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METHODS

Glycol methacrylate: the art of embedding and serial sectioning

Edward C. Yeung and Colin K.W. Chan

Abstract: Glycol methacrylate (GMA) is a popular embedding medium used for high resolution light microscopy. Since the introduction of the GMA embedding method by Feder and O'Brien (1968, Am. J. Bot. **55**: 123–142), improvements have been made to this technique. The purpose of our work is to detail some important advancements in the GMA embedding method and discuss different approaches to ensure successful processing and serial sectioning of GMA blocks. The best sectioning method is to combine the use of Ralph glass knives with a rotary microtome with a retraction return stroke. Moreover, softening the polymerized blocks with the addition of polyethylene glycol 400 during polymerization allows the blocks to be sectioned using a conventional rotary microtome with a disposable knife system.

Key words: glycol methacrylate, sectioning, Technovit, Ralph knife, disposable steel knife.

Résumé : Le méthacrylate de glycol (MAG) est un milieu d'inclusion populaire utilisé en microscopie optique à haute résolution. Depuis l'introduction de la méthode d'inclusion au MAG par Feder et O'Brien (1968, Am. J. Bot. **55**: 123–142), des améliorations ont été apportées à cette technique. Le but du travail des auteurs consiste à énumérer quelques avancées importantes dans la méthode d'enrobage au MAG et discuter de différentes approches qui garantissent le succès de la polymérisation et la coupe en série des blocs de MAG. La meilleure méthode de coupe consiste à combiner l'utilisation de couteaux de verre de Ralph et d'un microtome rotatif possédant une course de retour à rétraction. En outre, l'amollissement des blocs polymérisés par l'ajout de polyéthylène glycol 400 lors de la polymérisation permet aux blocs d'être coupés à l'aide d'un microtome rotatif conventionnel possédant un système de couteaux jetables. [Traduit par la Rédaction]

Mots-clés : méthacrylate de glycol, coupe, Technovit, couteau de Ralph, couteau en acier jetable.

Introduction

Nearly half a century ago, Feder and O'Brien (1968) published a landmark paper in the American Journal of Botany, which drew attention to the usefulness of the glycol methacrylate (GMA) embedding technique for the study of plant histology. This has since become the number one citation for the American Journal of Botany (Jernstedt 2014) and is a clear indication that the GMA technique has drawn a lot of interest for the study of plant structures. GMA has many excellent properties when used as an embedding medium for light microscopy (see Feder and O'Brien 1968; Bennett et al. 1976; Gerrits and Horobin 1996). The polymerized blocks are hard, therefore sections as thin as 2–3 μm thick can be readily obtained. Having thin sections greatly improves the resolution of the specimen when compared with the thicker paraffin-embedded sections (compare figs. 3 and 4 in Yeung and Peterson 1972). GMA does not react with tissue groups important in staining and because it is hydrophilic, many histological and histochemical staining methods developed for paraffin sections can be used with some modifications.

Since its introduction in the 1960s, many improvements on the original formulation have appeared in the literature, especially during the 1970s (Bennett et al. 1976). The highly exothermic reaction of polymerization was minimized, and improved sectioning methods were proposed, leading to the increased popularity of the GMA embedding method. One of the main drawbacks at that time was that serial sections were very difficult or impossible to obtain. Serial sections are important for locating a structure of interest such as the single-cell zygote within a developing seed (e.g., Lackie and Yeung 1996) and one cannot judge the relationship between various structures based on a few sections alone (Reid 1975). Furthermore, serial sections are essential for the three-dimensional reconstruction of an object (Wu et al. 2011). In the 1970s and early 1980s, to obtain serial structural information of a specimen, sections had to be cut and transferred onto a drop of water on a slide one at a time. Only by arranging the water droplets serially on a slide and placing the sections individually, was one able to obtain serial sections of a specimen. Although this sectioning process was time consuming, the quality of the images obtained was rewarding (e.g., Yeung and Peterson 1972 and Yeung et al. 1981).

The purpose of this work is to summarize some of the improvements in the GMA embedding method since Feder and O'Brien (1968), and to discuss different technical issues and possible remedies for each step of the process. The serial-sectioning techniques using the Technovit 7100 (T7100) resin will be emphasized. The

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*In June 2014, Dr. Edward Yeung was awarded the Canadian Botanical Association Lawson Medal in recognition of his lifetime contributions to Canadian botany. The Lawson Medal is the most prestigious award of the Association and was established "to provide a collective, formal expression of the admiration and respect of botanists in Canada for excellence in the contribution of an individual to Canadian botany". It is named in honour of Dr. George Lawson, who is generally regarded as Canada's first professional botanist.

Table 1. Advantages and disadvantages of different types of knives for glycol methacrylate sectioning.

Method	Advantage	Disadvantage
Ralph knives	Sharp, therefore produces sections easily Constant and reliable ribbon formation	Requires practice in knife making Commercial knife maker is expensive
Triangular knives	Sharp, therefore produces sections easily Constant and reliable ribbon formation	Larger blocks cannot be sectioned
Steel knives	Relatively cheap to replace Compatible with many different microtomes If used properly, can produce sections that rival the Ralph and triangular knives in quality	The block must be modified slightly by adding PEG 400

capacity to more easily produce serial sections is a major advantage of using this embedding medium for a histological study. The techniques presented are based on experience accumulated over many years by the first author. We hope that the information presented will encourage interested scientists to study specimens using high resolution light microscopy employing the GMA embedding method.

