Chapter 4
The Glycol Methacrylate Embedding Resins—Technovit 7100 and 8100

Edward C. Yeung and Colin K. W. Chan

4.1 Introduction

Methacrylate resin was first introduced as an embedding medium for electron microscopy by Newman et al. [1]. Due to the instability and fragility of the polymerized resin under an electron beam during viewing, methacrylates were subsequently replaced by much more stable epoxy resins. Although not suitable for use in electron microscopy, methacrylates have many desirable properties that make them excellent embedding media suitable for light microscopic examinations. The rigidity of the methacryate polymer permits thinner sections to be made, allowing for greater resolution at the light microscope level, especially when compared to conventional paraffin sections (compare Fig. 4.1a and b). This feature led to the introduction of the water miscible resin 2-hydroxyethyl methacrylate or glycol methacrylate (GMA) as embedment for light microscopy [2, 3]. Feder and O'Brien [4] first drew attention to the use of GMA as an embedding medium for botanical specimens. Since its introduction, many of the initial problems associated with this embedding method, that is, having a highly exothermic polymerization temperature and difficulty in making serial sections, have now been resolved [5]. In conjunction with the use of a microtome with a retraction return stroke and Ralph type glass knives or disposable steel knives, serial sections with large block face can easily be obtained [5, 6]. With proper equipment and techniques, the GMA embedding method can be a routine procedure in any histology laboratory.

E. C. Yeung (✉) · C. K. W. Chan
Department of Biological Sciences, University of Calgary, 2500 University Drive NW., Calgary, Alberta T2N 1N4, Canada
e-mail: yeung@ucalgary.ca

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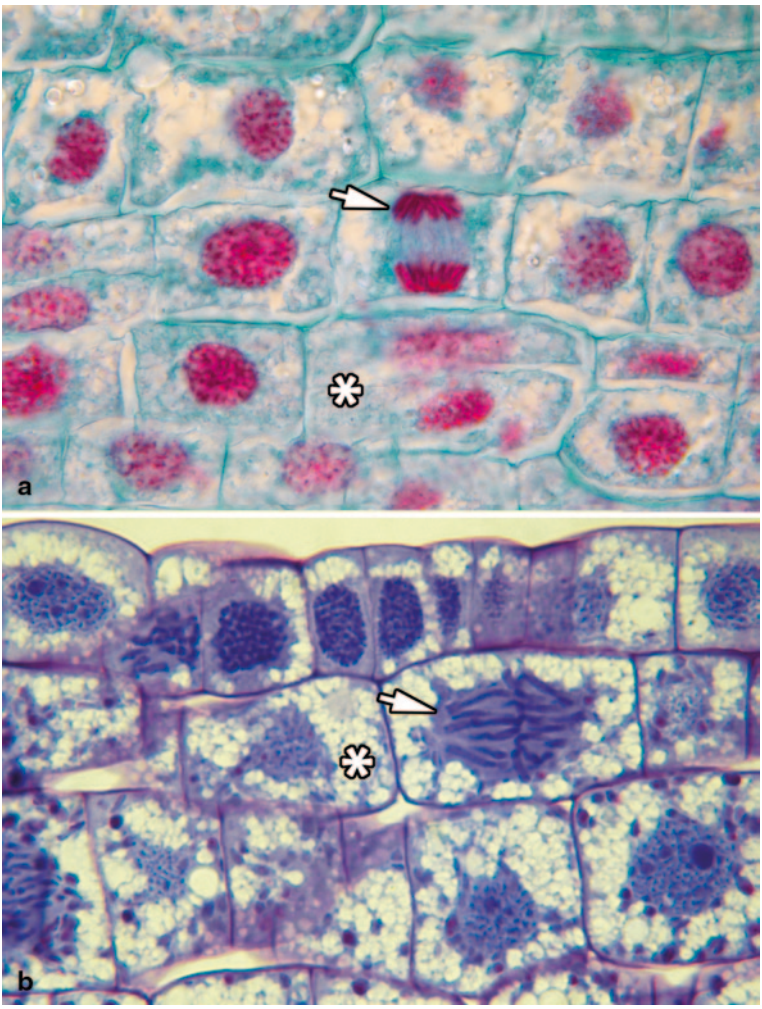

Fig. 4.1 Cotyledon sections of Radiata pine. **a** Paraffin-embedded section showing poor cytoplasmic details, that is, vacuoles (*) and nuclear features *(arrow)* are not distinct. **b** GMA-embedded section showing clear vacuolar (*) and nuclear features *(arrow)*. Scale bars = 20 μm

In this chapter, the embedding protocol of two popular GMA embedding media, that is, Technovit 7100 (T7100) and Technovit 8100 (T8100) will be detailed. To provide a complete description of the protocol, besides detailing the embedding process, we will begin by discussing the fixation step and also demonstrate the sectioning techniques using different combinations of microtomes and knives. For a more detailed discussion on recent advances on the GMA embedding methods in the study of plant histology, *see* Yeung and Chan [5].

