Life science sample preparations for scanning electron microscopy

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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 review is to equip researchers, post graduate students and technicians with the essential knowledge required to prepare samples for scanning electron microscopy investigations in the life sciences.

Keywords: Scanning electron microscopy, hexamethyldisilazane, critical point drying, air drying technique, freeze-drying technique.

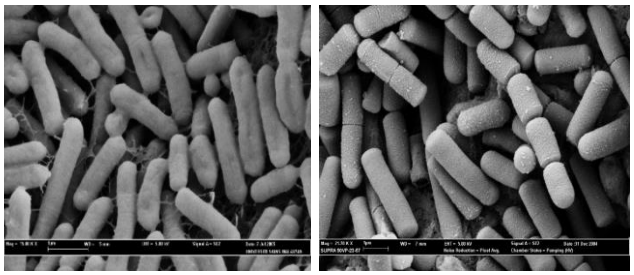
Preparación de muestras en ciencias de la vida para microscopia electrónica de barrido

RESUMEN
La preparación de muestras es esencial para la microscopía electrónica de barrido. Una preparación ineficiente de muestras puede comprometer la calidad de los resultados y generar falsas conclusiones. Por lo cual, el objetivo de este review es aportar a investigadores, estudiantes de posgrado y profesionales, los conocimientos indispensables sobre la preparación de muestras de investigación en ciencias de la vida para su microscopía electrónica de barrido.

Palabras claves: Microscopía electrónica de barrido, hexametildisilazano, secado de punto crítico, técnica de secado al aire, técnica de liofilización.

INTRODUCTION

Obtaining acceptable scanning electron microscopy (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.



(a) **(b)**

Fig. 1. *Bacillus cereus* (Courtesy of EM Unit, Universiti Sains Malaysia).

PRIMARY CONSIDERATION IN SCANNING ELECTRON MICROSCOPY PREPARATIONS

Collection of Samples

In the laboratory, collecting samples for scanning electron microscopy (SEM) preparations generally involves the dissection of an animal or incision of 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

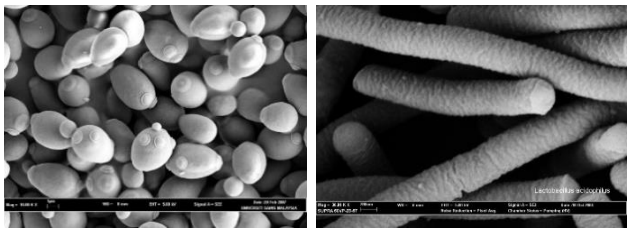
them in suitable physiological saline and fixed soon after at the earliest possible time. All incisions must be performed with fresh sharp blades to avoid deformation of tissues from the undue physical forces needed with blunt blades.

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. However, for fungal samples growing on leaves and barks, immersion fixation may not be suitable due to collapsing of the fluffy hypha structures when they come in contact with liquids. Vapour fixation followed by freeze-drying 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 minimize 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 which were brought to the laboratory from nearby trees within the campus and was minimized only by employing the method of transfer described above [1-4].

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 bio-safety cabinets only after the fixation process. The fixatives can be added directly into the culture plates or broth cultures as needed. Avoid the selection of organisms in the death phase of their growth curve or overgrown cultures except when doing comparative studies (Fig. 2a,b). Many students leave their cultures in a refrigerator for a long period of time and produce disappointing results in the SEM [5,6].



(a) **(b)**

Fig. 2. (a) Yeast - *Saccharomyces cerevisiae*, (b) *Lactobacillus acidophilus* (Courtesy of EM Unit, Universiti Sains Malaysia).

Fresh Water and Marine Samples

Organisms from freshwater habitats can be fixed in the routine electron microscopy fixatives. However, for marine samples (examples in Fig. 3), the fixatives should be prepared in filtered or artificial seawater with its osmolarity matching their environment [1,2,4,8]

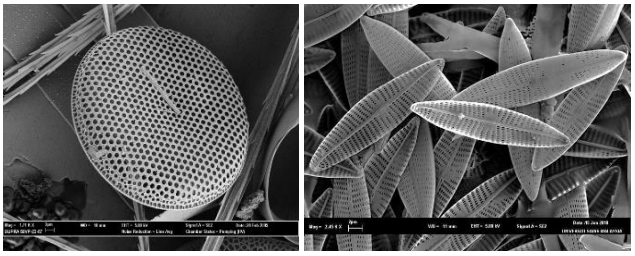


Fig. 3. Marine Diatoms(Courtesy of EM Unit, Universiti Sains Malaysia)

