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Abstract
Sample preparations are essential in scanning electron microscopy. Flawed sample preparations can undermine the quality of results and lead to false conclusions. Thus, the aim of this chapter is to equip researchers, post graduates and technicians with essential knowledge required to prepare samples for scanning electron microscopy (SEM) investigations in the life sciences.

Keywords: Scanning electron microscopy, sample preparation

1. Introduction

Obtaining acceptable SEM images with good ultrastructural preservation requires careful application of the SEM sample preparation methods. The image in Fig. 1a can be easily accepted as a reasonable image for publication. However, a better sample preparation technique would have produced an image as in Fig. 1b. The bacterial cells in Fig. 1a are flattened and shrunken. The non-removal of the mucus layer has obscured the surface structures, unlike the cells in Fig. 1b.

2. Primary consideration in SEM sample preparations

2.1. Collection of samples

In the laboratory, collection of samples for SEM preparations generally involves the dissection of an animal or incision of a plant material to remove tissues from the main...
body. Immediate fixing of the removed samples is very important to avoid autolysis, putrefaction and drying effects, which may destroy the ultrastructural integrity of the tissues. It is recommended that the organs are flooded with the primary fixative before incisions are made to remove the tissues. Once removed, the tissues should be immediately placed in a vial containing the fixative until the start of the processing protocol. If it is not possible for the samples to be placed in a fixative immediately, for example biopsies removed in a surgical theatre, then, the alternative could be to leave them in a suitable physiological saline and fixed soon after at the earliest possible time. All incisions must be performed with fresh sharp blades to avoid deformation of tissues from the undue physical forces needed with blunt blades. Once the samples have been placed in the vial containing the primary fixative, the same vial should be used throughout the sample preparation protocol until the ‘specimen mounting’ stage prior to viewing the sample in the SEM. Simply decant or pipette out the changes without any form of physical contact on the surface of the sample. If the fixed sample could not be processed on the same day, then it is advisable to leave the vial with the sample in a refrigerator (but never in a freezer or freezing compartments!). Every effort must be made to keep the tissue moist till the ‘drying’ processes at the end of all sample preparation protocols. For field collections, the researchers should have vials of fixative in hand for immediate immersion of the collected samples in the fixative. Immersion fixation may not be suitable for fungal samples growing on leaves and barks, due to the curling and collapsing of the fluffy hypha structures when they come in contact with liquids. Vapour fixation followed by freeze drying, as described in Section 4.3, gives better results. These field collected fungal samples must be carefully placed in a closed container space, kept moist by placing wet filter papers within, to minimise the drying of the fine structures, while being taken to the laboratory for vapour fixation. Students working with such fungal specimens in the author’s laboratory have encountered these drying problems even for the samples that were brought to the laboratory from nearby trees within the campus and was minimised only by employing the method of transfer described above [1, 2, 3, 4].

Figure 1. (a) Bacillus cereus. The cells were washed with distilled water and oven-dried at 40°C. The cells are flattened, shrunken and covered with a layer of dried mucus. (b) Bacillus cereus. The cells were air-dried using the HMDS protocol as described in 4.1.2b. The cells appear well rounded and fulsome while the dividing cells are well exposed.
2.2. Sample from cultures

Microbial cultures of bacteria and fungus need strict safety measures for the fixation process. Biosafety cabinets should be used wherever possible. The sample should be removed from the biosafety cabinets only after the fixation process. The fixatives can be added directly into the culture plates or broth cultures as needed. Generally, fresh microbial cultures give better results (Figs. 2a and 2b). Avoid the selection of organisms in their death phase of their growth curve or overgrown cultures except when doing comparative studies. Many students leave their cultures in a refrigerator for long period of times and produce disappointing results in the SEM [5, 6].

![Image](image_url)

**Figure 2.** (a) A fresh culture of yeast *Saccharomyces cerevisiae*, prepared using the air drying method with HMDS as described in 4.1.2c. (b) A fresh culture of *Lactobacillus acidophilus*, prepared using the air drying method with HMDS as described in 4.1.2b.

2.3. Fresh water and marine samples

Organisms from fresh water habitat can be fixed in the routine EM fixatives. However, for marine samples, the fixatives should be prepared in filtered or artificial seawater with its osmolarity matching their natural environment (Figs. 3a and 3b) [1, 2, 4, 7]

![Image](image_url)

**Figure 3.** Marine diatoms fixed in 4% glutaraldehyde prepared in seawater, followed by the air drying protocols with HMDS as described in 4.1.2b, showing good structural integrity.
2.4. Samples with heavy mucous films

The presence of mucous films on sample surfaces can obstruct the clarity of the surface ultrastructures (Fig. 1a). This problem is often encountered when processing samples like mucous producing organisms and parasites removed from organs. Although clearing the mucous layer is sometimes achieved by using gentle centrifugation, alcohol, glycerol or enzymes, the application methods need to be worked out very carefully to avoid any consequential damaging effect on the fine structures of the sample. In the absence of the availability of suitable chemicals to remove these mucous layers, washing of the samples with a suitable physiological saline before fixation does help. However, this has to be done by employing numerous changes of the physiological saline solution accompanied with very gentle agitations for every change of the saline solution. If the organisms are small, performing these washings in an embryo dish, under a stereomicroscope can be useful. Students have reported to the author that parasites removed from guts needed about 50 times of washings before the fine structures on these parasites were rendered visible (Figs. 4a and 4b). It has to be stressed here that the washings have to be done before fixation. Once the organism or tissue has been fixed, the removal of the mucous can be extremely difficult [4, 8].

