Chapter 3
Paraffin and Polyester Waxes

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3.1 Introduction

Infiltration of an embedding medium into a tissue provides proper support of cells and tissues for the subsequent sectioning procedure. Paraffin wax is likely the most common embedding medium used to date. Klebs introduced this embedding medium in 1869 [1]. Initially, wax was applied around the tissue and subsequent infiltration of wax into the tissue greatly enhanced the quality of sections [1]. Over the years, the paraffin embedding method has been improved and become a routine technique for different applications. When properly executed, good-quality images can be obtained. For example, our knowledge of the phloem tissue was based initially on the excellent images produced by Professor Katherine Esau using the paraffin embedding technique. Many embedding media were introduced in the past century for different applications. Steedman [2] developed a formulation based on polyester wax which augments the conventional paraffin embedding method. The purpose of this chapter is to provide basic information on paraffin and polyester waxes and associated protocols that enable successes in using them as embedding media at the light microscopy level. Additional details, especially on the paraffin embedding method can be found in the literature [3–9].

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3.1.1 Paraffin Waxes

Paraffin wax is a long chain hydrocarbon with a sizable molecular weight. If cells and tissues with a dense cytoplasm are "over-fixed" with fixatives containing glutaraldehyde, wax infiltration can be poor and the tissue becomes difficult to section. In order to improve sectioning, different manufacturers modified the paraffin media by mixing different types of waxes, adding plasticizers and penetration-enhancing compounds such as dimethyl sulfoxide. This results in waxes having different melting points, hardness, and sectioning quality allowing investigators to choose the appropriate mix for their own experiments. The most desirable feature of paraffin wax is that the wax blocks are easy to section, forming ribbons readily. Serial sections allow for a detailed examination of a specimen and three-dimensional (3D) reconstruction is possible. Furthermore, when compared with other embedding methods, the overall cost is relatively low. Once the system is set up, this can be a routine procedure for any laboratory.

There are a number of drawbacks with the paraffin embedding method. Waxes are hydrophobic compounds, with a relatively large molecular size; hence, proper infiltration into a tissue requires the use of a transitional fluid. The transitional fluid must be miscible and, better yet, must have a relatively good solubility of wax so that it can infiltrate into the specimens gradually. Unfortunately, many transitional fluids such as toluene and xylene are flammable and toxic. The use of xylene can result in further extraction of lipidic substances from cells and may weaken cellular structure. Another key disadvantage of the paraffin wax is that in order to keep it in a molten state, the specimens are subjected to a high temperature (about 60 °C) for a relatively long period of time. This results in shrinkage in the size of the specimen.

3.1.2 Polyester Waxes

Because of the high melting point of wax and potential shrinkage of specimen, Steedman in 1957 [2] introduced polyester wax (polyethylene glycol 400 distearate) as an alternative to paraffin wax. Polyester waxes are fatty acid esters of polyethylene glycol and have different chemical properties from paraffin waxes. The advantage of polyester wax is that the melting point is about 40 °C and specimen shrinkage is less than that occurring in the paraffin embedding method. In addition, a special transitional fluid is not needed as the wax is soluble in ethanol. The use of polyester wax allows for the study of heat-labile macromolecules so cytoskeletal elements and proteins can be evaluated through immunostaining methods. The main drawback of this embedding method is that one has to take extra care in sectioning and mounting of sections on slides in order to obtain consistent results. Since the publication by Steedman, many protocols have been developed to study different aspects of plant cell biology [10–13], especially the cytoskeletal elements.

3.2 Technical Comments

In this section, the basic techniques of wax embedding are discussed and additional hints are provided in the Methods and the Notes sections of this chapter. Readers are urged to consult other chapters in this volume for addition information.

3.2.1 Fixation

The objective of each specific experiment determines which fixative is to be used and the size and orientation of the specimens to be fixed. It is important to collect and fix the samples at the same time (*see* Chap. 2 for additional information). Before excising tissues, ensure that all the necessary tools and fixatives are available, and the specimen containers are properly labeled. If immediate fixation of samples is not possible, the tissue should be stored in ice and fixed immediately upon arrival in the laboratory.

The traditional formalin–acetic acid–alcohol (FAA) formulation works well for a majority of botanical specimens and it is the most common general fixative used today. This fixative penetrates quickly, even with large tissue samples, and the material will not severely harden over time. In fact, for long-term storage of botanical specimens, FAA can serve as both fixative and storage fluid. Subsequent processing does not require extensive washing. The main drawback of FAA is that plant cells tend to be plasmolyzed with the combination of fixing agents in this fixative. One can minimize plasmolysis by using a buffered formalin-based fixative and, if necessary, a very low percentage of glutaraldehyde can be added to harden the tissue further. For specimen with a dense cytoplasm, it is important to note that glutaraldehyde is not compatible with the paraffin embedding medium. The large paraffin molecules cannot penetrate into a well cross-linked matrix of cells and tissues, and this can result in excessive shrinkage of specimens [14].

Many other fixatives for paraffin embedding are available in the literature. The most notable alternative to FAA is CRAF (chromium salt, alcohol, and formalin), a fixative which preserves the cytology of cells even better than FAA [4]. The most common formulation is CRAF III [4, 8]. The stock solutions are mixed just before use, and the tissues require a longer fixation time as solutions penetrate into the tissue slower than FAA. After fixation, the tissues need to be washed carefully before further processing. For immunological studies, 4 % paraformaldehyde in a buffer is adequate. Additional additives can be added to the fixative to optimize the fixation of specific organelles and tissues (*see* Chap. 2). One needs to evaluate carefully the merit of each fixative before use.

Depending on the objective of the experiment, to minimize extraction of macromolecules, the fixation and subsequent dehydration steps can be performed at 4 °C before subjecting them to a higher temperature during infiltration of the embedding

medium. The duration of fixation depends on the fixative used and on the size of the specimen, ranging from a few hours to several days. For a prolonged fixation, it is advisable to keep the specimens at 4 °C to minimize extraction of substances from cells. In order to achieve effective fixation, a minimum ratio of 1:10 specimen to fixative volume should be maintained.