Improvements in the GMA embedding method

Significant improvements in the GMA recipe came in 1983 with a publication by Gerrits and Smid (1983). They published a formula that enabled the polymerization of GMA at a lower temperature using less toxic chemical components. Serial sectioning became possible with the addition of a plasticiser, polyethylene glycol 400 (PEG 400). This formulation is manufactured by Kulzer & Co. (Germany) and named as Technovit 7100 resin. T7100 is primarily used for histology, especially for the study of soft tissues. In North America, a similar product was marketed as the Liljeholmens, Kema, Bryggerier (LKB) Historesin, which has subsequently been renamed as Leica Historesin.

Soon after the production of T7100, the Technovit 8100 (T8100) embedding kit became available. In this embedding medium, in addition to the main ingredient GMA, a small amount of ethylene glycol dimethacrylate is added as a crosslinker (Gerrits and Horobin 1996). T8100 is primarily designed for use in immunohistochemical (IHC) and enzymatic studies, as this plastic can be polymerized at 4 °C. For cutting hard specimens such as bones, Technovit 9100 is now available with methyl methacrylate as the main component.

Improvements in microtome design and knives

Improvements in microtome design for light microscopy also played an important role in serial-sectioning techniques. The key improvement was the introduction of a specimen arm that could retract during a return stroke, thus preventing physical contact of the specimen block face with the back of the knife and reducing the chance of dislodging the sections from the front of a knife. As a result, it is easier to obtain a ribbon of sections, i.e., a single chain of serial sections. Additional features such as automatic advance and retraction of the specimen arm and semi-automated to fully automated sectioning were also developed. These types of microtomes became readily available in the early 1980s. However, they are more expensive than a conventional rotary microtome.

Aside from the utilization of a high-quality microtome, another key prerequisite for successful sectioning is the knife. Good quality triangular glass knives are commonly used, as they are readily available in the majority of histology laboratories. Although the knife angle is large, because the linear back surface is similar to a D-profile knife, the clearance angle is small (approximately 2°–4°). This combination allows sectioning of hard plastic blocks. Although triangular glass knives can be used for sectioning, its narrow width (6–6.4 mm) limits the size of the block that can be sectioned. The Ralph glass knife was formally introduced by Bennett et al. (1976) for sectioning GMA blocks and overcomes the triangular knife's disadvantage, as it is possible to section a block close to the width of the glass strip used to make the knife. Bennett et al. (1976) detailed a method for making glass knives manually. To make sharp, reproducible Ralph knives quickly and easily, it is best to use a glass-knife maker. The original LKB Histoknife maker served such a purpose in our laboratory; unfortunately, this knife maker is no longer manufactured. Similar knife makers can be purchased from other manufactures at present; however, knife making requires some practice and one has to ensure that the knives made are compatible with the knife holder for a particular model of microtome. The other alternative is to use a disposable steel knife system. Kulzer & Co. produces a holder specifically for a high-profile, thick-steel knife. This system is excellent for sectioning GMA blocks. However, the holder has a thick base and it may not fit all makes of microtomes. Besides the Kulzer disposable knife system, we also tested other knife systems and found that the combination of a universal knife holder with a high-profile disposable steel knife also gives satisfactory results. High-profile steel knives are designed to section hard specimen blocks, as the knives are thicker and wider than conventional low-profile disposable knives. The advantages and disadvantages of different types of knives are summarized in Table 1.

Polyethylene glycol 400

The hardness of the GMA polymer allows thinner sections to be made, therefore increasing the resolution of the images. However, the trade-off is that sectioning is difficult. Yeung and Law (1987) proposed a modified T7100 embedding medium suitable for sectioning using steel knives by adding 0.6 mL of polyethylene glycol 400 (PEG 400) to 15 mL of the embedding solution prior to embedding. PEG 400 is a plasticizer, which slightly softens the GMA block (Gerrits and Smid 1983). As a result, GMA blocks with a large block face (e.g., wider than 15 mm) can be sectioned with disposable steel knives.

Technovit 7100 embedding kit

The technical information and composition of the T7100 embedding kit is detailed by Gerrits and Smid (1983), Gerrits and Horobin (1996), and Montheard et al. (1992), but briefly, the T7100 embedding kit consists of three components: 500 mL Basic resin with GMA as its main component; Hardener I (five 1 g packages of dibenzoyl peroxide); and Hardener II (40 mL), a barbituric acid derivative that serves as an accelerator for polymerization. The infiltration solution is prepared by dissolving one package of Hardener I into 100 mL of Basic resin, but if a larger volume is needed, all five packages of dibenzoyl peroxide can be added to the entire bottle of the Basic resin, stirred to dissolve, and stored at 4 °C. Unused solution can be stored at –20 °C to prolong the life of the infiltration solution. When needed again, the infiltration solution is removed from the freezer and allowed to reach room temperature before use.

The embedding solution is prepared just before use, because polymerization begins as soon as the components are combined and the solution will solidify within 2 h at room temperature. Polymerization is triggered by Hardener II containing free radical producing agents and initiates the polymerization of GMA monomers into linear chains (Gerrits and Horobin 1996). For proper polymerization to take place, oxygen needs to be excluded from the medium, as it will interfere with the generation of the free radicals necessary for the chain reaction to take place.