Samples with heavy mucous films

The presence of mucous films on sample surfaces can obstruct the clarity of the surface ultrastructure. This problem is often encountered when processing samples like mucous producing organisms and parasites removed from organs. Although clearing the mucous is sometimes achieved using enzymes, the specificity of these enzymes and the application method needs to be worked out carefully

to avoid any consequential damaging effect on the fine structures on the sample.

In the absence of the availability of a suitable enzyme to digest off 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 helpful. Students had 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 (Fig. 4). It has to stress 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 [4,9].

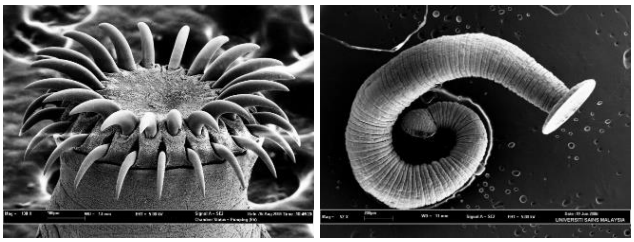


Fig. 4. Intestinal Parasites. Washed 25-50 times with physiological saline for the removal of the surface mucous layer (Courtesy of EM Unit, Universiti Sains Malaysia).

Avoiding Stress in Samples

Small organisms 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 a 5% magnesium chloride or dilute alcohol solutions before fixation [4,7,10].

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 preservation.

It should be realized that samples that are already dry cannot be reprocessed to bring it back to its original state. However, some temperature dried samples do provide valuable data, for which the techniques for particulate and bulk samples can be employed [4,7,10].

Handling of Samples

Scanning electron microscopy, unlike transmission electron microscopy, 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 tissue destroying the fine structures permanently. In the author's unit, performing these tasks are always under a stereomicroscope with the use of insect forceps (storkbill forceps) and fresh sharp blades [4,7,10].

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 (although the vapours do not penetrate more than a millimetre or two and are unlikely to cause permanent damage).

Some workers prefer to use a double layer of gloves during the fixation process. Hexamethyldisilazane and tetramethylsilane 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 strictly 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 [1-4].

FACTORS INFLUENCING SCANNING ELECTRON MICROSCOPY SAMPLE PREPARATION

Fixation

Fixation of tissues 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 their living state. The process should also prevent autolysis and putrefaction in the cells. This is usually 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. However, 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. McDowell-Trump fixative is the preferred fixative for SEM in the author's unit for its better penetrative properties which allow larger samples to be processed. Although there is a notion that penetration of fixatives is a lesser concern in SEM due to its surface analysis mode, it must be realized 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 [1-4,11,12].

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 to 7.4 while the concentrations of the buffers are adjusted between 0.5 and 1.0 molar solutions. For marine samples, osmolarity is achieved by using seawater instead of buffers. Hypertonic solutions give rise to cell shrinkage while hypotonic solutions result in cell swelling and poor fixation [1-4].

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 they may produce electron-dense precipitates in the presence of calcium ions. Precipitation is of lesser concern for cacodylate buffer but its formula contains arsenic which is hazardous and proper safety measures should be employed in the use and disposal of these solutions [1-4].

Temperature of Fixation

Fixation is routinely carried out at room temperature, although it is believed that fixation at 0-4 ^{o}C reduces the possibility of the extraction of cytoplasmic elements from cells [1-4].

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 '1 hr per 1 mm' of the tissue for most fixatives. Since SEM is for surface analysis, the width of the sample may not be a limiting factor but is largely dependent on the width of SEM sample holder available. However, the thickness of the 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 1 mm^3, a fixation period of 2 hours at room temperature or in a refrigerator may be sufficient. For larger samples, 6-24 h are maybe necessary [1-4].

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 strictly 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 [1-4].

The samples could not be processed immediately, can be kept in the glutaraldehyde-based fixative (McDowell-Trump Fixative, Karnovsky's Fixative or 4% Glutaraldehyde in phosphate 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 [1-4].

