

Chapter 2

Chemical and Physical Fixation of Cells and Tissues: An Overview

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2.1 Introduction

In order to keep the cellular components as “lifelike” as possible, it is essential that all biochemical and proteolytic processes are inactivated and structures are immobilized and locked in space by a “fixation” step. Two approaches are normally used to “fix” biological samples: chemical and physical fixation methods. Chemical fixation is the most common approach used in specimen preservation. The tissues are immersed in a fixative that kills and stabilizes the cell contents. Physical fixation is accomplished by microwaving and cryopreserving the samples, procedures that rapidly inactivate cellular activities using microwave (MV) energy and low temperatures, respectively. Chemical and physical fixation methods have their own merits and limitations. There is no ideal chemical fixative that can fix and preserve all cellular components. One has to be aware of the limitations of the fixing agents used, processing methods, and interpret the results accordingly. While rapid immobilization of cellular content through physical fixation minimizes artifact formation, typically, only samples of smaller sizes can be adequately processed, especially for ultrastructural studies. The application of physical methods in fixing vacuolated plant cells and tissues remains of limited value when compared to animal studies [1]. The combined use of chemical and physical fixation is recommended for optimal results [2], and this can lead to further improvements in fixation and processing of biological specimens.

Since fixation is the most important step in tissue preparation, it is not surprising to see many reviews and monographs [3–12] written on this topic. For example,

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Hayat [6, 7] provides an excellent discussion on fixation for both animal and plant tissues, and Kuo [12] updated this topic in the recently published third edition of the monograph *Electron Microscopy*. Many protocols related to physical methods of fixation are described in detail. Readers are urged to consult the literature prior to embarking on a histological study that involves fixation. The purpose of this chapter is to provide an introduction to the general concept of fixation and discuss issues related to fixation of botanical specimens at the light and electron microscope levels. Additional information can be found in other chapters of this volume.

2.2 Chemical Fixation for General Histological Studies

Chemical fixation is the most common approach used in the fixation of biological specimens for light microscopy (LM) and electron microscopy (EM). Relatively large specimens can be fixed when compared to the physical methods. Chemical fixation requires a fixative which is composed of fixing agent(s) and vehicle(s) [8]. Fixing agents are traditionally classified as coagulants and non-coagulants [13], but they can also be classified as additive and nonadditive, and acidic or basic [14, 15]. Coagulant fixing agents such as ethanol, methanol, and acetone can cause protein denaturation. These fixing agents precipitate proteins by replacing water, resulting in conformation change of protein molecules and their solubility. The non-coagulant fixing agents such as aldehydes and osmium tetroxide (OsO_4) react with proteins and other components, forming intermolecular and intramolecular cross-links resulting in a better retention of the cellular organization.

A vehicle is usually a buffer solution with additional compounds such as inorganic salts added to optimize fixation of targeted organelles such as cytoskeletal elements [8, 16], if necessary. A buffer is used to maintain the desired pH regardless of the chemical nature of the fixing agent(s) selected. The consideration in the selection of a buffer is based on its ability to maintain a constant pH in the desired pH range during fixation. The buffer chosen has to be compatible with other components of the fixative and stains. For example, one would not use an amine buffer such as TRIS when fixing with an aldehyde because the two would covalently react, minimizing the desired effects of each component. The fixative also needs to have a suitable osmotic concentration compatible with cells and tissues, so that biological materials will neither swell nor shrink during fixation. Furthermore, the buffer should not be toxic to the cells or induce morphological changes during fixation.

2.2.1 Common Fixing Agents and Their Properties

A great variety of chemical compounds can be used as fixatives [8]. In recent years, the most commonly used fixatives for botanical specimens are ethanol, methanol, acetone, formaldehyde, glutaraldehyde, and osmium tetroxide (OsO_4). Their properties and use in tissue fixation are summarized below.

Ethanol and methanol are coagulant fixing agents. They denature proteins by replacing water in the tissue environment and are usually combined with other fixing agent(s) as a fixative for LM. The alcohols, especially ethanol, are also used as dehydrating solvents for both LM and EM. When comparing results on cytological smears, both ethanol and methanol give similar results, that is, good preservation of nuclear morphology [17]. Ethanol is an important component of the Farmer's fixative (ethanol:acetic acid; 3:1, v:v) for the preservation of the nucleus and cytological studies. It is important to note that lipids are not preserved by methanol and ethanol, and some lipids may be extracted during the course of fixation and later processing.