![Figure 4](image.png)

Figure 4. The removal of the mucous layer in these intestinal parasites was achieved by 25–50 gentle washings with physiological saline. The number of washings has to be experimentally worked out for the type and intensity of the mucous covering.

2.5. Avoiding stress in samples

Small organism such as worms, water insects and zooplanktons tend to show some forms of stress or even struggle when immersed into the fixative. These stress effects may result in a change or loss of some of the ultrastructural components of the sample. It may be advisable to narcotize or slow down the organism before fixation. Some researchers leave the samples in a fridge at about 5–8°C for a short period of time before fixation while others immerse the samples in magnesium chloride solutions or dilute alcohol solutions before fixation [4, 7, 9].

2.6. Dry samples

The sample processing techniques to be discussed here, namely air drying, critical point drying and freeze drying involves the drying of the samples to ensure maximum structural preser-
vation. It should be realised that samples which are already dry cannot be reprocessed to bring it back to its original state. However, some temperature dried samples (Figs. 14a and 14b) do provide useful data, for which the techniques for particulate and bulk samples as described in Sections 7.0 and 8.0 can be employed.

2.7. Handling of samples

SEM, unlike TEM, allows the processing of larger organisms and tissues. However, if the samples are too large, it would be wise to cut them into smaller areas of interest, while ensuring that the surface areas of interest are not touched in the process. Even the slightest grazing of your tool on the sample surface of interest can introduce mechanical damage to your sample destroying the fine structures permanently. In the author’s unit, these tasks are always performed under a stereomicroscope with the use of storkbill forceps (Fig. 5) and fresh sharp blades.

Figure 5. Storkbill (or Insect) forceps, which are made of flexible materials, aid in careful handing of samples.

2.8. Safety issues

All fixatives are volatile and harmful to living cells. Even their vapours can fix epithelial cells of the mouth, nose, hand and corneal membranes. Avoid any form of exposure by using gloves and fume hoods. Some workers prefer to use a double layer of gloves during the fixation process. HMDS and TMS are highly volatile and flammable liquid should be used only in well-ventilated areas or in a fume hood. The preparation and use of osmium tetroxide should be very strict in a fume hood. The vial containing the sample should only be taken out of the fume
hoover after the ‘washings’ with buffer or distilled water as the protocol prescribes. Please refer Section 9.0 for the protocols to prepare osmium tetroxide stock solutions [1, 2, 3 and 4].

3. Factors influencing SEM sample preparation

3.1. Fixation

Fixation of samples is probably the most crucial step in SEM sample preparation protocols, which could determine the eventual quality of the images obtained. Ideally, the fixation process should preserve and stabilize the structures of the cells, tissues or organisms keeping them structurally as faithful as possible to its living state. The process should also prevent autolysis and putrefaction in the cells. This is normally achieved by placing the sample in a chemical fixative formulation while also providing an optimal environmental condition in terms of pH, temperature and osmolarity. Routine chemical fixation for SEM involves the use of fixative formulations containing glutaraldehyde, a protein cross-linker and osmium tetroxide, a lipid cross-linker. Formaldehyde is used in combination with glutaraldehyde, due its capability to penetrate faster into the tissue, although it is not known to be a good fixing agent on its own for electron microscopy. Although, there are numerous fixative formulations discussed in literatures and books, one may start with the fixative formulations routinely used in the author’s laboratory, which are 4% glutaraldehyde, Karnovsky’s fixative, McDowell-Trump fixative and 1% osmium tetroxide. (Please refer 9.0 for the recipe). McDowell-Trump fixative is the preferred fixative for SEM in the author’s unit for its better penetrative properties, which allows larger samples to be processed. Although there is a notion that penetration of fixatives is of lesser concern in SEM due its surface analysis mode, it must be realised that poorly fixed internal structures may result in inward shrinking or even collapsed surface structures. A general guideline for the volume of fixative to be used is to ensure that the volume of fixative should be about 15–20 times greater than the volume of the tissue [1, 2, 3, 4, 10 and 11].

3.2. pH and osmolarity

The choice of an appropriate buffer solution serves to keep the pH and osmolarity of the fixing solution within the physiological range as required for the sample, while acting as a vehicle for the fixing agent. The pH of buffers for the ultrastructure preservation is generally adjusted between pH 7.2 and 7.4 while the concentrations of the buffers are adjusted between 0.5 and 1.0 molar solution. For marine samples, osmolarity is achieved by using seawater instead of buffers (Figs. 3a and 3b). Hypertonic solutions give rise to cell shrinkage while hypotonic solutions result in cell swelling and poor fixation. The most commonly used buffers for electron microscopy are phosphate and cacodylate buffers. Phosphate buffers are safer to use and thought to be closer to cytoplasmic environments of most biological samples although it may produce electron dense precipitates in the presence of calcium ions. Precipitation is of lesser concern for cacodylate buffers but its formula contains arsenic, which is hazardous, and proper safety measures should be employed in the use and disposal of these solutions [1, 2, 3, 4].
3.3. Temperature of fixation

Fixation is routinely carried out at room temperature although it is believed that fixation at 0–4°C reduces the possibility of the extraction of cytoplasmic elements from cells [1, 2, 3 and 4].

