In Vitro Cell. Dev. Biol.—Plant 35:137–143, March–April 1999
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1054-5476/99 $05.00 + 0.00

THE USE OF HISTOLOGY IN THE STUDY OF PLANT TISSUE CULTURE SYSTEMS—SOME PRACTICAL COMMENTS

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(Accepted 22 March 1999; editor T. A. Thorpe)

SUMMARY

Histological methods have contributed significantly to our understanding of in vitro culture systems. A good histological study based on anatomical and histochemical changes provides insight into cellular processes and provides clues that allow for the proposal of hypotheses for further experimentation. This article serves to draw attention to the use of a histological approach to one's experimental system. Some of the common mistakes in the handling and processing of explants are discussed. A protocol for the plastic embedding method is detailed.

Key words: histology; paraffin and plastic embedding methods; photomicrography; staining.

INTRODUCTION

Histological techniques are widely used in many areas of research. Structural analysis is an important first step in the study of the organization and changes in the plant body, and it is an extremely useful approach in the study of plant morphogenesis (*see* Wetmore and Wardlaw, 1951). Different histological methods have contributed significantly to our understanding of in vitro culture systems. A cursory survey of tissue culture-related journals indicates that micrographs are often included. The purpose of the micrographs is obvious. It allows us to "see" changes that take place in our experimental system. A good histological study based on histological changes provides additional insight into the cellular process and provides clues and allows for the proposal of hypotheses for further experimentation. In recent years, it appears that fewer and fewer students are getting proper training in this "classical" method. Investigators often have little respect for the process and the purpose of carrying out a good histological investigation. Microscopists are often asked to help with getting one or a few micrographs to "dress up" the manuscript.

The purpose of this article is to draw attention of investigators, especially graduate students to: (1) how to conduct a structural study involving an in vitro culture system at the light microscope level and (2) how to examine one's own preparations. This article essentially stems from my instructions to students and researchers alike. It is by no means comprehensive, as it is impossible to cover all aspects of histological techniques and different experimental systems. Readers are referred to the following texts for a variety of cellular and histological techniques, namely Jensen (1962), Berlyn and Miksche (1976), O'Brien and McCully (1981), Gahan (1984), Harris and Oparka (1994), and Sanderson (1994). Additional practical information is provided in the appendix.

KNOW YOUR SYSTEM

Before you begin a histological investigation, it is imperative that you have a good understanding of your own culture system. If you are interested in a process such as somatic embryogenesis or shoot histogenesis, it is advisable to optimize the system before initiating an anatomical study. Initial observation and optimization of the system hold the key to a successful anatomical study. Careful observation of the changes of the explants will pinpoint the area in which events begin. This information is necessary because it identifies the area of activity. This is the part that should be excised and fixed for structural studies. This is also the area that one should focus on when examining prepared slides with a microscope. Furthermore, optimization of the culture system is essential to provide some semiquantitative changes occurring within the explant. For example, the frequency of shoot formation in vitro provides useful information when one looks for a similar number of changes occurring within the explants, especially before actual shoot formation. Never underestimate the usefulness of examining living cultures. Whenever possible, simple dissection, free hand sections and staining (Yeung, 1998), and photomacrography (Bracegirdle, 1995) of the "living" explants should be carried out as the essential first step to a quality histological or any other type of study.

PLANNING AND PREPARATION

Once the system has been optimized, one can begin to plan a histological study. In general, one of the main purposes of a structural investigation is to study the ontogenetical events and structural changes. In planning for this type of study, it is important to estimate the number of explants needed. This depends on the number of sampling points. Changes begin to occur after placement on an induction medium. In my laboratory, we usually sample frequently during the first few d of culture and then gradually lengthen the sampling time until the shoots, root, etc., appear. For each sampling point, we harvest a minimum of 10 explants. As a result, for each experiment, more than 100 explants are needed. Control treatments, e.g., explants cultured in maintenance medium without growth substances, should also be fixed and processed. The entire procedure should be repeated at a later date to ensure reproducibility of the observations.

