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

Plant Microtechniques and Protocols

Springer

Plant Microtechniques and Protocols

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

Plant Microtechniques and Protocols

Springer

Editors
Edward Chee Tak Yeung
Department of Biological Sciences
University of Calgary
Calgary
Alberta
Canada

Michael John Sumner
Department of Biological Sciences
University of Manitoba
Winnipeg
Manitoba
Canada

Claudio Stasolla
Department of Plant Sciences
University of Manitoba
Winnipeg
Manitoba
Canada

Bing Quan Huang
Center for Basic Research in Digestive Disease
Mayo Clinic
Rochester
Minnesota
USA

ISBN 978-3-319-19943-6 ISBN 978-3-319-19944-3 (eBook)
DOI 10.1007/978-3-319-19944-3

Library of Congress Control Number: 2015949269

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Science+Business Media (www.springer.com)

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
Claudio Stasolla
Michael John Sumner
Bing Quan Huang

Contents

Contributors

Ferhan Ayaydin Cellular Imaging Laboratory, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

Belay T. Ayele Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada

Michael G. Becker Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Shahanara Begum Faculty of Agriculture, Bangladesh Agricultural University, Mymensingh, Bangladesh

Mark F. Belmonte Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Alessandra Celant Department of Environmental Biology, Sapienza University, Rome, Italy

Colin K. W. Chan Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

C. C. Chinnappa Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Mei-Chu Chung Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, ROC

Simon D. X. Chuong Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Gemma Coccolini Department of Environmental Biology, Sapienza University, Rome, Italy

France Conciatori Centre for Forest Interdisciplinary Research (C-FIR), Departments of Biology/Environmental Studies and Sciences, University of Winnipeg, Winnipeg, Manitoba, Canada

Annie Deslauriers Département des Sciences Fondamentales, Université du Québec à Chicoutimi, Chicoutimi, Québec, Canada

Federico Di Rita Department of Environmental Biology, Sapienza University, Rome, Italy

Ryo Funada Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-Tokyo, Japan

Fengli Guo Stowers Institute for Medical Research, Kansas City, Missouri, USA

Subhash C. Hiremath Department of Applied Genetics, Karnataka University, Dharwad, Karnataka, India

Bing Quan Huang Center for Basic Research in Digestive Disease, Mayo Clinic, Rochester, Minnesota, USA

Peter Kitin Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, USA

Brian Kooyman Department of Anthropology and Archaeology, University of Calgary, Calgary, Alberta, Canada

Ján Kováč Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

Kayo Kudo Institute of Wood Technology, Akita Prefectural University, Noshiro-Akita, Japan

Soujanya Kuntam Cellular Imaging Laboratory, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

Yung-I Lee Biology Department, National Museum of Natural Science, Taichung, Taiwan, ROC

Eryuan Liang Key Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau Research, Academy of Sciences, Beijing, China

Mi Qi Liu Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Shiu-Cheung Lung Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

School of Biological Sciences, University of Hong Kong, Hong Kong SAR, China

Alexander Lux Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

Donatella Magri Department of Environmental Biology, Sapienza University, Rome, Italy

Jenna L. Millar Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Shalini Mukherjee Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada

Satoshi Nakaba Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-Tokyo, Japan

Widyanto Dwi Nugroho Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia

Sergio Rossi Département des Sciences Fondamentales, Université du Québec à Chicoutimi, Chicoutimi, Québec, Canada

Marcus A. Samuel Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Subramanian Sankaranarayanan Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Sarah Schoor Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Jose M. Seguí-Simarro COMAV-Universitat Politècnica de València, CPI, València, Spain

Masaki Shimamura Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Japan

Dustin Sigurdson Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Bonnie Smith Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Claudio Stasolla Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada

Francesca Romana Stasolla Department of Humanities, Sapienza University, Rome, Italy

Michael John Sumner Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Jacques C. Tardif Centre for Forest Interdisciplinary Research (C-FIR), Departments of Biology/Environmental Studies and Sciences, University of Winnipeg, Winnipeg, Manitoba, Canada

Marek Vaculík Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

Owen S. D. Wally Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada

Yusuke Yamagishi Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-Tokyo, Japan

Makoto Yanagisawa Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Edward C. Yeung Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Kelly Yeung Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

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]

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

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_1

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,

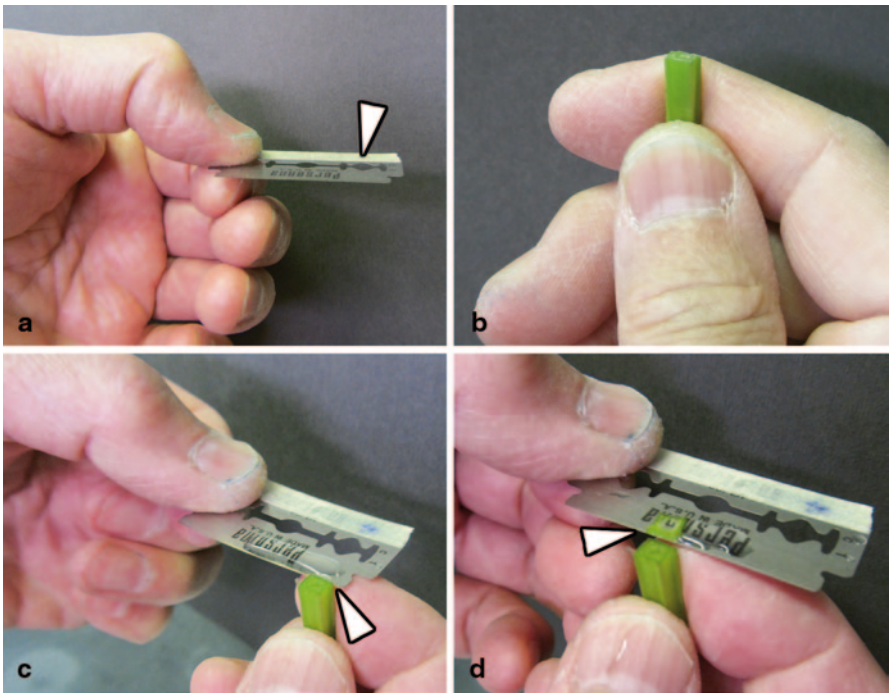

Fig. 1.1 Freehand sectioning procedure. **a** One side of the double-edged razor blade is covered by masking tape *(arrowhead)* to avoid accidentally cutting of one's finger. **b** In order to ensure quality transverse sections, the specimen must be held by the thumb, at right angle to the first finger. **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.

2. Proper illumination of the object is essential to quality photography. The new "mini-Light" from Electron Microscopy Sciences offers high light intensity with a small footprint that fits into most locations. A full range of devices can be found on the Electron Microscopy Sciences website (www.emsdiasum.com) for reference.
3. Digital cameras are now standard equipment and can be placed in the same light path as the microscope, using a trinocular head piece or by attaching the camera to one of the eye pieces. Scientific digital cameras are available from all major microscope vendors. There is a wide range of prices. Selection of a model is based on one's need and budget. The website from the Martin Microscope Company (www.martinmicroscope.com) provides useful information showing the different types of cameras available at a lower budget. If possible, test the camera with your microscope system before purchasing it to ensure compatibility. Software is usually purchased or included with the camera. It is important that the software be compatible with the computer system.
4. It is imperative that good quality dissecting tools are used such as fine forceps, microdissection knives and pins (*see* companies such as Fine Science Tools, www.finescience.ca, for selection). Quality dissecting tools prevent unnecessary physical damage to the explants during manipulation.
5. Keep plates in a refrigerator. They store well. Allow the plates to warm up before use. Excess water condensation can be removed using absorbent paper or a paper towel.
6. For freehand sections, if buffer solution is not available, dissolve 0.05% TBO in water and use it as the stain. The use of citrate buffer is preferred for better metachromicity [14]. The citrate buffer is prepared using citric acid and sodium citrate and adjust to pH 4.0 using HCl.
7. In order to retain phloroglucinol staining of lignin for a longer period of time, Speer [42] devised an improved method of staining. In essence, after initial phloroglucinol-HCl staining, the sections are mounted in glycerol and lactic acid together with a low concentration of phloroglucinol-HCl.
8. The staining of lipidic substances using Sudan dyes is a relatively simple procedure. The orange to red color of Sudan III and IV is easy to be discerned under a light microscope. Cuticular substances can be detected readily using these stains. However, for seed storage lipids, the black color of Sudan Black B gives a better contrast when viewed. Control treatment using a lipid solvent, for example, ethanol, methanol–chloroform prior to staining can be employed [9]. Other methods of lipid detection [9] should also be used to confirm the observation.
9. Some useful solutions related to the clearing techniques [11]: (a) Fixative FPA50—formalin, propionic acid, 50% ethanol; 5:5:90, v:v:v. (b) Lactic acid. (c) Lactic acid saturated with chloral hydrate. (d) 4½ clearing fluid—lactic acid, chloral hydrate, phenol crystals, clove oil, xylene; 2:2:2:2:1, by weight. (e) BB-4½ clearing fluid—4½ clearing fluid, benzyl benzoate; 9:1, by weight. (f) Hoyer's clearing solution, 9 g gum Arabic, 60 g chloral hydrate in 15 mL H_2O and add 6 mL glycerol. Chloral hydrate is a narcotic and is toxic, be sure to handle with care; wear gloves and avoid contact with skin.

10. The GUS buffer, once prepared, can be dispatched into 50 mL sterile conical tubes and keep at −20 °C to prevent microbial growth. Another method of preparing the staining buffer is to prepare the phosphate buffer, EDTA, and Triton X-100 at pH 7 as one solution and prepare separate 50 mM potassium ferro- and ferricyanide stock solutions. Add 1 mL each of the ferro- and ferricyanide stock to 100 mL of buffer solution. Prepare the staining buffer just before use. Since potassium ferrocyanide stock oxidizes over time, a new stock needs to be prepared regularly [32]. The combination of ferri- and ferrocyanide enhance the formation of the blue insoluble reaction product and prevent it from further oxidation to colorless or yellowish product [33].
11. Vitha [34] recommends that X-gluc be dissolved in methanol instead of *N*, *N*-dimethyl formamide as the latter inhibits GUS activity.
12. There are different mounting media available, each design for a specific purpose. Many suppliers have detail listings of mounting media in their website such as VWR (www.VWR.com) and Electron Microscopy Sciences (www.emsduasum.com).
13. An Optex portable photo studio and lighting kit is available from Gentec International (www.gentec-intl.com) for taking photos of specimens.
14. For beginner, one can avoid cutting the thumb by applying a small bandage over it while holding the specimen or cover the thumb using finger cots (Electron Microscopy Sciences; cat. no. 71130).
15. During sectioning, try not to aim for "complete" sections of a specimen. In general, since the stem and root usually have a radial symmetry, a small part is sufficient to view. For woody specimens, apply a bit more force during sectioning. Again, only a small piece of specimen is sufficient for observation.
16. Be creative in coming up with methods for hand sectioning. For example, although the leaf is thin and difficult to hold, one can gently roll the leaf into a rod and section as if it was a stem. In dealing with roots, instead of cutting one root, one can section a small clump of roots together.
17. Owing to the presence of HCl, be careful when handling the stain. Be sure that the stain is not in contact with any part of the objective and microscope stage as it is highly corrosive. Wash the brush or tools that have been in contact with the stain with running water to remove the acid after use.
18. Commercial formalin is approximately 37% solution formaldehyde and contains methanol as a stabilizer. The additive can have negative effects on enzyme activity. Hence, it is preferable to prepare fresh formaldehyde solution by depolymerizing PFA powder.
19. If leaf samples remained color, they can be bleached using commercial bleach without dilution. Once cleared, the samples can be treated with the sodium hydroxide solution.
20. As the solvent from the mountant evaporates, air pockets will appear surrounding the specimen. Additional mountant needs to be applied carefully under the coverslip to prevent air pocket formation. Instead of using conventional mountant, a polyester resin, Caroplastic from Carolina Biological Supply Co. (Burlington, NC) can be used. A protocol is detailed recently by Vasco et al. [43].