3.4. Duration of fixation

The time of fixation is dependent on the size and density of the sample to be fixed. A general rule for the penetration of the fixative is ‘1hr per 1mm’ of the sample for most fixatives. Since SEM is for surface analysis, the width of the sample may not be a limiting factor but largely dependent on the width of SEM sample holder available. However, the thickness of sample is best kept within about 3 mm to facilitate the penetration of fixatives. The shortest dimension of the sample determines the fixation time. If the sample is about 1mm³, a fixation period of 2 hours at room temperature or in a refrigerator may be sufficient. For larger samples, 6–24 hours may be necessary. Post-fixation of samples with osmium tetroxide is generally for 1–2 hours, but not any longer than 2 hours. The preparation and use of osmium tetroxide should be very strict in a fume hood. The vial containing the tissue should only be taken out of the fume hood after the ‘washings’ with buffer or distilled water as the protocol prescribes. Please refer Section 9.0 for the protocols to prepare osmium tetroxide. If the samples cannot be processed immediately, they can be kept in the glutaraldehyde-based fixative (McDowell-Trump Fixative, Karnovsky’s Fixative or 4% Glutaraldehyde in buffer) in a refrigerator for a few weeks, with changes of the fixative periodically. Storage of the fixed samples in the refrigerator for very long periods should be avoided as degradation of the samples may occur [1, 2, 3 and 4].

4. SEM sample preparation methods

Almost all living organisms are composed of significant water components in their bodies. However, image formation in an electron microscope requires a high vacuum environment. Thus, the drying of samples becomes a prerequisite for the viewing and obtaining of good images in normal high vacuum SEM systems. Although there are low vacuum SEM systems, which allow the viewing of wet samples, these systems are generally not known for very high resolution and high magnification images. Thus, the challenge posed in SEM biological sample preparation is to dry the samples without any structural damage or changes. The main cause of these structural damage are the ‘surface tension forces’ of the water during the drying process when water transforms into its gaseous phase from its original liquid phase. Thus, the drying methods employed are primarily developed to circumvent the effect of these ‘surface tension forces’. The three most common SEM sample preparation methods employed to minimise the structural changes associated with drying are ‘air drying’, ‘critical point drying’ and ‘freeze drying’[1, 2, 4, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22].

Generally, the techniques involve the following pathways:
Air Drying, Critical Point Drying and Freeze Drying – A Comparison

All the three methods are known to be equally effective in ensuring good preservation of the structural integrity by minimising or eliminating the impact of surface tension effects on the samples.
Different workers have their own preferences after assessing the effectiveness of one method over the other in relation to their own samples. However, the following are some pertinent points for consideration to assist in making a choice:

a. Air drying with hexamethyldisilazane (HMDS) or tetramethylsilane (TMS) is the newest of the three methods and is gaining popularity as a reliable technique. It requires limited expertise and equipment. No liquid gases are required. It has been reported to be suitable for even very soft tissues. It also allows larger samples to be processed as compared to the critical point drying and freeze drying, both of which are limited by the size of sample holders provided for the equipment (Figs. 10b and 12b)

b. Critical point drying with the use of liquid carbon dioxide (and freon in the 70s) has been the method of choice for biological samples for over 50 years. Some workers have observed that critical point drying is a better choice for some of their botanical samples.

c. Fungal hypha and similar soft structures tend to lose their rigidity (and curl up) irreversibly when they come in contact with liquids. This reaction can be compared to wetting a cotton wool with water, which would make the cotton wool to lose its fluffiness irreversibly that even drying may not restore its original fluffiness. This problem effectively rules out the use of air drying and critical point drying for fungal hypha and similar samples due to the need for immersion of the samples in various liquids. The freeze drying method, which employs vapour fixation and quick freezing in liquid nitrogen, offers the best solution for these types of samples (Figs. 11a and 11b)

d. The freeze drying method do not involve the immersion of the sample in any water- or alcohol-based solutions, which ensures minimal physical contact of the sample with any media until the end of the drying process (with the exception of liquid nitrogen). However, the only disadvantage in this circumstance is that, for unclean samples, any presence of dirt, salts and mucus on the sample surface remains on the sample till the end of the processing protocol and realized only at the SEM viewing stage. Some of these extraneous materials may obscure surface details. The air drying and critical point drying has an advantage on this aspect by providing a washing effect on the samples while undergoing many changes in the fixatives, buffers and alcohols [12, 13, 14, 18, and 20].

Standard Requirements for SEM Sample Preparation

Figure 7. (a) Double-sided carbon adhesive tape used extensively for sample attachment on sample stubs. (b) Double-sided carbon adhesive tabs also an excellent item for sample attachment. (c) SEM Sample stubs, shapes and sizes of which are dedicated to the SEM model in use. (d) Air dust blower, useful when preparing particulate samples, Section 7.
4.1. Air drying technique

4.1.1. General Concept

The air drying technique is based on the use of highly volatile organic compounds such as hexamethyldisilazane (HMDS) and tetramethylsilane (TMS), in the drying process to minimise the effect of surface tension forces on cell ultrastructures.