After fixation and before dehydration, it is advisable to remove the fixative by rinsing with an appropriate washing solution. For FAA, 50 % ethanol rinse is sufficient. If a metal ion-based formula is used, it is best to rinse with several changes of water before dehydration. If a buffer is used as a component of a fixative, the samples should be rinsed with the same buffer several times before dehydration. Improper removal of fixing agents may interfere with subsequent staining procedures.

3.2.2 Removal of Air from Botanical Specimens

In plants, intercellular air spaces are often present between cells. It is essential that air is removed from the specimen in order to facilitate the penetration of a fixative, solvents, and the embedding medium. This is best done by a dedicated vacuum system such as a vacuum oven or a desiccator connected to a vacuum pump with a sufficient suction to remove air from the tissues. A vacuum oven is preferred as the temperature can be regulated during the vacuuming step, especially when removing air/solvent from samples in molten wax at 40 °C for polyester wax and 60 °C for paraffin wax. From a safety standpoint, it is important to use a vacuum chamber that can withstand the force delivered by the vacuum pump. Otherwise dangerous shattering of the chamber can occur. Furthermore, if possible the entire system should be housed in a fume hood as the vacuum pump will remove volatile components from a fixative. Aldehyde and solvent fumes should not be inhaled. If a vacuum system is not available, one can generate a vacuum system using a laboratory water aspirator.

In general, it is best to allow the samples to be fixed for about an hour at room temperature, stabilizing the tissue before performing the first vacuum step. Depending on the tissues, some specimens remain afloat near the surface of the fixative. This is often due to the air present within the tissue and/or the hydrophobic cuticular surfaces. A few microliter of Tween 20 can be added to the fixative to reduce the surface tension and aid in air extraction of the sample, if necessary. Air is gently removed from the tissues during fixation. It is important to control the rate of vacuuming in order to avoid rapid extraction of air from within the tissue as this may result in the collapsing of cells. Once air bubbles cease to appear, vacuuming can be terminated. It is also advisable to replace fixative with fresh solution as some fixing agents would have evaporated under vacuum. Depending on the size of the tissue, the tissue is allowed to fix for an additional period of time usually about 24 h prior to dehydration.

To ensure complete removal of air from the samples, we routinely perform the vacuuming step two additional times, i.e., at the 100 % step of ethanol dehydration, and when the samples are in the pure molten embedding wax. Ethanol has a low density, trapped air within the samples, if any, can be easily extracted, ensuring the complete removal of air from the sample. The final vacuuming step ensures the removal of transitional fluid from the tissues. It is important to adjust the temperature of the vacuum oven so that the embedding medium remains at the molten stage during vacuuming.

3.2.3 Dehydration

Dehydration of samples is necessary as cellular water is not compatible with wax. Although different solvents such as acetone and methyl cellosolve can be used as dehydrating agents, alcohol is the most commonly used agent in histology. If FAA containing 50 % ethanol is used as a fixative, one can start the dehydration process at 50 % ethanol. If an aqueous fixative is used, after a brief water or buffer wash, dehydration commences at 30 % ethanol. Dehydration should be gradual, especially for vacuolated plant cells. A rapid dehydration can cause rapid diffusion resulting in plasmolysis and collapsing of cells. The duration of each dehydration step ranges from 30 min to overnight depending on the size of the fixed tissue. A rotary mixer can be used to facilitate fixation and the dehydration processes. If necessary, in order to maintain specimens at 4 °C, the rotary mixer can be housed inside a refrigerator during the entire course of fixation and dehydration.

One can take advantage of the dehydration steps to pre-stain the tissues. As tissues are being dehydrated, the pigments such as chlorophyll will be extracted and render the specimens colorless. This makes them difficult to locate within a wax block. By pre-staining the specimens at the 100 % step of dehydration, the specimens can be easily seen during embedding and subsequent sectioning. Pre-staining can be achieved by adding a 0.05 % of safranin or acid fuchsin stain to 100 % ethanol. Other stains can be used as long as they are not easily removed and do not interfere with the actual staining process.

3.2.4 Transitional Fluids and Wax Infiltration

A transitional fluid is necessary as it enables gradual infiltration of embedding medium into the tissue. For polyester wax, which is soluble in ethanol, ethanol can be used as a transitional fluid. A gradual increase in the concentration of polyester wax will eventually replace ethanol within the tissue. Ethanol is not a solvent for paraffin wax and it has to be replaced by a transitional fluid before infiltration of paraffin wax. A variety of transitional fluids can be used. For botanical specimens, tertiary butyl alcohol (TBA), xylene, and xylene substitutes such as Histoclear® are most common. Other transitional agents such as toluene and chloroform are also

suitable. Xylene is a good solvent for paraffin wax; hence, it is the best transitional fluid. It has a high refractive index and renders the specimen "clear." However, it is toxic and the liquid is dense, requiring a few more exchanges of pure wax in order to completely remove it from tissues. Because of the toxicity of xylene, xylene substitutes, such as Histoclear®, can be used. These are by-products of the citrus juice industry and appear to be safer alternatives. However, these compounds have a fixed shelf life and will deteriorate over time. Hence, for practical purpose, xylene is a more cost-effective product to use. Toluene is also a suitable transitional fluid, but because of its lower boiling point and flammability, from a safety standpoint, few investigators use this compound as a transitional fluid. Tertiary butyl alcohol is the most commonly used transitional fluid for botanical specimens. Wax is only slightly soluble in TBA at 60 °C but it is miscible with TBA. Although the infiltration of wax may not be as "smooth" as xylene, TBA is a less toxic solvent to use in a laboratory and is easily exchanged and removed from the tissues by evaporation. The main drawback is that TBA is a solid at room temperature. It needs to be kept at 28 °C to maintain it in a liquid state.

3.2.5 Polyester and Paraffin Wax

Polyester wax is composed of polyethylene glycol distearate and cetyl alcohol (1-hexadecanol) in a 99:1 ratio (w:w) [2]. To reduce variability between batches of wax and to improve sectioning properties, more 1-hexadecanol can be added to the mixture [7]. Brown and Lemmon [15] provide a detailed protocol in making the Steedman's wax with a 9:1 ratio. At present, the original formulation of polyester wax can still be purchased from Electron Microscopy Sciences, Hatfield, Pennsylvania. Other polyester wax formulations have to be "homemade." For homemade polyester wax, only a small amount should be prepared at one time and kept at room temperature. A sufficient amount of polyester wax is melted per experiment, as prolonged storage in an oven appears to alter the quality of polyester wax [2].