4.1.1 T7100 Embedding Medium

This embedding medium is designed primarily for histological studies and serial sectioning of specimens. T7100 is manufactured by Kulzer and Company from Germany, based on a formulation published by Gerritis and Smid [7]. The T7100 embedding kit is supplied in three components: 500 mL Basic resin, Hardener 1 (5 × 1 g package), and 40 mL Hardener II. The infiltration solution is prepared by dissolving one package of Hardener I into 100 mL Basic resin and it is used to infiltrate the tissues. At the time of embedding, the embedding solution is prepared by adding Hardener II to the infiltration solution in a ratio of 1:15. The embedding solution is prepared just before use. Once mixed, polymerization begins and the mixture will solidify within 2 h at room temperature. Compared with earlier formulations [5, 8], the key feature of the T7100 embedding medium is that it is composed of new, less toxic initiator–accelerator chemicals. This new system contained in Hardeners I and II, allows polymerization to be initiated at a lower temperature and with a reduction in the temperature generated during the polymerization process, thus reducing potential thermal damage to the specimens.

The inclusion of a plasticizer, polyethylene glycol 400 contributes to improve serial sectioning quality of the polymerized blocks [7, 8]. The ability to produce long chains of serial sections is the most desirable feature of T7100. Serial sections enable the researcher to search for specific cells or structures within a specimen and at the same time, the structural relationship among cells and tissues can also be studied, for example, *see* [9]. The information obtained also provides insight into one's experimental system. Another added advantage of this formulation is that large blocks of tissues can be sectioned. Molding cups with a size of 13 × 19 mm can readily be sectioned [5] allowing for a large area of a specimen to be studied.

4.1.2 T8100 Embedding Medium

Soon after the introduction of T7100, the Kulzer and Company introduced another methacrylate formulation specific for immunohistochemical (IHC) studies, that is, T8100. Although GMA is the main component in T8100, a small amount of ethylene glycol dimethacrylate is added as a cross-linker [8]. Furthermore the accelerator, *N,N,3,5*-tetramethylaniline is less toxic and more suitable for enzyme and IHC studies, when compared to the chloride ion/barbituric acid system of T7100 [10]. With the new initiator–accelerator system, the embedding medium can polymerize at a lower temperature (4 °C), allowing for better preservation of enzymatic activities and antigenicity of cells and tissues. For botanical studies, T8100 is a popular embedding medium for IHC studies, for example, *see* [11]. In contrast to T7100, serial sections are more difficult to obtain. Fortunately, for IHC studies, long ribbons of serial sections are usually not required. Similar to T7100, large blocks of

tissue can be sectioned. Hence, if ultrastructural IHC studies are not contemplated, T8100 is the plastic of choice, instead of the LR White resin (*see* Chap. 6), as one can visualize a large area of tissues for the distribution of antigens.

4.2 Practical Considerations for the Handling of Botanical Specimens in GMA Embedding

Similar to any other embedding methods, before embarking on a procedure, it is imperative that one has a sound theoretical understanding of the entire procedure, beginning with fixation. Readers are urged to consult published texts [12–16] and other relevant chapters in this book for additional information and references. Several key steps are essential to the success of GMA embedding and sectioning and are discussed below. It also needs to be emphasized that components of the embedding media are toxic and cause skin irritations [8]. Proper precaution is needed in the handling of chemicals and embedding media.

4.2.1 Vacuuming of Samples at Different Stages of Processing

Air is often present in intercellular spaces of botanical specimens and needs to be removed. If air is not extracted from the sample, it will impede the penetration of fixative, dehydrating solvents and the Technovit resins. Furthermore, oxygen inhibits the generation of free radicals, resulting in poor polymerization. Thus, air has to be extracted using a vacuum system at the time of fixation and at the end of the dehydration step. It is also advisable to vacuum the samples prior to embedding in order to ensure that the dehydrating solvent no longer remains within the tissue (*see* Chap. 3 and [5] for additional details), ensuring proper polymerization of the resins.