However, the use of HMDS and TMS has to be preceded by the ethanol dehydration process of using increasing ethanol concentrations to gradually remove or rather dilute the water in the tissue, until the water component in the cells is completely replaced with 100% ethanol. It should be noted that the protocols for air drying and critical point drying techniques are same up to the 100% ethanol stage (Fig. 6a) [12, 13, 17, 18, 21].

![Figure 8](https://example.com/figure8.png)

Courtesy of EM Unit, Universiti Sains Malaysia

**Figure 8.** (a) Mosquito egg prepared using the air drying method with HMDS as described in 4.1.2a. (b) Staphylococcus aureus cells prepared using the air drying method with HMDS as described in 4.1.2b.

4.1.2. Standard protocols for the Air Drying Technique

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fix in McDowell-Trump fixative (alternatives: Karnovsky's fixative or 5% Glutaraldehyde) prepared in 0.1M phosphate buffer, pH 7.2, at 4°C 2–24 hours</td>
</tr>
<tr>
<td>2.</td>
<td>Wash in buffer (Use the same buffer as in step 1) 3 x 10 minutes</td>
</tr>
<tr>
<td>3.</td>
<td>Postfix in 1% Osmium tetroxide prepared in the same buffer as above at room temperature. 1–2 hours</td>
</tr>
<tr>
<td>4.</td>
<td>Wash in distilled water 2 x 10 minutes</td>
</tr>
<tr>
<td>5.</td>
<td>Dehydrate the sample as follows: 1. 35% Ethanol 1 x 15 minutes 2. 50% Ethanol 1 x 15 minutes 3. 75% Ethanol 1 x 15 minutes</td>
</tr>
</tbody>
</table>
95% Ethanol 2 x 15 minutes
Absolute Ethanol 3 x 20 minutes

6. Immerse the dehydrated samples in 1–2 ml of hexamethyldisilazane (HMDS) or tetramethylsilane (TMS) for 2 x 10 minutes

7. Decant the HMDS from the sample vial. Leave the sample vial with the samples in a desiccator to air-dry at room temperature overnight

8. The dried samples are then mounted on to a SEM sample stub (Fig. 7c) with a double-sided sticky tape

9. Sputter the sample with gold and view in the SEM (please refer Section 6.0 on conductive coating of samples)

10. It should be noted that the protocols for Air Drying and Critical Point Drying techniques are the same up to the 100% Ethanol stage.

b Protocol for Cultured Micro-organisms (Loose or Loosened Cells)

1. For liquid cultures, centrifuge the cells (pellet formation) and discard the supernatant 10 minutes

2. Resuspend the pellet in McDowell-Trump fixative (alternatives: Karnovsky’s fixative or 5% Glutaraldehyde) prepared in 0.1M phosphate buffer, pH 7.2
   After 30 minutes, centrifuge and discard the supernatant

2a For solid agar cultures, The fixatives can be added directly into the culture plates. After a fixing period of about 30 minutes, the cells adhering to the agar are suspended into the fixative solution by using a cell scraper. The suspension can then be transferred into a vial. Centrifuge and discard the supernatant

3. Resuspend the pellet in 0.1M phosphate buffer. Centrifuge and discard the supernatant 2 x 10 minutes

4. Resuspend the pellet in 1% Osmium tetroxide prepared in 0.1M phosphate buffer
   After 1 hour, centrifuge and discard the supernatant
   **Fume Hood !**

5. Resuspend the pellet in distilled water.
   Centrifuge and discard the supernatant
   **Fume Hood !**

6. Dehydrate the sample through the ethanol series and HMDS as follows, centrifuging and discarding the supernatant for each change:

<table>
<thead>
<tr>
<th>Dehydration Stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>35% Ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>75% Ethanol</td>
<td>10 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 x 10 minutes</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>2 x 10 minutes</td>
</tr>
<tr>
<td>Hexamethyldisilazane (HMDS)</td>
<td><strong>Note:</strong> Centrifugations are not necessary if the cells settle easily at the bottom of the tube and the ethanol changes do not result in excessive loss of the cells. Just necessary</td>
</tr>
</tbody>
</table>

7. Discard the supernatant of the second HMDS and leave the sample vial with the cells in Overnight, a desiccator to air-dry at room temperature.
8. The dried cells are then mounted on to a SEM sample stub (Fig. 7c) with a double-sided sticky tape. (please refer Section 7.0 on 'Techniques for Particulate Samples')

9. Sputter the sample with gold and view in the SEM (please refer Section 6.0 on conductive coating of samples).

**c Protocol for Cultured Micro-organisms (On Solid Agar and other Substrates)**

1. Cut out about 5mm x 5mm piece of the growth colony with a sharp blade as carefully as possible without disturbing the cells on the surface. Perform this in a biosafety cabinet if necessary.

2. Place the piece into a 6cm diameter glass petri dish. Add McDowell-Trump fixative into the petri dish to wet the agar without reaching the colony surface but enough to allow the fixatives to diffuse through the substrate to reach the colonies. Cover the petri dish. Allow 1–2 hours.

3. Pipette out the fixative gently and replace it with 0.1M phosphate buffer and again wet the agar without reaching the colony surface. Allow 2 x 20 minutes.