Different paraffin waxes are available commercially. The commercial paraffin is clean, ready to use, and if necessary, can be filtered in an oven at the melting point of wax before use. A dedicated oven for paraffin embedding should be used and the temperature fixed at 2–3 °C above the melting point of the selected wax. All wax containers and sample vials should be placed on trays to collect spills as waxes are a fire hazard. In order to prevent rapid cooling of wax during embedding as the oven door will be opened and closed a number of times, the oven temperature can be increased by another 2–3 °C during the embedding process.

3.2.6 Embedding

Embedding is a process in which the tissue is surrounded with a medium to support it during sectioning. The most important step during the embedding process

is arranging the specimens in such a way that the desired orientation can easily be obtained during sectioning. Disposable embedding molds of various sizes are available commercially. One can also prepare paper boats or use simple aluminum dishes for embedding as long as solidified wax blocks can be removed easily. Paper boats are easy to prepare, inexpensive, and different sizes can be made to suite one's need [3, 7].

Once removed from the oven, wax begins to solidify at room temperature, especially for paraffin wax. One needs to work fast in order to arrange all the specimens properly in a desirable orientation before solidification of wax. If a paper boat is used, practice by embedding a few specimens at first; with improving skills, more specimens can be embedded at one time.

Overall, polyester wax solidifies slower than paraffin wax. Once embedding is complete, the wax molds are allowed to solidify at room temperature before storing them either at room temperature or in a box with a desiccant in a refrigerator.

3.2.7 Mounting of Wax Blocks

Depending on the types of embedding molds used, the wax block can be removed from the mold and mounted directly onto a suitable mounting chuck. If a paper boat method is used with a number of tissue pieces in it, the tissues need to be separated by cutting using a hot knife/spatula. The temperature of the knife minimizes the forces needed to cut the wax block, preventing uncontrolled "cracking" of the block. Traditionally, simple wood cubes can be prepared to suit a particular microtome for mounting purposes. The newly made wood cubes need to be "cured" by submerging it in molten wax for a few days before use in order to create a better bonding between it and the specimen. In this chapter, a plastic mounting ring method is illustrated in the Methods section.

3.2.8 Microtomy

Before embarking on sectioning, it is imperative that one has a good understanding and working knowledge of the microtome to be used. Read the manufacturer's instructions for proper maintenance and care of a microtome. If an older type rotary microtome such as the AO Spencer microtome is used, one needs to oil the recommended parts regularly for a smooth operation. For wax sectioning, a conventional rotary microtome is adequate for routine work. Microtomes with retraction during the return stroke are currently available. The retraction function prevents the wax block from touching the back of the knife during a return stroke, enhancing ribbon formation. Unfortunately, the cost of a retraction-type microtome is double that of a conventional one. It is important to keep the working area clean. If the paraffin sections are intended for RNA in situ hybridization studies, extra procedures are needed to prepare the work area to avoid RNase contamination.

The knife is the soul of a microtome. Without good, sharp knives it is simply not possible to obtain quality sections. Different types of microtome knives and knife sharpeners are available commercially. With proper care and regular re-sharpening, a microtome knife can last a long time. It is important to note that knives with different profiles have different cutting properties. The wedge (C-type) or universal knife is the most common type as it can section a variety of embedding media [5, 8]. A proper selection of knives is needed in order to obtain the best results. The alternative to re-sharpening of knives is to purchase disposable knife holders and disposable blades. There are low- and high-profile blades with different sizes, thicknesses, and coatings. The low-profile blades are narrower and thinner and are designed to cut soft materials. The high-profile blades are wider and thicker and are designed for harder tissues. For plant materials, the high-profile knives are preferred.

There are a number problems associated with paraffin wax sectioning. The most common problem is the generation of static electricity during sectioning, especially in a dry environment. This results in a ribbon of sections sticking to different parts of the microtome or upon itself. An increase in the humidity around the microtome by a vaporizer, or placing the wax block and the knife assembly in the fridge for a short time before sectioning to condense moisture on the block face and the knife will minimize the generation of static electricity. Polyester wax, on the other hand, will not generate static electric charges during sectioning.

The correct setting of the knife angle is key to successful sectioning. If one notices a buildup of wax at the back of the knife and the block face appears crushed, this is due to improper adjustment of the knife angle. A knife angle has to be just large enough so that the block face only touches the knife-edge as it sections. The angle should not be too large that it "scrapes" the surface of the block instead of making a sharp clean cut. The knife angle setting is usually between 5 and 10°. The smaller the block face, the smaller the knife angle. One needs to find the best angle for the type of wax, tissue, and the knife used. If a microtome with a retractable return stroke is used, this problem is minimized.

An improper cutting speed can cause wrinkling of ribbons. Sectioning too fast can cause compression of wax sections as the block passes across the knife. If this occurs, one needs to reduce the speed of sectioning. Highly compressed sections are difficult to expand evenly during section mounting.

If one notices that sectioning is not consistent, having skipped or missing sections or the sections are of uneven thickness, be sure to check all clamps and fittings. The aforementioned problems are most likely due to loose parts or the specimen block is not tightly secured. The knife may be dull as well.

In order to economize the space on a slide for research purpose, it is desirable to place as many sections on a slide as possible. This can be achieved only if the ribbon is straight. Furthermore, it is useful to trim away excess wax and unnecessary tissue prior to sectioning to maximize the number of sections that can be placed on a slide. In order to obtain a straight ribbon during sectioning, the top and bottom edges of the block have to be parallel to the knife-edge.

Lines on sections are due to scratches caused by a damaged knife. The knife can be damaged due to the presence of ergastic substances such as oxalate crystals from

within the tissue. If this is the case, move the knife to a new spot and continue sectioning. Scratches can also appear due to dirt particles adhering to the knife-edge. In this case, one can try to clean the knife by using a Kimwipe® with some xylene and wipe upward towards the knife-edge. This way, one can clean the knife without damaging its edge. Again, if scratches persist, the blade needs to be moved to a new spot. Care should be taken when moving the blade as the edge is easily damaged.