Methodology in sample preparation, embedding, and serial sectioning

This section highlights key elements of the GMA embedding method, with emphasis on serial sectioning. However, prior to embarking on any procedure, it is imperative to have a good theoretical understanding of the methods. Readers are urged to consult texts by Reid (1975), Hayat (1981), O'Brien and McCully (1981), Horobin (1982), Glauert and Lewis (1998), and Ruzin (1999) for additional information on fixation, processing, sectioning, and staining. Information concerning the handling and processing of in vitro culture explants for histological studies have been detailed (Yeung 1984, 1999, 2012; Yeung and Saxena 2005). The key suppliers for the materials and supplies are listed in the Supplementary data[1].

Handling of plant specimens for histological study

Proper planning is necessary before embarking on a histological study. To obtain the desired images, one has to excise the specimens with the proper plane of section in mind. It is important to remember that the embedding solution is a liquid with a low viscosity and the specimen will not be able to remain upright if the long axis exceeds its diameter. The tissue is best cut with a new double-edged razor blade. This step should be done quickly. It is best to excise and trim the tissue in a pool of fixative. However, because of the toxicity of aldehyde fumes, the specimens can be trimmed in a buffer or even in water, before being quickly transferred into vials containing the fixative. Although the specimen can always be re-trimmed in the infiltration solution just before being embedded, this is not recommended because it is very brittle, making it difficult to make a precise cut. The maximum length and width of a specimen will be limited to the cup size of the embedding mould, but the best thickness is between 1 and 3 mm so that proper polymerization can occur.

Leaves, woody stems, and mature seeds can pose problems during embedding and sectioning. Leaf surfaces are covered by a cuticle, which tends to be hydrophobic and, therefore, incompatible with the hydrophilic GMA resin. During sectioning, even though leaves are soft, because the plastic does not bond to the cuticle of leaves, the entire specimen can come out of the plastic. Hence, for leaf material, it is advisable to cut it into pieces to minimize this problem. Examples of woody materials include thick-walled parenchyma cells, tracheids, vessels, and fibers. The lignified secondary walls can impede the penetration of the infiltration solution into the cell lumen, especially in imperforated tracheids and fibers. The thick lignified walls can also interfere with polymerization and prevent the proper formation of the GMA polymer. In the absence of the polymerized embedding medium, the tissue appears powdery and cannot be sectioned. If one wishes to section woody tissues, the samples need to be sliced into thin pieces of no more than 1 mm in thickness and followed by several vacuuming steps: during fixation, at the 100% step of dehydration, and in 100% infiltration solution. For the study of woody specimens, other sectioning techniques such as the use of a sliding microtome may be beneficial. Small seeds, such as mature *Arabidopsis* seeds, can be technically challenging. The main problem is the relatively thick seed coat. Similarly to woody tissues, the thick sclerified cells of the seed coat often prevent proper polymerization of the plastic. As a result, the enclosed embryo simply collapses during sectioning, as there is no embedding medium supporting it. The only remedy is to gently remove the seed coat during the fixation process or to puncture the seed coat to allow for the penetration of fluids during processing and embedding, to ensure the proper polymerization of GMA.

Tissue fixation and processing

Fixation is the most important step in any tissue processing. The theoretical aspects of chemical fixation have been reviewed by Hayat (1981) and Eltoum et al. (2001). At present, there is no universal formula for the fixation of biological specimens (Eltoum et al. 2001). The optimal fixation for any particular specimen must be determined by trial and error.

For histological studies at the light-microscope level, a variety of tissues have been successfully fixed in the authors' laboratory by using a combination of 1.6% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (pH 6.9). Fixatives should never be stored with other tissue-culture chemicals or biochemicals, as the aldehyde fumes can interact with them and destroy many expensive chemicals. All fixatives and vials containing tissues undergoing fixation should be housed in their own refrigerator. Because formaldehyde and glutaraldehyde will self-polymerize upon storage (Culling et al. 1985), electron microscope grade glutaraldehyde is required and the paraformaldehyde solution must be prepared fresh just prior to use. For adequate fixation, the amount of fixative used per amount of tissue should be at least a 10:1 (*v*/*v*) ratio. In general, it is best to allow the samples to be fixed for approximately 1–2 h at room temperature to stabilize the tissue before performing the first vacuuming step. Vacuuming is imperative to the fixation process to extract air from the tissues. In plants, intercellular air spaces are often present between cells. It is essential that air be removed from the specimen to facilitate the penetration of fixative, solvents, and the infiltration solution, and for the proper polymerization of GMA. This is best completed in a dedicated vacuum system (i.e., a vacuum chamber or a desiccator connected to a vacuum pump) with sufficiently strong suction to remove air from the tissues. If a dedicated vacuum system is not available, a mild vacuum can be generated using a water aspirator system.