Why do we need so many explants? It is common sense that accurate observation cannot be based on a few samples. Although structural observation usually yields qualitative observation, we have to follow standard statistical sampling techniques. The importance of handling and sample size have been discussed by Jakstys (1988). Furthermore, since biological specimens are three-dimensional objects, one can easily draw erroneous conclusions based on the "two-dimensional" sections, if the observer is not careful in examining slides (Sanderson, 1994). By examining a large sample of specimens, error in interpretation can be avoided. During the course of development, certain events that take place within the cell can be difficult to capture histologically. For example, if an unequal cell division indeed takes place in the formation of the structure of interest, one should not base one's observation simply on two cells of unequal size. The larger cell may be a result of more rapid cell expansion after a mitotic division; it would be better to "catch" a cell during cytokinesis, i.e., having the cell plate located near one end of the newly divided cell to prove that indeed the cell division is unequal (Yeung et al., 1981). What is the chance of "catching" such an event? It is not easy because the M phase of the cell cycle is usually completed in a short time and you may not have selected the proper time for the fixation of the samples. However, if the unequal cell division is an important event that leads to histogenesis or organogenesis, then with a large number of samples, it is likely that you will be able to document such an event.

Another common mistake in the literature with regard to embryogenesis and shoot histogenesis is the failure to demonstrate a well-organized shoot meristem. Simply showing a structure having a shape of an embryo is not good enough. It is known that many somatic embryos cannot convert due to the absence of a properly organized shoot meristem (Kong and Yeung, 1992; Nickle and Yeung, 1993). It is imperative that through proper sampling and sectioning, a well-organized shoot meristem be shown to be indeed present. This information together with conversion frequency is a proper means to demonstrate good quality somatic embryos. Conversion frequency provides a quantitative assessment of functional shoot and root meristems. Similarly, to show successes in shoot histogenesis, it is essential to show a shoot primordium with a well-organized shoot meristem with flanking leaf primordia (*see* Yeung et al., 1981; Nour et al., 1993). Simply showing "bumpy" surfaces with leaf-like structures is not an adequate demonstration. During shoot histogenesis, leaf-like structures can be produced without a functional shoot (*see* Gaba et al., 1999). To demonstrate the presence of a well-organized meristem, perfect median longitudinal sections through somatic embryos or explants with shoot primordia are needed. Since the biological specimen is a three-dimensional object, the chance of obtaining perfect sections is not high. Thus, many explants are required. In many of our studies, it is not surprising that we often process many explants and this results in making a few thousand slides per project.

Fixation and Processing

The first step in the processing of a sample is fixation. Fixation is the most important step in the entire process. The sample should be harvested and fixed at the same time. The quality of the final preparation depends on how it is fixed. It is important that you understand the principle of fixation and the chemical properties of the fixing agents used in the fixative. For a critical discussion in relation to the properties of fixing agents used in plant tissues, readers are referred to the articles by O'Brien et al. (1973), Mersey and McCully (1978), Coetzee (1985), and Dong et al. (1994). Many recipes for fixation are available in the literature. It is important to remember that recipes are not formulae. Recipes can be changed or modified to suit one's own need. The willingness to try to experiment with different recipes is the key to good quality fixation.

One process that tends to be overlooked during the fixation process is the proper trimming of the explants. Explants can be large, especially after a lengthy culture period. It is important that the size be reduced. Trimming the specimen facilitates the penetration of fixative and the embedding medium. Organogenetic events may occur in a specific region of the explant and fixing the entire explant may not yield useful information regarding the event of interest. Excess tissue will lengthen processing time and waste resources. For example, with large tissue blocks, only a few sections can be placed on a slide. As a result, more slides are required and the processing time will also be lengthened.

