixation with glutaraldehyde, CPD specimens were gradually dehydrated with ethanol or acetone and critical-point dried with CO_2 at 40°C. The specimens were coated with gold in a sputter-coater. Wet surfaces cannot be coated. However, due to the high conductivity of glycerol this is not necessary.

Scanning electron microscopy was carried out using a Cambridge Stereoscan S-200; Fig. 4(b) was made with a Stereoscan 360 FE with a field-emission cathode. Accelerating voltages of 3 to 30 kV were used for the glycerol-substituted, uncoated specimens. Some objects provided distinctly better contrast at 5 kV as compared to higher voltages. Specimen observation time is restricted by the evaporation of the glycerol (*c.* 30 to 120 min at room temperature), after which charging becomes apparent. The use of a cooled (273–253 K) specimen holder (simply a massive, thermally isolated metal bar which gains heat very slowly in vacuum) extends observation time significantly and is particularly useful for the examination of small specimens. Samples may be re-wetted with glycerol for further investigation. When specimen surfaces are wet, it may take some time (10 to 40 min) before they have dried off sufficiently under high vacuum to be ready for observation.

3. Materials

To test the preparation methods, more than 100 specimens from a variety of origins were studied. Emphasis was given to botanical objects: delicate epidermal surfaces, glandular hairs with secretions, leaf and shoot transections, pollen and flagellae. Zoological objects included frog skin, various rat tissues, and human erythrocytes. From the field of food technology, fat-containing samples like sausage, cheese and curd were examined. Of these objects only a few specimens were selected for illustration (see captions to Figs. 2–6, 8).

4. Results

4.1. Behaviour of glycerol in the SEM

The evaporation rate of glycerol under high vacuum is very slow because of its low vapour pressure. Glycerol-infiltrated samples can thus be examined for some time in the SEM without drying up. The gradual evaporation of glycerol is advantageous since wetted surfaces dry up after a short time (Fig. 2). Glycerol in the tissue of the samples evaporates so slowly that changes are only observed after 30–120 min.

Hitherto, the evaporation of the glycerol inside the specimen chamber has not caused any malfunction of the vacuum system in our SEMs. Nevertheless, the samples should not remain in the SEM longer than necessary.

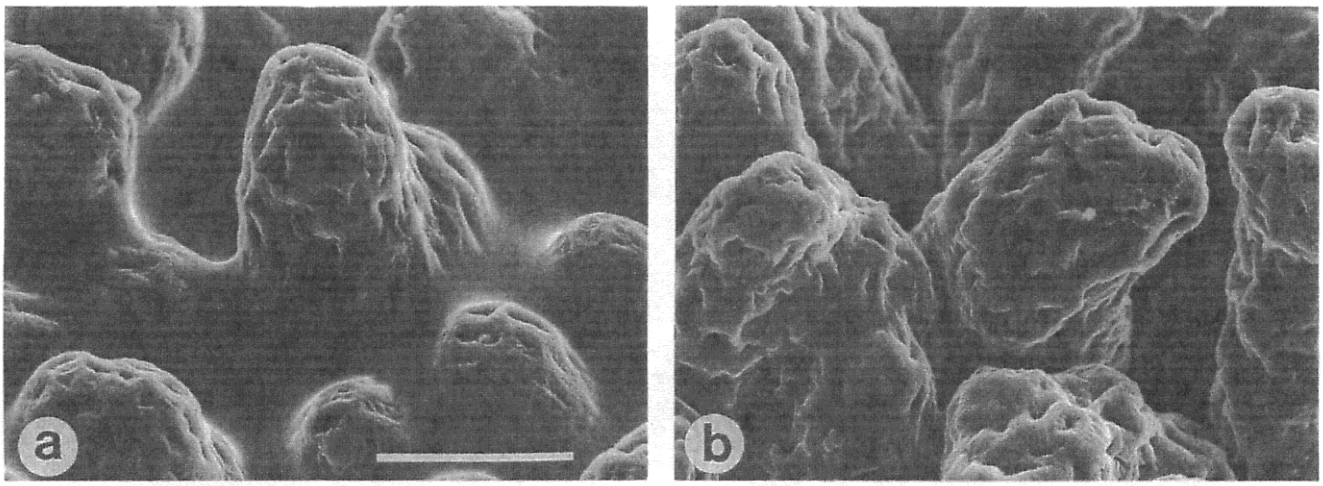

Fig. 2. Surface of a petal of *Aristolochia arborea* (after LS, uncoated). (a) Initially, the hydrophilic surface is covered by glycerol which slowly evaporates. (b) After 40 min under vacuum the sample is superficially dry and details of the cells become evident. Scale bar = 50 μm.