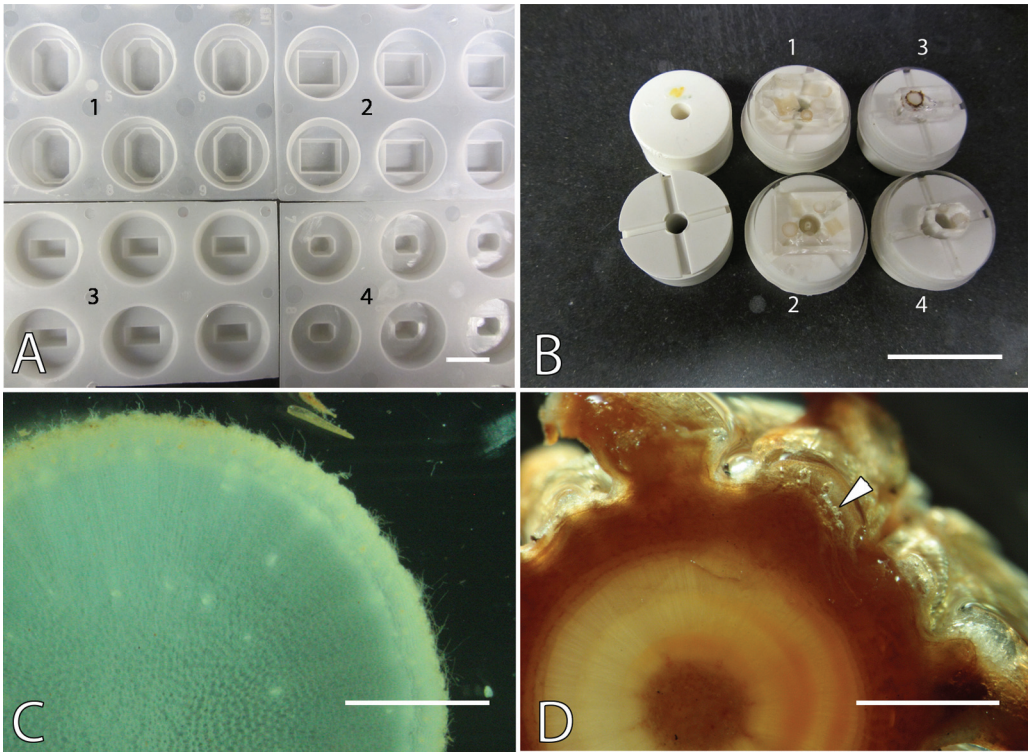
It is important to control the rate of vacuuming to prevent the rapid extraction of air from within the tissue, as this may result in cell collapse. Once air bubbles subside or cease to appear, vacuuming is terminated. At this time, it is also advisable to replace the solution with fresh fixative because some fixing agents will have been extracted during vacuuming. Because of the evaporation of toxic aldehyde and solvent fumes, the entire vacuum system should be housed in a fume hood. It is also important to use a vacuum chamber that can withstand the negative pressure generated by a vacuum pump to prevent shattering of the chamber, thereby preventing the risk of injury to laboratory personnel.

After the vacuuming step, the tissue should be allowed to fix for an additional period of time (usually approximately 24 h). The duration of fixation depends on the size of the specimen being fixed and the type of fixative used. Paraformaldehyde penetrates quickly, stabilizing the cells and tissues, while glutaraldehyde, being a bifunctional fixative, serves to further cross-link the cellular components (Eltoum et al. 2001). Tissues should not remain in a glutaraldehyde-containing fixative for longer than 48 h, as over-fixed samples can become too hardened and difficult to section. For IHC studies, the samples are usually fixed using freshly prepared paraformaldehyde in a buffer with additives such as an osmoticum and (or) inorganic salts (e.g., Tung et al. 2000). Fixation is carried out at 4 °C and for no longer than 24 h.

Typical samples are fixed for several hours including the vacuuming step at room temperature before being transferred to 4 °C overnight, with dehydration occurring on the following day. Dehydration is carried out using an ethanol series instead of the methyl cellosolve-ethanol series used by Feder and O'Brien (1968). Although methyl cellosolve is compatible with water, it can quickly extract plant pigments, such as chlorophyll (E. Yeung,

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2014-0177.

Fig. 1. Specimen embedding. (A) Moulding trays with different sizes of moulding cups in mm: (1) 13 × 19 × 5, (2) 12 × 16 × 5, (3) 12 × 6 × 5, and (4) 6 × 8 × 5 hexagon. Scale bar = 1.5 cm. (B) Round specimen holders with polymerized blocks and tissues. 1, 2, 3, and 4 correspond to the size of the moulding cups in Fig. 1A. Scale bar = 2.5 cm. (C) A properly infiltrated specimen is translucent and is relatively uniform in colouration. Scale bar = 1.5 mm. (D) If air is still present at the end of the infiltration step, silvery reflections and (or) air bubbles (arrowhead) can be seen under a stereomicroscope. Scale bar = 3 mm. (This figure is available in colour online.)



unpublished observations, 2000). If the T8100 embedding medium is used, an acetone dehydration series is required for the proper polymerization of this plastic. The time required for each dehydration step can range from 30 min to several hours. To facilitate the remaining dehydration and infiltration processes, the vials can be placed in a rotary mixer at 4 °C. This will increase the efficiency of processing and minimize the extraction of macromolecules.

To ensure the complete removal of air from the samples, we routinely vacuum the samples two additional times: after the 100% step of ethanol dehydration and when the samples are in the 100% infiltration solution. Because ethanol has a low density, any remaining air from within the samples can be easily extracted. The final vacuuming step ensures the removal of ethanol from the tissues, thereby improving the penetration of the infiltration solution.