Small explants and embryos appear to be soft and easy to process. On the contrary, cuticular material is often present at exposed surfaces of the embryo and explant, and often causes problems in the processing and sectioning of these samples. Some somatic embryos accumulate a large amount of storage material, which also presents problems for the infiltration of the embedding medium and the subsequent sectioning. Trimming of explants and embryos will facilitate the fixation process and minimize problems associated with the processing of samples.

Proper trimming also determines the orientation for embedding and subsequent sectioning. Proper orientation is the key to obtaining a median section through structures such as embryos, shoots, and roots. Thus, care should be taken at the time of fixation for the selection and trimming of explants. This will reduce the number of slides needed for the project.

Paraffin and Plastic Embedding Methods

Serial sectioning is essential to a quality histological study. This method allows you to trace changes in the explant and make a three-dimensional reconstruction of the object of interest, if desired. Many protocols regarding processing and embedding are available in the literature (*see* references in the Introduction). In general, there are two common embedding methods that will allow for serial sectioning, i.e., wax and glycol methacrylate embedding procedures. The wax embedding protocol such as the one detailed by Jensen (1962) is the most common procedure. Readers are urged to consult books for further details. When carefully processed, paraffin sections will provide information quickly. This method is especially useful for large explants. The cost associated with the paraffin embedding method is relatively low in comparison to the plastic embedding method.

Feder and O'Brien (1968) drew attention to the use of glycol methacrylate (GMA) as an embedding medium for botanical specimens. Since that time, different formulations based on GMA for embedding are now available. Further improvements of the catalyst used led to the development of a formulation that enables serial sectioning of this plastic (Gerrits and Smid, 1983). This embedding medium is sold under the trade name Technovit 7100 (Kulzer & Co. Gmbh, Bereich Technik, D-6393 Wehrheim, Germany). In North America, the same embedding medium is marketed by Leica under the trade name, Historesin. This, together with the improvement of the micro-

