Edward Chee Tak Yeung · Claudio Stasolla
Michael John Sumner · Bing Quan Huang
Editors

Plant Microtechniques and Protocols

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Preface

Old and new techniques are essential for any research program. New instrumentation, techniques, methodologies, and ideas continue to appear in the literature. Some will have a profound influence on our future research activities. At present, many traditional methods are still routinely in use in many laboratories, and simple methods such as hand sections and related techniques are extremely useful in botanical research. It is unfortunate, however, that many traditional methods are not being taught. Students often lack a clear understanding of the methods used and, therefore, cannot take full advantage of the different microtechniques available for their experimental studies. The usefulness of many histological methods is that by providing basic structural information, they help in generating questions and hypotheses needed to advance research.

The purpose of putting together a volume related to plant microtechniques is to gather the commonly used methods and update their procedures using a simple and fully understandable approach. Although, many similar monographs have been published in the past, unfortunately, the majority of them are out of print. We hope this book can serve as a handy resource for scientists familiar with the protocols, and as a guide for the novices, especially students just beginning to learn about various structural methods for the first time.

In terms of organization, it is not possible to include all methods in a single volume. Many related techniques including those used for the study of animal biology, can be found in the Protocol Book Series. Readers are urged to look for specific methods by checking the "Protocol" website from Springer (www.springerprotocol.com). In the first section of this volume, we have selected the more commonly used embedding methods, with emphasis on the preparative methods for light and electron microscopy. A number of cell biology-related protocols are compiled in Sect. 2 to showcase the usefulness of various techniques based on different processing and staining methods. Section 3 highlights some common and recent procedures in wood preparation. The last section includes botanical methods related to archaeological uses of plant materials. A special chapter on field and herbarium procedures is also included to serve as a guide for students interested in plant collection and taxonomic studies. It is our aim to include a range of topics in order to generate cross

talks among scientists in different research disciplines. We realize that the methods selected are incomplete and hope to update and include new methods in the future.

We would like to thank Mr. Douglas Durnin for his careful proofreading of manuscripts and Mr. Colin Chan for his help in editing the figures and graphics. Finally, we are grateful to all authors for their contributions to this book and their patience and cooperation during the course of preparation and editing.

Edward Chee Tak Yeung
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Part I
Fixation, Processing, Embedding and Staining of Botanical Specimens

Chapter 1
A Guide to the Study of Plant Structure with Emphasis on Living Specimens

Edward C. Yeung

1.1 Introduction

Morphological and anatomical investigations are essential to the study of the biology of an organism. Information gathered provides background knowledge of the experimental system and allows the generation of further questions and/or hypotheses. Many feel that morphological and histological studies are just simple observations with subjective interpretations. On the contrary, with proper designs, one can answer important questions about the experimental system, and at a minimum, the information can augment other experimental designs. To be successful in any histological and histochemical investigations, one needs to think in combined terms of structure, physiology, cell biology, and biochemistry [1, 2]. Be creative and think "outside the box" when examining a preparation. Morphological and anatomical studies are highly "dynamic", rewarding, and invaluable to any research program.

A large number of techniques and protocols are currently available to study macroscopic and anatomical features of the plant body. Many techniques that are currently used were developed more than a century ago. The classical methods such as the paraffin embedding method have proven to be reliable and are still being used. Moreover, new techniques and protocols continue to be developed, which further our understanding of the cellular and histological organization of the plant body and provide new insight into the cellular and biochemical processes. For example, confocal microscopy had become very popular in past decades and it has contributed significantly in many areas of cell biological studies (for methods and protocols, *see* refs. [3, 4]). New cell imaging techniques such as matrix-assisted laser desorption/ionization (MALDI) imaging [5] and atomic force microscopy [6]

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provide new approaches to study biological specimens. In a recent issue of the journal *Protoplasma*, new emerging techniques in light and electron microscopy are discussed [7]. It is important that we continue to keep abreast of current development and test new methods whenever possible.

The purpose of this chapter is to provide introductive approaches and simple methods used in the study of botanical specimens. Some of the procedures have been documented in earlier publications [8, 9]. This chapter serves to provide an overview as well as a guide, especially to students who plan to study their experimental system using a morphological and anatomical approach for the first time.

1.2 Approaches Used in the Study of Botanical Specimens

At present, a number of books focusing on different aspects of botanical microtechniques are available [10–17]. Furthermore, protocols have been published on the Springer website and are useful sources for a variety of techniques. Readers are urged to consult the monographs cited and the Springer website for further details and additional methods.

To be successful in applying any technique, it is essential that we have a proper theoretical understanding of the process used in a protocol. One should consult the original literature dealing with a specific method whenever possible. Furthermore, protocols are not formulae; often, one has to modify an existing protocol to suite one's need. To make a successful adaptation, it is essential to have a proper understanding of every step of a protocol, irrespective of how seemingly simple it appears. Although many protocols will give results, it is important to note that the mechanisms involved in some of the techniques are not well understood. Hence, we have to interpret the results with care and within the parameters of the method.