4.2.2 Tissues Need to be Thin in One Dimension for Proper Polymerization

The ability to section relatively large specimens is one of the desirable features of Technovit resins. However to be successful, the specimens need to be kept at a thickness between 1–3 mm for proper polymerization. Polymerization of plastic blocks is initiated with the addition of "Hardener II," an accelerator solution containing free radical producing agents. The generation of free radicals and the polymerization process have been discussed by Hand [16]. The initiator/accelerator system initiates the polymerization of GMA into linear chains [8]. The polymerization process will be hindered by lignified secondary walls of sclerenchyma and

xylem tissues resulting in blocks that cannot be sectioned. Thick, soft tissue slices or pieces (3–4 mm), such as embryos and tissue culture explants can be processed readily. However, woody tissues should be sliced thin, ensuring successes in sectioning. It is possible to process large tissue blocks by modifying the embedding process. In a study of rat skull–brain specimens, Quester et al. [17] allowed initial polymerization to take place at −20 °C. The slow polymerization enables the polymerization of a large block of tissue with a size of 4 × 2 × 2 cm. A similar approach should be tested on botanical specimens.

4.2.3 Rinsing of Specimens in Embedding Solution

A proper ratio of initiator–accelerator is essential to the polymerization process as these chemicals control the rate of polymerization and the final hardness of the block. To ensure that the embedding solution is not diluted during the final embedding steps, rinse the infiltrated samples in the embedding solution once, before embedding and the placement of the specimen adapter (for details, *see* Sect. 4.4.1.4). However, the manipulation needs to be done quickly and carefully. Polymerization begins the moment the embedding solution is prepared. For beginners, do not polymerize too many tissue pieces at one time. One can also slow the polymerization process by using cold infiltration solution in preparing the embedding solution.

4.2.4 Addition of Extra PEG 400 to Soften the Polymerized Blocks

Although polymerized blocks are hard, they can be readily sectioned using Ralph knives on a microtome with a retraction return stroke [5]. If disposable steel knives are used, it is advisable to soften the blocks slightly by adding a small amount of polyethylene glycol (PEG) to the embedding solution [5, 18]. Steel knives may appear hard, but they are not as sharp as Ralph knives or triangular glass knives. The addition of a small amount of PEG 400 to the embedding solution renders the blocks more amiable to sectioning using a disposable steel knife. With practice, it is possible to obtain sections or short ribbons of sections using a conventional rotary microtome [5].

4.2.5 Staining of GMA Sections

One of the advantages of GMA sections is that the plastic is clear and it does not need to be removed prior to staining [8]. Many histological and histochemical staining protocols used for paraffin sections can readily be applied to GMA sections.

This is most likely due to the fact that GMA is hydrophilic and it is nonreactive towards ionogenic groups within the tissues [8]. The background plastic does not need to be removed, thus greatly reducing the amount of organic waste when compared to the staining of paraffin sections. In this chapter, staining protocols of GMA sections have not been included as they are readily available in the literature [4, 12, 13, 19–21]. Some staining recipes can be found in Chaps. 9 and 24 and a sample of staining outcomes can be found in [5].

4.3 Materials

4.3.1 Laboratory Equipment and Embedding Supplies

1. Microtomes with or without retraction return stroke (different suppliers; *see Note 1)*
2. Ralph knife makers *(see Note 2)*
3. Vacuum chamber/oven and vacuum pump *(see Note 3)*
4. Disposable knife holder and blades—Electron Microscopy Sciences (EMS, www.emsdissum.com), standard microtome blade holder (#63050), and disposable steel blades (#63061-01)
5. Histoknife glass strips (different suppliers such as EMS)
6. Molding cup trays *(see Note 4)*
7. Specimen adapter for round "HistoMold" from Leica Biosystems (part number: 14702218310; *see Note 5)*
8. Rotary mixer (Pelco R1 Single Speed Rotator)
9. General laboratory supplies such as dissecting tools, razor blades, beakers, flasks, measuring cylinder, forceps, disposable pipettes, slides, and coverslips

4.3.2 Embedding Kits and Chemicals

1. T7100 and T8100 embedding kits (Kulzer and company, and also from different suppliers such as EMS and VWR Canada)
2. Polyethylene glycol 400 (Sigma-Aldrich 202398)
3. 25 % glutaraldehyde solution, EM grade (EMS, cat. no. 16400)
4. Paraformaldehyde (Sigma-Aldrich P6148)
5. Gelatin
6. Chromium potassium sulphate
7. Common laboratory chemicals such as hydrochloric acid, sodium hydroxide, acetone, and ethanol

4.3.3 Preparation of Embedding Medium, Fixatives, and Solutions

4.3.3.1 Preparation of Infiltration and Embedding Media

The T7100 kit consists of three components: 500 mL Basic resin, Hardener 1 (5 × 1 g package), and 40 mL Hardener II. Prepare the infiltration solution by dissolving one package of Hardener I into 100 mL Basic resin. If a larger volume is needed, all five packages can be added to the bottle of the Basic resin, stir to dissolve, and keep at 4 °C *(see Note 6)*. Unused solution can be stored in a freezer (−20 °C); this will prolong the "life" of the solution. To avoid condensation, allow it to rewarm to room temperature before use. Prepare an appropriate volume of embedding solution at the time of embedding. Add Hardener II to the infiltration solution in a ratio of 1:15. Be sure to mix the solutions well by stirring or shaking.