As one begins to section, it is often difficult to generate a ribbon. The sections tend to curl up and may fall off from the knife-edge. When this happens, increase the initial cutting speed; make a few quick turns of the microtome handle; and use a wet brush to hold down the sections. This will avoid initial curling of the sections and generate a ribbon. Try not to break the ribbon during sectioning as it may be difficult to reform a ribbon easily.

Polyester wax sectioning requires care. In order that polyester wax sections can expand and adhere to a slide quickly and properly during mounting, one needs to avoid unnecessary compression of sections during sectioning. Adjust the cutting speed to ensure that the sections are smooth with minimal compressions. Reduce the block face by trimming away unnecessary wax and tissues. A smaller block face makes a ribbon easier to obtain.

3.2.9 Flattening of Sections on Slides

Proper stretching of sections is essential for specimen examination. For paraffin sections, this is carried out using a warm water bath, or float sections directly on slides with water on a slide warmer or hot plate. We prefer the latter method as the sections are on the slide and do not require to be "fished out" from a water bath. The purpose of stretching is to remove wrinkles (compressions) generated during sectioning. If wrinkles are not removed, the cells will not appear flat and will be out of focus when viewed. Temperature is key to the stretching of ribbon. The temperature of water needs to be about 45 °C and clean distilled water should be used. If stretching is carried out using the slide method, one must ensure that the ribbon(s) are floating on water and do not touch the dry part of the slide as the section will melt and adhere to the slide and not be able to stretch. Once all wrinkles disappear, which can take a few minutes, the water is drained and the slides are placed on another slide warmer at about 30 °C to dry. Drying at high temperature can result in bubble formation due to vaporization of residual moisture underneath the sections and melting of wax.

Stretching of polyester wax sections requires extra care. This wax expands rapidly when in contact with water. With its low melting point, sections can be stretched at room temperature. Depending on the size of the ribbon, a small amount of water is placed on the slide. As the ribbon is placed over it, it will begin to expand rapidly. Once the compression is gone, the water is quickly removed by draining it away from the sections; otherwise, the section can start to disintegrate. It is important to practice the stretching of the ribbon and prepare one slide at a time.

The quality of microscope slides differs depending on the manufacturer. If possible, use Swiss glass slides, since they are perfectly clear; however, the price is higher than for borosilicate glass slides. Slides with different dimensions and coatings are available for different types of microscopic work from different manufacturers such as Fisher HealthCare (www.fishersci.com). For general histological studies and if the slides are not subjected to extensive washing and high-temperature treatments, we find that good quality precleaned slides can be used as such, without the need of prewashing and coating with a binding agent. Otherwise, the slides should be cleaned followed by coating with an adhesive, such as gelatin–chrome alum [17] or silane [8]. Formulae for subbing solutions are given in the next section. For the ease of operation, slides with a frosted end are preferred as one can label the slide with pencil instead of etching the information using a diamond pen.

3.2.10 Staining

After fixation, embedding, and sectioning, structural details within cells and tissues are difficult to be resolved. In order to examine the structural organization, sections need to be stained. The purpose of staining is simply to increase the visibility of biological specimens. There are numerous staining recipes for paraffin sections available in the literature. For detailed discussion and protocols, readers are urged to consult Jensen [3], Berlyn and Miksche [4], Ruzin [8], and Yeung and Sexena [16].

3.2.11 Safety

There are a number of safety concerns with the wax embedding method. First and foremost, the chemical reagents and solvents used such as aldehydes and xylene are hazardous and toxic. Be sure to understand the chemical properties of the reagents used by reading the Material Safety Data Sheets before using them. Avoid contact by wearing gloves and prepare fixatives and solutions of xylene inside a fume hood if possible. Be sure to dispose the fixative, xylene waste, and xylene–wax mixture in proper waste containers in a fume hood according to the protocols at one's institution. It is important to note that some fixatives contain heavy metal ions such as mercuric and chromium ions. Care is needed in handling the fixatives and proper disposal is required.

The microtome blades are extremely sharp and their edges damage easily. One needs to exercise care when handling microtome blades. The blade needs to be removed from the holder when not in use.

Be sure to clean the work area used for wax sectioning. A vacuum cleaner is best to remove small pieces of wax or wax ribbons. Waste wax can result in a slippery floor and can be dangerous to walk on.

3.3 Materials

3.3.1 Equipment

Rotary microtome, vacuum oven or a vacuum desiccator, vacuum pump, tissue rotator or rotary mixer (PELCO® R2 Rotator #1050 with rotator head PELCO® #1051 for 24 mL vials and PELCO® #1054 for 4 mL vials), a dedicated refrigerator for histology use only, laboratory ovens for waxes, and slide warmers.

3.3.2 Laboratory Supplies

Glass vials, pipettes, razor blades, forceps, needle and scalpel, brushes, Peel-A-Way® Disposable Histology Molds (different sizes), embedding ring, wood blocks for mounting wax tissue blocks, metal tray, Bunsen burner, slide boxes, plain glass microscope slides (Fisherbrand® #12–550 A3), slides with adherent surface: Superfrost® Plus Microscope Slides (Fisherbrand® 12-555-15), and coverslips.

3.3.3 Embedding Waxes

Paraplast® Plus Tissue Embedding Medium (Fisher Scientific #23-021-400), premade polyester wax (Electron Microscopy Sciences #19312), components for making polyester wax: polyethylene glycol distearate (Sigma #9005-08-7) and cetyl alcohol (Sigma #68824).

3.3.4 Chemical Reagents

Formalin, paraformaldehyde (Sigma #30525-89-4), absolute (100%) and 95% ethanol, formalin (Sigma-Aldrich #F15587), glacial acetic acid, tert-butyl alcohol (TBA) (Sigma-Aldrich #471712), xylene, safranin stain, gelatin, chromium potassium sulfate, aminopropyltriethoxysilane.