How to look at a botanical preparation? The answer is to ask questions when examining a specimen. Asking questions focuses one's attention on a problem. As an example, in the study of *Arabidopsis* reproductive development, one often looks for morphological changes associated with a treatment or a mutation. Are there notable changes associated with a treatment or a mutation? For the initial observation, one can look at inflorescence morphology, flower number, abnormality in flower development, presence of pollen abortion, changes in silique number, number of seeds within a silique, and seed germination characteristics. Answers to the above questions through macroscopic examinations provide clues for subsequent experiments.

In carrying out a procedure, it is important to remember that it takes time to learn and perfect a technique. Never underestimate the time needed for a histological study. To deliver quality results, one needs to aim for perfection, for example, presenting sections with the appropriate plane of section and at a specific stage of development. Although this can be time consuming, perfect macroscopic photographs or micrographs give readers the confidence of the work presented.

There are different approaches and methods to study biological specimens. The following section provides an overview of the methods used, emphasizing the study

of living botanical specimens and explants. This is followed by protocols of selected methods. Additional methods and protocols on fixation, embedding, and staining can be found in subsequent chapters of this book.

1.2.1 Direct Macroscopic Examinations

Direct examination of the experimental material is simple and yet a powerful approach in determining how plants or in vitro explants respond to the experimental treatment. This simple approach tends to be overlooked. Direct observation does not require elaborate equipment. As described earlier [9], one can obtain information simply by carefully examining a specimen using a hand lens, a stereomicroscope, or simple methods such as freehand sections and related techniques with the help of a light microscope. The information can be recorded using a personal digital camera, if necessary. It is important to stress that successes of many microtechniques hinge on the proper use of a light microscope. Direct and careful examination of the specimen should always be the first step in any experimental design.

1.2.2 Hand Sections

After the initial observation, additional simple methods can be used to study the system further. Owing to the firmness of the plant material, hand sections can be prepared to study gross structural changes if any, during the course of the experiment [2, 11, 15, 16, 18]. Sections can also be obtained using a hand-held or a sliding microtome if available. These quick sectioning methods allow one to examine the specimen in a few minutes [e.g., ref. 19]. Sectioning of smaller explants is possible but requires some practice. Patience and practice are key factors in obtaining good-quality hand sections. The added advantage of freehand sections is that fixation of specimen is usually not required. Chemical fixation and subsequent processing usually lead to extraction of some chemical components and pigments from the plant material and this generates artifacts (*see* Chap. 2).

Besides providing a quick assessment of the anatomical organization of the specimen, hand sections may also provide the histochemical determination of certain cell inclusions such as starch and wall components, for example, lignin and suberin. If a fluorescence microscope is available, the autofluorescence characteristics can be studied. Chlorophyll and phenolic compounds will autofluoresce when present. The autofluorescence characteristics of living cells can be analyzed further to determine the chemical nature of the compounds [20] when study using confocal laser scanning microscopy and the information obtained can also aid cell–cell contacts and cell interaction studies [21]. In addition to the study of the primary fluorescence, the specimen can also be stained using fluorochromes which can further our understanding of plant cell biology. In addition, the birefringent components within the plant tissue can be viewed using a polarizing microscope.

Hand sections can be ideal for the histochemical localization of enzymes. Enzyme histochemistry provides a link between morphology and biochemistry [22]. The results provide information on metabolic activities of cells and tissues. Publications are available in the literature providing details on various enzymatic staining protocols [22–24]. A study by Vreugdenhil and Sergeeva [25] clearly demonstrates the usefulness of fresh sections in the investigation of carbohydrate metabolism in plant tissues.

1.2.3 Whole-Mount Staining, Clearing, and Maceration Methods

Instead using hand sections, whole tissues can be stained to gain a better perspective on the three-dimensional distribution of the subject of interest. One of the most commonly used enzyme histochemical stains is the localization of the β-glucuronidase (GUS) activity in transgenic plants. To study the regulation of gene expression, one of the strategies is to fuse an isolated promoter of the gene of interest to the *GUS* gene of *Escherichia coli*. *GUS* serves as a reporter gene and will be expressed if the gene of interest is activated. If GUS is present, it will hydrolyze the substrate [5-bromo-4-chloro-3-indole β-D-glucuronide (X-gluc)] and will produce a blue colored product through the oxidation and dimerization of the primary reaction product, 5-br-4-Cl-3-indolyl. The presence of the blue precipitate indicates the location of the enzyme (*see*, e.g., ref. [26]). Since the introduction of this method by Jefferson et al. [27], different protocols have been published [e.g., 27–34]. Kim et al. [33] and Vitha [34] provide a careful evaluation and discussion of the GUS staining procedures.