Acetone works in a similar fashion to ethanol. It denatures proteins and causes rapid precipitation of proteins and dehydration of tissues. It is also an effective lipid solvent but makes the tissue more brittle. Acetone can also be used for fixation in histochemical and immunofluorescence studies [18]. Because of its rapid action, it is possible to maintain enzyme activity using cold acetone fixation. Acetone can also be used as a dehydrating solvent for the epoxy embedding method. Since it is miscible with epoxy resins, the use of a transitional fluid, that is, propylene oxide is not needed. Acetone is a highly volatile and flammable liquid and should be handled with care.

Acetic acid is considered as a non-coagulated fixing agent; however, it causes coagulation of nuclear proteins and indirectly stabilizes and helps to prevent the loss of nucleic acids [14]. Acetic acid does not react with proteins. It is commonly used in conjunction with ethanol as a cytological fixative and aids in the preservation of nucleic acid. When it is used alone, it can cause swelling of cells. Hence, acetic acid is used to counteract the shrinkage effect of other fixing agents such as ethanol. It penetrates fast into tissue and is used as a fixing agent at the light microscope level.

Formaldehyde has a long history of use as a fixing agent [19]. The chemical properties and its reactions towards proteins and other macromolecules are numerous and complex [4, 5]. Its molecular weight is 30. The small size allows it to penetrate into the tissue quickly. The aldehyde group can react with protein nitrogen, primarily the basic amino acid lysine, to form methylene bridges. The initial binding to protein is relatively fast, but the formation of methylene bridges occurs slowly [10]. Therefore, if formaldehyde is used alone, a longer fixation time is needed for proper fixation. Otherwise, the unfixed proteins will be coagulated by the dehydrating solvent such as ethanol. The reversibility of reactions with amino acids and proteins upon washing has been a subject of discussions [4, 5], but once the methylene bridges are formed with proteins, they appear to be stable [20]. A proper understanding of the reactions of formaldehyde towards proteins and other macromolecules underlies our understanding and its use in the fixation of biological specimens. Commercial formalin solution is a saturated solution of formaldehyde (about 37% by weight) in water. In order to stabilize formaldehyde in solution and prevent self-polymerization, methanol is added as a stabilizer. Upon prolonged storage, formic acid is also formed [21]. Therefore, it is difficult to know the exact and effective concentration of the monomeric form of formaldehyde present in a formalin solution. Methanol and formic acid can have negative effects on the fixa-

tion of tissues. Hence, it is always recommended that a fresh formaldehyde solution is made from paraformaldehyde (PFA) powder and used immediately.

Glutaraldehyde was introduced by Sabatini et al. in 1963 as an especially useful fixative for ultrastructural studies [22]. This compound has two aldehyde groups separated by three methylene bridges. Glutaraldehyde is a much more efficient cross-linker for proteins than formaldehyde. Its reactions with macromolecules are believed to be irreversible [4, 5] and also inhibits enzyme activity more than formaldehyde [23]. However, the rate of penetration (0.34 mm/h) is slower than formaldehyde [23]. Similar to formaldehyde, self-polymerization occurs in solution. In order to ensure the quality of the stock solution, that is, having mainly the monomeric form, an “electron microscope grade” glutaraldehyde solution should be used. Excess or unreacted aldehyde groups can generate nonspecific staining reactions, such as the periodic acid–Schiff’s reaction. This can be remedied by pretreating sections with sodium borohydride before staining [24]. Furthermore, glutaraldehyde-fixed specimens have a high level of fluorescence that can interfere with immunofluorescence studies. A remedy is provided by Tagliaferro et al. [25] based on the Schiff-quenching procedure.

Osmium tetroxide also has a long tradition as a fixing agent for light and electron microscope studies [8]. It reacts with unsaturated lipids and results in the reduction of osmium during the cross-linking process with the formation of dark brown to black compounds. When used alone, not all proteins can be fixed and results in the loss of protein during fixation. The key drawback of this compound is that it penetrates tissue very slowly (0.25 mm/h [6]), and therefore only small pieces of tissues can be fixed. As a result, this compound is seldom used as a fixing agent at the LM level and is now mainly used as a secondary fixing agent for postfixation after aldehyde in an ultrastructural study. Since OsO_4 is electron dense, it also serves as a “stain”, imparting contrast when viewed with an electron microscope. Osmium tetroxide is usually sold as a crystalline solid in a sealed glass ampule. It is well known that OsO_4 crystals sublime into vapor from a solid state. Exceptional caution is recommended when working with this fixative because unexpected exposure to OsO_4 vapor can lead to blindness and other ailments. Therefore, OsO_4 solution should be prepared and used in a fume hood and stored in double-sealed containers.