3.3.5 Solutions for Wax and Polyester Wax Preparation

1. Formalin–acetic acid–alcohol (FAA): Prepare 400 mL of FAA fixative in a beaker by adding 200 mL of 95% ethanol to 140 mL of water. Bring the beaker into the fume hood and add 40 mL of 37% formaldehyde solution and 20 mL of

glacial acetic acid. Mix, transfer the contents to a glass bottle with a secure cap. Store the fixative in the fridge.
2. A 4% paraformaldehyde solution: Prepare 500 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4. Warm 400 mL of water to 60 °C on a hot plate and add 20 g of paraformaldehyde *(see Note 1),* a few drops of 1 N sodium hydroxide. Stir to dissolve. Bring the volume to 500 mL with water and filter before use.
3. PBS buffer (NaCl, KCl, Na_2HPO_4, KH_2PO_4): Prepare 1 × PBS by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of water. Adjust the pH to 7.2 with HCl and bring the volume to 1 L with water.
4. Polyester wax preparation: Prepare 500 g of polyester wax by melting 480 g of polyethylene glycol distearate overnight in an oven set at 40 °C. In another oven melt 20 g of cetyl alcohol at 60 °C; once fully melted combine the two together, mix well, and pour into a plastic or aluminum tray. Once solidified, the wax can be stored at room temperature.
5. TBA dehydration solutions: $\mathbf{D_1}$ (50 mL H_2O, 40 mL 95% ethanol, 10 mL TBA), $\mathbf{D_2}$ (30 mL H_2O, 50 mL 95% ethanol, 20 mL TBA), $\mathbf{D_3}$ (15 mL H_2O, 50 mL 95% ethanol, 35 mL TBA), $\mathbf{D_4}$ (45 mL 95% ethanol, 55 mL TBA), $\mathbf{D_5}$ (25 mL 100% ethanol, 75% TBA), $\mathbf{D_6}$ (100% TBA), $\mathbf{D_7}$ (100% TBA). Safranin (0.05%) can be added to D5 to pre-stain the tissue.
6. Ethanol series: 30, 50, 70, 85, 95, and 100% ethanol.
7. Chrome alum slide subbing solution [17]: Dissolve 0.5% gelatin in distilled water at 35 °C and add 0.05% chromium potassium sulfate. First clean slides by soaking in acidic 70% ethanol overnight, then wash in deionized water followed by distilled water. Dip individual slides into the subbing solution, dry vertically.
8. Silane subbing solution [8]: Prepare a 1% silane in 100% EtOH and a 10% acetic acid solution. Mix silane and acetic acid in a ratio of 1:3 (v:v). Dip clean, dry slides into the subbing solution and air dry. Rinse the slides in 70% EtOH and allow them to dry. The slides are ready to use.

3.4 Methods

3.4.1 Paraffin Wax Embedding Method

3.4.1.1 Fixation

1. Before fixing the material, aliquot about 15 mL of FAA fixative into glass vials. Cap the vials and store on ice.
2. Using a scalpel (or a razor blade) and forceps, cut the tissue (a few mm) and place it immediately in the fixative *(see Note 2)*. For hard material, such as seeds, it might be necessary to scarify the surface and/or make several incisions to allow the penetration of the fixative.

3. Place the vials with loosen caps in a small tray or box filled with ice. Transfer the tray/box to a vacuum chamber and gently vacuum (about 24" Hg) the samples for 15–20 min *(see Note 3)*. Gradual evacuation of air will cause air bubbles to form and a majority of tissues will sink to the bottom of the vial. If this does not happen, re-vacuum for another 15 min. Low-density tissue, or tissue covered in water-repellent cuticle such as leaves, may remain floating and alternative infiltration methods might be necessary *(see Note 4)*.
4. Release the vacuum and replace the fixative with fresh solution, tighten the caps, and transfer the vials to a rotary mixer with gentle rotation overnight at 4 °C.

3.4.1.2 Dehydration

1. With a pipette gently remove the fixative from the glass vial, rinse several times with 50 % ethanol, and add 15 mL of solution $\mathbf{D_1}$ *(see Note 5)*. The volume of the solution must be in excess to cover the samples (10:1; liquid:tissue). Incubate on the rotary mixer overnight at 4 °C.
2. Repeat the procedure with the remaining solutions $\mathbf{D_{2–7}}$, the time of dehydration per step ranges from 30 min to overnight for each solution *(see Note 6)*.

3.4.1.3 Infiltration of Paraffin Wax

1. Fill a beaker (500 mL) with pellets of Paraplast® Plus tissue embedding medium. Melt the wax in an oven, set at a temperature of 56–60 °C *(see Note 7)*. As it might take a long time to melt such a large volume of wax, it is recommended to start the procedure in advance, during the last steps of dehydration.
2. Remove the vials from the rotary mixer and discard excess TBA from the vials but ensure that sufficient TBA is left to cover the tissue. Fill the vials with molten wax.The volume of wax should be in excess of the TBA and tissue. At this time, the wax will solidify.
3. Cap the vials tightly and place them into an oven just about 2 °C above the melting point of wax. Incubate in the oven for 12 h to overnight. The tissues will sink to the bottom of the vials.
4. Remove the vials from the wax oven, quickly decant the TBA–wax mixture into a waste container in a fume hood. Refill the vials with fresh molten wax and return them to the oven, without cap.
5. Leave the uncapped vial in the oven overnight to allow the residual TBA to evaporate.
6. Repeat the changes with melted wax (Step 6) twice.
7. To ensure no residual TBA and air are present within the samples, a vacuum step can be performed with the temperature of the vacuum oven set at about 4 °C above the wax melting temperature. If there is no visible sign of air bubbles, the vials can be returned to the wax oven, ready for embedding.

3.4.1.4 Embedding of Large Samples

1. Pre-warm the Peel-A-Way® Disposable Histology Molds and a spatula in the 56–60 °C oven or use home-made paper boats *(see Note 8)*.
2. Gently swirl the glass vial and quickly pour its content, i.e., the samples in molten wax, into the molds (Fig. 3.1a, *see Note 9*). If the volume of wax is too large, pour some of the excess wax in the waste container. Using forceps and spatula place the samples at the bottom of the mold. If possible, separate the samples 4–5 mm apart and away from the edge of the mold. This procedure has to be done quickly as the wax will tend to harden *(see Note 10)*.
3. Once the samples are arranged at the bottom of the molds in the desired position/orientation, place the molds directly into a tray containing water and ice for about 30 min, ensuring the blocks are quickly solidified. Once fully solidified, the molds can be stored in a plastic bag in the fridge. Be sure to label the molds.