A modified T7100 Embedding medium suitable for sectioning using disposable steel knives is prepared by adding 0.6 mL of polyethylene glycol 400 (PEG 400) to 15 mL of embedding medium just prior to embedding [17].

The T8100 kit consists of 500 mL Basic resin, Hardener I (5 × 0.6 g packages), and 30 mL Hardener II. Prepare the infiltration solution by dissolving one package of Hardener I in 100 mL Basic resin. Just prior to embedding, prepare an appropriate volume of embedding solution by adding Hardener II to the infiltration solution in a ratio of 0.5:15. Be sure to mix the solution well before use.

4.3.3.2 Preparation of General Fixatives

Prepare a general fixative consisting of 1.6% paraformaldehyde and 2.5% glutaraldehyde in a 0.05 M phosphate buffer at pH 6.9. A 16% stock solution of paraformaldehyde is prepared by adding an appropriate weight of paraformaldehyde powder into a beaker containing distilled water at 60 °C in which a few drops of 1 N KOH have been added *(see Note 7)*. Stir to dissolve. After about 5 min, the solution should be clear with a few undissolved particles. Adjust the final volume with water to make a 16% stock solution. Filter the solution before use. To prepare 100 mL of fixative, mix 50 mL of 0.1 M phosphate buffer, pH 6.9 with 10 mL each 16% paraformaldehyde and 25% glutaraldehyde stock solutions *(see Note 8)* and 30 mL of distilled water. For IHC studies, prepare a fixative containing 4% paraformaldehyde in a buffer with appropriate additives.

4.3.3.3 Preparing Subbed Slides

Clean slides from the suppliers can be used directly without the need of cleaning *(see Note 9)*. If the "pre-cleaned" slides look dusty and feel greasy, these slides can be cleaned by soaking overnight in a 70% EtOH solution containing 0.5% of 1 N

HCl, followed by thorough washing with distilled water. The cleaned slides are then taken directly (without drying) from distilled water, dipped in an adhesive solution, and placed vertically in a dust-free area to dry. Prepare the adhesive solution by dissolving 5.0 g gelatin in 1 L of warm distilled water and adding 0.5 g chrome alum (chromium potassium sulfate). After the solution has cooled, filter the solution through Whatman no. 1 filter paper. Preferably, this solution should be used at once, but may be stored at 4 °C for up to 48 h [22].

4.4 Methods

4.4.1 General Fixation and Processing

4.4.1.1 Collection and Fixation of Plant Tissues

Carefully excise and trim selected plant parts to the desired orientation with a sharp double edge razor blade *(see Note 10)*. The size of the specimens should not be larger than the size of the molding cup used for embedding. For woody specimens, the samples should not exceed a thickness of 1 mm. Leaf specimens also need to be sliced thin *(see Note 11)* and seed coat of mature seeds should be removed for better infiltration of solution *(see Note 12)*. It is best to excise and trim the tissue in a pool of fixative. However, due to the toxicity of aldehyde fumes, the tissue can be excised and trimmed in its own medium or buffer and quickly transferred into vials containing the fixative. Label the vials properly *(see Note 13)*.

Different fixatives can be used depending on the objective of the experiments (for fixative selection, *see* Chap. 2). For general histological studies, a combination of 1.6% paraformaldehyde, 2.5% glutaraldehyde, in phosphate buffer, pH 6.9 is suitable. For IHC studies, a freshly prepared 4% buffered paraformaldehyde solution is commonly used. Fix the tissues at room temperature for 1–2 h prior to a vacuuming step. After vacuuming, replace the fixative and transfer the vials to a refrigerator. Depending on the size of the specimen, the total fixation time ranges from 12 to 48 h. Over-fixation can render the tissues hard and can be difficult to section. All subsequent steps are preferably carried out at 4 °C in a rotary mixer.