4. After pipetting out the phosphate buffer, place 1 or 2 pieces of dry filter paper beside the 1 hour agar colony piece. Wet these filter papers with drops of 1% osmium tetroxide. Close the petri dish and seal it with parafilm. Use of fume hood is essential. Alternatively, a few drops of the 1% osmium tetroxide can be placed to wet the agar but without reaching the colony surface. Allow 1 hour.

5. Remove the filter papers with osmium tetroxide and wash the sample with distilled water in the same manner as in Steps 2 and 3. Allow 2 x 20 minutes.

6. Dehydrate the sample through the ethanol series and HMDS in the same manner as in Steps 2 and 3:

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>35% Ethanol</td>
<td>1 x 30 minutes</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>1 x 30 minutes</td>
</tr>
<tr>
<td>75% Ethanol</td>
<td>1 x 30 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 x 30 minutes</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>3 x 30 minutes</td>
</tr>
<tr>
<td>Hexamethyldisilazane (HMDS)</td>
<td>2 x 30 minutes</td>
</tr>
</tbody>
</table>

Allow 30 minutes or more per step for thorough diffusion through the agar blocks and colonies.

7. Once the second change of HMDS has been pipetted out, leave the sample in a desiccator with the petri dish cover slightly open to air-dry at room temperature. The dried samples are then mounted on to a SEM sample stub (Fig. 7c) with a double-sided sticky tape.

8. Sputter the sample with gold and view in the SEM (please refer Section 6.0 on conductive coating of samples).

**d Technique for a) preparations with few cells b) fragile cells that may not withstand centrifugation and c) if pelleting is to be avoided**

1. Prepare a solution of 0.1% poly-L-lysine in phosphate-buffered saline (pH 7.2).

2. Place a drop of the poly-L-lysine solution on a coverslip (or microscope slide) and place it in a covered petri dish lined with moist filter paper for about 1 hour.
3. Rinse the poly-L-lysine coated coverslip with distilled water and place a drop of the cell suspension on the coated area of the coverslip (in the covered petri dish lined with moist filter paper) for about 30–60 minutes to allow the organisms to settle on the sticky material. The smaller the organism, the longer it takes to settle.

4. Carefully touch on the side of the drop with a fresh piece of cut filter paper to remove the liquid and immediately place a drop of EM fixative to replace the earlier fluid. (Do not let it to dry completely in between the step). Leave it alone for about 10 minutes.

5. Repeat the steps with the standard SEM sample preparation protocol through buffer, OsO₄, distilled water, graded alcohols and HMDS, BUT in the covered petri dish lined with DRY filter paper. (The times can be shortened to 5–10 minutes in each step.)

6. Important: Ensure that the liquids DO NOT dry up completely in between the changes

7. At the 2nd HMDS stage, once the HMDS has dried completely, the coverslip/slide can be attached to the SEM sample stub, sputtered and viewed in the SEM.

8. Note: This technique can also be used for cells grown on coverslips, if the cells are well adhered on to the coverslip.

4.1.3. General Precautions

a. Once the samples have been placed in the vial containing the primary fixative, the same vial should be used throughout the sample preparation protocol until the ‘sample mounting’ stage prior to viewing the sample in the SEM. Simply decant or pipette out the changes without any form of physical contact on the surface of the sample.

b. The need for centrifugation is needed only to form a loose pellet and therefore low speed centrifugations of 500–1500 should suffice.

c. Never let the samples to dry at any stage (till the samples are immersed in the HMDS).

d. Strict safety measures should be observed for the fixation of pathogenic materials. Fixatives can be added directly into the culture plates or broth cultures as a safety measure.

e. Times recommended are for small pieces of samples (smaller than 1–2 mm³). For larger pieces, times may have to be varied accordingly.

f. Buffers: Normally, 0.1M of pH 7.2, but requirements may vary for different samples. Refer to published papers.

4.2. Critical point drying technique

4.2.1. General Concept

Critical point drying method is based on the principle that by increasing the pressure and temperature of a wet sample, it is possible to dry the sample by achieving the ‘critical point’ of that liquid at which the phase boundary between its liquid and gaseous states are eliminated. At this state, the gas is released slowly thus minimising the effect of surface tension forces on cell ultrastructures.
However, the critical point for water is extremely high at 228.5 bar (3212 psi) and 374°C, which could instantly destroy the samples. Critical point of ethanol is 60.8 bar (882 psi) and 241°C. These properties of water and alcohol make them unsuitable for the critical point drying method.

CO$_2$ liquid with a critical point at 73, 8 bar (1072 psi) and 31°C is generally used. Since CO$_2$ is not miscible with water, acetone is used as a transitional (intermediate) fluid in the process.

Just as has been done in the air drying technique, the use of the critical point dryer has to be preceded by the ethanol dehydration process of using increasing ethanol concentrations to gradually remove or rather dilute the water in the sample tissue, until the water component in the cells is completely replaced with 100% ethanol. The size of the samples should be determined carefully in view of the sample holder sizes provided with critical point dryer (Fig. 10b). Some CPD manufacturers provide sample holders for cells grown on coverslips or wafer materials. However, good adherence of the cells on these substrates must be confirmed before attempting this drying technique. This should be done to avoid the excessive loss of cells during the drying cycle. The use of poly-l-lysine helps in the adherence of most of the cells on these substrates (refer 4.1.2d) [1, 14, 15, 16, 19].