Other techniques that can be performed readily to augment hand section studies are clearing and maceration procedures. Clearing techniques aim to render the specimen translucent through the removal of cytoplasmic content and further treatment using reagents with a high refractive index renders the tissues translucent, having uniform refractive properties. The cellular details can be discerned with the help of a phase contrast or differential interference contrast microscope, as these optics can intensify small refractive differences of organelles and other cellular components, allowing for their identification [35, 36]. Depending on the objective of the experiment, if one aims at studying the cell wall features and vascular tissue arrangement, he or she can aim to remove the protoplasmic content of cells as much as possible. Direct treatment of specimens with sodium hydroxide solution, lactic acid, or lactic acid saturated with chloral hydrate may be sufficient. However, if one wishes to study the internal cellular organization of structure, such as ovules and embryo sac, the tissues would need to be fixed briefly using a fixative such as FPA (formalin, propionic acid, and ethanol), precleared with lactic acid saturated with chloral hydrate followed by solvents that can render the tissues translucent such as the 4½ or the BB4½ clearing fluid [33, *see Note 9*]. Lux et al. ([37], also *see* Chap. 10) detailed a protocol that further enhances the image quality of hand sections by clearing.

Chemical maceration and enzymatic methods of cell separation primarily weaken the middle lamella between cells, enable the cells to separate from one another, thereby allowing the identification of cell types and the size and shape of cells to be determined. Maceration procedures can be carried out using fresh and certain dry specimens and have been proven useful in archeological and ethnobotanical research [38]. Enzymatic cell separation using wall digestive enzymes allows the study of living cells when properly prepared and aseptically cultured (*see* Chaps 11 and 12). A maceration method is detailed in Sect. 1.4.8. Additional methods are documented by Berlyn and Miksche [10]. Although all these methods can be considered as classical, they are still extremely useful in providing information in a botanical investigation.

1.2.4 Different Embedding Methods

There are limitations of the hand sectioning methods and the aforementioned procedures. This is primarily due to the relatively low resolution power of each method. For example, hand sections are thick and cannot provide clear resolution of cellular content. For tiny and soft objects, such as root tips, good-quality hand sections are difficult to obtain. As a result, tissues need to be fixed and different embedding methods need to be used to study detailed histological and histochemical changes of a specimen. Tissue processing and embedding methods are detailed in subsequent chapters. It is important to note that each embedding method has its own merit and it is essential to have a good understanding of the pros and cons of each embedding medium before selecting an appropriate embedding method to study the system further.

1.2.5 Staining of Specimens

A majority of plant tissues have little color; hence, little contrast is present to show surface and internal features. One way to increase the contrast of a specimen is through staining. Furthermore, proper selection of staining methods can provide specific histochemical information and define cellular components clearly. Staining theories and mechanisms of staining are detailed by Horobin [39] and Horobin and Kiernan [40], and many staining methods are available in the literature [*see* 10–12, 14]. One has to have a clear understanding of the theory of a selected protocol before applying it to one's own studies. Furthermore, the fixation and embedding methods used can influence the staining outcome; it is important to understand the limitation of the methods used before drawing conclusion from staining observations.

In the following section, protocols for macroscopic examination, hand sectioning procedure, clearing and maceration methods, and a GUS staining protocol are detailed. This section is an updated version of earlier publications [8, 9].

1.3 Materials

1.3.1 Equipment and Supplies for Macroscopic Observation

1. Hand lens and/or a good quality stereomicroscope preferable with a digital camera attached (*see Note 1*).
2. Lighting accessories: Ring light that attaches directly to the lens of the stereomicroscope, gooseneck fiber optics (*see Note 2*).
3. Digital camera: Commercial hand-held digital camera with macro-function, digital cameras designed for photomicrography, and camera accessories such as tripods can be useful (*see Note 3*).
4. Dissecting tools: Different instruments can be used as dissecting tools depending on the size and the nature of the specimen. In general, fine forceps, dissecting knives, razor blades, needles, and scissors are useful (*see Note 4*).
5. Black agar plates: Add approximately 4 % (w:v) charcoal powder (e.g., Sigma cat. no. 161551) to a 2 % agar solution before solidification (*see Note 5*).
6. A hair dryer to remove condensation on the surface of culture plates and containers.