4.4.1.2 Dehydration

After fixation, wash the specimens 3× with a buffer and dehydrate with an ethanol series. If T8100 is used as the embedding medium, samples are dehydrated in an acetone series. The duration of dehydration depends on the size of the specimen. Each step can range from 30 min to several hours. Dehydration should take place at 4 °C to minimize extraction of macromolecules from cells. To ensure that there is

no more air present within the specimen, vacuum the specimens one more time after the first change of 100% EtOH or acetone *(see Note 14)*.

4.4.1.3 Infiltration of Samples

Infiltrate the tissues gradually with a mixture of 100% EtOH/acetone and infiltration solution in a ratio of 2:1, 1:1, and 1:2 before transferring to the pure infiltration solution. Like dehydration, the duration of infiltration depends on the size and the density of the specimens. For embryos with a large amount of storage products and woody tissues, a longer infiltration time is necessary. In general a 24-h period is sufficient in each of the intermediate solutions. A rotary mixer can be used to facilitate the infiltration process. A final vacuuming step should be performed once the specimens are in the pure infiltration solution prior to embedding. Examine the specimen with a stereomicroscope to ensure no air bubbles or silvery reflections are present before embedding [5]. A well-infiltrated specimen is somewhat translucent. If silvery reflections are present, the specimens need to be trimmed to a smaller size and subject to additional vacuuming steps prior to embedding.

4.4.1.4 Embedding

1. Select a molding tray with an appropriate cup size (Fig. 4.2a). Routinely, we use a molding tray with cup size of 6 × 12 × 5 mm. Fill the well of each cup halfway with infiltration solution. Then, transfer selected specimens from the Petri dish into a cup mold (Fig. 4.2b). The tissues should not be exposed to air in order to avoid trapping of air bubbles. Since Hardener II has not been added, this step can be done slowly. If more than one piece of tissue is included in the same cup, it is important that they do not overlap with one another. Once all suitable specimens have been transferred, the entire molding cup tray can be vacuumed briefly one more time, ensuring no air bubbles are trapped during the transfer process.
2. Once the tissues are in place, prepare the embedding solution. This solution should be used immediately because polymerization begins as soon as the Hardener is added. Remove the infiltration solution quickly using a disposable pipette. Rinse the sample once with the embedding solution, then refill each cup with the new embedding solution (Fig. 4.2c). Proper orientation and positioning of the specimen is essential in the final analyses *(see Note 15)*. Therefore, quickly reexamine the specimen under a stereomicroscope to check their orientation. Reorient the samples if necessary. Finally place a plastic round specimen holder on top of each molding cup (Fig. 4.2d). Apply sufficient embedding solution to fill to the rim of the cup (Fig. 4.2e). This ensures that the medium can polymerize around the specimen holder securely *(see Note 16)*.