Glycerol may accumulate in the oil of the vacuum pumps which, as a result, may need to be exchanged more often than usual. The glycerol reliably prevents electric charging in infiltrated samples, so that uncoated specimens may be investigated without difficulty. Images may be made of wet surfaces and even of the glycerol itself, which cannot be coated with metal (Figs. 2 and 7).

4.2. *Application of LS to selected examples*

4.2.1. Examination of epicuticular wax and contaminations on plant surfaces. Since hydrophobic surfaces remain dry during preparation, LS is an excellent method for the examination of waxes and other secretions and attached particles (e.g. pollutants) on plants. Wax crystalloids are often studied on air-dried specimens (Barthlott & Wollenweber, 1981) despite tissue shrinkage, because CPD results in damage to the wax structure, ranging from insignificant to total dissolution of the wax, which is caused by the dehydration solvents. Such artefacts are avoided in most specimens by preparation with LS (Fig. 3). The inherent high electrical conductivity of LS samples allows for the application of very thin metal coatings, enabling high-resolution imaging, whereas dried specimens require a thicker coating to prevent charging, particularly on rough surfaces, which may obliterate fine details (Fig. 4). However, the stability of structures which are highly susceptible to damage in the electron beam (e.g. wax crystalloids) increases with coating thickness.

The use of different metals to produce more finely grained coatings than gold may further enhance the resolving power (e.g. Peters, 1985; Wepf *et al.*, 1991).

Attached particles and contaminations are not affected or washed off during LS (Fig. 5), and, since a metal coating is not necessary, LS samples are ideally suitable for examination using X-ray microanalysis (EDX) and backscattered electron (BSE) imaging.

4.2.2. Small specimens, unicellular organisms. The main advantage of applying LS to small specimens is that the procedure is very easy to perform. For studies at high magnifications, gold coating is advisable after drying of the surface to improve stability, contrast, and resolution (Fig. 6a). Uncoated LS specimens mounted on a transparent, conductive specimen support (such as glycerol-soaked gelatine film) permit light microscopy after SEM (Fig. 6b,c).

4.2.3. Imaging of fatty substances. LS may also be applied to samples containing fat as well as water (food substances or animal tissues). Both water and glycerol, being polar liquids, prevent fat from diffusing and spreading over the entire surface, which tends to occur with air drying. With CPD dehydration, fatty substances are totally extracted. After LS, fat-containing samples may be subjected to EDX.

4.2.4. Study of fluorescence-labelled liquids with cathodoluminescence (CL) imaging. After staining the glycerol with fluorecent dyes (e.g. auramine) the behaviour of the liquid may be observed by CL imaging. Figure 7(d) shows mixing and diffusion processes in a glycerol droplet, and aqueous and lipid phases can be distinguished in Fig. 8.

5. Discussion

Liquid substitution using glycerol or triethylene glycol is a simple method for preparing many biological specimens for SEM and offers an alternative to other preparation techniques. All standard methods pose some disadvantages with certain types of specimens. While low-temperature