3.4.1.5 Embedding of Small Samples

For large samples, embedding and orientation of samples can be done rapidly with practice. However, the procedure described in Sect. 4.1.4 may be difficult to perform for small size samples, such as embryos, which are difficult to orient in a short period of time. The following steps are recommended as it allows more time for embedding.

1. Transfer one vial with tissues, a pre-warmed mold, spatula, and forceps from the oven to a hot plate (or slide warmer) which was previously set at 60 °C.
2. Carefully swirl the vial and pour some samples into the mold, so that only one-third of the mold is filled.
3. Use the pre-warmed spatula and/or forceps (which can be rewarmed using a Bunsen burner or an alcohol burner) to orient the samples at the bottom of the mold depending on the desired plane of sectioning. A magnifying lens can be used at this stage to help with the orientation *(see Note 11)*.
4. Once the samples are in place, transfer the partially full mold onto a flat cold surface and let the bottom layer of wax solidify for a few seconds. Gently fill the whole block with more molten wax.
5. Place the mold on a tray containing water and ice for about 30 min to allow the remaining wax to solidify completely. Store the molds in the fridge until further use.
6. Repeat Steps 2–5 with the remaining vials.

3.4.1.6 Mounting of Paraffin Blocks

1. A properly trimmed wax block needs to be mounted onto a microtome chuck in order that it can be sectioned. We make use of the plastic embedding ring as our embedding chuck by filling their cavity with melted wax (this needs to be done

at least a day in advance). Adhesive tape can be used to seal one end of the rings while wax is being poured. Once solidified, the wax in the embedding ring will provide a flat support for the wax block (Fig. 3.1b).
2. Using an alcohol burner or Bunsen burner, heat a spatula which will be used to reduce the height of the wax block and flatten its surface opposite to the samples by melting away excess wax. This procedure is preferably done in a fume hood to avoid inhaling smoke from melting wax and carried out on a sheet of aluminum foil for easy clean up. If samples are embedded in a large paper boat, the wax block will need to cut into smaller piece using a hot spatula.
3. Remove the plastic rings with wax (Fig. 3.1c). Warm both sides of the spatula and place one side on top of the wax support of the embedding ring. Quickly place the sample-containing block of wax on the other side (Fig. 3.1d). As soon as the wax on either side starts melting, slide the spatula away and allow the two surfaces to melt together by gently pressing the top of the wax block. Release the pressure after a few seconds. Reheat the spatula and melt the edges together one more time ensuring the block is secured to the plastic microtome chuck.
4. Store the blocks in the fridge to ensure all the wax solidifies. Depending on the procedure, the blocks can be stored in the refrigerator or at room temperature for several months.

3.4.1.7 Sectioning and Flattening of Sections

1. Using a single-edged razor blade trim the block to the appropriate size and ensure that the top and bottom edges are parallel to one another and the trims are clean. This ensures the formation of a "straight" ribbon. The surface of the trimmed block can be a trapezoid (Fig. 3.1e).
2. Insert the plastic ring in the holder of a rotary microtome. Depending on the size of the block face, the cutting angle should be set at 5–10 while the section thickness can be set between 5 and 10 μM. Generally speaking, the smaller the block face, the smaller the angle, and the thinner the sections that can be obtained.
3. Set the slide warmer at 37 °C, label the slides *(see Note 12)*, and place them on the warmer. With a pipette gently add water onto each slide.
4. During sectioning the first sections can be discarded if they do not contain any sample. With practice, a ribbon of sections will form (Fig. 3.1f). A small wet brush is used to stick and hold the ribbon at one end, gently lift it up from the knife without detaching it from the knife-edge. Continue to section. Once the desired length is reached, using another wet brush gently lift the entire ribbon and place it on a black colored cardboard (Fig. 3.1g) with the dull side of the ribbon facing up. The other side of the ribbon is shiny and smooth as it is the side cut by the knife. Using a sharp single-edge razor blade divide the ribbon into segments of about two-thirds the length of a slide.
5. Using a wet brush gently apply each ribbon segment to a slide with water on the slide warmer. Depending on the size of the ribbon, if there is space for a second row, an additional ribbon can be placed next to it *(see Note 13)*. Be sure to place