![Figure 9](image-url) Figure 9. (a) Callus of Pomelo prepared using the critical drying method as described in 4.2.3. (b) Critical Point Dried House Fly prepared using the critical drying method as described in 4.2.3.

### 4.2.2. Materials

![Figure 10](image-url) Figure 10. (a) Critical Point Dryers (CPD). The CO$_2$ gas cylinders are not shown. It is advisable to use a CO$_2$ gas of at least 99.8% purity and the gas cylinder is fitted with a siphon tube to draw liquid CO$_2$ from the bottom of a cylinder. (b) CPD Sample Holders, which are supplied with the CPD apparatus.
4.2.3. Standard protocol

General Protocol

1. Fix in McDowell-Trump fixative (alternatives: Karnovsky's fixative or 5% Glutaraldehyde) prepared in 0.1M phosphate buffer, pH 7.2, at 4°C 2 – 24 hours

2. Wash in buffer. (Use the same buffer as in step 1) 3 x 10 minutes

3. Postfix in 1% Osmium tetroxide prepared in the same buffer as above at room temperature. 1–2 hours

4. Wash in distilled water. 2 x 10 minutes

5. Dehydrate the sample as follows:
   - 35% Ethanol 1 x 15 minutes
   - 50% Ethanol 1 x 15 minutes
   - 75% Ethanol 1 x 15 minutes
   - 95% Ethanol 2 x 15 minutes
   - Absolute Ethanol 2 x 15 minutes
   - Acetone 1 x 15 minutes

6. Transfer the samples into the CPD specimen holder or baskets (Fig. 10b). The sample holder should contain (or immersed in) enough acetone to cover the samples.

7. Perform the critical point drying as described in the instruction manual for the apparatus (Fig. 10a)

8. The dried samples are then mounted on to a SEM sample stub (Fig. 7c) with a double-sided sticky tape.

9. Sputter the sample with gold and view in the SEM (please refer Section 6.0 on conductive coating of samples).

4.2.4. General Precaution

The exchange and release of the CO₂ liquid and gas should be performed in a controlled manner to avoid turbulence within the CPD, which may be detrimental to the sample structures.

4.3. Freeze drying technique

4.3.1. General concept

Freeze drying method is based on the principle of sublimation by shifting a solid phase directly into its gaseous phase in a vacuum, bypassing the liquid phase in the process, thus eliminating the problems associated with surface tension forces, which are dynamic only during 'liquid to gas' phase change [1, 14, 15, 16, 19].
4.3.2. Materials

Figure 12. (a) SEM Freeze Dryers are generally fitted with thermoelectric peltier cooled stages, adjustable to -60°C to enable careful sublimation of frozen samples under vacuum. The vacuum pump is not shown. (b) Aluminium Planchettes, useful to hold samples for freeze drying. After the freeze drying and sputtering processes, the planchette with the sample can be attached on the SEM stub in one piece for viewing. (c) Tissue-Tek, generally used in cryostat sectioning, aids in the firm attachment of frozen samples on the planchette when used together with double-sided carbon adhesive tapes or tabs.

4.3.3. Standard protocol

Freeze Drying

1. Prepare a planchette with double-sided sticky tape and a thin layer of ‘Tissue-Tek’ on the tape (Fig. 12b and 12c). Label the sample positions carefully. *(The sticky tape tends to detach from the planchette if used on its own. Similarly, ‘Tissue-Tek’ sometimes slide off with the sample once frozen when used on its own. In the author’s unit, the sticky tape, preferably carbon, is firmly attached on to the planchette followed by a thin layer of ‘Tissue-Tek’ covering the sticky tape and beyond. The sample can then be placed on the ‘Tissue-Tek’ layer for vapour fixation and the liquid nitrogen plunge.)*
Freeze Drying

2. Cut out a small piece of agar/substrate containing the bacterial/ fungal growth (about 5mm x 5mm) and quickly place it on the double-sided sticky tape with ‘Tissue-Tek’. [FUME HOOD]

3. Place the planchette in a filter paper lined petri dish; wet the filter paper with a few drops of 2% osmium tetroxide, away from the planchette and close the petri dish immediately. Leave it alone in the fume hood for about 1–2 hours. This process is known as ‘vapour fixation’. [FUME HOOD]

4. Once the sample has been ‘vapour fixed’, the planchette is plunged into ‘slushy nitrogen (-210˚C) and transferred on to the ‘peltier-cooled’ stage of the Freeze Dryer (Fig. 12a) and left to freeze dry for about 10 hours. (Refer to manual for the operation of the freeze dryer.)

5. After the freeze drying process, the planchette with the sample must be kept in a desiccator (if the sample cannot be viewed immediately).

6. The planchette is then mounted on to a SEM sample stub (Fig. 7c) with a double-sided sticky tape. Sputter the sample with about 5–10 nm of gold before viewing it in the SEM.

4.3.4. General Precaution

Keep the sample in a moist environment at all times till the freezing process. If the sample is already dry or partially dry (i.e. if some removal of water from the sample has occurred prior to vapour fixation), structural alteration or deformation is to be expected.