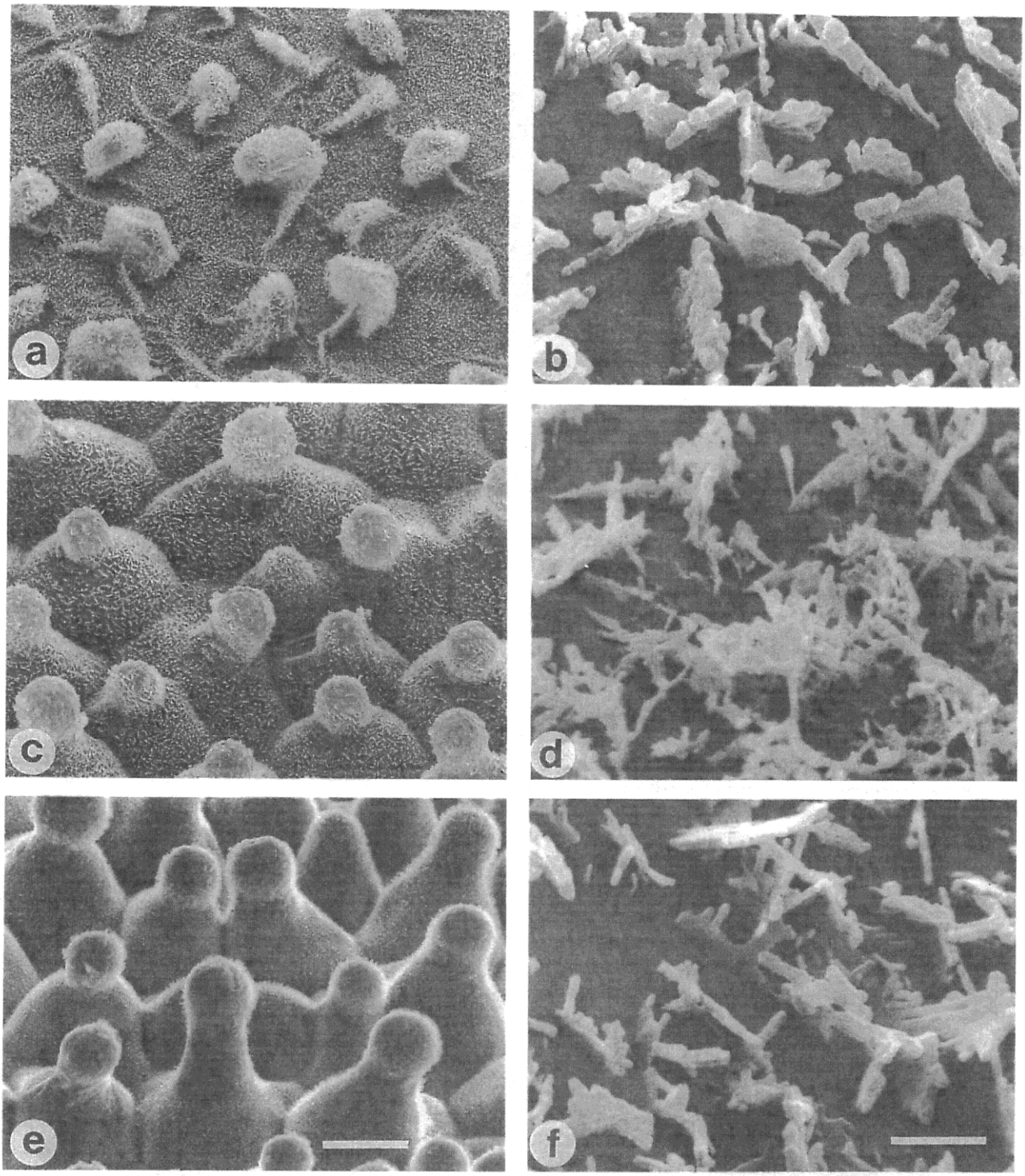

Fig. 3. Comparison of different preparation procedures applied to leaves of *Xanthosoma* sp. The surface of the papillar cells of the lower epidermis is covered with wax scales (a–d, f: Au-coated, e: non-coated). (a, b) Air-dried: wax scales well preserved, but papillae collapsed. (c, d) CP-dried: papillae well preserved, but wax scales partially dissolved and altered. (e, f) LS, surface unmoistened: papillae and wax scales well preserved. Contrast of the wax scales is poor in uncoated sample (e). Scale bars = (a,c,e) 20 μm, (b,d,f) 1 μm.