21. After maceration treatment, the cell wall properties have changed; hence, metachromatic colors will not be present.

Acknowledgments I am grateful to the Natural Sciences and Engineering Research Council of Canada for the continuous funding of my research program.

References

1. Wetmore RH, Wardlaw CW (1951) Experimental morphogenesis in vascular plants. Annu Rev Plant Physiol 2:269–292
2. Jensen WA (1962) Botanical histochemistry. Freeman, San Francisco
3. Paddock SW (1999) Confocal microscopy methods and protocols, vol 122. Humana Press, Totowa (Meth Mol Biol)
4. Paddock SW (2013) Confocal microscopy methods and protocols, vol 1075, 2nd edn. Humana Press, Totowa (Meth Mol Biol)
5. Meding S, Walch A (2013) MALDI imaging mass spectrometry for direct tissue analysis. Meth Mol Biol 931:537–546 (Taatjes DJ, Roth J (eds) Cell imaging techniques: methods and protocols, 2nd edn)
6. Braga PC, Ricci D (2004) Atomic force microscopy: biomedical methods and applications, vol 242. Humana Press, Totowa (Meth Mol Biol)
7. Robinson DG, Borst JW (2014) Emerging techniques in light and electron microscopy. Protoplasma 251:273–458
8. Yeung EC (1998) A beginner's guide to the study of plant structure. In: Karcher SJ (ed) Tested studies for laboratory teaching, vol 19. Proceeding of the 19th workshop/conference of the Association for Biology Laboratory Education (ABLE). Purdue University, Lafayette, IN, pp 125–141
9. Yeung EC (2012) The study of *in vitro* development in plants: general approaches and photograph. Meth Mol Biol 877:95–108 (Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant cell culture protocols, 3rd edn)
10. Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. Iowa State University Press, Ames
11. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi, Melbourne
12. Gahan PB (1984) Plant histochemistry and cytochemistry—an introduction. Academic Press, London
13. Sanderson JB (1994) Biological microtechnique. BIOS Scientific Publishers Ltd., Oxford
14. Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, New York
15. Cutler DF, Botha T, Stevenson DW (2008) Plant anatomy—an applied approach. Blackwell, Oxford
16. Peterson RL, Peterson CA, Melville LH (2008) Teaching plant anatomy. NRC, Canada
17. Zarsk V, Cvrckova F (2014) Plant cell morphogenesis: Methods and protocols, vol 1080. Humana Press, Totowa (Meth Mol Biol)
18. Soukup A, Tylova E (2014) Essential methods of plant sample preparation for light microscopy. Meth Mol Biol 1080:1–23 (Zarsk V, Cvrckova F (2014) Plant cell morphogenesis: Methods and protocols)
19. Mayo JM, Legge AH, Yeung EC, Krupa SV, Bogner JC (1992) The effects of sulphur gas and elemental sulphur dust deposition on *Pinus contorta x Pinus banksiana*: cell walls and water relations. Environ Pollut 76:43–50
20. Hutzler P, Fischbach R, Heller W, Jungblut, et al. (1998) Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J Exp Bot 49:953–965

21. Roschchina V (2012) Vital autofluorescence: application to the study of plant living cells. Int J Spect 2012:14 (article ID 124672)
22. Yeung EC, Blackman SJ (1987) Histochemical localization of alcohol dehydrogenase in developing bean seeds. Am J Bot 74:1461–1465
23. Lojda Z, Gossrau R, Schiebler TH (1979) Enzyme histochemistry. A laboratory manual. Springer, Heidelberg
24. Van Fleet DS (1952) Histochemical localization of enzymes in vascular plants. Bot Rev 18:354–398
25. Vreugdenhil D, Sergeeva LI (2002) In situ staining of activities of enzymes involved in carbohydrate metabolism in plant tissues. J Exp Bot 53:361–370
26. Bassuner BM, Lam R, Lukowitz W, Yeung EC (2007) Auxin and root initiation in somatic embryos of *Arabidopsis*. Plant Cell Rep 26:1–11
27. Jefferson RA, Burgess SM, Hirsh D (1986) B-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci U S A 83:8447–8451
28. De Block M, Debrouwer D (1992) *In-situ* enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. Plant J 2:261–266
29. Liedl BE, McCormick S, Mutschler MA (1993) A clearing technique for histochemical location of GUS activity in pollen tubes and ovules of *Lycopersicon*. Plant Mol Biol Rep 11: 194–201
30. Vitha S, Benes K, Phillips JP, Gartland KMA (1995) Histochemical GUS analysis. Meth Mol Biol 44:185–193 (Gartland KMA, Davey MR (eds) Agrobacterium protocols)
31. Rech P, Grima-Pettenati J, Jauneau A (2003) Fluorescence microscopy: a powerful technique to detect low GUS activity in vascular tissues. Plant J 33:205–209
32. Cervera M (2004) Histochemical and fluorometric assays for uidA (GUS) gene detection. Meth Mol Biol 286:203–213 (Pena L (ed) Transgenic Plants: Methods and Protocols)
33. Kim K-W, Franceschi, VR, Davin LB, Lewis NG (2006) β-Glucuronidase as reporter gene. Meth Mol Biol 323:263–373 (Salinas J, Sanchez-Serrano JJ (eds) Arabidopsis protocols, 2nd edn)
34. Vitha S (2007) Histochemicallocalization of B-glucuronidase (GUS) reporter activity in plant tissues. Microscopy and Imaging Center, Texas A & M University online resource: microscopy.tamu.edu/lab-protocols/light-microscopy-protocols.html
35. Herr JM Jr (1993) Clearing techniques for the study of vascular plant tissues in whole structures and thick sections. In: Goldman CA, Hauta PL, O'Donnell MA, Andrews SA, van der Heiden R (eds) Tested studies for laboratory teaching, 5. Proceedings of the 5th Workshop/ Conference of the Association for Biology Laboratory Education (ABLE), pp 63–84
36. Gardner RO (1975) An overview of botanical clearing technique. Stain Tech 50:99–105
37. Lux A, Morita S, Abe J, Ito K (2005) An improved method for clearing and staining free-hand sections and whole-mount samples. Ann Bot 96:989–996
38. Molares S, Ladio AH (2014) Plant anatomy in ethnobotanical research: micrographic techniques and application. In: Albuquerque UP, Cruz da Cunha LVF, Lucena RFP, Alves RRN (eds) Methods and techniques in ethnobiology and ethnoecology, Springer protocols and handbooks, vol 15. Springer, New York, pp 321–333
39. Horobin RW (1982) Histochemistry. Gustav Fischer, Stuttgart
40. Horobin RW, Kiernan JA (eds) (2002) Conn's biological stains. BIOS Scientific, Oxford
41. O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
42. Speer EO (1987) A method of retaining phloroglucinol proof of lignin. Stain Technol 62: 279–280
43. Vasco A, Thadeo M, Conover M, Daly DC (2014) Preparation of samples for leaf architecture studies, a method for mounting cleared leaves. Appl Plant Sci 2:1400038

Chapter 2
Chemical and Physical Fixation of Cells and Tissues: An Overview

Bing Quan Huang and Edward C. Yeung

2.1 Introduction

In order to keep the cellular components as "lifelike" as possible, it is essential that all biochemical and proteolytic processes are inactivated and structures are immobilized and locked in space by a "fixation" step. Two approaches are normally used to "fix" biological samples: chemical and physical fixation methods. Chemical fixation is the most common approach used in specimen preservation. The tissues are immersed in a fixative that kills and stabilizes the cell contents. Physical fixation is accomplished by microwaving and cryopreserving the samples, procedures that rapidly inactivate cellular activities using microwave (MV) energy and low temperatures, respectively. Chemical and physical fixation methods have their own merits and limitations. There is no ideal chemical fixative that can fix and preserve all cellular components. One has to be aware of the limitations of the fixing agents used, processing methods, and interpret the results accordingly. While rapid immobilization of cellular content through physical fixation minimizes artifact formation, typically, only samples of smaller sizes can be adequately processed, especially for ultrastructural studies. The application of physical methods in fixing vacuolated plant cells and tissues remains of limited value when compared to animal studies [1]. The combined use of chemical and physical fixation is recommended for optimal results [2], and this can lead to further improvements in fixation and processing of biological specimens.

Since fixation is the most important step in tissue preparation, it is not surprising to see many reviews and monographs [3–12] written on this topic. For example,

B. Q. Huang (✉)
Center for Basic Research in Digestive Disease, Mayo Clinic, 17th Floor Guggenheim Building, 200 1st Street SW, Rochester, Minnesota 55905, USA
e-mail: huang.bing@mayo.edu

E. C. Yeung
Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_2

Hayat [6, 7] provides an excellent discussion on fixation for both animal and plant tissues, and Kuo [12] updated this topic in the recently published third edition of the monograph *Electron Microscopy*. Many protocols related to physical methods of fixation are described in detail. Readers are urged to consult the literature prior to embarking on a histological study that involves fixation. The purpose of this chapter is to provide an introduction to the general concept of fixation and discuss issues related to fixation of botanical specimens at the light and electron microscope levels. Additional information can be found in other chapters of this volume.

2.2 Chemical Fixation for General Histological Studies

Chemical fixation is the most common approach used in the fixation of biological specimens for light microscopy (LM) and electron microscopy (EM). Relatively large specimens can be fixed when compared to the physical methods. Chemical fixation requires a fixative which is composed of fixing agent(s) and vehicle(s) [8]. Fixing agents are traditionally classified as coagulants and non-coagulants [13], but they can also be classified as additive and nonadditive, and acidic or basic [14, 15]. Coagulant fixing agents such as ethanol, methanol, and acetone can cause protein denaturation. These fixing agents precipitate proteins by replacing water, resulting in conformation change of protein molecules and their solubility. The non-coagulant fixing agents such as aldehydes and osmium tetroxide (OsO_4) react with proteins and other components, forming intermolecular and intramolecular cross-links resulting in a better retention of the cellular organization.