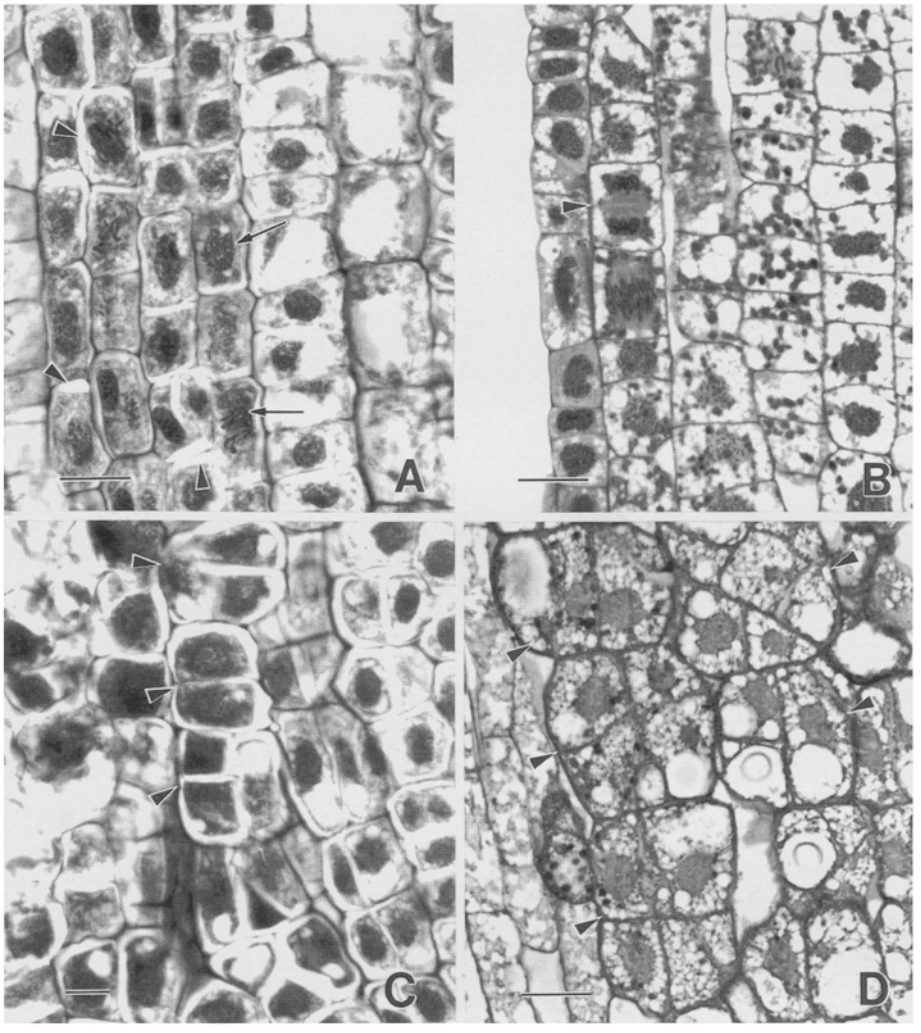

FIG. 1. Light micrographs showing structural features of radiata pine cotyledon explants using two different embedding methods. *A*, Although chromosomes (*arrow*) and mitotic figures are clearly visible with the paraffin embedding method, the cells tend to be plasmolysed (*arrowheads*), and smaller vacuoles are not well preserved. The sections were stained with safranin, basic fuchsin, and crystal violet and counterstained with fast green. *B*, A plastic-embedded specimen of the same material. More cellular details can be discerned with this procedure. Mitotic figures and the newly formed cell plate (*arrowhead*) are clearly visible. Small vacuoles are better preserved in the cytoplasm. The sections were stained with the PAS procedure and counterstained with toluidine blue O. *C*, At a later stage of development, meristemoids begin to form before shoot histogenesis. Although meristemoids (*arrowheads*) can be found, they are not readily detected by the paraffin embedding procedure. *D*, A plastic-embedded specimen of the same material. As the sections were 2 μm thick, more features can be resolved. In this micrograph, meristemoids (*arrowheads*) are easily discerned. All *scale bars* are 20 μm.

tome which allows for retraction during the return stroke, enables us to serially section plastic blocks. The improvement in the quality of fixation, together with a reduction in section thickness, improves the resolution of the specimen. This greatly enhances the quality of histological studies. Fig. 1 provides a comparison between paraffin- and plastic-embedded specimens of a same sample. It is clear from the micrographs that the plastic embedding specimens provide more cellular details. As a result, the plastic embedding procedure is certainly the method of choice. A modified protocol for preparing the Historesin embedding medium was described by Yeung and Law (1987), and a more detailed description of the entire procedure can be found in the appendix.

To carry out serial plastic sectioning of specimens, major capital investments such as a good quality microtome with a retractable return stroke (i.e., the Leica 2040 and the Zeiss HM 360) and a Ralph glass knife maker are required. The consumables, such as the embedding medium and glass strips, are relatively expensive as compared with those in the paraffin embedding method.

Although it is recognized that plastic sections are more superior than paraffin sections, many studies using the plastic embedding method are often disappointing. I suspect that the main reason is due to the lack of serial sections. Serial sectioning is difficult without a microtome with a retractable return stroke. Through the use of a conventional microtome fitted with a triangular glass knife, only small tissue blocks can be sectioned. Using this setup, few investigators are willing to section a large number of blocks to overcome the deficiency of serial sections. As a result, median sections are often missing and cannot be included. In addition, many stages are also missing from the study. If this is the case, careful processing with the paraffin embedding method may be better or a combination of both methods can be used to complement the shortcomings of each procedure.

STAINING

There are numerous recipes for staining of specimens. Readers are referred to the text by Jensen (1962) and O'Brien and McCully (1981). It is beyond the scope of this article to detail problems and artifacts dealing with the processing and staining of specimens. I would like to draw attention to the fact that fixation, processing, and the type of embedding medium used could extract and alter the staining properties of the specimen. Care should be taken to interpret one's own finding. For a detailed discussion, *see* Horobin (1982).