The above fixing agents can be used alone or combined with others in a fixative. For high resolution LM and transmission electron microscopy (TEM), the common design is to combine formaldehyde with glutaraldehyde as the primary fixative. This combination was introduced by Karnovsky [26] with the advantage of the rapid penetration and stabilization of tissue by formaldehyde molecules and the stable cross-links generated by glutaraldehyde that come later. Since OsO_4 penetrates too slowly for the bulky specimen at the light microscope level, and some of the embedding media such as glycol methacrylate are not compatible with it, OsO_4 is used mainly as a secondary fixing agent for TEM only.

The Farmer’s fixative, a combination of ethanol and acetic acid, causes rapid coagulation of nuclear proteins and aids in the conservation of nucleic acid. Hence, this is a common fixative to study the cytology of cells, and more recently it has been selected as the preferred fixative for laser-capture microdissection methods (*see* Chap. 20).

Formalin–acetic acid–alcohol (FAA) is the most common botanical fixative for the paraffin-embedded method. The fixative penetrates tissue quickly, and the shrinkage effect of ethanol is counterbalanced by the swelling effect of acetic acid. The tissue is stabilized by the cross-linking property of formaldehyde. Although glutaraldehyde serves as a better cross-linking agent, it is not compatible with the paraffin-embedding medium, as the large paraffin molecules cannot penetrate into a well-cross-linked matrix of cells and tissues. Excessive shrinkage of specimens also occurs [21].

Other organic and inorganic compounds can be used as fixing agents [8, 21]. Due to the toxicity of formaldehyde, safer alternatives are preferred [27]. Glyoxal has been introduced as a “formalin substitute,” and this compound gives a pleasing effect in histology and immunohistochemistry [28]. Carbodiimides are also found to be effective as tissue fixatives and for immunohistochemical studies [4].

2.2.2 Common Buffers and Their Properties

Although many buffers can be used in conjunction with a fixing agent(s) [8, 21], for plant histology, phosphate, cacodylate, PIPES, and HEPES buffers are the most common.

Phosphate buffer is the most common buffer used for LM and EM, as it is non-toxic and has a stable buffer capacity at the physiological pH range. It is relatively inexpensive to make, and once prepared, it is stable for several weeks at 4°C. Changes in temperature have little effects on its pH. The disadvantage of a phosphate buffer is that it can form precipitates in the presence of calcium and other metallic ions, and it can be contaminated with microorganisms during storage. A new buffer solution needs to be prepared regularly.

Sodium cacodylate is a sodium salt of dimethylarsenic acid and is a highly toxic compound. This buffer is easy to prepare and can be stored for a long time without contamination, as arsenic can serve as a preservative. No precipitation occurs in the presence of low concentrations of calcium and magnesium salts. It is mainly used for EM when phosphate buffer cannot be used and for cytochemical staining protocols. The main disadvantage of this buffer is that it may cause a redistribution of cellular material along osmotic gradients, and arsenic is toxic. One needs to avoid it from mixing with acid which causes arsenic gas formation [7]; this buffer should be prepared in a fume hood.

Good’s buffers, that is, PIPES and HEPES, are excellent for physiological pHs. These Zwitterionic buffers are nontoxic to cells and are compatible with divalent cations. They appear to resist chemical extraction of cell components. Desired results have been reported at the ultrastructural level for the study of algae [16] and plant tissues [29, 30]. PIPES buffer is often used as the buffer for the fixation of cytoskeletal elements [31]. These buffers may interfere with the amine-aldehyde reaction [18]. Good’s buffers are relatively more expensive to prepare.