A vehicle is usually a buffer solution with additional compounds such as inorganic salts added to optimize fixation of targeted organelles such as cytoskeletal elements [8, 16], if necessary. A buffer is used to maintain the desired pH regardless of the chemical nature of the fixing agent(s) selected. The consideration in the selection of a buffer is based on its ability to maintain a constant pH in the desired pH range during fixation. The buffer chosen has to be compatible with other components of the fixative and stains. For example, one would not use an amine buffer such as TRIS when fixing with an aldehyde because the two would covalently react, minimizing the desired effects of each component. The fixative also needs to have a suitable osmotic concentration compatible with cells and tissues, so that biological materials will neither swell nor shrink during fixation. Furthermore, the buffer should not be toxic to the cells or induce morphological changes during fixation.

2.2.1 Common Fixing Agents and Their Properties

A great variety of chemical compounds can be used as fixatives [8]. In recent years, the most commonly used fixatives for botanical specimens are ethanol, methanol, acetone, formaldehyde, glutaraldehyde, and osmium tetroxide (OsO_4). Their properties and use in tissue fixation are summarized below.

Ethanol and methanol are coagulant fixing agents. They denature proteins by replacing water in the tissue environment and are usually combined with other fixing agent(s) as a fixative for LM. The alcohols, especially ethanol, are also used as dehydrating solvents for both LM and EM. When comparing results on cytological smears, both ethanol and methanol give similar results, that is, good preservation of nuclear morphology [17]. Ethanol is an important component of the Farmer's fixative (ethanol:acetic acid; 3:1, v:v) for the preservation of the nucleus and cytological studies. It is important to note that lipids are not preserved by methanol and ethanol, and some lipids may be extracted during the course of fixation and later processing.

Acetone works in a similar fashion to ethanol. It denatures proteins and causes rapid precipitation of proteins and dehydration of tissues. It is also an effective lipid solvent but makes the tissue more brittle. Acetone can also be used for fixation in histochemical and immunofluorescence studies [18]. Because of its rapid action, it is possible to maintain enzyme activity using cold acetone fixation. Acetone can also be used as a dehydrating solvent for the epoxy embedding method. Since it is miscible with epoxy resins, the use of a transitional fluid, that is, propylene oxide is not needed. Acetone is a highly volatile and flammable liquid and should be handled with care.

Acetic acid is considered as a non-coagulated fixing agent; however, it causes coagulation of nuclear proteins and indirectly stabilizes and helps to prevent the loss of nucleic acids [14]. Acetic acid does not react with proteins. It is commonly used in conjunction with ethanol as a cytological fixative and aids in the preservation of nucleic acid. When it is used alone, it can cause swelling of cells. Hence, acetic acid is used to counteract the shrinkage effect of other fixing agents such as ethanol. It penetrates fast into tissue and is used as a fixing agent at the light microscope level.

Formaldehyde has a long history of use as a fixing agent [19]. The chemical properties and its reactions towards proteins and other macromolecules are numerous and complex [4, 5]. Its molecular weight is 30. The small size allows it to penetrate into the tissue quickly. The aldehyde group can react with protein nitrogen, primarily the basic amino acid lysine, to form methylene bridges. The initial binding to protein is relatively fast, but the formation of methylene bridges occurs slowly [10]. Therefore, if formaldehyde is used alone, a longer fixation time is needed for proper fixation. Otherwise, the unfixed proteins will be coagulated by the dehydrating solvent such as ethanol. The reversibility of reactions with amino acids and proteins upon washing has been a subject of discussions [4, 5], but once the methylene bridges are formed with proteins, they appear to be stable [20]. A proper understanding of the reactions of formaldehyde towards proteins and other macromolecules underlies our understanding and its use in the fixation of biological specimens. Commercial formalin solution is a saturated solution of formaldehyde (about 37% by weight) in water. In order to stabilize formaldehyde in solution and prevent self-polymerization, methanol is added as a stabilizer. Upon prolonged storage, formic acid is also formed [21]. Therefore, it is difficult to know the exact and effective concentration of the monomeric form of formaldehyde present in a formalin solution. Methanol and formic acid can have negative effects on the fixa-

tion of tissues. Hence, it is always recommended that a fresh formaldehyde solution is made from paraformaldehyde (PFA) powder and used immediately.

Glutaraldehyde was introduced by Sabatini et al. in 1963 as an especially useful fixative for ultrastructural studies [22]. This compound has two aldehyde groups separated by three methylene bridges. Glutaraldehyde is a much more efficient cross-linker for proteins than formaldehyde. Its reactions with macromolecules are believed to be irreversible [4, 5] and also inhibits enzyme activity more than formaldehyde [23]. However, the rate of penetration (0.34 mm/h) is slower than formaldehyde [23]. Similar to formaldehyde, self-polymerization occurs in solution. In order to ensure the quality of the stock solution, that is, having mainly the monomeric form, an "electron microscope grade" glutaraldehyde solution should be used. Excess or unreacted aldehyde groups can generate nonspecific staining reactions, such as the periodic acid–Schiff's reaction. This can be remedied by pretreating sections with sodium borohydride before staining [24]. Furthermore, glutaraldehyde-fixed specimens have a high level of fluorescence that can interfere with immunofluorescence studies. A remedy is provided by Tagliaferro et al. [25] based on the Schiff-quenching procedure.

Osmium tetroxide also has a long tradition as a fixing agent for light and electron microscope studies [8]. It reacts with unsaturated lipids and results in the reduction of osmium during the cross-linking process with the formation of dark brown to black compounds. When used alone, not all proteins can be fixed and results in the loss of protein during fixation. The key drawback of this compound is that it penetrates tissue very slowly (0.25 mm/h [6]), and therefore only small pieces of tissues can be fixed. As a result, this compound is seldom used as a fixing agent at the LM level and is now mainly used as a secondary fixing agent for postfixation after aldehyde in an ultrastructural study. Since OsO_4 is electron dense, it also serves as a "stain", imparting contrast when viewed with an electron microscope. Osmium tetroxide is usually sold as a crystalline solid in a sealed glass ampule. It is well known that OsO_4 crystals sublimate into vapor from a solid state. Exceptional caution is recommended when working with this fixative because unexpected exposure to OsO_4 vapor can lead to blindness and other ailments. Therefore, OsO_4 solution should be prepared and used in a fume hood and stored in double-sealed containers.

The above fixing agents can be used alone or combined with others in a fixative. For high resolution LM and transmission electron microscopy (TEM), the common design is to combine formaldehyde with glutaraldehyde as the primary fixative. This combination was introduced by Karnovsky [26] with the advantage of the rapid penetration and stabilization of tissue by formaldehyde molecules and the stable cross-links generated by glutaraldehyde that come later. Since OsO_4 penetrates too slowly for the bulky specimen at the light microscope level, and some of the embedding media such as glycol methacrylate are not compatible with it, OsO_4 is used mainly as a secondary fixing agent for TEM only.

The Farmer's fixative, a combination of ethanol and acetic acid, causes rapid coagulation of nuclear proteins and aids in the conservation of nucleic acid. Hence, this is a common fixative to study the cytology of cells, and more recently it has been selected as the preferred fixative for laser-capture microdissection methods (*see* Chap. 20).

Formalin–acetic acid–alcohol (FAA) is the most common botanical fixative for the paraffin-embedded method. The fixative penetrates tissue quickly, and the shrinkage effect of ethanol is counterbalanced by the swelling effect of acetic acid. The tissue is stabilized by the cross-linking property of formaldehyde. Although glutaraldehyde serves as a better cross-linking agent, it is not compatible with the paraffin-embedding medium, as the large paraffin molecules cannot penetrate into a well-cross-linked matrix of cells and tissues. Excessive shrinkage of specimens also occurs [21].

Other organic and inorganic compounds can be used as fixing agents [8, 21]. Due to the toxicity of formaldehyde, safer alternatives are preferred [27]. Glyoxal has been introduced as a "formalin substitute," and this compound gives a pleasing effect in histology and immunohistochemistry [28]. Carbodiimides are also found to be effective as tissue fixatives and for immunohistochemical studies [4].

2.2.2 Common Buffers and Their Properties

Although many buffers can be used in conjunction with a fixing agent(s) [8, 21], for plant histology, phosphate, cacodylate, PIPES, and HEPES buffers are the most common.

Phosphate buffer is the most common buffer used for LM and EM, as it is nontoxic and has a stable buffer capacity at the physiological pH range. It is relatively inexpensive to make, and once prepared, it is stable for several weeks at 4°C. Changes in temperature have little effects on its pH. The disadvantage of a phosphate buffer is that it can form precipitates in the presence of calcium and other metallic ions, and it can be contaminated with microorganisms during storage. A new buffer solution needs to be prepared regularly.

Sodium cacodylate is a sodium salt of dimethylarsenic acid and is a highly toxic compound. This buffer is easy to prepare and can be stored for a long time without contamination, as arsenic can serve as a preservative. No precipitation occurs in the presence of low concentrations of calcium and magnesium salts. It is mainly used for EM when phosphate buffer cannot be used and for cytochemical staining protocols. The main disadvantage of this buffer is that it may cause a redistribution of cellular material along osmotic gradients, and arsenic is toxic. One needs to avoid it from mixing with acid which causes arsenic gas formation [7]; this buffer should be prepared in a fume hood.

Good's buffers, that is, PIPES and HEPES, are excellent for physiological pHs. These Zwitterionic buffers are nontoxic to cells and are compatible with divalent cations. They appear to resist chemical extraction of cell components. Desired results have been reported at the ultrastructural level for the study of algae [16] and plant tissues [29, 30]. PIPES buffer is often used as the buffer for the fixation of cytoskeletal elements [31]. These buffers may interfere with the amine-aldehyde reaction [18]. Good's buffers are relatively more expensive to prepare.

2.2.3 Achieving Good Quality Chemical Fixation

Chemical fixation prepares cells for a whole series of rather traumatic events. Ideally, the fixing agents and additives selected should alter the protoplasm structure into a stabilized elastic gel by cross-linking. It should retain the spatial relationship of all organelles in their "natural state," stabilize the phospholipids, which form the frame work of the cell and render the chemical constituents insoluble [32]. Unfortunately, there is no ideal fixative. Not all substances and macromolecules can be "fixed" by the fixing agents. Components such as nucleic acids are indirectly stabilized through protein fixation. Furthermore, the vacuole compartments, different types of cell walls, and ergastic substances can pose challenges to a fixation protocol. Compromises have to be made in selecting a protocol that is best suited for the objective of the experiment. In order to achieve good quality fixation, it is important to consider the following variables during chemical fixation:

1. *Rate of Penetration* The size of the sample should be appropriate for the selected fixative, the embedding method, and the objective of the experiment. For example, FAA is a common fixative for botanical specimens at the LM level. Penetration of fixing agents is fast and will not overly harden the tissues. As a result, if FAA is selected as the fixative, large specimens of close to a centimeter in size can be fixed and stored for immediate or future processing. If specimens are intended to be used for TEM studies, due to the slow penetration rate of fixing agents, that is, glutaraldehyde and OsO_4, the size of tissue blocks should be approximately 1 mm^3 or less. Other factors such as the porosity and the density of tissue need to be considered in deciding the appropriate size of a tissue to be fixed for a particular protocol.
2. *Concentration of Fixing Agents and Additives* Different cellular components react differently to different concentrations of fixing agents and additives. Depending on the objective of the experiment, one needs to choose proper concentrations of components within a fixative. Low concentrations of fixing agents require a longer time of fixation, hence some enzymatic activities can be maintained. However, this can cause extraction of cellular materials, diffusion of enzymes, and shrinkage or swelling of cells and organelles. On the other hand, high concentrations of fixing agents can destroy enzyme activities and damage cellular fine structures. Mitochondria appear to be more sensitive to an increase in buffer concentration, whereas endoplasmic reticulum (ER) is sensitive to low concentration of fixative [33]. Normally, the optimal concentration for PFA and glutaraldehyde are 2.5−4% and 1–2% for OsO_4.
3. *Length of Fixation* The optimal time for fixation is determined empirically. If fixation time is too short, penetration of fixative and crosslinking of macromolecules will not be adequate. Longer fixation time may cause over-cross-linking, making the sample brittle. Leaching of ions and other cellular components may also occur. For TEM studies, tissues with a size of 1 mm^3, a 4 h fixation each at room temperature or overnight at 4 °C, is adequate for both the primary and

secondary fixation. For LM, since the size of the specimen is usually larger than those for TEM studies, an overnight fixation in a refrigerator is recommended.