SCANNING ELECTRON MICROSCOPY 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 that 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 damages 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 minimize the structural changes associated with drying are 'air drying', 'critical point drying' and 'freeze drying' [1,2,4,13-23]. Generally, the techniques involve the following paths (figures 5 and 6):

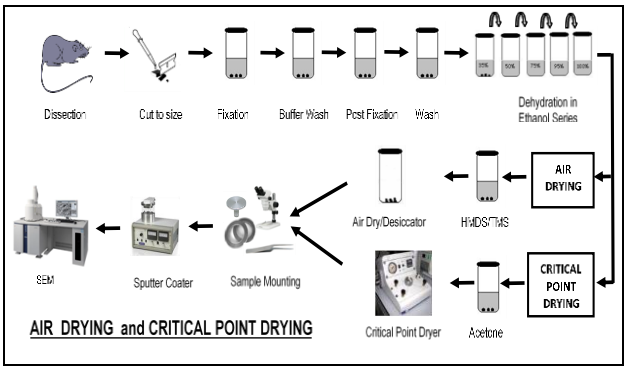


Fig. 5. Air drying and critical point drying sample preparation.

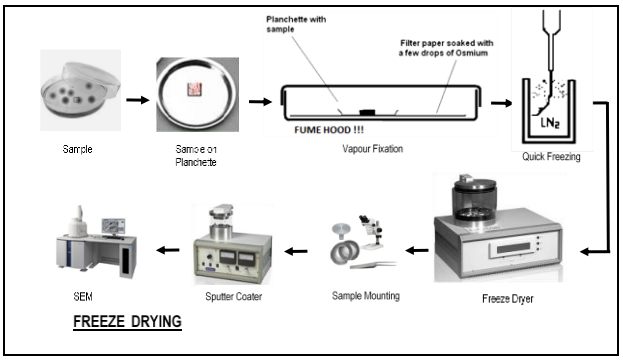
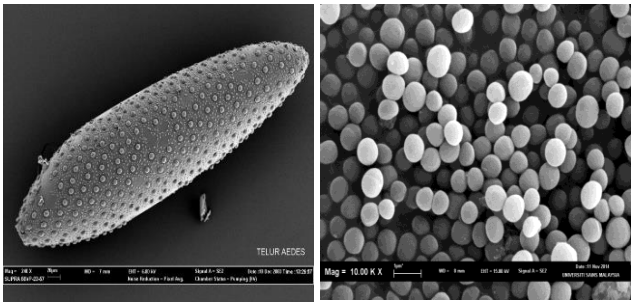


Fig. 6. Freeze drying sample preparation.

AIR DRYING TECHNIQUE

The air-drying technique is based on the use of highly volatile organic compounds such as hexamethyldisilizane (HMDS) and tetramethylsilane (TMS), in the drying process to minimize the effect of surface tension forces on cell ultrastructure (fig. 7a,b).



(a) **(b)**

Fig. 7. (a) Air Dried Mosquito Egg, (b) Air Dried Staphylococcus aureus (Courtesy of EM Unit, Universiti Sains Malaysia).

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 with100% ethanol. [13,14,18,19,22].

It should be noted that the protocols for air drying and critical point drying techniques are same up to the 100% ethanol stage [13,14,18,19,22].

SAMPLE PREPARATION PROTOCOLS FOR THE AIR-DRYING TECHNIQUE

Protocol for tissues, insects and organisms which can be held or picked up with forceps

1. Fix in McDowell-Trump fixative (alternatives: Karnovsky's fixative or 5% Glutaraldehyde) prepared in 0.1 mol/l phosphate buffer pH 7.2 for 2-24 h at 4 °C.
2. Wash in buffer for 3×10 min. (Use the same buffer as in step 1).
3. Postfix in 1% Osmium tetroxide prepared in the same buffer as above for 1-2 h at room temperature.
4. Wash in distilled water for 2×10 min.
5. Dehydrate the sample as follows:

a. 35 % Ethanol	1×15 min
b. 50% Ethanol	1×15 min
c. 75% Ethanol	1×15 min
d. 95% Ethanol	2×15 min
e. Absolute Ethanol	3×20 min

6. Immerse the dehydrated tissues in 1-2 ml of hexamethyldisilazane (HMDS) or tetramethylsilane (TMS) for 10 minutes.
7. Decant the HMDS from the specimen vial. Leave the specimen vial with the tissues in the desiccator to air-dry at room temperature overnight.
8. The dried specimens are then mounted on to a SEM specimen stub with a double-sided sticky tape (or silver paint).
9. Sputter the sample with gold and view in the SEM.
10. It should be noted that the protocols for Air Drying and Critical Point Drying techniques are same up to the 100% Ethanol stage.