2.2.3 *Achieving Good Quality Chemical Fixation*

Chemical fixation prepares cells for a whole series of rather traumatic events. Ideally, the fixing agents and additives selected should alter the protoplasm structure into a stabilized elastic gel by cross-linking. It should retain the spatial relationship of all organelles in their “natural state,” stabilize the phospholipids, which form the frame work of the cell and render the chemical constituents insoluble [32]. Unfortunately, there is no ideal fixative. Not all substances and macromolecules can be “fixed” by the fixing agents. Components such as nucleic acids are indirectly stabilized through protein fixation. Furthermore, the vacuole compartments, different types of cell walls, and ergastic substances can pose challenges to a fixation protocol. Compromises have to be made in selecting a protocol that is best suited for the objective of the experiment. In order to achieve good quality fixation, it is important to consider the following variables during chemical fixation:

1. *Rate of Penetration* The size of the sample should be appropriate for the selected fixative, the embedding method, and the objective of the experiment. For example, FAA is a common fixative for botanical specimens at the LM level. Penetration of fixing agents is fast and will not overly harden the tissues. As a result, if FAA is selected as the fixative, large specimens of close to a centimeter in size can be fixed and stored for immediate or future processing. If specimens are intended to be used for TEM studies, due to the slow penetration rate of fixing agents, that is, glutaraldehyde and OsO_4 , the size of tissue blocks should be approximately 1 mm^3 or less. Other factors such as the porosity and the density of tissue need to be considered in deciding the appropriate size of a tissue to be fixed for a particular protocol.
2. *Concentration of Fixing Agents and Additives* Different cellular components react differently to different concentrations of fixing agents and additives. Depending on the objective of the experiment, one needs to choose proper concentrations of components within a fixative. Low concentrations of fixing agents require a longer time of fixation, hence some enzymatic activities can be maintained. However, this can cause extraction of cellular materials, diffusion of enzymes, and shrinkage or swelling of cells and organelles. On the other hand, high concentrations of fixing agents can destroy enzyme activities and damage cellular fine structures. Mitochondria appear to be more sensitive to an increase in buffer concentration, whereas endoplasmic reticulum (ER) is sensitive to low concentration of fixative [33]. Normally, the optimal concentration for PFA and glutaraldehyde are 2.5–4% and 1–2% for OsO_4 .
3. *Length of Fixation* The optimal time for fixation is determined empirically. If fixation time is too short, penetration of fixative and crosslinking of macromolecules will not be adequate. Longer fixation time may cause over-cross-linking, making the sample brittle. Leaching of ions and other cellular components may also occur. For TEM studies, tissues with a size of 1 mm^3 , a 4 h fixation each at room temperature or overnight at 4°C , is adequate for both the primary and

secondary fixation. For LM, since the size of the specimen is usually larger than those for TEM studies, an overnight fixation in a refrigerator is recommended.

4. *Temperature* The sample should be fixed at the same temperature at which the plants are grown or maintained. This is to avoid temperature shock and is especially important if cultured cells are studied. For LM studies, after initial fixation at room temperature, the specimens are usually transferred to a refrigerator at 4 °C for an overnight fixation. On the other hand, if the primary objective of the experiment is to stabilize proteins for immunological or enzymatic studies, a lower temperature is preferred.
5. *Tonicity* An isotonic solution will cause neither swelling nor shrinkage of cells in the solution. Isotonic solutions give an osmotic pressure equal to that of the cell cytoplasm. Hypertonic solutions cause shrinkage, while hypotonic solutions induce swelling. The tonicity can be adjusted by adding electrolytes (e.g., NaCl) or nonelectrolytes (e.g., sucrose). Most of the fixatives are slightly hypertonic [7]. SEM specimens should be fixed under or near isotonic condition, while TEM samples should be fixed using a slightly hypertonic fixative. For plant protoplast fixation, a careful adjustment of osmoticum concentration is needed in order to avoid bursting or shrinkage of protoplasts (*see* Chap. 12).
6. *Cuticle and Air Spaces* Many exposed plant surfaces are protected by a cuticle, and intercellular air spaces are common between cells. These two features alone can impede the penetration of the fixative into the specimen. Plant specimens with cuticle and large intercellular spaces typically float on the fixative solution. Since the cuticular surface is hydrophobic, the fixative will not be able to access the internal compartment of the specimen. In order to improve fixative penetration into specimens such as a leaf, the samples need to be sliced thin. The cut surfaces are exposed directly to the fixative to allow penetration. A vacuuming treatment can extract air from within the sample, allowing better penetration of the fixative. This will also improve fixation quality and aid in the subsequent dehydration and embedding processes.