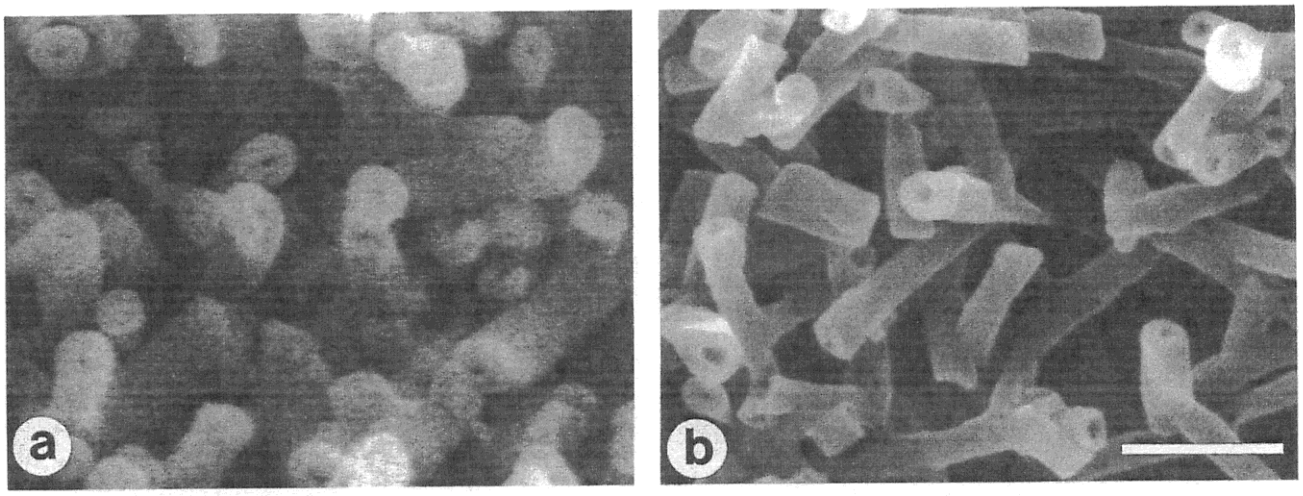

Fig. 4. Tubular wax crystals at high magnification on leaves of *Tropaeolum majus*. (a) Delicate structures are masked by normal gold coating (30 nm thick). (b) Following LS, a very thin gold coating (3 nm) suffices to eliminate charging. Thus, the ultrastructure is clearly visible. (This view was made with a field-emission SEM.). Scale bar = 0·5 μm.

SEM (LTSEM) and environmental SEM (ESEM) require expensive equipment, the different drying methods may cause various types of artefacts (Boyde, 1978). LS with its very different properties may help to avoid some of these problems.

For specimens with a dry, unwettable surface, such as plant surfaces covered with epicuticular wax, LS displays optimal results because the surface remains unaffected. Cryo methods (LTSEM, freeze-drying) are a more expensive solution, and damage to delicate surface structures cannot always be avoided when immersing the specimen in the cooling agent. However, this is largely avoided with LS. Therefore, LS can be recommended as a simple procedure for the investigation of the impact of environmental stresses on plants as well as for the analysis of surface contamination.

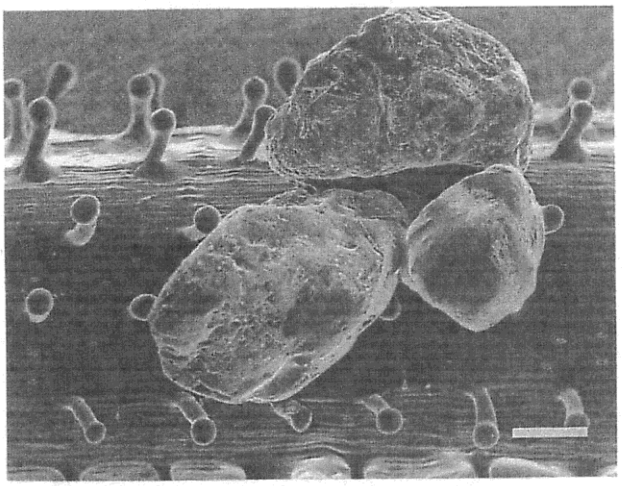

Fig. 5. Leaf of *Albuca viscosa* with sand grains clinging to sticky secretions. Only after LS can these loosely attached particles be illustrated *in situ*. Scale bar = 200 μm.

LS specimens with wet surfaces (e.g. animal tissues) still often give satisfactory results after superficial layers of free glycerol have dried up in the SEM. Shrinkage and distortions are minimal, but soft structures may collapse, and some fine details on the surface (e.g. microvilli) are hardly visible, as they are flattened during the drying. Surfaces appear clean, without artificial structures, which often occur after CPD. Of course, other methods (CPD, freeze-drying, LTSEM) frequently give superior results. In many instances it depends on the aim of the investigation as to whether a more natural, wet condition of the specimen is favoured, or whether the relevant details are only discernible after drying, extraction of fatty substances, dissolving of mucilages or other alterations (see Danilatos & Postle, 1982, p.9).

The main benefit of LS in the preparation of unicellular organisms is the simple handling and the fact that no specimens are lost during the procedure. The results are often good, although some shrinkage may occur.