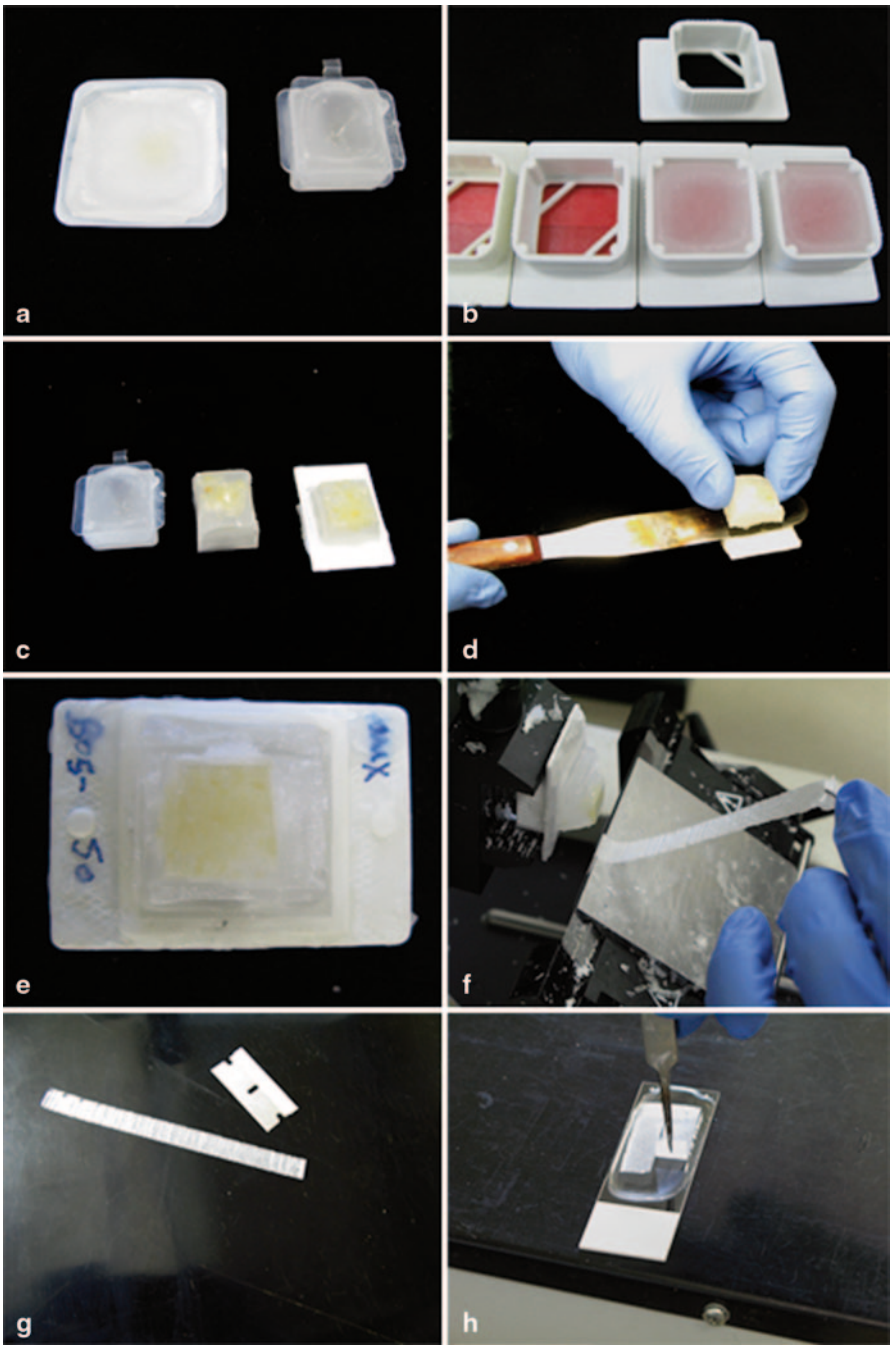

Fig. 3.1 **a** Embedding and orientation of the samples can be achieved using either weighing boats (*left*) or Peel-A-Way® Disposable Histology Molds (*right*). Melted wax containing the samples is first poured in the respective molds, and the samples are then placed at the bottom of the molds

the shiny side down onto the slide as it is smooth and will adhere to the slide surface better when dry. When applying the ribbon to the slide, start by placing one end of the segment on the edge of the slide and then rapidly, with a lateral movement, drop the whole ribbon segment which will float on the water on top of the slide (Fig. 3.1h). The ribbon will expand on the warm water removing the compression marks generated during sectioning.

6. Place the remaining ribbon segments on the consecutive slides. Each segment will expand in water. Be sure that the ribbon is free floating. If necessary, facilitate its expansion by adding additional water at the edge of the segment.
7. Once the ribbon segments are fully expanded (it will take a few minutes) drain the water off from each slide on a paper towel with the help of a small brush. Do not allow the slide to dry on a "hot" slide warmer, as this will cause bubble formation and/or melting of wax, destroying the specimen.
8. Once the excess water has been removed, place the slides onto another slide warmer set at 30 °C overnight. Just before drying, if necessary, the sections can be examined using a microscope. Once fully dry, the slides can be stored in a slide box and used in further procedures.

3.4.2 Polyester Wax Embedding Method

Many steps for the utilization of paraffin wax are also common to polyester wax. The section below outlines differences in the procedure unique to polyester wax.

3.4.2.1 Fixation

Although FAA and a combination of paraformaldehyde and glutaraldehyde are commonly used in general histological procedures, a paraformaldehyde solution is often used in immunofluorescence studies because of its moderated cross-linking characteristics and reduced tissue autofluorescence.

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and oriented as desired. The molds are then placed on a solid surface to allow the wax to solidify. **b** To provide support for the samples, plastic embedding rings are prepared by filling their cavity with melted wax after one end of the ring is sealed with tape. **c** After removing the plastic of the Peel-A-Way® Disposable Histology Molds (*left*), the exposed wax block (*center*) can be mounted on the embedding ring (*right*). **d** Mounting the wax block onto the embedding ring can be achieved by warming a spatula which is then placed between the wax support of the ring and the block of wax. As soon as the wax on both sides of the spatula starts to melt, the spatula is gently retracted and the two surfaces then melt together. **e** Formation of a "straight" ribbon is ensured by trimming the surface of the block as a trapezoid using a single-edged razor blade. **f** Using a retraction microtome, ribbons of wax containing sections can be easily obtained. **g** The ribbon is placed on a flat surface and segments of about two-thirds the length of the microscope slides can be produced using a single-edge razor blade. **h** The ribbon segments are laid onto water on a microscope slide on a slide warmer (50 °C). The ribbons will soon expand removing the compression marks generated during sectioning

1. Before fixing the material, aliquot about 15 mL of the fixative in glass vials. Cap the vials and store in ice.
2. Continue with Sect. 3.4.1.1 Steps 2–4 of fixation for paraffin wax.

3.4.2.2 Dehydration

1. Remove fixative from vials, for aqueous fixative such as 4% paraformaldehyde solution, the tissues are washed with an appropriate buffer such as PBS several times and dehydrated using an ethanol series commencing with 30% ethanol. The volume of the solution must be in excess to cover the samples. Incubate on the rotary mixer for 1 h to several hours at 4 °C, depending on the size of the sample.
2. Repeat the procedure to 100% ethanol. A vacuum step can be performed between the two 100% ethanol changes.

3.4.2.3 Infiltration of Polyester Wax

1. Fill a plastic beaker (500 mL) with solidified polyester wax. Melt the wax in an oven set at 40 °C. As it might take a long time to melt such a large volume of wax, it is recommended to start the procedure in advance, during the last steps of dehydration.
2. Remove the vials from the rotator, decant about one-fourth of the 100% ethanol and replace it with a similar volume of melted polyester wax. Cap the vial and place it in the 40 °C oven for 2 h. Manually agitate the vials occasionally during all infiltration steps.
3. With a pipette gently remove half of the wax/ethanol solution and add an equal volume of melted wax into the vial. Remove the cap and leave the uncapped vial in the oven overnight; this will allow the ethanol to evaporate.
4. Decant all the wax/ethanol solution into a waste beaker and add melted wax into the vial. Leave the uncapped vial in the oven overnight.
5. Repeat the changes with melted wax (Step 4) twice. A third vacuuming step can be performed in a vacuum oven before embedding.