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

1. *For liquid cultures,* centrifuge the cells (pellet formation) for 10 min and discard the supernatant.
2. Resuspend the pellet in McDowell-Trump fixative (alternatives: Karnovsky's fixative or 5% Glutaraldehyde) prepared in 0.1 mol/l phosphate buffer, pH 7.2 for 30 minutes. After 30 min, centrifuge and discard the supernatant.
   a. *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 scrapper. The suspension can then be transferred into a vial. Centrifuge and discard the supernatant.
3. Resuspend the pellet in 0.1 mol/l phosphate buffer for 2×10 min. Centrifuge and discard the supernatant.
4. Resuspend the pellet in 1% Osmium tetroxide prepared in 0.1 mol/l phosphate buffer for 1 h. After 1 h, centrifuge and discard the supernatant (Fume Hood).
5. Resuspend the pellet in distilled water for 2×10 min. 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:

a. 35 % Ethanol	10 min
b. 50% Ethanol	10 min
c. 75% Ethanol	10 min
d. 95% Ethanol	10 min
e. Absolute Ethanol	2×10 min
f. HMDS	2×10 min

   Note: 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.
7. Discard the supernatant of the second HMDS overnight and leave the specimen vial with the cells in a desiccator to air-dry at room temperature.
8. The dried specimens are then mounted onto a SEM specimen stub with a double-sided sticky tape (refer to section on 'Techniques for Particulate Samples').
9. Sputter the sample with gold and view in the SEM.

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

1. Cut out about 5×5 mm piece of the growing 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 for 1-2 h. Cover the petri dish.
3. Pipette out the fixative gently and replace it with 0.1 mol/l phosphate buffer, and again wetting the agar without reaching the colony surface.
4. After pipetting out the phosphate buffer, place 1 or 2 pieces of dry filter paper beside the agar colony piece. Wet these filter papers with drops of 1% osmium tetroxide. Close the petri dish and seal it with parafilm. The use of a 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.
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 for 2×20 min.
6. Dehydrate the sample through the ethanol series and HMDS in the same manner as in Steps 2 and 3:

a.	35 % Ethanol	1×30 min
b.	50% Ethanol	1×30 min
c.	75% Ethanol	1×30 min
d.	95% Ethanol	2×30 min
e.	Absolute Ethanol	3×30 min
f.	HMDS	1×30 min

7. Allow 30 min or more per step for thorough diffusion through the agar blocks and colonies.
8. 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 specimens are then mounted on to a SEM specimen stub with double-sided sticky tape.
9. Sputter the sample with gold and view in the SEM.

Special case technique

Suitable for samples with few cells, fragile cells that may not withstand centrifugation and cells that is not suitable for pelleting.

1. Prepare a solution of 0.1% poly-L-lysine in phosphate-buffered saline (pH 7.2).
2. Place a drop ofthe 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 h.
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 for about 30-60 min 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 min.
5. Repeat the steps with the standard SEM sample preparation protocol through the buffer, OsO_4, 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 min 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 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 to the coverslip.

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 ‘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.

B. The need for centrifugation is needed only to form a loose pellet and therefore, low-speed centrifugations of 500-1500 g should suffice.

C. Never let the tissues 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 tissues (smaller than 1-2 mm^3). For larger pieces, times may have to be varied accordingly.

F. Buffers: normally 0.1 mol/l of pH 7.2, but requirements may vary for different tissues.

CRITICAL POINT DRYING TECHNIQUE

Critical point drying (CPD) method is based on the principle that by increasing the pressure and temperature of a wet specimen (fig. 8a,b), 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 stage, the gas is released slowly thus minimizing the effect of surface tension forces on cell ultrastructure. But, the critical point for water is extremely high at 22.850 MPa and 374 °C which would instantly destroy the specimen. The critical point of ethanol is 6.08 MPa and 241 °C. CO_2 liquid with a critical point at7.38 MPa 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 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 tissue until the water component in the cell is completely replaced with 100% ethanol.