To ensure gradual penetration of the infiltration solution, three intermediate infiltration solutions are used (2:1, 1:1, 1:2, (*v*/*v*) ethanol:infiltration solution) before the specimens are placed in pure infiltration solution. Similar to dehydration, the time required for infiltration depends on the size of the specimen. For specimens with abundant storage products within cells, such as mature seeds, a longer infiltration time is necessary. Normally a 24 h period is sufficient in each of the intermediate solutions. The entire process can be expedited if the vials containing the samples are placed in a rotary mixer at 4 °C.

Embedding of specimens

Embedding is a process in which the tissue is surrounded by a supporting medium so that it may be sectioned. The most important step during the embedding process is arranging the specimens so that the desired orientation can easily be obtained during sectioning.

For embedding, moulding trays with various cup sizes are available (Fig. 1A). We routinely use a moulding tray with a cup size of 12 mm × 6 mm × 5 mm in conjunction with the round plastic embedding specimen holders from Leica Microsystems (Fig. 1B). The round specimen holder fits different moulding cups, and forms an effective barrier to air, allowing the proper polymerization of the embedding solution. Just prior to use, the moulding cup is filled with infiltration solution and degassed briefly to remove any air trapped at the bottom of the mould.

Just prior to embedding, the tissues can be poured into a small Petri dish and examined under a stereomicroscope to determine the extent of infiltration. Properly infiltrated samples are somewhat translucent with a relatively uniform in colouration (Fig. 1C). If air is still present, it will appear as a silvery reflection and (or) small bubbles within or between cells (Fig. 1D). If this occurs, it can be remedied by trimming off the affected parts of the tissue and vacuuming again. If the specimen is larger than the embedding moulding cup, it should be trimmed using a sharp double-edged razor blade so that it fits the mould.

When embedding, the mould cups should be filled with infiltration solution before transferring the select infiltrated tissues to the cups. It is important to make sure that the tissue pieces are in their correct orientation and form a single layer at the bottom of the mould. Once all suitable specimens have been transferred, the entire moulding cup tray can be vacuumed one more time to ensure that no air is re-introduced into the tissues and trapped while embedding.

After the tissues are in place, the embedding solution can be prepared. This solution should be used immediately, because polymerization begins as soon as Hardener II is added. To ensure a consistent and uniform polymerization, the specimens are rinsed quickly with the embedding solution. The infiltration solution is

removed using a disposable plastic pipette, the samples are rinsed once with the embedding medium followed by refilling each mould with fresh embedding medium. During rinsing, specimens can move around, therefore the specimens need to be quickly inspected under a stereomicroscope for their orientation and reoriented quickly, if necessary. Finally, a round specimen holder is placed on top of the moulding cup. A sufficient amount of embedding medium is applied to fill to the rim of the cup, which ensures that the medium can polymerize around the specimen holder securely. Gerrits and Horobin (1996) suggest a different way of rinsing the specimen. Instead of rinsing the specimens inside a moulding cup, the specimens are rinsed in a separate dish containing the complete embedding solution before transferring into a moulding cup filled with the embedding solution. To exclude air completely from the embedding solution, they propose adding a layer of molten wax around the specimen block holder. Once embedding is complete, the entire cup tray is allowed to polymerize at room temperature undisturbed for at least 2 h. For T8100, because the intention of using this embedding medium is primarily for IHC or enzymatic staining, the entire tray can be polymerized on ice at 4 °C overnight.

The polymerized blocks are stored at room temperature. In high-humidity environments, it may be preferable to store the specimen blocks in a desiccator. GMA blocks are hygroscopic and they can absorb moisture from their surroundings; the block will change from being solid and transparent to being rubbery and white. Rubbery blocks cannot be sectioned.

Sectioning

A variety of tissues, both soft and hard, and plastic blocks of different sizes can be sectioned by using different combinations of microtome and knife (Figs. 2A–2D). Despite the range of moulding cup sizes, which create different sizes of block faces, generally, 3 μm thick sections can be produced and are suitable for most studies. Because the moulding cup has parallel edges, trimming the block for evenness is usually unnecessary. It is important to ensure that the specimen block and the knife are firmly secured into their respective holders. Any unsecured parts will cause vibrations during sectioning which can result in unevenness in section thickness or missing of sections. The glass knife also should not extend beyond the recommended position and the edge of a steel knife should not extend more than 1 mm from the edge of the holder (Fig. 2E) to further prevent unnecessary vibration during sectioning. Table 2 summarizes common problems associated with sectioning and suggests possible remedies.

Prior to sectioning, set the clearance angle between 4 and 6 degrees. The proper setting of the clearance angle ensures that the block face does not touch any part of the knife assembly except the cutting edge of the knife. However, this angle has to be as small as possible to reduce the compression of sections during sectioning. The knife assembly is advanced carefully and 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 to, but not touching, the block face. We prefer manual advance as this allows for better control of sectioning to prevent cutting thick sections. Cutting sections thicker than 5 μm carries the risk of chipping the block and (or) the glass knife. Hence, wearing safety glasses during sectioning is recommended. Once the section covers the entire block face, sections can be collected. To ensure consistent section thickness, a steady force needs to be applied, as the block passes through the knife edge during sectioning. This is best achieved by gripping the entire handwheel securely with the palm resting against the wheel instead of merely holding onto the wheel handle (Fig. 2F). This way, the operator is in full control of the sectioning speed, applying the necessary force to cut a section.