1.3.2 Equipment and Supplies for Freehand Sections, Clearing, Maceration Techniques, and GUS Staining

1. General laboratory equipment: incubating ovens, −20 °C freezer, balance, pH meter
2. General laboratory supplies: double-edged razor blades, small paint brushes, section lifter, dissecting needles, slides and coverslips, watch glass or the lid of Petri dishes, vials, absorbing paper, disposable pipettes, dropper bottles for staining solution, and large wash bottles

1.3.3 Chemical Reagents and Solutions for Freehand Sections, Clearing, Maceration Techniques, and GUS Staining

1.3.3.1 Staining Solutions for Freehand Sections

1. Toluidine blue O (TBO) staining solution: Dissolve 50 mg TBO in 100 mL 0.05 M citrate buffer, pH 4.0 (*see Note 6*).
2. Phloroglucinol-hydrochloric acid (HCl) staining solution: There are various procedures to prepare the staining solution but commonly it is prepared as a saturated

solution of phloroglucinol in 20 % HCl. *Be sure to handle the solution with care. Wear gloves during solution preparation and prepare this solution in the fume hood*. First dissolve phloroglucinol (2.0 g) in 80 mL of 20 % ethanol solution and then slowly add 20 mL of concentrated HCl (12 N) to it (*see Note 7*).

3. Iodine-potassium iodide (IKI) staining solution: First dissolve 2 g of KI in 100 mL of distilled water, and add 0.2 g of iodine into the KI solution. This solution should be prepared ahead of time as it takes time for iodine to dissolve completely. Iodine sublimates at room temperature; hence, cap the bottle tightly and store in the dark.
4. Sudan dyes: Prepare a saturated solution of Sudan III, Sudan IV, or Sudan Black B in 70 % ethanol. Filter before use (*see Note 8*).

1.3.3.2 Solutions for the Clearing Procedures

1. A 4 % sodium hydroxide solution
2. Methyl cellosolve (2-methoxyethanol)
3. Safranin staining solution: safranin, basic fuschin, and crystal violet—0.5, 0.2, 0.2 % (w:v), respectively, in 50 % alcohol
4. Ethanol series (50, 70, 85, and 95 % in distilled water, and 100 % ethanol)
5. Xylene reagent for making permanent mount of specimen: 100 % ethanol: xylene (1:1, v:v), xylene
6. For additional formulations of clearing solutions, *see Note 9*

1.3.3.3 Solutions for Maceration Procedures

1. The maceration fluid is prepared by combining one part of a 30 % solution of hydrogen peroxide, four parts of distilled water, and five parts of glacial acetic acid. Be sure to use a clean glass bottle and prepare this solution in the fume hood just before use. Avoid contact with the solution, wear gloves if necessary.
2. The Haupt's adhesive: Dissolve 1 g gelatin in 100 mL of water at 90 °C. Cool this mixture to room temperature, and add 15 mL of glycerol. Two grams of phenol may be added as a preservative if one intends to keep the adhesive for a few weeks.
3. TBO staining solution for maceration: Dissolve 0.1 g per 100 mL water.
4. Ethanol series for dehydration and xylene as a clearing agent for permanent mount.

1.3.3.4 The GUS Staining Solution

1. GUS staining buffer: 100 mM $NaH_2PO_4 \cdot H_2O$, 10 mM $Na_2EDTA \cdot 2H_2O$, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and Triton X-100 (1 mL/L). Adjust to pH 7.0 with NaOH (*see Note 10*).

2. X-Gluc (5-bromo-4-chloro-3-indole glucuronide, Paul Gold or other suppliers) stock solution: Dissolve 40 mg/mL of X-Gluc in *N*, *N*-dimethyl formamide (Sigma), aliquot into 100 µL portion, and store at −80 °C freezer (*see Note 11*).
3. GUS staining solution: Prepare a 10 µL X-Gluc stock/mL of GUS staining buffer just prior to use.
4. Paraformaldehyde (PFA) solutions: First prepare a 10 % stock solution of PFA by adding 10 g of PFA powder into a beaker containing 70 mL distilled water at 60 °C in which a few drops of 1 N KOH have been added. The solution is stirred continuously with heat to dissolve the powder. After about 5 min, the solution should be clear with a few undissolved particles. The final volume of the solution is adjusted to make a 10 % stock solution. To prepare a 1 % PFA in a 0.05 M phosphate buffer, mix 10 mL of the 10 % PFA stock with 50 mL 0.1 M phosphate buffer and 40 mL distilled water. The stock PFA solution and the fixative should be prepared inside a fume hood.

1.3.3.5 Mounting Solutions for Temporary or Permanent Slide Preparations

1. Temporary mounting solutions: A 30–50 % glycerol solution in water or in a buffer such as phosphate buffer and the slide can be sealed using nail polish if needed.
2. Permanent mounting solutions: Xylene- or toluene-based synthetic mounting medium such as the Acrytol® mounting medium (Electron Microscopy Sciences) (*see Note 12*).

1.4 Methods

1.4.1 Photography of Large Specimens

1. Plant materials such as an entire small plant, for example, *Arabidopsis* or large explants from in vitro cultures can be examined directly with an unaided eye or with the help of a stereomicroscope. The specimen can be photographed using a hand-held digital camera. The macro-function of the camera enables the capture of close-up features of interest.
2. Before photography, in order to obtain professional-looking photographs, it is useful to design a proper background using colored cardboards or cloths. Arrange the lighting equipment to highlight the point of interest and to avoid obvious shadow formation (*see Note 13*).
3. Label the plant material and indicate the treatment if necessary.
4. A scale such as a ruler should be photographed together with the object to indicate the size of the specimen.