4. *Temperature* The sample should be fixed at the same temperature at which the plants are grown or maintained. This is to avoid temperature shock and is especially important if cultured cells are studied. For LM studies, after initial fixation at room temperature, the specimens are usually transferred to a refrigerator at 4 °C for an overnight fixation. On the other hand, if the primary objective of the experiment is to stabilize proteins for immunological or enzymatic studies, a lower temperature is preferred.
5. *Tonicity* An isotonic solution will cause neither swelling nor shrinkage of cells in the solution. Isotonic solutions give an osmotic pressure equal to that of the cell cytoplasm. Hypertonic solutions cause shrinkage, while hypotonic solutions induce swelling. The tonicity can be adjusted by adding electrolytes (e.g., NaCl) or nonelectrolytes (e.g., sucrose). Most of the fixatives are slightly hypertonic [7]. SEM specimens should be fixed under or near isotonic condition, while TEM samples should be fixed using a slightly hypertonic fixative. For plant protoplast fixation, a careful adjustment of osmoticum concentration is needed in order to avoid bursting or shrinkage of protoplasts (*see* Chap. 12).
6. *Cuticle and Air Spaces* Many exposed plant surfaces are protected by a cuticle, and intercellular air spaces are common between cells. These two features alone can impede the penetration of the fixative into the specimen. Plant specimens with cuticle and large intercellular spaces typically float on the fixative solution. Since the cuticular surface is hydrophobic, the fixative will not be able to access the internal compartment of the specimen. In order to improve fixative penetration into specimens such as a leaf, the samples need to be sliced thin. The cut surfaces are exposed directly to the fixative to allow penetration. A vacuuming treatment can extract air from within the sample, allowing better penetration of the fixative. This will also improve fixation quality and aid in the subsequent dehydration and embedding processes.

The aforementioned discussion illustrates the complexity of the chemical fixation process. There are many variables that can alter the quality of fixation of a specimen. In order to ensure that the images obtained are "true to life," the only way is to compare results with the study of living cells [13]. Comparative studies using different methods need to be done, especially by using the physical methods of fixation in order to validate the results.

2.3 Physical Methods of Fixation

Although chemical fixation is the most common approach used to immobilize biological processes in cells and tissues, the quality of fixation is sometimes not ideal or even poor, especially for those difficult-to-fix plant tissues such as pollen, embryo sac, or cells undergoing rapid structural changes, such as during the

fertilization process [34, 35]. Since the penetration rate of fixing agents is slow, active cellular process cannot be stopped instantaneously in every part of the same cell and within the tissue [36]. Large plant vacuoles often rupture prior to being fixed. The release of vacuolar content can alter the cellular organization prior to fixation of cells [36]. Physical methods, that is, cryofixation (CF) and microwave fixation (MF), overcome the deficiencies of chemical fixation; however, the procedures and instrumentation can be technically challenging.

2.3.1 Cryofixation

At the LM level, CF and sectioning of tissue are more successful with animal tissues than with plant tissues. The presence of vacuoles in plant cells make them prone to ice damage during freezing, and the inherent properties of plant cell walls also make sectioning difficult [37, 38]. However, the damage to cells by ice crystal formation during freezing provokes further studies and improvements to the methodology. The current successes in CF, especially at the EM level, result from culminated efforts by many researchers over the past years. Earlier methods related to plant tissue cryotechniques are discussed by Gahan [38].

CF arrests cell activities instantaneously at low temperatures (−180°C). Low temperature vitrifies cell water and prevents ice formation and, together with subsequent freeze substitution (FS) techniques and embedding processes, produce better quality images which can be seen at the ultrastructural level. At the LM level, samples are pretreated and protected by a cryoprotectant, frozen in liquid nitrogen, and sectioned using a cryostat and processed accordingly [38, 39].

For ultrastructural studies, four main methods have been applied to cryopreserve the plant samples: (1) plunge freezing [40], (2) spray freezing [41], (3) propane-jet freezing [42, 43], and (4) high-pressure freezing (HPF) [42–45]. A detailed theoretical discussion and methodologies can be found in the review by Gilkey and Staehelin [42]. Methods 1, 3, and 4 are common methods used for plant cells and tissues. For the plunge freezing method, samples are loaded on a grid or directly on a membrane support or other carrier and plunged rapidly into either liquid propane or helium at −180 to −190°C in a freezing system (either self-made or commercial). Well-preserved layers of plant tissue may be obtained up to a thickness of 15 μm. In HPF, samples are held in a metal carrier, and a high pressure at 2100 bar is applied just before freezing. This method changes the freezing properties of water. Nucleation and crystal formation of water molecules are slowed, allowing thicker samples (up to 600 μm thick) to be well preserved without crystal damages [1]. The key advantage of HPF is that larger samples can now be fixed when compared to other freezing methods. It is important to note that specimen preparation and handling before freezing are the most important steps in order to achieve successes using the HPF technique [44].

After initial freezing, a FS procedure follows. FS is the process in which an organic solvent with or without fixing agents (such as OsO_4, uranyl acetate, or glutaraldehyde) is used to replace the vitrified water within cells at a temperature

of about −80 to −90°C. If a fixing agent is included in the substitution fluid, the cells will be fixed once the temperature is raised during further processing. Once fixed, an embedding medium such as London Resin White (LR White), LR Gold, or Lowicryl HM20 resin can infiltrate into the tissue at low temperature (0–20°C for LR White/Gold and −20 to −40°C for HM20), or the specimen is allowed to warm slowly to room temperature followed by infiltration of a selected embedding resin. The added advantage of this method is that chemical fixation is not necessary if immunostaining is desired. CF alone maintains antigenicity of cells allowing for improved immunostaining of the specimen [45]. For additional information on CF and recent protocols, see [44, 46–49] and Chap. 7.

With the potential benefit of the CF technique over the conventional chemical fixation methods, new approaches and modifications to current techniques continue to appear in the literature. In a recent study, McDonald and Webb [50] published a protocol on FS that can be completed in 3 h or less after HPF using a variety of biological samples including leaves of *Nicotiana benthamiana*. On the other hand, the FS procedure is not necessary if cryo-ultramicrotoming is followed by observation using a cryo-electron microscope [51]. The advantage of "cryo-electron microscopy of vitreous section" (CEMOVS) is that there is no chemical treatment and/or fixation needed and this "preserves the sample close to the native state" [51]. Several protocols related to cryo-EM can be found in [12].

2.3.2 Microwave Fixation

Although CF takes only milliseconds to immobilize the tissue, the subsequent processing time can be long, up to several days. The technique of MF was introduced by Mayers [52] in 1970, and a review on early research and methods of MF is summarized by Login and Dvorak [53]. With improved instrument design, MF has become more popular in recent years [54]. MW irradiation accelerates fixation and processing of biological materials [53]. MW energy causes excited molecules to rotate, generating a quick and homogeneous heat within a sample, thus greatly speeding up chemical reactions during fixation and subsequent processing steps. It also improves the accessibility of fixatives and reagents to cells and maintains antigenicity of cell components [52, 55, 56]. Since MW generates heat for fixation, if it is not well controlled, it may damage cells and tissues. The new MW systems, for example, Pelco BioWave Pro (Tel Pella, Inc.), have the capability of maintaining a uniform environment with a ColdSpot, an enclosed bed of circulated water, which continuously absorbs all generated MW energy. The inclusion of a vacuum chamber in the MW cavity further enhances penetration of fixatives and other solvents. This feature is especially beneficial to the processing of botanical specimens. The vacuum–MW combination enhances the diffusion of fixative and improves the quality of the cell morphology [57]. With standardized equipment and techniques, MF not only shortens the fixation and processing time but also produces comparable results to conventional processing methods. Methods for MW-assisted processing and embedding for TEM are now well established [58]. The procedures are less demanding

when compared to CF protocols. Hence, MF and processing are becoming routine procedures in many histology laboratories.

Reports on the use of MF in processing plant samples are few compared to animal studies. Schichnes et al. [59] documented a MW paraffin technique for botanical specimens and another procedure for in situ localization of KNOTTED messenger ribonucleic acid (mRNA) [60]. The samples were microwaved with FAA as the fixative. Other studies focused on structural and antigenic preservation of samples. For example, Zellnig et al. [61] recently established a MW-assisted plant sample preparation protocol for rapid immunohistochemical diagnosis of tobacco mosaic virus.

2.4 Comments on Practical Issues of Fixation

2.4.1 Fixatives and Fixation of Botanical Specimens at the Light Microscope Level

The most important considerations in any successful fixation protocol are specimen handling, choice of fixative, and fixation conditions. As indicated previously, plant cells have cell walls, intercellular spaces, large vacuoles, and some have cuticular substances on their surface. Therefore, the methods used in sample collection and fixation vary depending on the tissue of interest. For good quality fixation, it is important to collect and fix the tissue immediately and at the same time. Therefore, prior to fixation, all necessary equipment, tools and fixative need to be prepared.

The following suggestions on tissue handling will help to ensure the quality of morphological preservation: (1) One should avoid physical damages to the specimen during dissecting; the tissues should be dissected or excised using sharp knives or double-edged razor blades. (2) At the time of dissection, the specimen needs to be trimmed carefully with the final image and the correct plane of section in mind in order that the specimen can be embedded easily. (3) Tissues should be immersed in the fixative or buffer at all times during the excision, if possible. The cut surfaces should be in contact with the fixative as soon as possible. (4) The final size of specimens for fixation depends on the choice of fixative, the embedding medium, and the objective of the experiment. Retrimming of tissue blocks after dehydration or prior to embedding is not recommended, as the tissues will be very brittle and it is difficult to make precise cuts. (5) For those tissues covered by cuticular materials, the specimens have to be cut open to allow for the penetration of the fixative. If necessary, for LM, a small drop of detergent (e.g., Tween 20) can be added to the fixative to disrupt the surface tension of the cuticle. (6) All botanical specimens should be subjected to a vacuum treatment to ensure that air is removed from intercellular spaces. It is always advisable to vacuum the sample for 5–15 min to extract air from the tissues in order to improve on fixative penetration and subsequent infiltration of the embedding medium. We also routinely vacuum the samples at the first 100 %

step of dehydration and when the samples are in pure embedding medium prior to embedding.