The size of the samples should be determined carefully in view of the sample holder sizes available for the brand of critical point dryer to be used. 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 adherence of most of the cells on these substrates [1,15-17,20].

(a) **(b)**

Fig. 8. (a) Critical Point Dried Callus of Pomelo, (b) Critical Point Dried House Fly (Courtesy of Dr. S. Sasidharan, Universiti Sains Malaysia).

Protocol for Critical Point Drying technique

1. Fix in McDowell-Trump fixative (alternatives: Karnovsky's fixative or 5% Glutaraldehyde) prepared in 0.1 M phosphate buffer, pH 7.2 for 2-24 h, at 4 °C.
2. Wash in the buffer for 3×10 min (use the same buffer as in step 1).
3. Postfix in 1% Osmium tetroxide prepared in the same buffer as above for 1-2 h at room temperature.
4. Wash in distilled water for 2×10 min.
5. Dehydrate the sample as follows:
   a. 35 % Ethanol 1×15 min.
   b. 50 % Ethanol 1×15 min.
   c. 75 % Ethanol 1×15 min.
   d. 95 % Ethanol 2×15 min.
   e. Absolute Ethanol 2×15 min.
   f. Acetone 1×15 min.
6. Transfer the tissues into the CPD specimen holder or baskets. The specimen holder should contain (or be immersed in) enough acetone to cover the tissues.

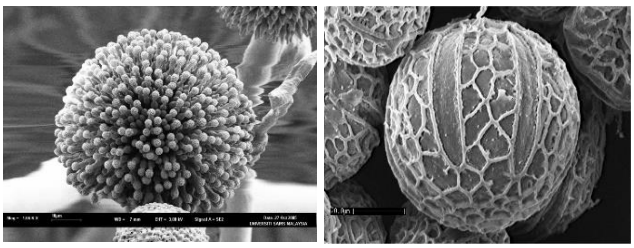
7. Perform the critical point drying as described in the instruction manual for the apparatus.
8. The dried specimens are then mounted onto a SEM specimen stub with double-sided sticky tape.
9. Sputter the sample with gold and view in the SEM.

General Precaution

The exchange and release of the CO_2 liquid and gas should be performed in a controlled manner to avoid turbulence within the CPD, which may be detrimental to the sample structures.

FREEZE DRYING TECHNIQUE

The 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 (figs. 9a,b) which are dynamic only during 'liquid to gas' phase change [1,15-17, 20].



(a) **(b)**

Fig. 9.(a) Freeze Dried Aspergillus niger, (b) Freeze Dried Pollen of Orthosiphonstamineus (Courtesy of Ms. Wardah Abd. Rahman, Universiti Sains Malaysia).

Protocol for freeze-drying technique

1. Prepare a planchette with double-sided sticky tape "Tissue-Tek". 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 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).
2. Cut out a small piece of agar/substrate containing the bacterial/ fungal growth (about 5×5 mm) and quickly place it on the double-sided sticky tape "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 h. 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 to the 'peltier-cooled' stage of the Freeze Dryer (Emitech K750) and left to freeze-dry for about 10 h (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 is not be viewed immediately).

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. some removal of water from the sample has occurred prior to vapour fixation), structural alteration or deformation is to be expected.

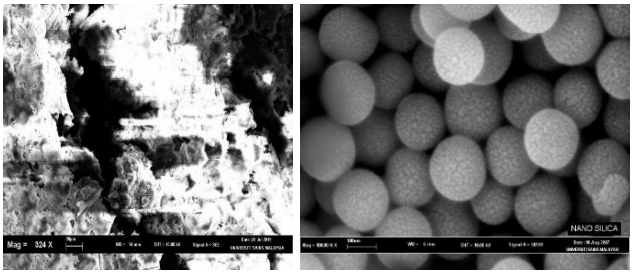
MOUNTING OF SAMPLES

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 heart-breaking 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. Insect forceps (or storkbill forceps), fine spatulas, double-sided carbon adhesives, Leit-C, conductive paint and sharpened softwood sticks are some of the tools which could come handy when performing the mounting process. Factors

that need to be considered while mounting are sample orientation, sample stability and ensuring good adherence on the SEM stub. Leit-C can aid in placing the sample in the required orientation [1,2,4].