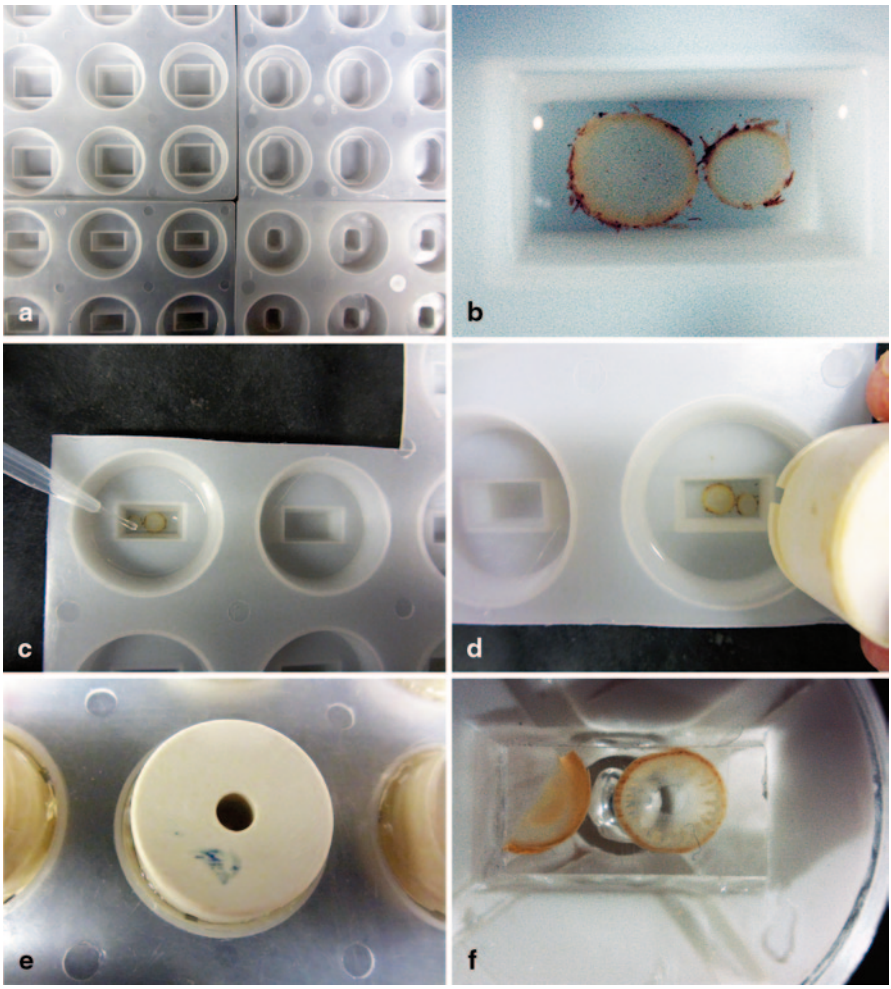

Fig. 4.2 Embedding procedures. **a** Different sizes of embedding mold. **b** Tissue pieces are transferred into a molding cup with infiltration solution. **c** Once the tissues are properly arranged, the infiltration solution is removed using a disposable pipette and the specimens are rinsed once with the embedding solution before refilling with embedding solution. **d** Apply a round specimen holder to the molding cup. **e** Ensure that the embedding solution is filled to the rim, allow the solution to polymerize for at least 2 h before sectioning. **f** A proper polymerized block is relatively clear and the tissues are visible

3. Allow the entire cup tray to polymerize at room temperature for at least 2 h before sectioning. A proper polymerized block has a clear background with samples clearly visible at the surface (Fig. 4.2f). It is advisable to leave the entire molding tray overnight prior to sectioning the next day. Depending on the nature of the experiment, especially for T8100, the entire tray is allowed to polymerize in a refrigerator overnight. In countries where the humidity is high, it is preferable to store the polymerized specimen blocks in a desiccator at room temperature.

4.4.2 Sectioning

4.4.2.1 Sectioning Using Ralph Glass Knife on a Microtome with a Retraction Return Stroke

1. Insert and secure the specimen block into the mounting chuck. Select the thickness to be sectioned. [For (10 x 6 x 5 mm) molding cup], 3 µm thickness works the best *(see Note 17)*.
2. Insert the glass knife into the knife holder and set the clearance angle to about 5°. The knife edge should be set at the recommended height of the knife holder. Overextended knives can generate vibrations *(see Note 18)*.
3. Align the top and bottom edges of the block parallel to the knife edge. Advance the knife forward slowly towards the block face. If the microtome is equipped with automatic advance and retraction controls, press the appropriate button and bring the knife very close but not touching the block face. Begin sectioning by rotating the hand wheel. Grab the entire hand wheel securely and not by the handle [5]. When turning the wheel, apply force with the wrist rather than the fingers, this allows better control of the sectioning process.
4. Manual advance allows for better initial control of sectioning, and avoids cutting thick sections. Advancing too quickly can chip the specimen block and/or the glass knife *(see Note 19)*. Once the section covers the entire block face, begin to collect sections as they come off the knife and aim to generate a ribbon of sections.
5. To aid in serial sectioning, apply a small amount of liquid PEG 400 to the top and bottom edges of the specimen block to make the edges slightly stickier. Allow the PEG to soak into the block for 2 min and remove the excess using a Kimwipe before resuming sectioning. Hold the forceps with the left hand and as a section comes off halfway from the knife edge, gently grab the corner of the section with a fine forceps to prevent it from rolling back. Follow through and finish cutting the section by turning the hand wheel with the right hand. At this time, hold the section, lift it up slightly but do not dislodge it from the knife edge. Quickly make several sections by turning the hand wheel and simultaneously, gently pull on the sections with the forceps preventing it from sticking to the glass knife. Once a ribbon has been initiated, section at a constant speed. Try not to break the ribbon until a desired length is reached.
6. The long ribbon can be cut into 3 cm long pieces (about 7 sections). The short chains of sections are placed on water on a regular glass slide or subbed slides coated with chrome alum. The plastic sections stretch once they are in contact with water *(see Note 20)*. The excess water is drained off and slides are then allowed to dry using a slide warmer at 40 °C.
7. Serial sectioning of T8100 tissue blocks is more difficult compared to T7100. With practice, short ribbons are possible. Since this embedding medium is used primarily for IHC studies, long ribbons are usually not required.
8. Once the sections are dried, the slides are ready to be stained.