With regard to fat-containing samples LS offers a simple means of examining fat distribution and morphology. Of course, LTSEM is the optimal method (Sargent, 1988), but this requires expensive equipment.

The particular advantage of LS is the lack of specimen charging at both high and low beam accelerating voltages, facilitating the study of uncoated or thinly coated samples. Other methods of preventing specimen charging all suffer from shortcomings.

1. The 'OTO method' (Seligman *et al.*, 1966; Kelley *et al.*, 1973) which increases the bulk conductivity of specimens by impregnation with osmium uses highly toxic OsO_4. In addition, the EDX signal is disturbed by osmium.

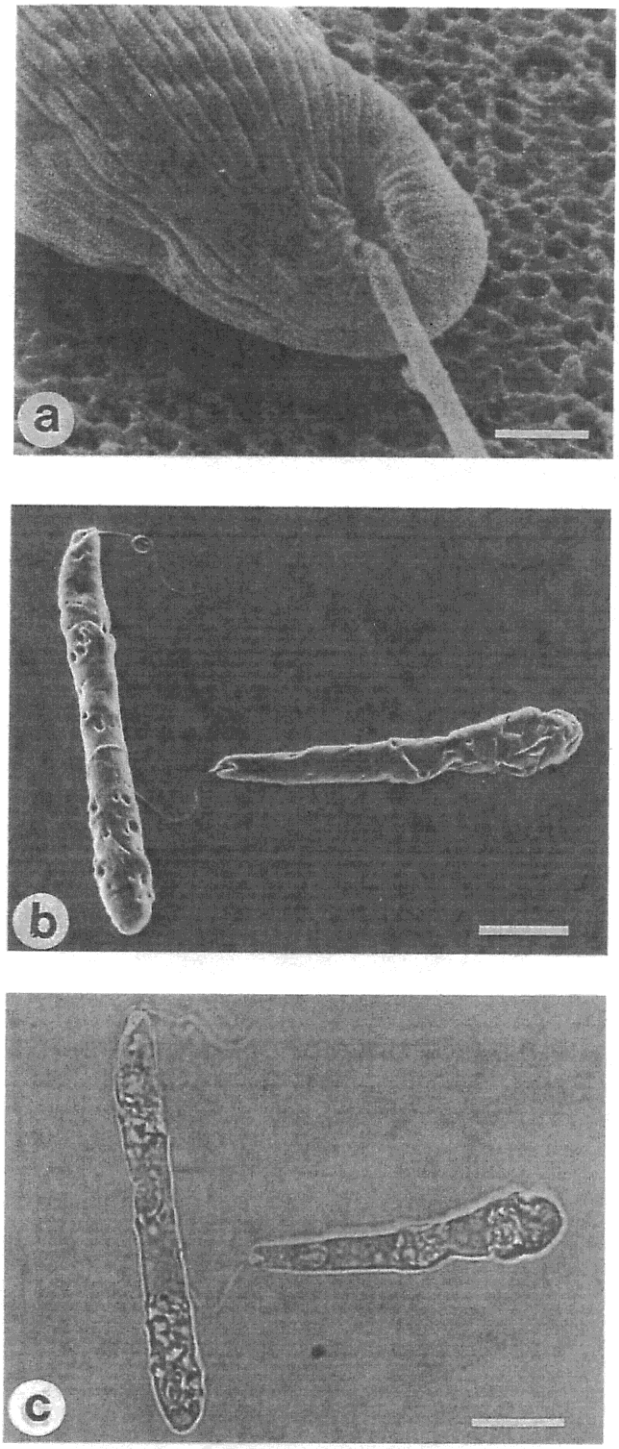

Fig. 6. Illustrations of single-celled specimens after LS. (a) *Euglena gracilis* on membrane filter, Au-coated (30 nm). (b, c) *Euglena gracilis*, uncoated: after LS the flagellates were transferred to a glycerol-soaked gelatine membrane. Photographs were first taken in the SEM (b) and afterwards in the LM (c). Scale bars = (a) 1 μm, (b,c) 10 μm.

2. SEM at low acceleration voltages may avoid charging, but at the expense of resolution and with altered contrast. Furthermore EDX analysis is not possible.
3. SEM using a low-vacuum specimen chamber (ESEM, WETSEM) requires special equipment, although charging may be inhibited by ionized gases in the vicinity of the specimen.