5. After photographing the object, check the digital files at once to ensure the images are sharp, showing the desired features. Otherwise, retake the photos at once before storing or discarding the specimen.

1.4.2 Photography Through a Stereomicroscope

For a detailed examination of the object, careful study using a stereomicroscope is preferred. The “zoom” feature of a stereomicroscope easily allows the change of magnifications. The image can be captured using a digital camera directly attached to the stereomicroscope.

1. For in vitro culture explants, one can examine the explants without removing them from the culture vessels. Using this method, one can follow the developmental changes through the course of the experiment without disturbing the in vitro culture environment. Photographs of the specimens can be taken directly through Petri plates or culture vessels. Condensation often occurs on the lid of a Petri plate impeding observation. It is possible to remove the condensation by using a hair dryer to evaporate the condensate from the surface of the Petri dish or culture vessel just prior to photography.
2. For better close-up observations, especially for smaller specimens such as floral parts or small in vitro culture explants, it is advisable to take a closer look without the obstruction of the Petri plate or the container. Selected explants can be removed and placed directly on a black agar plate. The black agar plate provides a better contrast for the specimen. It also prevents the specimen from drying during examination. Since agar is soft, the specimen can be partially submerged to allow for a sharper focus of the surface features.
3. A small scale bar should be placed close to the specimen to provide information about the size of the specimen. It can be created by photocopying a ruler, cut off a centimeter portion of the image and place next to the specimen.
4. Proper lighting is important as it provides optimum light intensity as well as creating shadowing effects, highlighting the structures of interest. Ring light provides an even illumination over the entire specimen when examining the specimen using a stereomicroscope. The positioning of the gooseneck arms and/or any other lights can create shadows and provides a better contrast of the specimen. Try different positions by moving the gooseneck arms or other light sources to obtain the best desirable image that showcases the feature of interest.

1.4.3 Photomicrography Through a Light Microscope

For more detailed studies of living cells, small cell aggregates, and hand sections, a compound light microscope is preferred as it has a better resolving power than a stereomicroscope.

1. The specimens can be examined without staining. Transfer a very small amount of material or a few hand sections to a slide and surround the cells or sections with liquid medium or water. Quickly place a coverslip over it. It is imperative that no liquid should overflow from the slide. The excess liquid can corrode the microscope stage and the condenser.
2. Unstained specimens can be examined using phase contrast or interference contrast optics, if available. Close down the iris diaphragm to enhance the contrast for better viewing.
3. If the microscope is equipped with a fluorescent lighting system, the autofluorescence features of the specimens can be studied.
4. Specimens can also be stained before viewing. Small cell aggregates and hand sections can be stained with different stains, rinsed and mounted as before (*see* Sect. 1.4.5 for more details). Again, besides the staining protocols listed in Sect. 1.4.5 a number of fluorescent stains are available and are suitable for the staining of hand sections (*see* Chap. 9).
5. Capture images with a digital camera. Be sure to record the information, especially the objective used in capturing the image.
6. The exact size of the object can be determined using a calibrated eyepiece micrometer or photographs of a stage micrometer can be taken for each objective from the photomicroscope and then use them to calibrate the size of the object of interest.

1.4.4 Freehand Sections

1. Obtain a new double-edged razor blade. For students, in order to minimize the risk of cutting oneself, cover one edge of the razor blade with masking tape (Fig. 1.1a, *see Note 14*). Rinse the blade with warm tap water to remove traces of grease from the surface of the blade if necessary.
2. Hold the plant material firmly but not too tight. The material should be held against the side of the first finger of the left hand (or right hand) by the thumb. The first finger should be kept as straight as possible, while the thumb is kept well below the surface of the material out of the way of the razor edge. For good quality transverse sections, the plant material should be held at right angle to the first finger (Fig. 1.1b). As long as the sections are not cut obliquely, "thick" sections are useful.
3. Flood the razor blade with water. This will reduce the friction during cutting as sections can float onto the surface of the blade. Take the razor blade in the right hand (or left hand) and place it on the first finger of the left hand (or right hand), at a right angle to the specimen (Fig. 1.1c). Hold the razor at the edge and not too tightly.
4. Draw the razor across the top of the material in such a way as to give the material a drawing cut (about 45 ° in the horizontal direction). This results in less friction as the razor blade passes through the specimen (Fig. 1.1d). As the razor is sharp,