3.4.2.4 Embedding

The steps for preparing polyester wax blocks are the same as those listed in the preparation of paraffin wax blocks. However, the temperature of the oven and hot plate/slide warmer should be set at 40 °C. Special care must also be exercised during the preparation of the blocks (Fig. 3.1d). Due to the low melting point of the polyether wax it is important not to overheat the spatula. If the room temperature of the working environment is warm, wood blocks are preferred.

3.4.2.5 Sectioning and Flattening of Sections

1. Prepare the wax block for sectioning as in Sect. 3.4.1.7.
2. The low melting point of the polyester wax can pose some challenges during sectioning. We found that low temperature helps the process. Cool the blocks, the microtome knife holder, and the knife in the fridge for at least 1 h before sectioning.
3. Insert the embedding ring or wood block in the holder of a rotary microtome. The cutting angle should be set at 5–10° while the section thickness should be of 7–10 μm.
4. Work at room temperature. Label the slides and place them on the bench. With a pipette gently add water onto each slide.
5. Prior to sectioning, remove excess wax, leave about 1 mm of wax around the specimen. Section at a slower speed compared with paraffin sectioning to reduce compression of sections.
6. Ribbon handling is similar to that described in Sect. 3.4.1.7 except that a dry brush or forceps with a wide tip are needed.
7. Place the ribbon on a dark cardboard (Fig. 3.1g), and using a sharp razor blade divide it into segments of about half the length of the slide.
8. There are different techniques for expanding the ribbon on the slide. One can place a ribbon segment on water as described for the paraffin sections. The polyester wax sections will expand quickly. Once the compressions (wrinkles) disappear from the section, the water has to be removed to retain the integrity of the sections. Another method is to place sections on a dry slide, tip the slide slightly, and run some drops of water underneath the sections. As the water passes through, the sections will expand. Once the desired stage is reached, the water is completely drained. This method allows a more controlled expansion of the ribbon.
9. Allow the slides to air dry at room temperature in a dust-free area for at least a day before staining. For routine staining, clean plain slides can be used. For immunostaining, coated slides such as the Superfrost Plus slides are preferred.

3.5 Notes

1. When preparing this buffer caution must be exercised in handling the paraformaldehyde powder. The whole preparation, including the weighing of paraformaldehyde, should be carried out in a fume hood using a hot plate to dissolve the paraformaldehyde powder.
2. As the purpose of fixation is to immobilize proteins and cellular components in order to retain the structural integrity of the tissue it is imperative to perform this step rapidly. Depending on the desired sections and regions of interest, additional trimming might be required. A flat surface would allow the tissue to lie at the bottom of the mold and facilitate its positioning during embedding.

3. In the absence of vacuum chamber/oven, the vacuum infiltration step can be performed using a vacuum desiccator or a vacuum aspirator connected to a water tap.
4. A small syringe (10 mL) can be used as an alternative for difficult-to-vacuum samples. After removing the piston, add the samples and 4 mL of fixative by covering the outlet with a gloved finger. Reinsert the piston and invert the syringe with the outlet facing up. Gently remove the air by pressing the piston. When all the air is removed, re-cover the outlet with the gloved finger and pull the piston in order to create a vacuum. Shake the syringe vigorously to remove the forming air bubbles from the tissue. Continue this procedure for 5–10 min.
5. For small samples, such as cultured cells, a nylon mesh can be used at the tip of the pipette to prevent the sample from being sucked into the pipette while changing solutions.
6. The length of the dehydration steps can be shortened to a few hours for small (a few millimeters) and soft samples. The incubation time should not be shortened for larger samples or samples enriched in cuticle and/or hard substances. Additional cuts along the edges of these samples can facilitate the penetration of the solutions.
7. The temperature should not exceed 60–62 °C as elevated temperatures alter the properties of the wax.
8. A large variety of Peel-A-Way® Disposable Histology Molds is commercially available. One important characteristic is the bottom area of the mold supporting the sample. Tapered molds with smaller bottom areas (*see* the Materials section) are recommended for small samples or if only a few samples are used.
9. This step must be performed quickly as the wax will solidify rapidly. If dealing with many vials, working with a few of them at one time and closing the oven door in between might facilitate the process.
10. If a large number of samples are available, weighing boats can be used instead of Peel-A-Way® Disposable Histology Molds. This is particularly useful for embedding cells generated from suspension cultures. The large wax blocks obtained with weighing boats will need to be cut into smaller blocks to fit the embedding rings.
11. Orienting the sample in the block is a crucial step of the whole procedure. Generally, longitudinal sections are easier to obtain, as the sample can be placed flat at the bottom of the mold. Cross-sections, on the other hand, require a more tedious handling of the tissue, especially for elongated samples such as roots. The vertical orientation of the sample can be achieved in a layer of wax which is only partially solidified and which can provide support. The use of a hotplate can facilitate the process.
12. Depending on the procedure, different types of slides are available. For general histology Economy Plain Glass Microscope Slides (Fisherbrand® #12–550 A3) can be used. Slides, which electrostatically "charged" glass without adhesive and/or protein coating (Superfrost® Plus Microscope Slides, Fisherbrand® 12-555-15), are also available for specific procedures such as immunological analyses.

13. It is easier to trim the wax block so that the width of the ribbon is half the width of the slide. This will ensure that two ribbon segments can be placed in one slide (Fig. 3.1h).

3.6 Concluding Remarks

Both waxes are useful embedding media for light microscopy. Results can be obtained quickly and the overall cost of operation is relatively low compared with other embedding methods. Hence for basic anatomical studies, the wax embedding methods are the methods of choice. The added advantage for polyester wax is that because of its low melting point, immunological procedures can be carried out. The fact that both waxes are relatively soft, thin sections of 2 μm or less cannot be cut, thus reducing the resolution power of the images. For high-resolution studies, it is best to use acrylate or epoxy embedding methods. In general, it is advisable to study the gross anatomical organization of the plant body using the wax embedding methods before proceeding to the plastic embedding procedures.

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