A number of applications are based on the capability of imaging fluids such as glycerol in the SEM. Imaging of liquid water and wet specimens is usually possible only in special scanning electron microscopes (MEATSEM, ESEM), in which the specimen chamber is held at a gas pressure closer to ambient than normal, allowing, in some cases, the observation of live specimens (Shah & Beckett, 1979; Danilatos & Postle, 1982). Using glycerol or TEG the following observations can be performed in any SEM: drying processes of biological samples, physical processes like fluid flow, diffusion or wetting. This includes the capability of controlling evaporation rate or viscosity by temperature adjustment.

LS also opens up new approaches for the investigation of charging phenomena since the conductivity of glycerol-infiltrated samples may be continuously changed by refrigeration until charging phenomena occur at about 253 K.

6. Conclusions

Liquid substitution is possible with low-vapour-pressure liquids allowing SEM examination of infiltrated samples as well as of the liquid itself. Advantages over usual SEM procedures are: the electrical conductivity of the liquid which makes coating unnecessary, only minor alterations of the samples during preparation, and the simplicity of the procedure itself. LS can be recommended in particular for investigations (EDX, BSE, CL) of uncoated specimens and improved resolution due to ultrathin coating; simple preparation of tiny specimens; preparation of samples containing wax, fat, or loosely attached particles (where CPD fails); and study of the behaviour of liquids (e.g. drying processes). Moreover LS is a low-cost method available for investigators without access to CPD or coating equipment. We assume that further applications may exist in other fields such as cytology (visualization of Au-labelled antibodies on uncoated cell surfaces, possibly by BSE imaging; Walther *et al.*, 1984), or in scanning tunnelling microscopy of biological specimens (glycerol infiltration could enhance specimen conductivity and thus enable the use of extremely thin conductive coatings).

Due to its advantages and versatility LS may have the potential to become a standard SEM specimen preparation technique, as it may help to overcome a number of problems inherent in other methods.