At the very least, sections can be collected one at a time and placed on a slide with distilled water. The advantage of embedding tissues in T7100 is that ribbons are readily obtained from the block, which greatly decreases the time spent sectioning and allows for an easier evaluation of the tissues after staining. The "stickiness" of a block is what allows the sections to connect to each other to form a ribbon. To further improve the "stickiness" of the block for serial sectioning, a small amount of liquid PEG 400 can be applied to the top and bottom edges of the block. The liquid is allowed to soak into the block for 2 min and the excess is removed using a Kimwipe before resuming sectioning. To generate a ribbon, 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 fine forceps to prevent it from rolling back, then follow through and finish cutting the section by turning the handwheel with the right hand. At this time, hold the section and lift it up slightly but do not dislodge it from the knife edge. Several sections are cut by turning the handwheel and, simultaneously, gently pulling on the sections with the forceps. This will prevent the sections from sticking to the glass knife, allowing a ribbon to form. Once a ribbon begins to form, sectioning should proceed at a constant speed and care should be taken to prevent breaking the ribbon until a desired length is reached. Our record length for a single ribbon is 1.5 m!

The long ribbon is then cut into 3.5 cm long pieces (approximately seven sections). The truncated ribbons are picked up using forceps and transferred to water on a regular clean glass slide. The sections stretch once they are in contact with water. When the sections have fully expanded, the excess water from the slide can be drained off carefully or the sections are simply allowed to dry on a slide warmer at 40 °C before storing them in slide boxes. For general histological staining, the sections can be mounted on clean slides without the need of a subbing treatment. Subbing of slides is a process by which clean slides are coated with a substance to enhance sticking of sections to slides. If the slides are dusty or greasy, they can be cleaned and coated with chrome alum gelatin prior to use (Pappas 1971). Other subbing solutions are also available (see Ruzin 1999).

Sectioning using Ralph type glass knives and a microtome with a retraction return stroke

The ideal combination for sectioning GMA blocks is to use Ralph knives, prepared using histoglass strips, 25 mm wide mounted on a microtome that retracts on the return stroke (Fig. 2A). It is easy to section a 12 mm × 6 mm × 5 mm block and ribbons can be obtained reliably. When sectioning with a Ralph knife, it is important that the knife is well made. A low-cost assembly using Ralph knives made from regular microscope glass slides has also been reported to cut thin sections of small gelatin capsule embedded tissues using a conventional rotary microtome (Semba 1979).

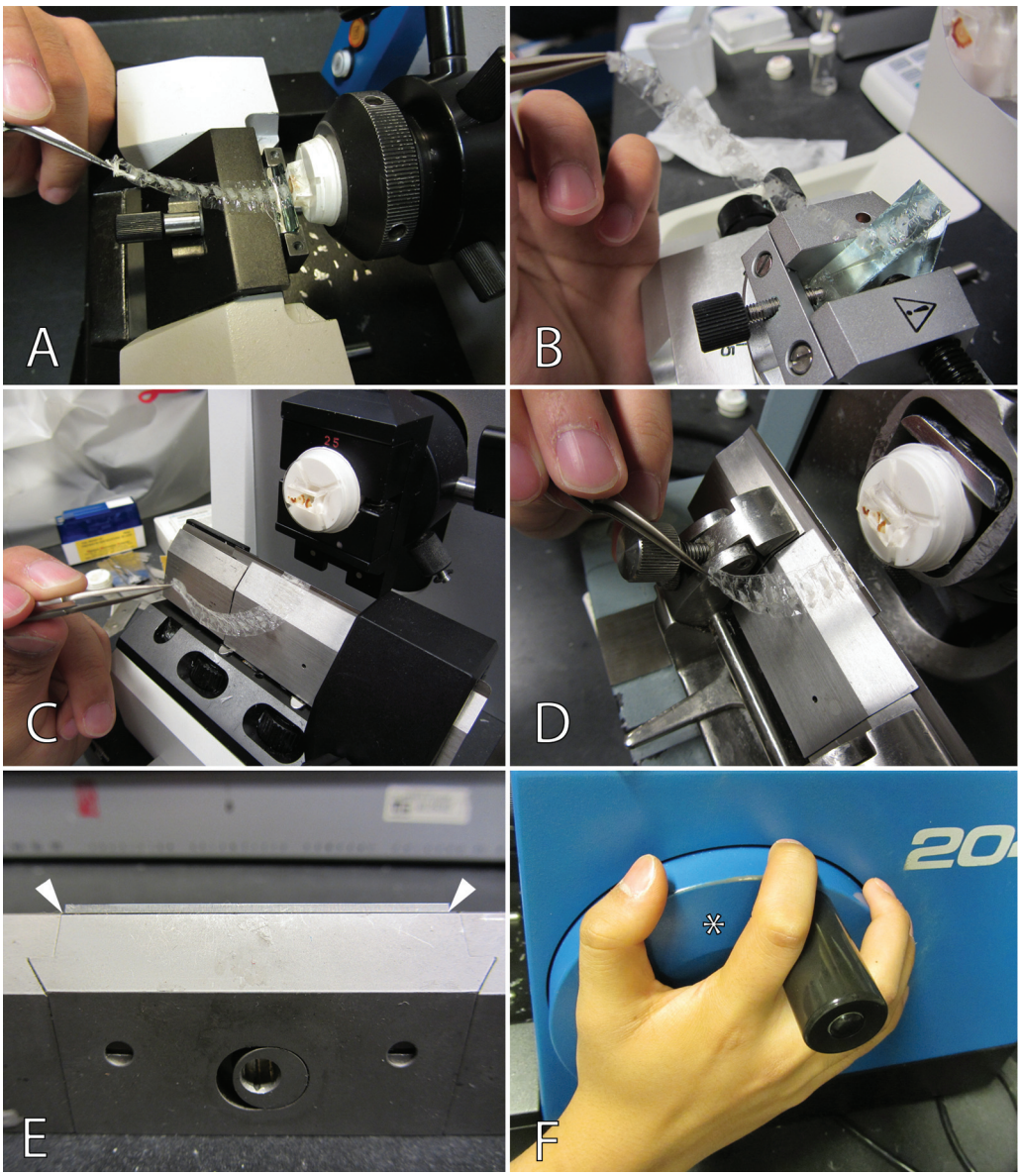
Sectioning using triangular glass knives