4.4.2.2 Sectioning Using Triangular Glass Knives on a Microtome with a Retraction Return Stroke

The sectioning of GMA blocks with triangular glass knives is similar to that described for the Ralph glass knife. Be sure the size of the tissue block is not larger than the knife edge. If necessary, reduce the size of the block face to fit the width of the knife used *(see Note 21)*.

4.4.2.3 Sectioning Using Disposable Steel Knife System on a Microtome With or Without a Retraction Return Stroke

Steel knives are not as sharp as glass knives. Therefore, when sectioning hard objects like GMA blocks, modifications need to be made. PEG 400 [18] can be added to the embedding medium prior to polymerization to soften the block slightly. This allows the blocks to be sectioned successfully. The basic method of sectioning is similar to that described for Ralph glass knife, except the sectioning speed must be reduced. When used in conjunction with a microtome with a retraction return stroke, ribbons of sections are readily obtained. With a conventional rotary microtome, single sections and short ribbons are possible.

1. Insert and secure the specimen block into the mounting chuck. Set the section thickness between 3–4 μm.
2. Insert and secure the knife holder into the microtome knife assembly. Carefully place a disposable knife into the holder starting at one end of the knife, and lock the knife in place *(see Note 22)*
3. Set the clearance angle at approximately 7°. The knife angle is usually larger than that of the glass knife due to the presence of the knife holder.
4. Move and lock the knife assembly very close to but not touching the block face. Apply PEG to the top and bottom edges of the specimen block similar to sectioning with the Ralph knife. Ensure that the top and bottom edges are parallel to the knife edge.
5. When using a conventional rotary microtome, advance the specimen block by rotating the hand wheel and be patient! When sections start to come off, grab the hand wheel securely and section slowly. Apply a constant cutting force as sections come off the knife edge. Pick up the sections with a forceps and always aim for a ribbon of sections.
6. The sections are stretched and dried as described in subheading 4.4.2.1

4.5 Notes

1. Different microtomes can be purchased from different vendors. Before purchasing, we recommend a careful evaluation of the microtome to determine if it is suitable for sectioning “large” GMA blocks. Some newer microtome models

may have too many additional attachments on the specimen arm, which can cause vibrations and make sectioning of large GMA blocks impossible.

2. Two different models of Ralph knife maker are available from EMS. Again, an evaluation of the equipment before purchasing is advised.
3. A good vacuum and associated vacuum chamber are recommended for any fixation and embedding protocols. It is important to ensure that the vacuum chamber can withstand the evacuating action of the pump. Preferably, the entire assembly should be housed in a fume hood as the extracted fumes can be harmful.
4. Molding cup trays of various sizes can be purchased from different manufacturers. The parallel edges of the molding cup, allowing straight ribbons to form readily. The trays can be washed and reused. Five different sizes of molding cup trays are available from EMS (cat. no. 70176). The Histomolds from Leica can be obtained from their consumable unit (http://www.leicabiosystems.com/specimen-preparation/consumables/). The cat. no. for the Leica Histomold are: 14702218313 (13 × 19 mm) and14702218311 (6 × 8 mm).
5. The round specimen adapters can be reused by soaking the adapter with the embedding plastic in water for a few days, then washing in running water; the resin will have swelled and can easily be separated from the adapter. The removal of T8100 resin is possible but requires more effort as it bonds to the adapter more tightly than T7100 resin.
6. Gerrits and Eppinger [23] demonstrated that there is a gradual decrease of the inhibitor concentration during storage. This reduces the shelf life of the GMA solution. Prepare only a suitable volume of infiltration solution and store the solution at 4 °C.
7. The preparation of the paraformaldehyde solution and fixative solution should always be carried out in the fume hood to avoid inhaling the toxic aldehyde fumes. Generally speaking, the phosphate buffer should be able to maintain the fixative pH at about 6.8. However, if too much hydroxide was used in preparing the paraformaldehyde solution, the pH of the final fixative may need to be adjusted. For adjusting the pH, 1 N sulfuric acid solution should be used instead of hydrochloric acid, as this can result in the production of a carcinogenic product [24]. Since the paraformaldehyde in the stock solution can repolymerize upon storage, a freshly prepared paraformaldehyde solution should be used in preparing a new batch of fixative solution.
8. An electron microscope grade glutaraldehyde solution should be used as glutaraldehyde exists in different polymerized forms. A good quality solution ensures proper fixation of tissues.
9. If the slides are very clean, such as the Micro Slides from Van Waters and Rogers (VWR) or the Superfrost® microscope slides from Fisher Scientific, they can be used without the need of any treatment. Sections will not detach from clean slides when routine staining is performed. For IHC studies, silane-coated slides such as the Superfrost® Plus slides from Fisher Scientific are preferred.
10. At the time of fixation, it is important to excise the specimen with the final orientation in mind. Because the embedding medium has a low viscosity, one

needs to trim the tissues so that they can lay at the bottom of the embedding mold with the desired orientation. For example, if cross sections are required, the specimens should be cut into small discs of appropriate thickness to ensure that it will not "tip-over" during embedding. Although trimming of specimens is possible at the time of embedding, the tissues are brittle and are more likely to be damaged.