For paraffin sections, the method of safranin and fast green is often used (*see* Jensen, 1962). This procedure usually is time-consuming as the safranin stain is rapidly replaced by the fast green stain. A modified safranin mixture, i.e., safranin, crystal violet, and basic fuchsin (0.5%, 0.2%, 0.2%, respectively, in 50% ethyl alcohol, wt/vol) can be used as an alternate to just safranin alone. This mixture will stain the specimen intensely and the staining time is reduced to 20–30 min. The result is quite pleasing (*see* Yeung and Peterson, 1972). Meristematic areas are easily identified as they always have a more intense red color.

Staining of plastic sections is simpler than paraffin sections as it is not necessary to remove the plastic embedding medium. Thus, one can avoid using organic solvents such as xylene. Furthermore, since GMA is hydrophilic, the sections can be stained easily as GMA is compatible with a majority of aqueous staining recipes. This greatly simplifies the staining procedures. The common staining procedures for GMA are detailed in Yeung (1984). Once stained, the sections can be dried and mounted with the desired mounting medium.

How to Examine the Slides?

At a first glance, when you are faced with many slides, the sheer number can be overwhelming! This appears to be a tremendous task. The question is, How to look? The following examples are approaches that one can use when looking at one's own preparations in the structural analysis of a problem. For an ontogenetic study of a certain structure or event such as shoot histogenesis, my advice is to go backwards in time, starting by the examination of the oldest explants. Mature structures such as shoots are easy to locate. Once the location is determined, one can trace the ontogenetic pattern backwards in time to seek changes in similar locations in "younger" specimens. For the next round of examination, one can then examine different sets of slides and go forward in time to confirm the observation.

After the preliminary pattern is determined, the next important task is to seek as much information as possible from the prepared slides by asking questions as you examine the specimens. Questions will need to be asked to determine the underlying events associated with a particular process. The answer is in front of you. In the case of shoot histogenesis, some of the questions are: Does the shoot originate from a single cell? Are meristemoids present? Does starch begin to accumulate before shoot differentiation? When seeking answers, it is essential that you search for them one at a time. It will require that you examine the same sections many times over. This approach appears to be tedious; however, it will provide confidence in your own findings. Through these examinations, additional information can be gained with your imagination! Students should always be trained to ask questions during their investigation.

Sections are two-dimensional objects. It is essential that during slide examination, you should perceive the object in three dimensions and imagine the dynamic processes that were taking place within the explants before their fixation. To extract the most information from your specimens, you have to be willing "to think in the combined terms of morphology, physiology, and biochemistry" (Jensen, 1962).

Show and Tell

During the course of slide examination, sections should be identified and/or photographed in preparation for future presentation and/or publication purposes. Photography is a good medium for keeping record. It is a good practice to take photos during the course of slide examination. Take as many as necessary to demonstrate structural changes regardless of how minor they seem. Try not to save film at this time. Once you have finalized the sequence, it is important to examine the results objectively to determine the quality of the images obtained and whether critical events from the observation are missing. If the results are not satisfactory, the experiment needs to be repeated and further improved.

For publication purposes, due to limited number of plates allowable per manuscript and the associated publication costs, only selected quality micrographs should be presented. The micrographs should demonstrate key events related to the objective(s) of the experiment. At this time, one should rephotograph the selected images from the initial observation if necessary. This will improve the quality of presentation, as you can deliver the information by using micrographs with the proper magnification for the plate size selected, and contrast can be improved by using contrast enhancement techniques to highlight the features of interest. Median sections of shoots, embryos, and other structures should be selected to show the maximum number of features on a single micrograph. The quality of information, as illustrated by a micrograph and how it is presented, is a key feature that will determine the acceptance or rejection of the manuscript. If the quality of the micrographs is poor and if claims are made in the text that are not substantiated by the micrographs, this will certainly lead to the rejection of the manuscript. The micrographs that you submit for publication should represent your best. If the quality is poor, the reviewer will certainly wonder about the rest of the slide collection. Quality micrographs require quality preparations. Micrographs should be grouped whenever possible into plate(s) and properly labeled. Scale bars should be used to indicate the magnifications of the micrographs. Only good quality plates, and not photocopies, should be sent with all copies of the manuscript.