The method of sectioning using a triangular knife is similar to the one described above for the Ralph knife (Fig. 2B). The main limitation is that the triangular knife is narrow; hence, the size of the GMA blocks must be reduced to fit the knife edge before they can be sectioned. The width of the knife is usually 6–6.4 mm; however, the newer triangular knife makers can break glass strips with a thickness of up to 10 mm.

Sectioning using disposable steel knives

Although steel knives may appear to be sufficiently sharp and hard, they are not as sharp as glass knives. The sectioning speed usually must be reduced when sectioning with a steel blade to minimize compression during sectioning. Because the steel knife is housed within a holder, the clearance angle of the knife assembly has to be slightly larger to avoid contact between the specimen block and the back of the knife holder. However, this increase in the clearance angle will further increase the compression of the block face while sectioning. One way to increase the ease of sectioning when using disposable steel knives is to reduce the hardness of

Fig. 2. Serial sectioning using different combinations of microtome and knife. (A) A ribbon is easily obtained using a microtome with a retraction return stroke and a Ralph glass knife. (B) A block with a smaller block face is easily sectioned using a triangular knife. (C) A ribbon can be obtained using a microtome with a retraction return stroke and a universal knife holder with a high profile disposable steel knife. (D) A ribbon can be obtained using a conventional rotary microtome with a universal knife holder with a high profile disposable steel knife. (E) A Kulzer knife holder with a disposable steel knife. The knife edge (arrowheads) is extended only 1 mm beyond the holder. (F) To control the speed of cutting, the entire handwheel (asterisk) is secured by the hand instead of simply holding onto the handle. (This figure is available in colour online.)



the plastic blocks. The addition of PEG 400 to the embedding medium (Yeung and Law 1987) slightly softens the polymerized blocks, making it more suitable for sectioning with the steel knives. Using this method, we have successfully sectioned blocks with a size of 13 mm × 19 mm × 5 mm.

When blocks made with PEG 400 added to the embedding medium are sectioned with a disposable knife system mounted on a microtome with a retraction return stroke, ribbons form readily (Fig. 2C). However, if disposable knives are used in conjunction with a conventional microtome (Fig. 2D), the cutting speed will need to be reduced. With practice, at the very least, individual sections can be obtained using this method. For softer tissues, short ribbons are possible (Fig. 2D).

The preceding sectioning methods also apply when embedding with T8100. In our hands, this plastic is easier to section than T7100, but ribbon formation is more difficult. Luckily, because this plastic is usually used for IHC studies, long ribbons are usually not required. Sections intended for IHC or enzymatic staining should be placed on silane-coated slides such as Superfrost Plus slides (ER4951PLUS) from Fisher Scientific to ensure that they do not detach during the prolonged staining and washing processes. A sample protocol can be found in Lee et al. (2013) using this embedding procedure to study protocorm formation in an orchid.

Staining

Because GMA is hydrophilic, many staining recipes used for paraffin sections can be applied to GMA sections with some modifications. Many staining recipes are detailed in the literature (e.g., Feder and O'Brien 1968; O'Brien and McCully 1981) and will not be repeated here. Some autofluorescent characteristics of plant cells

Table 2. Common technical issues associated with sectioning, possible causes, and their solutions.