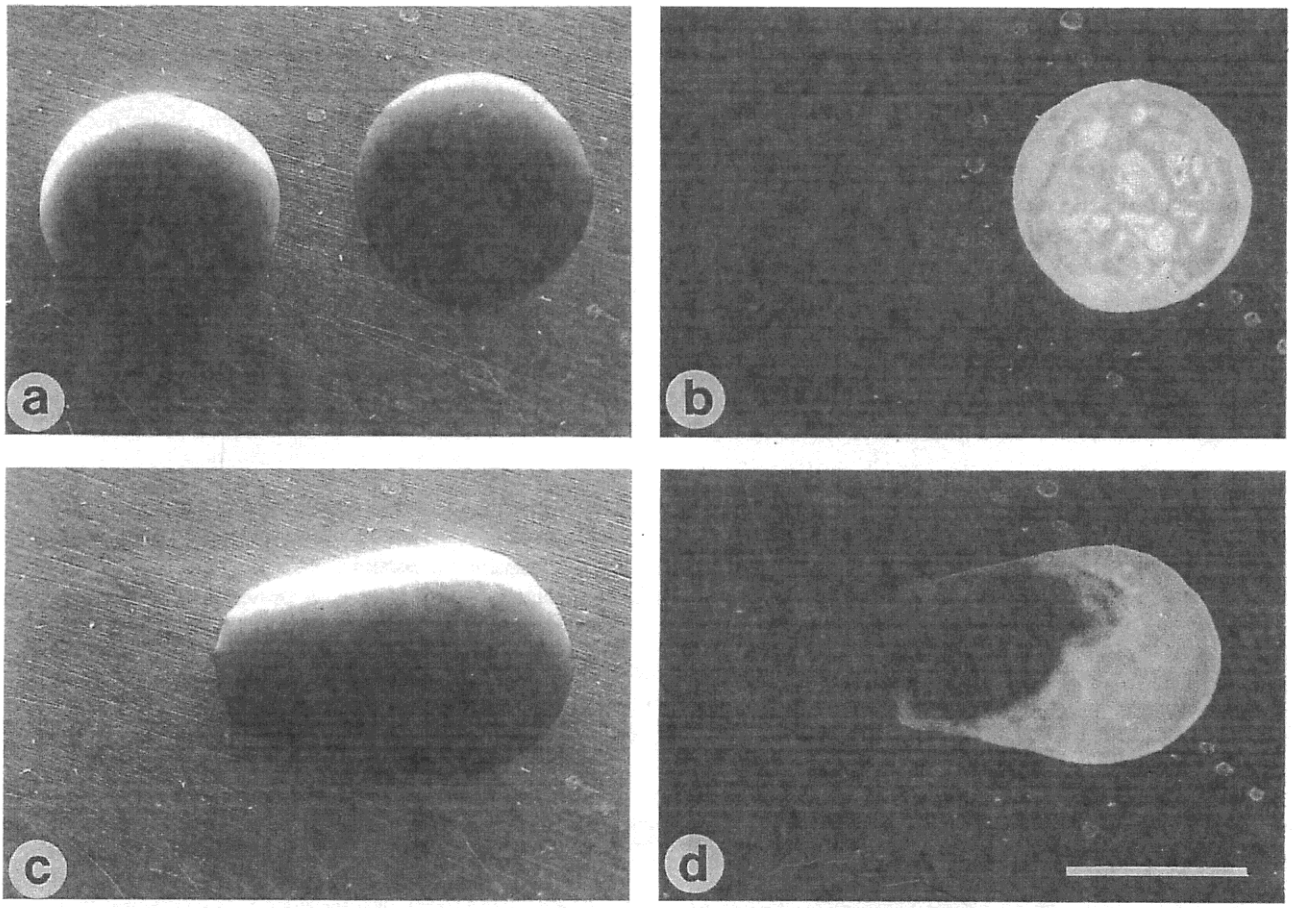

Fig. 7. Mixing processes of glycerol droplets; SE (a,c) and CL (b,d) imaging. Right-hand drop stained with 1·5% auramine, thus fluorescing in the CL image. (a,b) Droplets still separated. (In the SE image, the right-hand droplet displays a better wetting of the substrate.) (c,d) After confluence; mixing processes may be studied in detail with CL. Scale bar = 1 mm.

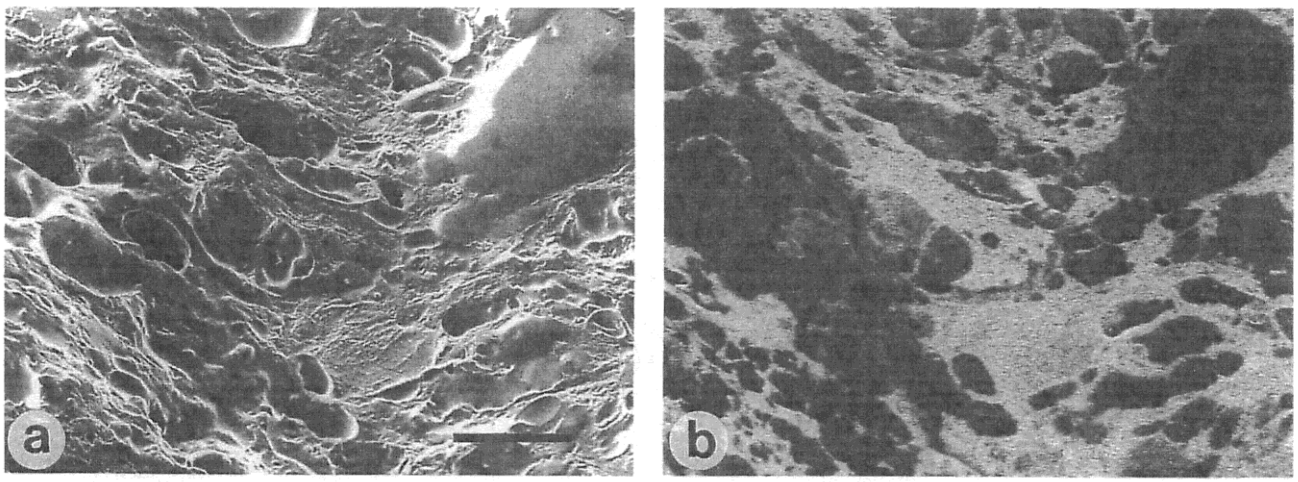

Fig. 8. Fracture of sausage ('Meica Bockwurst', uncooked) infiltrated with auramine-labelled glycerol. (a) Secondary electron (SE) image, (b) cathodoluminescent (CL) image: glycerol-infiltrated parts fluoresce while fat drops remain dark. Scale bar = 200 μm.

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