The aforementioned discussion illustrates the complexity of the chemical fixation process. There are many variables that can alter the quality of fixation of a specimen. In order to ensure that the images obtained are “true to life,” the only way is to compare results with the study of living cells [13]. Comparative studies using different methods need to be done, especially by using the physical methods of fixation in order to validate the results.

2.3 Physical Methods of Fixation

Although chemical fixation is the most common approach used to immobilize biological processes in cells and tissues, the quality of fixation is sometimes not ideal or even poor, especially for those difficult-to-fix plant tissues such as pollen, embryo sac, or cells undergoing rapid structural changes, such as during the

fertilization process [34, 35]. Since the penetration rate of fixing agents is slow, active cellular process cannot be stopped instantaneously in every part of the same cell and within the tissue [36]. Large plant vacuoles often rupture prior to being fixed. The release of vacuolar content can alter the cellular organization prior to fixation of cells [36]. Physical methods, that is, cryofixation (CF) and microwave fixation (MF), overcome the deficiencies of chemical fixation; however, the procedures and instrumentation can be technically challenging.

2.3.1 Cryofixation

At the LM level, CF and sectioning of tissue are more successful with animal tissues than with plant tissues. The presence of vacuoles in plant cells make them prone to ice damage during freezing, and the inherent properties of plant cell walls also make sectioning difficult [37, 38]. However, the damage to cells by ice crystal formation during freezing provokes further studies and improvements to the methodology. The current successes in CF, especially at the EM level, result from culminated efforts by many researchers over the past years. Earlier methods related to plant tissue cryotechniques are discussed by Gahan [38].

CF arrests cell activities instantaneously at low temperatures (-180°C). Low temperature vitrifies cell water and prevents ice formation and, together with subsequent freeze substitution (FS) techniques and embedding processes, produce better quality images which can be seen at the ultrastructural level. At the LM level, samples are pretreated and protected by a cryoprotectant, frozen in liquid nitrogen, and sectioned using a cryostat and processed accordingly [38, 39].

For ultrastructural studies, four main methods have been applied to cryopreserve the plant samples: (1) plunge freezing [40], (2) spray freezing [41], (3) propane-jet freezing [42, 43], and (4) high-pressure freezing (HPF) [42–45]. A detailed theoretical discussion and methodologies can be found in the review by Gilkey and Staehelin [42]. Methods 1, 3, and 4 are common methods used for plant cells and tissues. For the plunge freezing method, samples are loaded on a grid or directly on a membrane support or other carrier and plunged rapidly into either liquid propane or helium at -180 to -190°C in a freezing system (either self-made or commercial). Well-preserved layers of plant tissue may be obtained up to a thickness of $15\text{ }\mu\text{m}$. In HPF, samples are held in a metal carrier, and a high pressure at 2100 bar is applied just before freezing. This method changes the freezing properties of water. Nucleation and crystal formation of water molecules are slowed, allowing thicker samples (up to $600\text{ }\mu\text{m}$ thick) to be well preserved without crystal damages [1]. The key advantage of HPF is that larger samples can now be fixed when compared to other freezing methods. It is important to note that specimen preparation and handling before freezing are the most important steps in order to achieve successes using the HPF technique [44].

After initial freezing, a FS procedure follows. FS is the process in which an organic solvent with or without fixing agents (such as OsO_4 , uranyl acetate, or glutaraldehyde) is used to replace the vitrified water within cells at a temperature

of about -80 to -90°C . If a fixing agent is included in the substitution fluid, the cells will be fixed once the temperature is raised during further processing. Once fixed, an embedding medium such as London Resin White (LR White), LR Gold, or Lowicryl HM20 resin can infiltrate into the tissue at low temperature (0 – 20°C for LR White/Gold and -20 to -40°C for HM20), or the specimen is allowed to warm slowly to room temperature followed by infiltration of a selected embedding resin. The added advantage of this method is that chemical fixation is not necessary if immunostaining is desired. CF alone maintains antigenicity of cells allowing for improved immunostaining of the specimen [45]. For additional information on CF and recent protocols, see [44, 46–49] and Chap. 7.