At present, a large number of fixatives are available in the literature for fixation of specimens at the LM level [14, 15]. For large specimens, a fast penetrating fixative is preferred. Hence, FAA or a 4 % buffered PFA solution is sufficient. FAA fixation in conjunction with the paraffin-embedding method is detailed in Chap. 3. For high-resolution LM which involves the use of acrylate resins, in order to improve fixation quality, a combination of PFA (1–2 %) and glutaraldehye (2–3 %) solution is preferred. The specimen size is usually smaller than those fixed for paraffin sectioning. Furthermore, in order to avoid problems with polymerization of acrylate resins, the tissues need to be sliced thin, to a thickness of 1–3 mm (see Chaps. 4 and 6). Postfixation using OsO_4 is not required. In fact, OsO_4 is not compatible with the common glycol methacrylate embedding resin.

2.4.2 Fixatives and Fixation Considerations for TEM

As a general rule, prior to embarking on an ultrastructural study, it is imperative for the investigator to have a good knowledge of the histology of the tissues at the LM level. For TEM studies, many formulations related to fixation are available in the literature [6–11]. The tissues are fixed using a primary fixative consisting of glutaraldehyde alone or a combination of PFA and glutaraldehyde, followed by a secondary fixation using OsO_4. As glutaraldehyde and OsO_4 penetrate the tissue slowly, the blocks need to be small to guarantee adequate fixation. It is possible to enhance contrast of certain components within cells by incorporating additional compounds into the fixative. For example, the pectin in the walls of immature fiber cells of hypocotyl of flax seedlings can be stained using ruthenium red after formaldehyde fixation [62], or the inclusion of Alcian blue [63] to a glutaraldehyde fixative can improve mucopolysaccharide staining in the cells. The addition of malachite green can aid in the preservation of lipid containing granules [64]. Besides the commonly used fixation protocols, other fixing agent such as glyoxal and carbodiimides can be tested. One needs to be creative in any histological study. Comparative studies and testing of different fixing agents and protocols may yield additional useful information.

2.4.3 Fixatives and Fixation Considerations for Immunological Studies

Successes in immunohistochemical (IHC) studies in plant cells and tissues, especially immuno-electron microscopy (IEM), are determined by three factors: (1) good quality antibodies, (2) well-preserved antigenicity and cell and tissue morphologies of the specimen, and (3) accessibility of the antibodies to antigens. In general, there is no standard fixation protocol that fits all the plant cells and tissues

for an IHC study. Protocols need to be optimized depending on the tissue utilized. Fixation for well-preserved antigenicity and cell morphology is always a compromise since epitopes differ significantly in their sensitivity to fixation. Some protein epitopes are highly sensitive to glutaraldehyde, which should be used in limited amount, if at all, while some carbohydrate epitopes can even tolerate OsO_4 fixation [48]. As a rule, chemical fixation should be mild to ensure the epitopes are available to the antibodies, but at the same time strong enough to preserve the fine cell structures. To ensure a better chance of success, one should test the quality of antibody using western blots to estimate purity and sensitivity to tissue antigens prior to embarking on an IHC study.

For chemical fixation, freshly prepared PFA is a common fixing agent for IHC. PFA alone is an adequate fixing agent for IHC using LM, but a poor fixing agent of ultrastructure in IEM. Glutaraldehyde is superior to PFA as a fixing agent for preservation of cell structures; however, the use of glutaraldehyde in concentrations higher than 0.05 % may result in loss of antigenicity. Therefore, it is used at a very low concentration in combination with PFA to improve cell ultrastructure and yet maintain some native configuration of the protein. For immunofluorescence studies, glutaraldehyde should not be used as a fixative since it generates autofluorescence and interferes with the observation [65]. In general, PFA (2–4 %) is used as a fixing agent for immunofluorescence and IEM, or in combination with glutaraldehyde (0.05–0.2 %) for IEM only.

Besides the traditional chemical fixation protocols, other treatments can also enhance the fixation of macromolecules for IHC studies. Pretreatment using m-maleidobenzyol-N-hydroxysuccinimide ester, a protein cross-linking reagent followed by formaldehyde fixation allows the visualization of fine actin filaments [66]. Periodate-lysine-paraformaldehyde (PLP) is an alternative fixative used in IEM. It has less deleterious effects on epitopes and preserves acceptable morphology of the cells. The fixation by PLP is through stabilization of carbohydrate moieties by periodate oxidation followed by lysine cross-linking of the carbohydrate group [67]. A formaldehyde substitute, glyoxal, appears to give satisfactory results in IHC studies [4].

To ensure that the positive signals are indeed correct, control staining needs to be performed. The use of pre-immune serum and antibody serum pre-absorbed with tissue powder should give negative results. Under certain staining conditions, amyloplasts can give false positive results [68].

For IHC studies at the LM level, there are two immunolabeling methods: whole mount and sectioning. The whole mount method is superior to the sectioning method because the sample is able to be viewed globally and 3-D reconstruction is possible. However, plant cells have walls, and this prevents the accessibility of antibodies to the antigens. Before whole-mount immunostaining can be carried out, cell walls need to be digested or removed using wall digestive enzymes. The cell membranes also need to be treated by a detergent (e.g., Triton X-100) in order to permeabilize cells and favors the access of the antibody to antigens. If the treatments described above are too harsh, the cell structure will be damaged and results in poor morphology. However, if the treatment is too mild, the antibody will not be able to access the

antigens, and immunolabeling will fail. Therefore, wall digestion is the most critical step in the whole mount immunolabeling protocol (also *see* Chap. 11). In the study of immunolabeling on cytoskeleton of plant embryo sacs of maize [69], tobacco [70], and an orchid [71], the fixation time, enzyme composition, and digestion time for cell wall, and the concentration of Triton X-100 (or other detergents) for permeabilizing cell membranes, vary for each species and experiment. For example, since *Cymbidium sinense* embryo sac has thick cell walls, a harsher extraction procedure is needed to make the embryo sac permeable to the tubulin antibodies [71].

In spite of its limitation, the sectioning method is still the most common method used for IHC studies in LM because of the following reasons: (1) The cell wall digestion and cell membrane permeability steps for immunolabeling are not necessary, (2) the same tissue block can be used for both LM and TEM studies, and (3) it is easy to process and choose the area of interest for further ultrathin sectioning.

A freshly made 2–4% PFA is a sufficient fixative for immuno-LM studies. Chemical fixation coupled with methacrylate [72] and Steedman's wax [73] embedding can preserve and visualize cytoskeletal elements. When used in conjunction with the LR White embedding method, IHC studies can be carried out at both the light and electron microscope level [56].

Physical fixation methods are excellent approaches in the study of IHC of cells and tissues, especially at the ultrastructural level. CF followed by FS without fixation can be a useful procedure for antigen preservation and detection [45, 74]. A detailed discussion of using cryo-methods in conjunction with immunogold labeling can be found in Chap. 14. Successes in obtaining optimal structural and antigen preservation in botanical specimens using MW-assisted processing have been reported for a variety of plant tissues [54, 75–77].

2.4.4 Fixatives Used for Molecular Biology Studies

Histochemical localization of transcripts using in situ hybridization methods provides useful information about the temporal and spatial distribution of genes and their activities during the course of development. Many protocols are available in the literature concerning in situ hybridization of ribonucleic acid (RNA) probes (*see* Chap. 21 for additional information) and specific deoxyribonucleic acid (DNA) sequences in chromosomes, that is, the fluorescence in situ hybridization (FISH) technique (*see* Chap. 17 for additional information). Fixation is an essential initial step for the preservation of nucleic acids. Moreover, it is important to note that nucleic acids cannot be fixed using common fixing agents. For in situ hybridization of RNA probes, since structural integrity of the specimen is essential for the interpretation of results, tissues are usually fixed with FAA or buffered PFA. Formaldehyde is an essential component of the fixative as it serves to cross-link certain protein elements, stabilizing cellular structures for subsequent processing. For botanical specimens, FAA is commonly used as a fixative. Although the advantage of acetic acid and ethanol is not apparent, their fast penetrating and coagulation actions

may enable a better preservation of nucleic acids within the tissue, minimizing the potential deleterious effect of the vacuolar content during fixation. The combined use of formaldehyde, acetic acid, and alcohol provides a good combination in the fixation of tissues, especially for botanical specimens. It is important to note that although fixation is the most important step in the protocol, subsequent processing and handling of specimens can influence the outcome of experiment.

In recent years, laser microdissection of tissues for targeted profiling studies has become popular. Specific cells and tissues can be laser dissected, followed by RNA isolation and amplification for profiling studies using different molecular biological methods such as microarray, etc. (*see* Chap. 20 for additional information). Proper preservation of RNA is paramount to the success of this method. For botanical specimens, the Farmer's fixative (ethanol: acetic acid; 3:1 v:v) appears to be the most common fixative used [78, 79]. Rapid coagulation of cytoplasmic content enables "trapping" of nucleic acids. Since the cellular components are not cross-linked by formaldehyde, subsequent RNA extraction is relatively easy. In a recent article, this fixative was used in conjunction with MW processing and high-quality RNA was extracted from a variety of plant tissues after laser microdissection of paraffin sections [78]. In animal tissues, a formalin-based fixative was also found to be adequate to preserve RNA when compared to other fixing agents [80].

Further comparative studies and optimization of tissue fixation for plant molecular biology studies are needed. A combination of other techniques, such as CF and FS, is possible. In situ hybridization procedures have been successfully performed using FAA in conjunction with MW fixation [60].

2.4.5 Cryofixation of Botanical Specimens

Although the general principle of CF is not difficult to understand, there are challenges in instrumentation and the handling of samples during freezing and FS. It is important to note that specimen preparation and handling before freezing are the most important steps in order to achieve successes using the HPF technique [44]. The protocols for different individual samples or particular cell types and organelles should be optimized since there is no "standard" suitable protocol for every tissue and different cell types. Some practical aspects of plant CF methods have been discussed [81, 82]. When using cryoprotectants, fine structures of the plant samples in the range of 2 mm in diameter and up to 600 μm in thickness could be well preserved with HPF [83, 84]. In addition, the rapid freezing and low temperature embedding greatly improve the preservation of antigens for immunolabeling [83]. Quality results have been demonstrated in a variety of plant samples including root tip of *Nicotiana* and *Arabidopsis* [85, 86], ovules and embryo sacs of *Petunia* [87], pea seedling leaves [88], and rice leaves [89].

Careful preparation of samples is needed to ensure successes in CF. It is essential to use specimen carriers to accommodate the samples without causing any physical damages. If necessary, the samples should be trimmed to fit the cavity of the

specimen carrier. All enclosed spaces should be completely filled with the samples or cryo-filler to eliminate any air space [82]. The size and geometry of the specimen holder should be chosen and optimized according to sample size and one's need. Copper or aluminum Type A or Type B carriers are commonly used, which usually come with one "welled" type and a "flat" type. Different size wells can be generated by combining the "welled" or the "flat" type specimen carrier. Moreover, the larger-size carrier with more specimens to freeze will have a higher risk of ice crystal damage to the specimens.