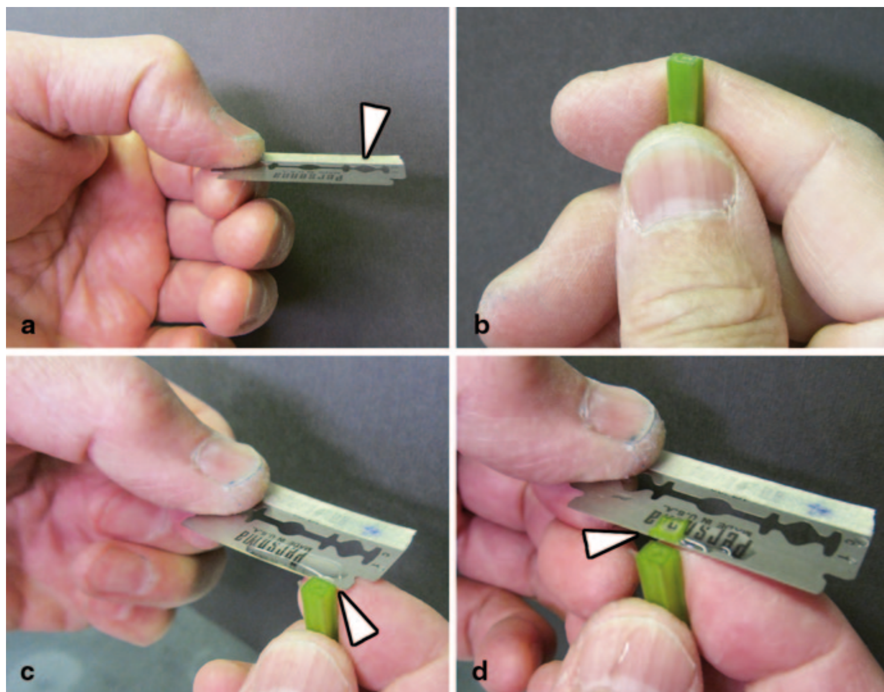

Fig. 1.1 Freehand sectioning procedure. **a** One side of the double-edged razor blade is covered by masking tape *(arrowhead)* to avoid accidentally cutting of one's finger. **b** In order to ensure quality transverse sections, the specimen must be held by the thumb, at right angle to the first finger. **c** The razor blade and the specimen should be wet to reduce friction during sectioning. The specimen is position at one end of the blade as indicated by the *arrowhead*. **d** A section is made by a drawing cut and it should appear at the other end of the razor blade *(arrowhead)*

simply allow the razor blade to glide over the surface of the specimen. Cut several sections at a time. Gently squeeze the thumb against the specimen; this will gently push the specimen upward. Continue to make a few more sections. Sections will certainly vary in thickness. However, there will be usable ones among the "thick" sections (*see Note 15*).

5. Transfer sections to a watch glass or the lid of a Petri dish containing water, always using a brush or a section lifter, not a forceps or needle.
6. Gently wash the sections by moving them around in water, using a brush. This is to remove phenolic compounds and slimy materials as they will interfere with subsequent staining procedures.
7. Select and transfer the thinnest sections (the more transparent ones) onto another watch glass/Petri dish and stain (*see* Sect. 1.4.5).
8. For delicate and hard to hold specimens, such as thin leaves and tiny roots, additional support can be used to facilitate hand sectioning. Place or insert the specimen into or between pith slices such as small pieces of carrot. Once the tissue is firmly in place, the hand sectioning technique can be applied (*see Note 16*).

1.4.5 Simple Histological and Histochemical Staining Methods

1.4.5.1 Toluidine blue O Staining of Hand Sections

TBO is the best stain for fresh botanical specimens, especially hand sections. It is a cationic, polychromatic dye that reacts differently to different chemical components of cells and results in a multicolored specimen. TBO will react with carboxylated polysaccharides such as pectic acid to give a purplish color, greenish blue or bright blue with polyphenolic substances such as lignin and tannins, and purplish or greenish blue with nucleic acids [for details, *see* ref. 39]. Hence, the colors generated can provide information on the nature of the cell and its walls. General expected results: pectin will be red or reddish purple; lignin, blue; other phenolic compounds, green to blue-green. Thin-walled parenchyma will be reddish purple; cells with lignified secondary walls usually appear blue; sieve tubes and companion cells, purple; middle lamella, red to reddish purple; callose and starch, unstained [41].

1. Transfer the suspension culture to a small container such as a small test tube. Remove excess culture medium using a disposable pipette with a fine tip. Apply the TBO stain to the cells and stains for 1–2 min. Gently remove the stain and wash the cells two times with water. Transfer the cells directly onto a clean slide, add a few drops of water as mountant, apply a coverslip, and examine with a light microscope.
2. For freehand sections, apply the TBO stain directly to the sections for 1–2 min. Gently remove the stain by using a fine tip pipette or a piece of filter paper. Wash the sections by flooding them with water followed by its removal. Repeat until there is no excess stain around the sections. Transfer the sections to slides, add a drop of clean water over the sections, and apply a coverslip. The slide is ready for examination.