The other consideration in presenting the micrographs in a publication is the selection of color versus black and white micrographs. In a majority of cases, black and white micrographs are adequate for most purposes. Furthermore, the publication cost is usually low and the investigators may not be charged for it. Color micrographs are useful only when they are absolutely essential in presenting results of the study. It is important to bear in mind that there are always costs associated with color plate production. Often after submitting the manuscript, a number of investigators request that the color plate be withdrawn and a black and white image be produced instead. This creates extra work for the editor and can also lead to the rejection of the manuscript. Therefore, plan ahead. If warranted, take both black and white and color photos and then select the appropriate medium when submitting the manuscript for publication.

The light microscope is the most misused piece of equipment in a laboratory. There is more to using it than just turning it on and pressing a button to take a picture. The final quality of micrographs depends on the quality of the specimens as well as the proper operation of a photomicroscope. It is essential that you understand the principle of operating a microscope and how to generate contrast and the contrast enhancement process. The proper selection of objectives, condenser, and even coverglass thickness can influence the final quality of the micrographs produced. The following books by Lacey (1989), Smith (1994), and Bradbury and Evennett (1995) provide basic information on photomicrography.

Concluding Remarks

Although the histological method is emphasized in this article, this is just one method of many in our research endeavors. Other approaches and methods are just as important, and different methods should be used whenever possible to complement a histological investigation.

In recent years, molecular biology and genetics have contributed new and exciting information concerning plant growth and development. Histological techniques are becoming more and more important, as they are used in conjunction with many molecular methods, such as in situ hybridization and immunohistochemistry. For example, in the generation of transgenic plants, success in a plant transformation protocol requires a better understanding of where and how target cells arise. Anatomical research will provide the necessary information that allows for further refinements in the selection of explants for transformation experiments. The GUS gene and the green fluorescent protein are markers used to illustrate success in the transformation process. Again, good knowledge of histological preparation is essential to accurate observation and conclusions. In this article, I hope I have demonstrated how to use a histological approach to study one's problem. When carefully used, this is a rewarding approach.

Appendix: The Technovit (Historesin) Embedding Procedure

The following is our routine procedure in preparing tissues for histological examination using the plastic embedding technique.

1. Before collecting and fixing specimens, prepare all necessary fixatives, solutions, and equipment needed. The tissue is usually fixed in a fixative containing a mixture of 1.6–2% paraformaldehyde and 2–2.5% glutaraldehyde in a 0.05 *M* phosphate buffer at pH 6.8. As an alternative, 3% glutaraldehyde in the phosphate buffer can also be used. A stock solution of paraformaldehyde (16 or 20%) is prepared by adding the appropriate weight of paraformaldehyde into hot distilled water (90° C) in which a few drops of 1 *N* KOH have been added. The solution is stirred continuously and extra heating may be needed to dissolve the powder. After about 10 min, a clear solution should appear. The final volume of the solution is adjusted with distilled water. The solution should then be filtered to remove a few undissolved particles. 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 (Goodbody and Lloyd, 1994). Furthermore, it is essential to carry out this procedure, i.e., weighing and stirring/heating, in the fume hood to avoid the inhalation of formaldehyde fumes. Preferably, a freshly prepared paraformaldehyde solution should be used in the preparation of fixatives. For glutaraldehyde, a good quality solution can be purchased directly from different suppliers. Whenever possible, "electron microscope grade" glutaraldehyde should be used. Fixatives should never be stored with other tissue culture chemicals as the aldehyde fumes can interact with and destroy many expensive chemicals. All fixatives and vials containing tissues should be housed in their own refrigerator. For adequate fixation, approximately 10 volumes of fixative should be used for 1 volume of tissue. It is important to remember that there is no universal formula for the fixation of biological specimens. High-quality fixation depends on trial and error.