For HPF CF, the critical elements for success are: (1) Specimens should first be wetted with buffer or filler in a Petri dish or specimen carrier. (2) Specimen loading should be done as quickly as possible to prevent samples from drying out. One should practice several times in order to be familiar with the techniques used. (3) Each carrier should contain an appropriate number of specimens without overcrowding to prevent the physical damage to the tissues. (4) If samples cannot fill the space of the carrier cavity, a suitable filler should be used with the tissue. For the majority of samples, the best choice of filler should have cryoprotection capability such as 0.15 M sucrose. There are two kinds of cryoprotectants: non-penetrating and penetrating. Among them, dextran (5–15 %), 1-hexadecene, and Ficol (5–15 %) belong to the non-penetrating category, and 0.15 M sucrose is an example of the penetrating type. (5) It is critical to avoid any air bubbles in the cavity of the carrier. Air bubbles can be removed either by using a pipette or forceps before applying the top freezing hat; air bubbles within the holder act as insulators and collapse at high pressure, thus damaging the samples. (6) After freezing, the sample should be kept in cryo- or glass-vials for FS. It is recommended to keep the sample in the same vial during processing. This would prevent the exposure of samples to air. (7) All tools for CF, dehydration agents, and infiltration resin must be prechilled before use. A number of detailed protocols are now available [45, 46]. Chapter 7 serves as an example of the CF process.

2.4.6 Microwave Fixation of Botanical Specimens

The success in MF is primarily due to the development of an instrument that creates the proper, controlled conditions for fixation. One needs to follow the general recommendations from the manufacturer in instrument operation. However, since different tissues require different fixation and processing conditions, it is essential that various parameters are optimized for one's own use. The following serve to illustrate the process of MF and processing.

Preparation and handling of plant tissues by MW-enhanced fixation are similar to those described for conventional fixation. Since the MW radiation and temperature can promote fixative penetration and infiltration, larger plant specimens can be well preserved, including those difficult-to-fix tissues [75].

For primary fixation, the power step is set at 150 W. Fixatives containing formalin (PFA) require an initial low-power step (83–250 W), followed by a step at a

higher power, that is, 450 W [52]. Non-formalin-containing fixatives require only a single low-power setting for the fixation step. The "power on" step promotes MW-assisted diffusion and the "power off" step provides time for fixative cross-linking within the tissues. The fixation process can be repeated for those difficult-to-fix tissues. For those cell pellets embedded in agarose, the radiation time of MW should be limited to 40 s, prolonging the time could cause the agarose to melt. For vacuum-MW processing, the specimens are placed in the "Vac" chamber and connected to the vacuum system within the MW oven before MF.

Buffer rinse can always be done outside the MW oven or with three 40 s MW steps at 150 W prior to OsO_4 postfixation. Fixation in OsO_4 can be done using the same time duration and condition as aldehyde fixation except OsO_4 fixation is carried out only at a low-wattage setting. Recommended settings are between 85 and 250 W—we set it at 150 W. Aqueous OsO_4 in concentration from 0.5 to 2% is recommended. Removal of OsO_4-fixed samples must be performed in the fume hood following by three distilled water washes prior to dehydration.

Dehydration does not require temperature control and is done under a low-power processing condition. If ethanol is used for dehydration, it is recommended that after the last 100% ethanol infiltration step, ethanol is replaced with acetone, especially if epoxy resin infiltration follows. Each dehydration step only requires 40 s of MW exposure at 150 W.

Resin infiltration in the MW oven can be done with or without vacuum. The use of vacuum is recommended for efficiency and time savings. Viscous resins, for example, Araldite or Epon 812 substitutes, are best processed at 250–350 W. Low-viscosity resins, for example, LR White and Spurr's resins, can be processed in the MW oven using a lower-power setting between 100 and 200 W. The last step of infiltration of samples is carried out at room temperature overnight in a desiccator under vacuum.

2.4.7 Laboratory Safety

Almost all fixing agents, certain buffers and histochemical-related compounds are toxic and require proper handling and storage. Many safety concerns related to the use of fixatives are detailed by Titford [90]. It is imperative to have the proper knowledge of the chemicals used and how the chemicals should be handled. As a general rule, fixing agents, fixatives, and tissue samples should be stored in their own refrigerator and not placed among other biochemicals and tissue culture chemicals. The volatile properties of some fixing agents and fixatives can destroy expensive biochemicals. It is advisable to conduct a majority of fixation and processing operations inside a fume hood. The handling of liquid gases and other cryogenic chemicals for CF requires extreme care. Improper handling can lead to cold burns. Rapid gas expansion can be explosive. Proper containers are needed for transportation and use in a laboratory. In a histology laboratory or any type of laboratory, a good safety practice has to be followed at all times.

2.5 Concluding Remarks

The purpose of fixation is to arrest cellular processes in an attempt to preserve the original cellular structures. Chemical and physical fixations are the two common approaches used to achieve this goal. For chemical fixation, the choice of fixatives and additives depends on the need and resolution level required for the experiment. In general, FAA and aldehyde fixatives are mostly used for LM studies, while aldehyde fixatives and OsO_4 are common for ultrastructural studies. Mild fixation of aldehyde is usually adopted for cross-linking of proteins for antigen preservation. Careful handling of the specimens and optimal conditions for fixation are critical issues to obtain high-quality morphological and antigenicity preservations. For physical fixation, MW-enhanced fixation combined with vacuum application greatly promotes the speed of fixation and shortens the overall processing time. Protocols for ultrastructural and cytochemical studies are now available for EM, but fixation steps for difficult-to-fix tissues may need to be repeated and optimized. CF has the fastest rate of fixation and a simultaneous stabilization of all cellular components, which are two distinct advantages over chemical fixation. Ice crystal damage for ultrastructure is the major concern during rapid freezing, thus the size of specimen has to be kept to a minimum. Specimen handling and choice of freezing carriers and cryo-protectants are all important factors that can affect the quality of ultrastructural preservation. In spite of these limitations, the preservation of fine structures is close to their native state, and the results are far superior than with chemical fixation. In addition, its applications for reconstructive tomography and cryo-EM have generated a great deal of interest. These new methods and approaches will further our understanding of plant and animal cell biology in the future.

Acknowledgments The authors thank Professor Jeffrey L. Salisbury at the Department of Biochemistry and Molecular Biology and Microscopy and Cell Analysis Core Facility in Mayo Clinic for critical reading and editing of the manuscript.

References

1. Karahara I, Kang BH (2014) High-pressure freezing and low-temperature processing of plant tissue samples for electron microscopy. Meth Mol Biol 1080:147–157
2. Ripper D, Schwarzt H, Stierhof YD (2008) Cryo-section immunolabeling of difficult to preserve specimens: advantages of cryofixation, freeze-substitution and rehydration. Biol Cell 100:109–123
3. Jones ML (2001) To fix, to harden, to reserve-fixation: a brief history. J Histotechnol 24: 155–171
4. Eltoum IE, Freenburgh J, Myers RB, Grizzle WE (2001) Introduction to the theory and practice of fixation of tissues. J Histotechnol 24:173–190
5. Eltoum I, Fredenburgh J, Myers RB, Grizzle WE (2001) Advanced concepts in fixation: 1. Effects of fixation on immunohistochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues. 2. Developmental methods of fixation. J Histotechnol 24:201–210

6. Hayat MA (1981) Fixation for electron microscopy. Academic, New York
7. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, New York
8. Glauert AM, Lewis PR (1998) Biological specimen preparation for transmission electron microscopy. Princeton University Press, Princeton
9. Bozzola JJ, Russel LD (1999) Electron microscopy, 2nd edn. Jones and Bartlett, Sudbury
10. Kiernan JA (1999) Histological and histochemical methods: theory and practice, 3rd edn. Butterworth-Heinemann, Oxford
11. Suvarna KS, Layton C, Bancroft JD (2013) Bancroft's theory and practice of histological techniques, 7th edn. Churchill Livingstone, London
12. Kuo J (ed) (2014) Electron microscopy, 3rd edn. Humana Press, Totowa, New Jersey
13. Baker JR (1966) Cytological technique, 6th edn. Chapman & Hall, London
14. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi Pty. Ltd., Melbourne
15. Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, New York
16. McFadden GI, Melkonian M (1986) Use of a Hepes buffer for microalgal culture media and fixation for electron microscopy. Phycologia 25:551–557
17. Kumarasinghe MP, Constantine SR, Hemamali RL (1997) Methanol as an alternative fixative for cytological smears. Malays J Pathol 19:137–140
18. Leong AS-Y (1994) Fixation and fixatives. In: Woods AE, Ellis RC (eds) Laboratory histopathology: a complete reference, vol 4. Churchill Livingstone, New York, pp 4.1-1-4,1–26
19. Fox CH, Johnson FB, Whiting J, Roller PP (1985) Formaldehyde fixation. J Histochem Cytochem 33:845–853
20. Puchtler H, Meloan SN (1985) On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. Histochem 82:201–204
21. Culling CFA, Allison RT, Barr WT (1985) Cellular pathology techniques, 4th edn. Butterwords, London
22. Sabatini DD, Bensch K, Barrnett RJ (1963) Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 17:19–58
23. Hopwood D (1967) Some aspects of fixation with glutaraldehyde. J Anat 101:83–92
24. Kasten FH, Lala R (1975) The Feulgen reaction after glutaraldehyde fixation. Stain Technol 50:197–201
25. Tagliaferro P, Tandler CJ, Ramos AJ, Saavedra JP, Brusco (1997) Immnofluorescence and glutaraldehye fixation. A new procedure based on the Schiff-quenching method. J Neurosci Meth 77:191–197
26. Karnovsky MJ (1965) A formaldehye-glutaraldehyde fixative of high osmolality for use in electron microscopy (abst). J Cell Biol 27: 137 A
27. Moelans CB, ter Hoeve N, van Ginkel J-W, ten Kate FJ, van Diest PJ (2011) Formaldehyde substitute fixatives. Amer J Clin Pathol 136:548–566
28. Dapson RW, Feldman AT, Wolfe D (2006) Glyoxal fixation and its relationship to immunohistochemistry. J Histotechnol 29:65–76
29. Salema R, Brandao I (1973) The use of PIPES buffer in the fixation of plant cells for electron microscopy. J Submicr Cytol 5:79–96
30. Lawton JR, Harris PJ (1977) Fixation of senescing plant tissues: sclerenchymatous fibre cells from the flowering stem of a grass. J Microscopy 112:307–318
31. Tung SH, Ye XL, Zee SY, Yeung EC (2000) The microtubular cytoskeleton during megasporogenesis in the Nun orchid, *Phaius tankervillae*. New Phytol 146:503–513
32. Dykstra MJ, Reuss LE (2003) Biological electron microscopy. Theory, techniques, and troubleshooting, 2nd edn. Springer, New York
33. Thornthwaite JT, Thomas RA, Leif SB, Yopp TA et al (1978) The use of electronic cell volume analysis with the AMAC II to determine the optimum glutaraldehyde fixative concentration for nucleated mammalian cells. Scan Electron Microscopy 2:1123