Issue	Possible cause(s)	Possible solution(s)
Inconsistent (on and off) cutting	A part of the microtome is loose or the block is too soft	Tighten the parts of the microtome Increase section thickness by 1 μm
Chattering — horizontal lines of varying thickness across sections	A part on the microtome is loose	Tighten the parts of the microtome Replace the knife
Scratches and (or) lines	The knife is damaged or chipped	Replace the knife Move to a new part of the knife
The sections curl into a cylinder	The sectioning speed is too fast	With forceps, pull the edge of the section gently as the block passes over the knife Reduce the sectioning speed
Sections are not uniformly thin and transparent	Dull knife	Replace with a new glass knife Move to a new part of a steel knife
Section thickness is inconsistent	The block is soft The block is being cut too quickly	Increase section thickness Reduce the sectioning speed
The block is rubbery after polymerization	The block did not polymerize properly Improper mixing of embedding solution	The blocks may be salvageable by placing in a 60 °C oven for several hours to harden the blocks further
The block has a dull, white colour and rubbery appearance	The block was exposed to too high a humidity and has turned to “rubber”	Discard the block and store future blocks in a desiccator
Ribbons are not forming	The sections are not sticking together properly	Paint a thin layer of PEG 400 over the top and bottom of the block and let it soak for about 2 min
Ribbons are not forming, sections dislodge from the edge of the knife	The block hits the back of the knife during the return stroke, knocking off the previous section	Turn the handwheel slowly; return the specimen arm to the uppermost cutting position at a slower speed Trim the block edges to ensures the edges are smooth
A corner or part of the section is missing	The block face is not even	Realign the block Continue sectioning until it disappears
The sections are only connecting at two points	Either the top or the bottom edges of the block is uneven	Trim the uneven edges of the block using a single edge razor blade
The tissue comes off from the section	The tissue is surrounded with a coating such as cuticle that is not compatible with glycol methacrylate	Slow down the cutting speed or decrease the thickness of the sections
The block separates from the plastic block holder	There is an air bubble between the block holder and the block, preventing proper adhesion	Remove the block from the specimen holder and glue it to the back of a round specimen holder using epoxy glue, allow to dry for 1h before resuming sectioning

can also remain even after processing, and fluorochromes can be used to detect macromolecules such as DNA and callose in plant cells as well (Supplementary Fig. S1[1]). Another advantage of GMA sections is that the plastic does not need to be removed prior to staining, thereby eliminating the use of organic solvents as would be used for paraffin sections. This greatly reduces the generation of organic wastes. However, background plastic staining can be present and requires proper washing. Our routine staining protocol consists of the periodic acid – Schiff's (PAS) reaction for total carbohydrates, and amido black 10B as a counter-stain and for proteins (Yeung and Saxena 2005). The PAS reaction highlights cell walls and starch, while amido black 10B reveals general cytoplasmic details. The two stains also tend not to overlap, thereby simultaneously providing excellent contrast and histochemical information. Another combination of stains that is commonly used is PAS and toluidine blue O, which gives better contrast to cell wall features (Yeung and Saxena 2005). Examples of stained sections can be found in Supplementary Fig. S1[1].

Because serial sectioning can generate many long ribbons which can be mounted on a large number of slides, to increase the efficiency of staining all of those sections on the slides, we use multiple plastic slide carriers when staining. The slide carriers we use are manufactured by Sakura (www.sakura.eu), and can each house up to 24 slides. One of the advantageous features of the GMA embedding medium is that slides can be examined without the application of coverslips. Although coverslips can improve image clarity, their cost continues to rise. Therefore, to conserve the use of laboratory consumables, coverslips may be applied to select slides for photographic purposes after a careful screening of all the slides for that particular project.

Discussion

In recent years, the formulations of GMA have been standardized with the production of different Technovit resins and the traditional JB4 embedding medium. These formulations are effective in embedding tissues with a range of hardness and allow them to be sectioned with ease. IHC and enzymatic studies at the light-microscope level can be carried out readily thanks to the GMA embedding method.

This paper presents updated information on the GMA embedding method and the specific techniques used in our laboratory.

The degree of challenge in obtaining large GMA sections is dependent on the microtome and the knife assembly. Although the use of a rotary microtome with retraction together with a Ralph knife is the best combination for the efficient generation of ribbons of sections, the purchase of these apparatuses is costly. We have demonstrated that GMA blocks can be sectioned with the use of a low-cost rotary microtome with disposable steel knives by softening the plastic block with the addition of PEG 400. In general, botanical specimens can be easily processed using the GMA embedding method. Relatively large and soft tissues do not pose any problems in processing and sectioning. For hard materials, as long as they are thin in one dimension, the GMA embedding method is also successful.

The most important feature of the GMA embedding method is that serial sections can be made, especially when embedding with the T7100 resin. Serial sectioning provides valuable information allowing for the accurate description of cells and their surrounding environment.

As with other techniques, these methods can certainly be modified as needed. In the study of large animal tissues, modifications to the procedures enable large blocks of tissues with block faces up to 2 cm^2 to be sectioned using a heavy duty sledge microtome (Quester et al. 2002; De Jonge et al. 2005).

Because the optimization of tissue processing, embedding, and sectioning protocols is often necessary owing to differences in starting biological material or laboratory resource availability, perhaps more than for most other projects, histological studies require exceptional commitment and patience on the part of the investigator(s) to overcome some of the challenges described in this paper. Practice and repetition are essential to produce reliable results. To deliver first-rate results, one needs to aim for perfection (e.g., presenting histological sections with the proper plane of section and at a specific stage of development, etc.). Although this can take a lot of time, well-made micrographs indicate the quality of the study and provide confidence in one's research.

Furthermore, for the successful completion of any histological investigation, an integrative knowledge of structure, physiology, cell biology, and biochemistry is usually required to accurately interpret the results. Although the entire process can be very time-consuming, well-made micrographs and the thorough interpretation of those micrographs can contribute to the validity of the research presented. A well-executed morphological and anatomical study is highly rewarding, and can provide background information of the experimental system and lead to the generation of further questions and (or) hypotheses to study the system.

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