1.4.5.2 Phloroglucinol-HCl Test for Lignin

Lignin is a common constituent in plant cell walls, especially the secondary walls. The cinnamaldehyde end groups of lignin appear to react with phloroglucinol-HCl to give a red-violet color [12]. Different forms of lignin give different staining intensity toward the phloroglucinol stain. Because of the ease of staining, this is the most common procedure to test for the presence of lignin in plant cell wall. General expected results: Lignified walls become red.

1. Transfer cells, cell clusters, or hand sections directly onto a slide.
2. Apply one or two drops of stain over the plant material and stain for at least 2 min.
3. Since the staining intensity varies and the color fades gradually, it is advisable to examine the specimen without removing the stain. Gently apply a coverslip over the specimen without washing. Examine the specimen at once (*see Note 17*).

1.4.5.3 Starch: Iodine-Potassium Iodide Test

The iodine-potassium iodide (IKI) stain is specific for starch. The length of the starch molecule determines the color of the reaction—the shorter the molecule, the more red the color; the longer the molecule, the more deep blue the color [12]. General expected results: starches will give a deep blue color in a few minutes. If the staining intensity is too high, it is advisable to dilute the stain. The color will fade over time.

1. Stain sections by applying a few drops of IKI solution directly on the sections.
2. Wait for 2 min, rinse sections in water, and transfer the sections to a slide with a brush. Apply a coverslip and examine with a microscope. Small blue-black bodies indicate the presence of starch.

1.4.5.4 Total Lipid: Sudan Dyes

The mechanism of staining is based on differential solubility. The Sudan dyes are more soluble in apolar solvents. As a result, they tend to dissolve more in structures such as the cuticle, lipid droplets, or suberin which are all hydrophobic substances. Different methods are available in preparing the Sudan dyes [2, 12]. General expected results: For Sudan III and IV, fats and oils will stain orange to red. Leaf cuticle, suberized walls of cork cells, and the Casparian strip, if present, will give a positive reaction. For Sudan black B, lipidic substances appear gray to black.

1. Apply the Sudan staining solution onto the cells or hand section in a small Petri dish. Stain the material for about 10–30 min.
3. Rinse the section in 50 % ethanol to remove excess stain.
4. Mount the section in 50 % glycerol and examine with a compound light microscope.

1.4.6 GUS Staining of Plant Specimens

In order to minimize diffusion of enzyme during staining and generating staining artifacts, the tissue is briefly fixed, followed by washing and staining. However, for preliminary testing to determine the presence or absence of GUS activity, the tissues can be stained without the fixation step.

1. Prepare and ensure all necessary solutions and tools are present prior to collecting the samples.
2. Depending on the size and the purpose of the experiment, small seedlings and plant parts can be stained whole or the specimen is cut into small pieces, a few millimeter in size just prior to staining. In order to allow for the penetration of stain and prevent localization artifacts due to limited penetration of the substrate,

large stems and roots need to be cut into disks or longitudinal slices of various sizes. The final optimum size of a specimen for staining is determined by trial and error.

3. Prefix tissues at once in freshly prepared 1% PFA in 0.1 M phosphate buffer, pH 7 at 4 °C for 1 h (*see Note 18*).
4. Wash tissues twice in 0.1 M phosphate buffer (pH 7) for 5–10 min each, rinse three times with GUS staining buffer. All solutions are kept at 4 °C.
5. After rinsing, the explants are stained for GUS activity with the X-Gluc staining solution for 4–24 h. Owing to the presence of intercellular air spaces between cells and in order to facilitate the penetration of substrate into the tissues, the samples are subjected to a vacuuming step to remove air and aid in the infiltration of substrate. The samples can be kept in the vacuum chamber for 1–2 h before transferring to a 37 °C incubating oven. In general, staining should not be longer than 24 h.
6. Examine the samples during the course of staining. To stop the staining reaction, transfer and re-fix the samples in 4% PFA in 0.1 M phosphate buffer for 24 h to stabilize the structures. The samples can be stored in the fixative for subsequent viewing. It is advisable to transfer the samples to phosphate buffer during reexamination in order to minimize exposure to PFA fume.
7. To get a better view of the site of localization, samples can be dehydrated using an ethanol series. The dehydration steps will also aid in the removal of chlorophyll. The samples can be cleared by transferring first to ethanol: xylene (1:1) and pure xylene; mount and examine using a microscope (*see* Sect. 1.4.7) or can be processed for paraffin or glycol methacrylate embedding methods [25], also *see* Chaps 3 and 4 for details.

1.4.7 Clearing

The choice of the most suitable clearing method depends on the types of tissues, their pigmentation, size, and the objective of the experiment. The following is an example on preparing permanent preparations of cleared leaves for the study of vascular tissue distribution. Methyl cellosolve extracts chlorophyll readily from leaves and can be used as the first step in the clearing process. The entire procedure can be carried out in a suitable size glass vial.