34. Parthasarathy MV (1994) Transmission electron microscopy: chemical fixation, freezing methods, and immunolocalization. In: Freeling M, Walbot V (eds) The maize handbook. Springer, New York, pp 118–134
35. Huang BQ, Strout GW, Russell SD (1993) Fertilization in *Nicotiana tabacum*: ultrastructural organization of propane-jet-frozen embryo sacs *in vivo*. Planta 191:256–264
36. Mersey B, McCully ME (1978) Monitoring of the course of fixation of plant cells. J Microscopy 114:49–76
37. Jensen WA (1962) Botanical histochemistry. Freeman, San Francisco
38. Gahan PB (1984) Plant histochemistry and cytochemistry—an introduction. Academic, London
39. Anderson G, Bancroft J (2002) Tissue processing and microtomy. In: Bancroft J, Gamble M (eds) Theory and practice of histological techniques. Churchill Livingstone, London, pp 85–107
40. Lancelle SA, Callahan DA, Hepler PK (1986) A method for rapid freeze fixation of plant cells. Protoplasma 131:153–165
41. Fields SD, Strout GW, Russell SD (1997) Spray-freezing freeze substitution (SFFS) of cell suspensions for improved preservation of ultrastructure. Micro Res Tech 38:315–328
42. Gilkey JC, Staehelin LA (1986) Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J Elect Microsc Tech 3:177–210
43. Ding B, Turgeon R, Parthasarathy MV (1991) Routine cryofixation of plant tissue by propane jet freezing for freeze substitution. J Electron Microsc Tech 19:107–117
44. McDonald KL, Morphew M, Verkade P, Muller-Reichert T (2007) Recent advances in high-pressure freezing. Meth Mol Biol 369:143–173
45. Monaghan P, Robertson D (1990) Freeze-substitution without aldehyde or osmium fixatives: ultrastructure and implications for immunocytochemistry. J Microscopy 158:355–363
46. Kaech A, Ziegler U (2014) High-pressure freezing: current state and future prospects. Meth Mol Biol 1117:151–172
47. King BH (2010) Electron microscopy and high-pressure freezing of *Arabidopsis*. Meth Cell Biol 96:259–283
48. Wilson SM, Bacic A (2012) Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. Nature Protoc 7: 1716–1727
49. McDonald KL (2014) Out with the old and in with the new: rapid specimen preparation procedures for electron microscopy of sectioned biological material. Protoplasma 251:429–448
50. McDonald KL, Webb RI (2011) Freeze substitution in 3 hours or less. J Microscopy 243: 227–233
51. Hurbain I, Sachse M (2011) The future is cold: cryo-preparatoon method for transmission electron microscopy of cells. Biol Cell 103:405–420
52. Mayers CP (1970) Histological fixation by microwave heating. J Clin Pathol 23:273–275
53. Login GR, Dvorak AM (1994) Methods of microwave fixation for microscopy—a review of research and clinical applications: 1970–1992. Prog Histochem Cytochem 27:1–119
54. Giberson RT, Demaree RS Jr (eds) (2001) Microwave techniques and protocols. Humana Press, Totowa
55. Zcehmann B, Zellnig G (2009) Microwave-assisted rapid plant sample preparation for transmission electron microscopy. J Microscopy 233:258–268
56. Leria F, Marco R, Medina FJ (2004) Structural and antigenic preservation of plant samples by microwave-enhanced fixation, using dedicated hardware, minimizing heat-related effects. Micros Res Tech 65:86–100
57. Russin WA, Trivett CL (2001) Vacuum-microwave combination for processing plant tissues for electron microscopy. In: Giberson RT, Demarce RS Jr (eds) Microwave techniques and protocols. Humana Press, Totowa, pp 25–35
58. Webster P (2014) Microwave-assisted processing and embedding for transmission electron microscopy. Meth Mol Biol 1117:21–37

59. Schichnes D, Nemson JA, Ruzin SE (2001) Microwave paraffin techniques for botanical tissues. In: Giberson RT, Demarce RS Jr (eds) Microwave techniques and protocols. Humana Press, Totowa, pp 181–189
60. Schichnes D, Nemson J, Sohlberg L, Ruzin SE (1999) Microwave protocols for paraffin microtechnique and in situ localization in plants. Microsc Microanal 4:491–496
61. Zellnig G, Mostl S, Zechmann B (2013) Rapid immunohistochemical diagnosis of tobacco mosaic virus disease by microwave-assisted plant sample preparation. Microscopy 62: 547–553
62. Jauneau A, Cabin-Flaman A, Morvan C, Pariot C, et al (1994) Polysaccharide distribution in the cellular junctions of immature fibre cells of flax seedlings. Histochem J 26:226–232
63. Beneŝ K (1968) On stainability of plant cell walls with alcian blue. Biol Plant 10:334–346
64. Lawton JR (2011) The use of malachite green during fixation of plant tissues for the preservation of lipids. J Microscopy 144:201–209
65. Hughes JE, Gunning BES (1980) Glutaraldehyde-induced deposition of callose. Can J Bot 58:250–258
66. Sonobe S, Shibaoka H (1989) Cortical fine actin filaments in higher plant cells visualized by rhodamine-phalloidin after pretreatment with m-maleimidobenzoyl N-hydroxysuccinimide ester. Protoplasma 148:80–86
67. McLean W, Nakane PF (1974) Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
68. Swatzell LJ, Edelmann RE, Makaroff CA, Kiss JZ (1999) Integrin-like proteins are localized to plasma membrane fractions, not plastids, in *Arabidopsis*. Plant Cell Physiol 40:173–183
69. Huang BQ, Sheridan WF (1994) Female gametophyte development in maize: microtubular organization and embryo sac polarity. Plant Cell 6:854–861
70. Huang BQ, Russell SD (1994) Fertilization in *Nicotiana tabacum*: cytoskeletal modifications in the embryo sac during synergid degeneration. A hypothesis for short-distance transport of sperm cells prior to gamete fusion. Planta 194:200–214
71. Huang BQ, Ye XL, Yeung EC, Zee SY (1998) Embryology of *Cymbidium sinense*: the microtubule organization of early embryos. Ann Bot 81:741–750
72. Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. Planta 187:405–413
73. Vitha S, Baluška F, Mews M, Volkmann D (1997) Immunofluorescence detection of F-actin on low melting point wax sections from plant tissues. J Histochem Cytochem 45:89–95
74. Griffiths G (1993) Fine structure immunocytochemistry. Springer, Berlin
75. Benhamou N, Noel S, Grenier J, Asselin A (1991) Microwave energy fixation of plant tissue: an alternative approach that provides excellent preservation of ultrastructure and antigenicity. J Electron Microsc Tech 17:81–94
76. Heumann HG (1992) Microwave-stimulated glutaraldehyde and osmium tetroxide fixation of plant tissue: ultrastructural preservation in seconds. Histochem 97:341–347
77. Carpentier A, Abreu S, Trichet M, Satiat-Jeunemaitre (2012) Microwaves and tea: new tools to process plant tissue for transmission electron microscopy. J Microscopy 247:94–105
78. Takahashi H, Kamakura H, Sato Y, Shiono K et al (2010) A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J Plant Res 123: 807–813
79. Belmonte MF, Kirkbride RC, Stone SL, Pelletier JM et al (2013) Comprehensive developmental profiles of gene activity in regions and subregions of the *Arabidopsis* seed. Proc Natl Acad Sci U S A 110:E435–E444
80. Páska C, Krisztina B, László S, Annamária T et al (2004) Effect of formalin, acetone, and RNAlater fixatives on tissue preservation and different size amplicons by real-time PCR from paraffin-embedded tissue. Diagn Molec Path 13:234–240
81. Hess MW (2003) Of plants and other pets: practical aspects of freeze-substitution and resin embedding. J Microscopy 212:44–52

82. Hess MW (2007) Cryopreparation methodology for plant cell biology. Meth Cell Biol 79: 57–100
83. Austin II JR (2014) High-pressure freezing and freeze substitution of *Arabidopsis* for electron microscopy. Meth Mol Biol 1062:473–486
84. Kuo J (2014) Processing plant tissue for ultrastructural study. Meth Mol Biol 1117:39–55
85. Kiss JZ, Giddings TH Jr, Staehelin LA, Sack FD (1990) Comparison of the ultrastructure of conventionally fixed and high pressure frozen/freeze substituted root tips of *Nicotiana* and *Arabidopsis*. Protoplasma 157:64–74
86. Stachelin LA, Giddings TH Jr, Kiss JZ, Sack FD (1990) Macromolecular differentiation of Golgi stacks in root tips of *Arabidopsis* and *Nicotiana* seedlings as visualized in high pressure frozen and freeze-substituted samples. Protoplasma 157:75–91
87. Thijssen MH, Mittempergher F, Van Aelst AC, Van Went JL (1997) Improved ultrastructural preservation of *Petunia* and *Brassica* ovules and embryo sacs by high pressure freezing and freeze substitution. Protoplasma 197:199–209
88. Kaneko Y, Walther P (1995) Comparison of ultrastructure of germinating pea leaves prepared by high-pressure freezing-freeze substitution and conventional chemical fixation. J Electron Microsc 44:104–109
89. Bourett TM, Czymmek KJ, Howard RJ (1999) Ultrastructure of chloroplast protuberances in rice leaves preserved by high-pressure freezing. Planta 208:472–479
90. Titford M (2001) Safety considerations in the use of fixatives. J Histotechnol 24:165–171

Chapter 3
Paraffin and Polyester Waxes

Claudio Stasolla and Edward C. Yeung

3.1 Introduction

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

C. Stasolla (✉)
Department of Plant Science, University of Manitoba, 222 Agriculture Bld,
Winnipeg, Manitoba R3T 2N2, Canada
e-mail: Claudio.Stasolla@umanitoba.ca