With the potential benefit of the CF technique over the conventional chemical fixation methods, new approaches and modifications to current techniques continue to appear in the literature. In a recent study, McDonald and Webb [50] published a protocol on FS that can be completed in 3 h or less after HPF using a variety of biological samples including leaves of *Nicotiana benthamiana*. On the other hand, the FS procedure is not necessary if cryo-ultramicrotoming is followed by observation using a cryo-electron microscope [51]. The advantage of “cryo-electron microscopy of vitreous section” (CEMOVS) is that there is no chemical treatment and/or fixation needed and this “preserves the sample close to the native state” [51]. Several protocols related to cryo-EM can be found in [12].

2.3.2 Microwave Fixation

Although CF takes only milliseconds to immobilize the tissue, the subsequent processing time can be long, up to several days. The technique of MF was introduced by Mayers [52] in 1970, and a review on early research and methods of MF is summarized by Login and Dvorak [53]. With improved instrument design, MF has become more popular in recent years [54]. MW irradiation accelerates fixation and processing of biological materials [53]. MW energy causes excited molecules to rotate, generating a quick and homogeneous heat within a sample, thus greatly speeding up chemical reactions during fixation and subsequent processing steps. It also improves the accessibility of fixatives and reagents to cells and maintains antigenicity of cell components [52, 55, 56]. Since MW generates heat for fixation, if it is not well controlled, it may damage cells and tissues. The new MW systems, for example, Pelco BioWave Pro (Tel Pella, Inc.), have the capability of maintaining a uniform environment with a ColdSpot, an enclosed bed of circulated water, which continuously absorbs all generated MW energy. The inclusion of a vacuum chamber in the MW cavity further enhances penetration of fixatives and other solvents. This feature is especially beneficial to the processing of botanical specimens. The vacuum–MW combination enhances the diffusion of fixative and improves the quality of the cell morphology [57]. With standardized equipment and techniques, MF not only shortens the fixation and processing time but also produces comparable results to conventional processing methods. Methods for MW-assisted processing and embedding for TEM are now well established [58]. The procedures are less demanding

when compared to CF protocols. Hence, MF and processing are becoming routine procedures in many histology laboratories.

Reports on the use of MF in processing plant samples are few compared to animal studies. Schichnes et al. [59] documented a MW paraffin technique for botanical specimens and another procedure for in situ localization of KNOTTED messenger ribonucleic acid (mRNA) [60]. The samples were microwaved with FAA as the fixative. Other studies focused on structural and antigenic preservation of samples. For example, Zellnig et al. [61] recently established a MW-assisted plant sample preparation protocol for rapid immunohistochemical diagnosis of tobacco mosaic virus.

2.4 Comments on Practical Issues of Fixation

2.4.1 *Fixatives and Fixation of Botanical Specimens at the Light Microscope Level*

The most important considerations in any successful fixation protocol are specimen handling, choice of fixative, and fixation conditions. As indicated previously, plant cells have cell walls, intercellular spaces, large vacuoles, and some have cuticular substances on their surface. Therefore, the methods used in sample collection and fixation vary depending on the tissue of interest. For good quality fixation, it is important to collect and fix the tissue immediately and at the same time. Therefore, prior to fixation, all necessary equipment, tools and fixative need to be prepared.

The following suggestions on tissue handling will help to ensure the quality of morphological preservation: (1) One should avoid physical damages to the specimen during dissecting; the tissues should be dissected or excised using sharp knives or double-edged razor blades. (2) At the time of dissection, the specimen needs to be trimmed carefully with the final image and the correct plane of section in mind in order that the specimen can be embedded easily. (3) Tissues should be immersed in the fixative or buffer at all times during the excision, if possible. The cut surfaces should be in contact with the fixative as soon as possible. (4) The final size of specimens for fixation depends on the choice of fixative, the embedding medium, and the objective of the experiment. Retrimming of tissue blocks after dehydration or prior to embedding is not recommended, as the tissues will be very brittle and it is difficult to make precise cuts. (5) For those tissues covered by cuticular materials, the specimens have to be cut open to allow for the penetration of the fixative. If necessary, for LM, a small drop of detergent (e.g., Tween 20) can be added to the fixative to disrupt the surface tension of the cuticle. (6) All botanical specimens should be subjected to a vacuum treatment to ensure that air is removed from intercellular spaces. It is always advisable to vacuum the sample for 5–15 min to extract air from the tissues in order to improve on fixative penetration and subsequent infiltration of the embedding medium. We also routinely vacuum the samples at the first 100 %

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