2. The explants, embryos, or other structures are collected from culture vessels. The appropriate part is carefully excised and trimmed to the desired orientation with a sharp, double-edge razor blade. Whenever possible, the explants are sliced into 2–3-mm-thick slices. This ensures proper fixation and infiltration of the embedding medium. This step should be done gently to avoid physical damage due to handling. 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 even in water and quickly transferred into vials containing the fixative. The tissues are fixed at room temperature for 2–4 h before their transfer to 4° C overnight. The total fixation time should be no more than 24 h. Overfixation can render the tissue extremely hard to section.

3. Most botanical specimens require a vacuum infiltration step during fixation because of the presence of air within the tissues. This step can be carried out at the end of the fixation step. Place the vials in a small ice bath, such as a beaker of ice, and evacuate the specimens. When no more air bubbles appear, the specimens can be removed from the vacuum chamber. Before using the vacuum and the vacuum chamber, be sure to understand the operating procedures of such devices. Also confirm that the pump and chamber are compatible with one another. The chamber has to be able to withstand the negative pressure generated by the vacuum pump.

4. After fixation, the specimen is dehydrated with methyl cellosolve followed by two changes of absolute ethanol. The duration of dehydration depends on the size of the specimen. Dehydration should take place at 4° C to minimize extraction of macromolecules. After the completion of dehydration, if the specimen will not be processed immediately, it can be stored in the freezer and used at a later date.

5. The Technovit/Historesin embedding kit consists of three components. One is the basic resin, a glycol methacrylate monomer containing Co catalyst XCl. The second is the activator (benzoyl peroxide powder, moistened with 20% H_2O), supplied in packets of 0.5 g. The third is the hardener (the accelerator solution containing a derivative of barbituric acid and dimethyl sulfoxide). Before the specimen is infiltrated, one must prepare the infiltration solution by dissolving one packet (0.5 g) of activator (component 2) in 50 ml of basic resin (component 1). After the powder is dissolved by gentle stirring with a magnetic stirrer at room temperature, the solution should be kept in a freezer (−20° C). It is essential to allow the bottle to warm to room temperature before its use in order to prevent condensation of water vapor from the air.

To ensure even penetration, three intermediate infiltration solutions are used (2:1, 1:1, 1:2, ethanol:infiltration solution) before transfer to the pure infiltration solution. Again, the duration of infiltration depends on the size and the density of the specimens. For dense specimens such as seed materials, a longer infiltration time is necessary. Normally a 24-h period is sufficient in each of the intermediate solutions. To further ensure proper infiltration of the specimen, a vacuum step in pure infiltration solution is advised. After that, the infiltration solution is changed once more and the tissue blocks are allowed to remain in it for a few d at 4° C before embedding. The entire process can be sped up if the vials containing the samples can be placed in a rotator housed in a refrigerator.

6. Just before embedding, the desired embedding molding cup trays should be selected. Plastic molding cups of different sizes can be purchased from Polysciences (Warrington, PA), Electron Microscopy Sciences (Fort Washington, PA) and Kulzer (Wehrheim, Germany). We routinely use the molding tray with the well size of 6 × 12 × 5 mm.

For embedding, the well is first filled with the infiltration solution. The tissue is then placed in the well. This is to avoid the trapping

of air. More then one piece of tissue or embryo can be added to the same well. Once the samples are in place, the embedding solution is prepared. This is done by mixing 15 ml of infiltration solution with 1 ml of the hardener (component 3). To increase the "stickiness" of the section, 0.6 ml of polyethylene glycol 400 can be added to 15 ml of the embedding medium (Yeung and Law, 1987). This solution should be used immediately because polymerization begins as soon as it is prepared.