1. Treat whole leaves or leaf segments with methyl cellosolve to extract pigments from the specimens at room temperature. Use 20× volume of methyl cellosolve to 1 volume of tissues. Place vials on a rotary mixer to facilitate pigment extraction. Replace methyl cellosolve if necessary (*see Note 19*).
2. Once the pigments have been extracted, replace methyl cellosolve with a 5% sodium hydroxide solution and place the vial in a 50 °C oven for 1–2 days. The volume of hydroxide solution should be at least 20× the volume of the leaf tissue. For large, thick leaves, it is advisable to change the sodium hydroxide solution at least once and leave them in the oven for 1 or 2 more days. The specimens

should be slightly translucent. The timing for each step depends on the size and thickness of leaves.
3. Remove the sodium hydroxide solution and gently rinse the specimens with several changes of water. Place specimens into 50 % ethanol for 30 min prior to staining.
4. Stain the leaf tissue with the safranin staining solution for about 30 min.
5. Remove the stain and quickly dehydrate the sample using 70, 90, and 100 % ethanol and transfer into ethanol/xylene mixture and then xylene. Since the stains are highly soluble in ethanol, the dehydration steps also serve to destain the specimen. The lignified xylem elements will be red while the background should have less color. The timing for each step depends on trial and error. If staining is perceived as not sufficient, the specimen can be restained by passing it through the ethanol series back to the stain. After restaining, repeat the dehydration step. The samples can be stored in xylene and will appear transparent with "red" vascular strands.
6. For the final permanent mount, first apply a few drop of mounting medium to a slide, place the specimen on the mounting medium. Be careful not to trap air bubbles. Apply one or two more drops of mounting medium on top of the specimen before applying a "large" coverslip over it. This procedure prevents the trapping of air bubbles.
7. In order to flatten the specimen, a small weigh such as the aluminum tissue mounting stubs from Electron Microscopy Sciences (cat. No. 70145) can be placed over the coverslip gently. Allow to mounting medium to solidify on a slide warmer for at least 24–48 h before examination and storage (*see Note 20*).

1.4.8 Maceration

The following procedure is a chemical method that weakens the middle lamella allowing the cells to separate. A variety of plant tissues ranging from soft pith tissues to woody xylem samples can be studied using this technique; however, the duration of treatment will vary.

1.4.8.1 Procedure for Making Temporary Preparations

1. Cut plant tissues into small pieces and place into vials containing the maceration fluid. The volume of fluid required is approximately 10–15 × the volume of the tissue.
2. Cap tightly and place the vials in an oven at about 56 °C for 1–4 days. The duration of maceration depends on the nature of the material. For soft tissues, such as the sunflower stem pieces, 24 h is sufficient.
3. Maceration is judged to be complete if the tissues appear whitish in color. The tissue stays intact at this time. If the tissues remain yellowish brown or if the

maceration fluid is colored, add fresh maceration fluid and leave it for an additional 1–2 more days.

4. When maceration is complete, gently rinse the tissues in three changes of water (an hour between each change) in order to remove the acetic acid. Perform these steps in the fume hood.
5. To separate cells, shake the vial vigorously until the water becomes clouded with cells.
6. Apply a small drop of the mixture to a glass slide, cover it with a coverslip, and examine with a compound light microscope. Alternatively, one can stain the preparation by adding a drop of TBO to increase the contrast. Apply a coverslip, examine the specimen without the need to remove the stain (*see Note 21*).

1.4.8.2 Procedure for Making Permanent Preparations

Depending on the project, it may be convenient to have permanent preparations of macerated specimens.

1. Prepare macerated samples as detailed above. Apply a small drop of cells to a glass slide which has been freshly coated with the Haupt's gelatin adhesive.
2. Spread the cells evenly on the slide. Dry the slide on a slide warmer at about 40 °C for an hour.
3. The slide can then be stained in a 0.05–0.1 % TBO solution for 2–3 min.
4. Pour off excess stain and rinse very gently with distilled water by dipping the slide in a beaker of distilled water. Dry the slides using a slide warmer at about 40 °C. Some cells will be lost during water rinse.

Perform steps 5 and 6 in a fume hood to avoid inhalation of xylene fumes.

5. Dip the slides into 100 % ethanol for 10–15 s. Then dip the slides into ethanol/xylene (1:1 mixture) for 10–15 s. Rinse thoroughly with two changes of xylene.
6. Drain excess xylene from the slide and quickly apply a permanent mounting medium such as the Acrytol® mounting medium. Place the slides horizontally on a slide warmer or in trays and allow the slides to set for at least a few hours before examination. Any air bubbles trap within the coverslip will expand and move to the edge of the coverslip.

1.5 Notes

1. Depending on the available budget, instruments with different price ranges can be found. Information can be acquired through local suppliers or the World Wide Web. The magnification power and the quality of the objective lens determine the price of a stereomicroscope. The presence of a transmitted light base is a useful feature, but will add to its cost.