5. Mounting of samples

Mounting of samples on the SEM stubs needs utmost care and the use of a low power stereomicroscope is highly recommended during the process. It can be heartbreaking to see the region of interest of a sample damaged by a simple unintentional graze with a tool, after all the hours spent on processing the sample. Storkbill forceps or insect forceps (Fig. 5), fine spatulas, double-sided carbon adhesives (Fig. 7a), Leit-C (Fig. 15a) and sharpened soft wood sticks are some of the tools which could come handy when performing the mounting process. Factors which need to be considered in the sample mounting process are sample orientation, sample stability and ensuring good adherence of the sample on the SEM stub. Leit-C can aid in placing the sample in the required orientation for larger samples. [1, 2, 4].

6. Conductive coating of samples for sem

Biological samples, being non-conductive, give rise to charging problems in the SEM as the bulk of primary electrons from the electron beam remain in the sample to form clouds of negative charges. This built-up charge interferes with the primary beam to bring about image distortions, loss of contrast with very bright and dark areas, known as ‘charging effect’ (Fig. 13b). To overcome these problems, a thin layer of metal is sputtered on the sample, thereby increasing the conductance on the sample to enable the absorbed electrons to find
their way to ground. A sputter coater (Fig. 13a) is used for this purpose. The preferred metals for sputtering are gold, gold-palladium, platinum and chromium. However, platinum and chromium are the choice metals for high resolution imaging (more than 50,000x) as gold sputtering results in visible graininess on the surface of the sample (Fig. 13c). Other materials used for sputtering are iridium and carbon. In the present state of art Field Emission SEMs, metal sputtering can be completely ignored with the use of low KV imaging techniques of less than 500 volts [1, 2, 4].

![Image](image.png)

**Figure 13.** (a) Sputter Coater – employs the physical vapour deposition principle by using argon ions to eject target material (Au, Au-Pd, Pt, Cr, etc.) from the target surface to sputter a thin film of the metal or alloy on the sample, in a vacuum. (b) Charging Effect on a wood sample in the SEM, thus metal sputtering is needed to enable the absorbed electrons to be dissipated. (c) Nano Silica Powder with visible graininess from gold sputtering. Chromium or Platinum would have been a better choice for this SEM magnification.

### 7. Technique for particulate samples

Particulate samples of dry powders (Fig. 14a), fine crystals (Fig. 14b), nanoparticles (Fig. 13c), dried bacterial cells and spores do not need any special sample preparation protocols. However, care must be taken to avoid the particles loosely ‘stacking’ one over the other. It should also be ensured that the particles are firmly stuck on the specimen stub.

**Procedure:**

1. **Sprinkle a little of the sample evenly but lightly on a SEM sample stub with the double-sided sticky tape.**
2. **Use a hand blower (Fig. 7d) to blow away the loose particles. Always blow away from yourself into a bin or sink. Employ increased safety procedures if the particles are known to be harmful**
3. The sample is now ready for viewing in the SEM. Sputter with Au, Pt or Cr, if necessary.
Stacking of particles is generally the main cause of image instability and charging problems. Sometimes, after the blowing away of the loose particles, the sticky tape may appear to be very clean without any particles left for SEM study. It will be noted that this type of preparation tends to give lesser agglomeration and lesser charging. It is possible for fine particles prepared on carbon sticky tape in this manner to be viewed employing the backscatter mode without the need for metal sputtering [1, 2, 4].

8. Technique for large and bulk samples

Samples of rocks, wood, plastics, metals and electronic components, which are large enough to be handled with general laboratory forceps do not need any processing. However, ensure the use of suitable large SEM sample holders and the sample height to conform with the specifications of the SEM (Fig. 15b). The samples must be secured and stuck firmly on the holder to avoid any possible movement or vibrations. For larger samples, securing of the samples should be at the base and also across the samples to hold down the sample firmly on the SEM stub, carefully avoiding the areas to be studied in the SEM (Fig. 15b). Double-sided carbon sticky tapes and tabs (Figs. 7a and 7b) have been found to be the most suitable for the attachment of the sample on the SEM sample stubs, although other materials such as colloidal silver paint, carbon paints and Leit-C (Fig. 15a) can be employed. Leit-C can be very useful in positioning the sample to obtain suitable orientations for imaging in the SEM. Sputtering of the sample is necessary for non-conductive samples [1, 2, 4].
Figure 15. (a) Leit-C, which is flexible like plasticine clay, is useful in positioning the sample to obtain the required orientation large samples for imaging. (b) Large sample on stub. Double-sided carbon adhesive tape has been used to secure the sample on the sample stub. (c) Copper wire with lead particles, secured on the SEM sample stub with double-sided carbon adhesive tape. No processing is required. Being a conductive sample, no metal sputtering is required. (d) Nanocomposite block material, secured on the SEM sample stub with double-sided carbon adhesive tape. Being a non-conductive sample, sputtered with gold, but no other processing is required.

9. Reagents

References for section 9 are from [1, 2, 3, 4, 10, 11]

1. **Phosphate Buffer: (Sörensen)**

To prepare a 0.2M phosphate buffer of pH 7.2 as follows:

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 20.44 \text{ g} \\
\text{NaH}_2\text{PO}_4 & \quad 6.72 \text{ g}
\end{align*}
\]

Add distilled water to a final volume of 1000 ml. Dilute to obtain other concentrations.