CONDUCTIVE COATING OF SAMPLES FOR SCANNING ELECTRON MICROSCOPY

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'. 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 the ground. A sputter coater 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. 10a,b). Other materials used are iridium and carbon. In the present state of art SEMs, metal sputtering can be completely ignored with the use of low KV imaging techniques of less than 500 volts [1,2,4].



(a) **(b)**

Fig. 10. (a) Charging effect on a Wood Sample in SEM, (b) Nano Silica Powder with visible graininess from gold sputtering(Courtesy of EM Unit, Universiti Sains Malaysia).

TECHNIQUE FOR PARTICULATE SAMPLES

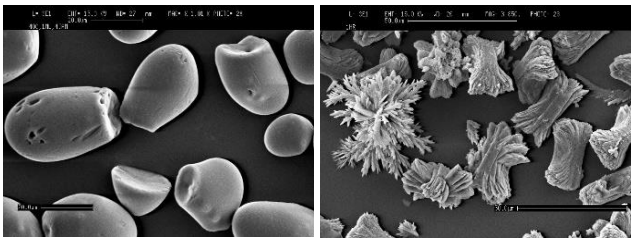
Particulate samples of dry powders, fine crystals, nanoparticles, dried bacterial cells and spores do not need any special sample preparation protocols. However, care must be taken to avoid the particles loosely 'stacking' one over the other. It should also be ensured that the particles are firmly stuck on the specimen stub. Procedure:

a) Sprinkle a little of the sample on the sample stub with the double-sided sticky tape, evenly but lightly

b) Use a hand blower 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

c) The sample is now ready for viewing in the SEM. Sputter with Au, Pt or Cr, if necessary.

The 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 (figs. 11a,b).It is possible for fine particles prepared on sticky carbon tape in this manner to be viewed employing the backscatter mode without the need for metal sputtering [1,2,4].



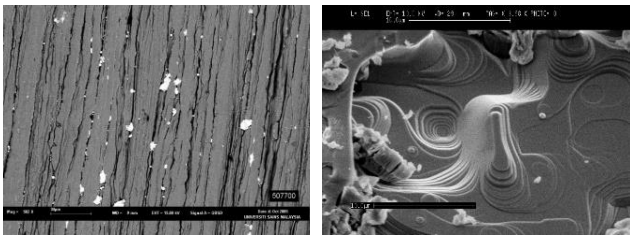
(a) **(b)**

Fig. 11. (a) Starch Powder, (b) Calcium Oxalate in dendritic forms (Courtesy of EM Unit&Zakaria Mohd. Amin, Universiti Sains Malaysia).

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.

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. 12).

Double-sided carbon sticky tapes have been found to be the most suitable for the attachment of the sample on the SEM specimen stubs, although other materials such as colloidal silver paint, carbon paints and Leit-C can be employed. Leit-C can be very useful in positioning the sample to obtain suitable orientation for imaging in the SEM.Sputtering of the sample is necessary for non-conductive samples [1,2,4].



(a) **(b)**

Fig. 12. (a) Lead particles on a copper wire, (b) Nano composite block material (Courtesy of EM Unit, Universiti Sains Malaysia).

CONCLUSION

In conclusion, sample preparations in scanning electron microscopy are crucial in producing high-quality results which will lead to new discoveries and accurate results. These technical notes will provide necessary inputs regarding the technical issues that should be considered before embarking in series of protocols starting from collection and handling of different nature of samples which including fixation process.

Three main types of scanning electron microscopy samples preparations; air drying, freeze and critical point drying methods were discussed elaborately regarding general inputs and its precautions before ending with mounting and conductive coating steps.

I Hope these technical notes will equip researchers, postgraduate students and technicians with the essential knowledge required to obtain acceptable scanning electron microscopy (SEM) images with good ultrastructural preservation.

LIST OF ABBREVIATION

- SEM Scanning electron microscopy.
- TMS Tetramethylsilane.
- HMDS Hexamethyldisilizane.
- EM Electron microscopy.
- CPD Critical point drying.

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Not applicable.

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