Quickly remove most of the infiltration solution from the tissue-containing wells. A small quantity of the embedding solution is added to each well and then immediately removed. This serves to rinse the tissue within the well. After the rinsing of tissues, the embedding solution is added so that it fills the wells close to their rim. The orientation of the tissues within the wells should be checked before addition of the plastic block holders. It is desirable to move the specimens to the center of the well. This will facilitate ribbon formation later. The specimens within the well can be moved with a toothpick. Once the tissues are properly arranged within the well, a plastic block holder is placed gently on top of each well to exclude air from the surface of the mold. Air will interfere with the polymerization process. These steps are repeated until all of the wells are processed. The entire tray is left on the bench for at least 2 h, by which time the embedding solution should be polymerized. Care should be taken when handling the infiltration and embedding solutions as the components can be toxic.

The polymerized blocks should be kept in the mold until they are ready to be sectioned. It is better to section the blocks as soon as possible, as it is easier to form ribbons. If the relative humidity is high, it is necessary to store the entire mold in a desiccator, since the absorption of moisture can turn the plastic into rubbery blocks which are impossible to section.

7. Sections can be obtained readily with a Ralph type glass knife (Bennett et al., 1976) and a microtome with a retractable return stroke such as the Leica 2040 and the Zeiss HM 360 microtomes. The long ribbon produced can be cut into 4-cm-long pieces. Occasionally, when ribbon fails to form, a small amount of liquid PEG 400 can be painted on the top and bottom edges of the block to aid in the formation of ribbon. The ribbon can be stretched in water on a regular glass slide. The slides are then allowed to dry using a slide warmer at 50° C or at room temperature. If the slides are very clean, such as the J. Melvin Freed Brand microscope slides (Cat. No. 301M) obtained from VWR Canlab, (Mississauga, Ontario, Canada), slides can be used without the need of any treatment. Sections will not detach from clean slides. However, many so called "pre-cleaned" slides are still quite dusty and sometimes feel greasy. In this case, these new slides can be cleaned by soaking overnight in a 70% ethyl alcohol 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 in a dust-free area to dry. The adhesive solution is prepared by dissolving 5.0 g gelatin in 1 liter of warm distilled water and adding 0.5 g chrome alum (chromium potassium sulfate). After the solution has cooled, it is filtered through Whatman no. 1 filter paper. This solution may be stored at 4° C for up to 48 h, but should be discarded after that time (Pappas, 1971). Further information on handling sections can be obtained in the papers by Bennett et al. (1976) and Yeung and Law (1987).

8. One of the advantages of the GMA-type embedding medium is that the plastic does not interfere with the staining procedure and therefore its removal is not necessary. In general, slides are stained with an appropriate stain, rinsed in distilled water, dried, and mounted. For detailed staining recipes, consult Feder and O'Brien (1968), O'Brien and McCully (1981), and Yeung (1984). For the staining of lipid using Technovit 7100, consult van Goor et al. (1986) for tissue processing and staining.

In general, we usually stain one-third of the slides with the periodic acid-Schiff (PAS)–toluidine blue O schedule, and one-third using the PAS–protein stain; we leave one-third of the slides unstained. The unstained slides can be used to check for other substances such as callose by other staining methods after the examination of the stained ones.

The cost of coverglasses continues to escalate. One of the nice features of the GMA embedding medium is that you can examine the slides without the need of coverglasses. Therefore, if resources are a problem, coverglasses can be applied to selected slides after a careful screening of all the slides for that project. Usually, a coverglass should be number 1.5 thickness, as a majority of objectives are designed to work with this thickness. Be sure to store all of the slides in slide boxes and label them properly for future reference.

Acknowledgments

I am grateful to the Natural Sciences and Engineering Research Council of Canada for the continuous funding of my research program. Thanks also to my teachers, colleagues, and students for their advice, suggestions, criticisms, and questions, all of which have made me a better biologist.

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