2. **Cacodylate Buffer:**

Prepare a 0.4M solution of Sodium Cacodylate with

\[
\text{Na(CH}_3\text{)}_2\text{AsO}_3\text{H}_2\text{O} \quad 21.4 \text{ g}
\]

Add distilled water to make 250 ml.

Prepare 0.2M Cacodylate Buffer, pH 7.2 (approx.)

0.4M Sodium Cacodylate ..... 50 ml
0.2M HCl..... 8 ml
Add distilled water to a final volume of 100 ml.
The pH of the buffer is adjusted, if necessary, to the required value with HCl.

3. **4% Glutaraldehyde:**

Prepare the fixative as follows:
0.2M buffer..... 50 ml
25% glutaraldehyde..... 16 ml
Add distilled water to a final volume of 100 ml

4. **McDowell and Trump’s Fixative (1976):**

Prepare the fixative as follows:
0.2M buffer..... 50 ml
37% formaldehyde..... 11 ml
25% glutaraldehyde..... 4 ml
Add distilled water to a final volume of 100 ml
This fixative contains 4% formaldehyde and 1% glutaraldehyde in 0.1M buffer (4F : 1G). Other concentrations are obtained by using different quantities of the constituents. The pH may change on adding the aldehydes.

5. **Karnovsky’s Fixative (1965):**

Prepare 20 ml of a 10% solution of paraformaldehyde by dissolving 2.0 g of paraformaldehyde powder in 20 ml of distilled water and heating to about 60°C (in a fume hood). Add a few drops of 1M Sodium Hydroxide until the solution becomes clear. Allow the solution to cool before use.

Prepare the fixative as follows:
0.2M buffer..... 50 ml
10% paraformaldehyde..... 20 ml
25% glutaraldehyde..... 10 ml
Add distilled water to a final volume of 100 ml
This fixative contains 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M buffer. Other concentrations are obtained by using different quantities of the constituents. The pH may change on adding the aldehydes.
If necessary, adjust the osmolarity of the fixative with sucrose, glucose or sodium chloride.
If required, add sufficient calcium or magnesium chloride to give a final concentration of 1–3 mM, taking care to avoid the formation of precipitates with phosphate buffers.
6. **4% Glutaraldehyde in Seawater**

Prepare the fixative as follows:

- 25% glutaraldehyde..... 16 ml OR
- 50% glutaraldehyde..... 8 ml

Add artificial seawater/membrane filtered seawater to a final volume of 100 ml

7. **Osmium tetroxide solutions**

**Notes:**

1. Osmium tetroxide is a contact and inhalation hazard. Do not break the ampoule in the open, as the crystals may sprinkle around and the vapour from the crystal is very harmful.

2. The preparation of osmium tetroxide must be performed in a chemical fume hood.

3. Solutions should be prepared in very clean bottles, as the presence of any organic substance tends to react with osmium (and changes colour over time).

4. Store in a fridge (5 deg), by placing the smaller 100 ml bottle containing the stock osmium solution in a larger wide mouth bottle with a tight screw cap, e.g. Kilner Jars.

5. If possible, prepare at least 1 day before use to ensure complete dissolution of the crystals

**Items needed to prepare 2% Aqueous Osmium tetroxide:**

- 1g Osmium tetroxide in an ampoule (or any other amount but recalculate for 2%)
- 50 ml distilled water in a clean glass measuring cylinder
- A clean 100 ml thicker walled glass bottle to contain the 2% stock solution
- A glass scorer (any types)
- A thick glass rod (about 8” long)
- Get ready all these items in the fume hood

**Method**

- Take a 1 g osmium vial and score the neck with a glass scorer (making sure not to score too hard such that the glass could break)

  **[If the ampoule has a label sticker on it, remove the sticker, wash the gum remnants on the glass with a little soap solution if necessary, and rinse the ampoule with a few changes of distilled water before proceeding to score and use. Gum material sticking on the glass would react with osmium to deteriorate the stock solution.]**

- From the measuring cylinder containing 50 ml of distilled water, add about 10 ml of the water into the 100ml glass bottle
- Place the ampoule into the bottle
• Use the glass rod to break the ampoule. Once broken, crush the ampoule with the glass rod into a couple of pieces.

• Now add the remaining 40 ml of distilled water into the bottle (while also washing down the glass rod into the bottle)

• Close the bottle tightly and swirl the bottle for a while to enhance the dissolving of the osmium in the water.

• Now place this 100 ml bottle containing the 2% Osmium into a larger wide mouth screw capped glass container and store it in a fridge.

• Before use the next day, swirl the bottle for about a minute. This swirling is needed only for using it for the first time.

• [It is alright to leave the ampoule glass pieces in the 100 ml bottle. Sometimes, when the 50 ml of osmium solution has been used up, I break another ampoule in the same bottle (as described above) without removing the earlier glass pieces. I do this for about 3 ampoules before washing and removing the glass pieces for a complete wash of the bottle, provided the osmium solutions remain clear all the time]

• [Sometimes, if the osmium is needed to be used on the same day as when prepared, the bottle containing the stock solution is left in the ultrasonic bath for a few minutes before use.]

• The working fixative is prepared just before use, by mixing equal parts of 2% aqueous stock osmium tetroxide and 0.2M phosphate buffer – resulting in 1% osmium tetroxide in 0.1 M phosphate. [However, there are some researchers known to use other concentrations of osmium tetroxide and buffers].

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