11. All exposed plant surfaces are covered with a cuticle. The hydrophobic cuticular material is not compatible with the Technovit resins. As a result, the embedding medium cannot "bind" with the cuticular surface and there is a tendency for the tissue to come off and separate from the surrounding plastic medium during sectioning. The only solution is to cut the leaf into small pieces, so that the embedding medium can penetrate the tissue through the cut edges. At the time of sectioning, the speed of sectioning needs to be reduced in order to minimize dislodging of tissues from the embedding medium.
12. The small size of tissue does not necessary make it easy to process and section. Seeds such as *Arabidopsis* can be technically challenging. The main reason is that the seed coat is composed of sclerified cells with thick walls and mature embryo cells are usually packed with storage products with little room for the embedding medium. The only solution to obtain good sections is to remove the seed coat at the time of fixation or poke tiny holes through the seed coat during fixation.
13. The vials can be labeled by directly writing on the outside of the vials using a permanent marker. To ensure that the ink is not washed off during processing, cover it with a piece of transparent adhesive tape to protect the writing.
14. Acetone has a low boiling point. Under vacuum, it will "boil" vigorously. Be sure to control the rate of vacuuming and never run the vacuum pump unattended.
15. Since the section is picked up at the edge using fine forceps, it is advisable to place the tissue away from the edges during embedding in order to avoid damaging the tissue during sectioning.
16. For a $10 \times 6 \times 5$ mm mold cup, it takes approximately 2 mL of embedding medium/cup to rinse and fill the cup. The necessary volume of embedding medium can be prepared according to the number of blocks prepared.
17. Section thickness depends on many factors such as the hardness and the size of the block. For a block with a small block face, that is, 5 mm^2, sections between 1–2 μm are possible. For the $12 \times 6 \times 5$ mm blocks, the ideal thickness is 3 μm. For softer blocks, section thickness has to be increased to 4–5 μm.
18. Many high profile blades are available commercially, it is important to select knives that extend no more than a millimeter beyond the edge of the knife holder. If the knife edge extends too far beyond the edge of the holder, it will result in vibrations and serial sectioning will simply be impossible.
19. During sectioning, turning the hand wheel too quickly can cause the glass knife and/or the plastic block to shatter. For beginners, especially, it is advisable to wear safety glasses during sectioning.

20. Placement of sections onto water requires practice. If not done properly, the sections simply crumple upon touching the water. Gently grip the edge of a ribbon at one end with a fine forceps and quickly slide the ribbon over the water in one swift motion. The ribbon expands at once in every direction. Another common problem is that the ribbon does not separate from the forceps. In this case, use a needle to dislodge the ribbon gently from the forceps.
21. Using a triangular knife requires smaller blocks that fit the width of the knife. To trim blocks, use a jewel saw to saw away the extra plastic instead of a razor blade.
22. One of the most common mistakes during sectioning is not securing the knife and specimen block to the microtome tightly. If not secure, the knife will vibrate and the resulting sections will have chatters or variation in section thickness. A squeaking sound during sectioning is an indication that certain parts of the microtome or the knife holder have not been tightened properly and the speed of sectioning needs to be reduced. When sectioning, it is also important to make sure that the knife is sharp. A sign of a dull or damaged knife is that the sections are opaque and not transparent to the naked eye and scratches may also appear on sections. The knife should be changed.

4.6 Concluding Remarks

To be successful in any technique, it is important to have a good theoretical understanding of process. Gerritis and associates [7, 8, 23, 25] have published a large number of articles focusing on various aspects of GMA embedding methods. Readers are urged to consult pertinent information from the literature. Further, it is important to "play" with different procedures in order to achieve optimal results. Different experimental materials pose different technical challenges. Modifications to an existing protocol are often necessary to achieve one's objective. The recommended protocol using disposable steel knives in combination with a conventional microtome is certainly not an ideal set up. But, at the very least, individual sections can be obtained. With patience and practice, it is possible to study histological changes of the specimens using high resolution light microscopy by the GMA embedding method.

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