E. C. Yeung
Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_3

3.1.1 Paraffin Waxes

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

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

3.1.2 Polyester Waxes

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

3.2 Technical Comments

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

3.2.1 Fixation

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

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

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

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

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

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

3.2.2 Removal of Air from Botanical Specimens

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

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

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

3.2.3 Dehydration

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

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

3.2.4 Transitional Fluids and Wax Infiltration

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

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

3.2.5 Polyester and Paraffin Wax

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

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

3.2.6 Embedding

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

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

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

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

3.2.7 Mounting of Wax Blocks

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

3.2.8 Microtomy

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

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

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

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

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

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

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

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

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

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

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

3.2.9 Flattening of Sections on Slides

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

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

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

3.2.10 Staining

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

3.2.11 Safety

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

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

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

3.3 Materials

3.3.1 Equipment

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

3.3.2 Laboratory Supplies

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

3.3.3 Embedding Waxes

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

3.3.4 Chemical Reagents

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

3.3.5 Solutions for Wax and Polyester Wax Preparation

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

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

3.4 Methods

3.4.1 Paraffin Wax Embedding Method

3.4.1.1 Fixation

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

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

3.4.1.2 Dehydration

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

3.4.1.3 Infiltration of Paraffin Wax

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

3.4.1.4 Embedding of Large Samples

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

3.4.1.5 Embedding of Small Samples

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

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

3.4.1.6 Mounting of Paraffin Blocks

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

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

3.4.1.7 Sectioning and Flattening of Sections

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

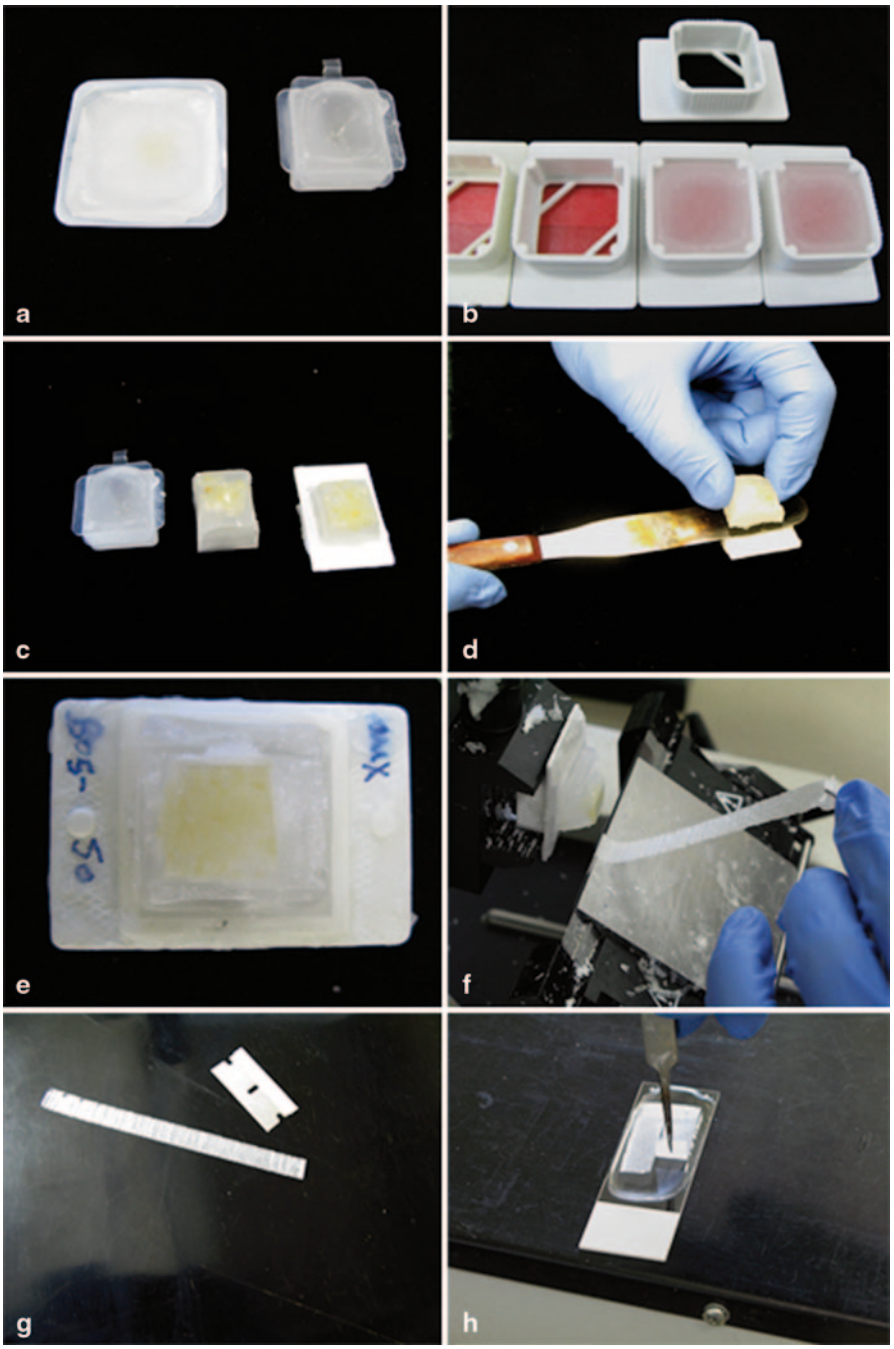

Fig. 3.1 **a** Embedding and orientation of the samples can be achieved using either weighing boats (*left*) or Peel-A-Way® Disposable Histology Molds (*right*). Melted wax containing the samples is first poured in the respective molds, and the samples are then placed at the bottom of the molds

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

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

3.4.2 Polyester Wax Embedding Method

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

3.4.2.1 Fixation

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

---

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

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

3.4.2.2 Dehydration

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

3.4.2.3 Infiltration of Polyester Wax

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

3.4.2.4 Embedding

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

3.4.2.5 Sectioning and Flattening of Sections

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

3.5 Notes

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

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

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

3.6 Concluding Remarks

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

References

1. Bracegirdle B (1978) A history of microtechnique. Cornell University Press, Ithaca
2. Steedman HF (1957) Polyester wax—a new ribboning embedding medium for histology. Nature 179:1345
3. Jensen WA (1962) Botanical histochemistry. Freeman, San Francisco
4. Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. Iowa State University Press, Ames
5. Sanderson JB (1994) Biological microtechnique. BIOS Scientific Publishers Ltd, Oxford
6. Anderson G, Bancroft J (2002) Tissue processing and microtomy. In: Bancroft JD, Gamble M (eds) Theory and practice of histological techniques. Churchill Livingstone, London, pp 85–107
7. Steedman HF (1960) Section cutting in microscopy. Blackwell Scientific Publications, Oxford
8. Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, New York
9. Winsor L (1994) Tissue processing. In: Woods AE, Ellis RC (eds) Laboratory histopathology: a complete reference. Section 4.2. Churchill Livingstone, London, pp 1–42
10. Thuy TT, Yeung EC, Chinnappa CC (2009) Differences in cellular structure regulate stem elongation of alpine and prairie ecotypes of *Stellaria longipes* grown under two contrasting temperature regimes. Can J Bot 87:1167–1176
11. Vitha S, Baluška F, Jasik J, Volkmann D, Barlow PW (2000) Steedman's wax for F-actin visualization. In: Staiger CJ, Baluška F, Volkman D, Barlow PW (eds) Actin: a dynamic framework for multiple plant cell functions. Kluwer Academic Publishers, Dordrecht, pp 619–636
12. Brown RC, Lemmon BE, Olsen O-A (1994) Endosperm development in barley: microtubule involvement in the morphogenetic pathway. Plant Cell 6:1241–1252
13. Ovečka M, Šamajová O, Baluška F, Šamal J (2014) Immunofluorescent localization of MAPKS in Steedman's wax sections. Meth Mol Biol 1171:117–130

14. Culling CFA, Allison RT, Barr WT (1985) Cellular pathology techniques, 4th edn. Butterworths, London
15. Brown RC, Lemmon BE (1995) Methods in plant immunolight microscopy. Meth Cell Biol 49:85–107
16. Yeung EC, Sexena PK (2005) Histological techniques. In: Jain SM, Gupta PK (eds) Protocols for somatic embryogenesis in woody plants. Springer, Dordrecht, pp 517–537
17. Pappas PW (1971) The use of a chrome alum–gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technol 46:121–124

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

Edward C. Yeung and Colin K. W. Chan

4.1 Introduction

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

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

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_4

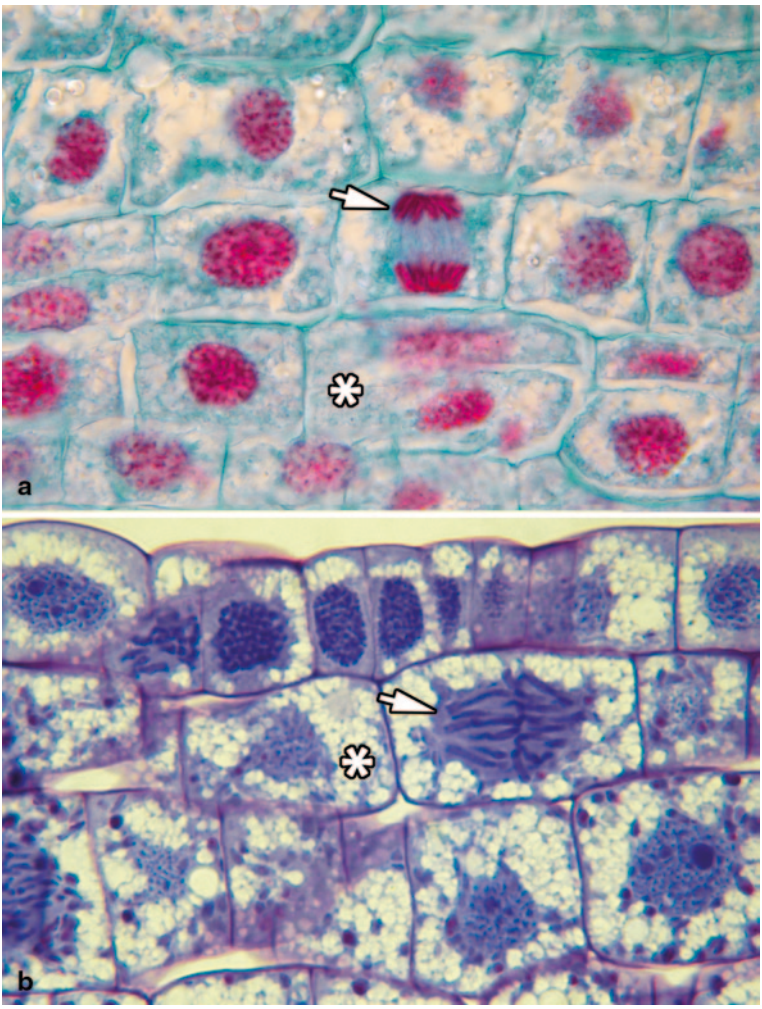

Fig. 4.1 Cotyledon sections of Radiata pine. **a** Paraffin-embedded section showing poor cytoplasmic details, that is, vacuoles (*) and nuclear features *(arrow)* are not distinct. **b** GMA-embedded section showing clear vacuolar (*) and nuclear features *(arrow)*. Scale bars = 20 μm

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

4.1.1 T7100 Embedding Medium

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

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

4.1.2 T8100 Embedding Medium

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

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

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

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

4.2.1 Vacuuming of Samples at Different Stages of Processing

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

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

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

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

4.2.3 Rinsing of Specimens in Embedding Solution

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

4.2.4 Addition of Extra PEG 400 to Soften the Polymerized Blocks

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

4.2.5 Staining of GMA Sections

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

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

4.3 Materials

4.3.1 Laboratory Equipment and Embedding Supplies

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

4.3.2 Embedding Kits and Chemicals

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

4.3.3 Preparation of Embedding Medium, Fixatives, and Solutions

4.3.3.1 Preparation of Infiltration and Embedding Media

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

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

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

4.3.3.2 Preparation of General Fixatives

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

4.3.3.3 Preparing Subbed Slides

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

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

4.4 Methods

4.4.1 General Fixation and Processing

4.4.1.1 Collection and Fixation of Plant Tissues

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

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

4.4.1.2 Dehydration

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

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

4.4.1.3 Infiltration of Samples

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

4.4.1.4 Embedding

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

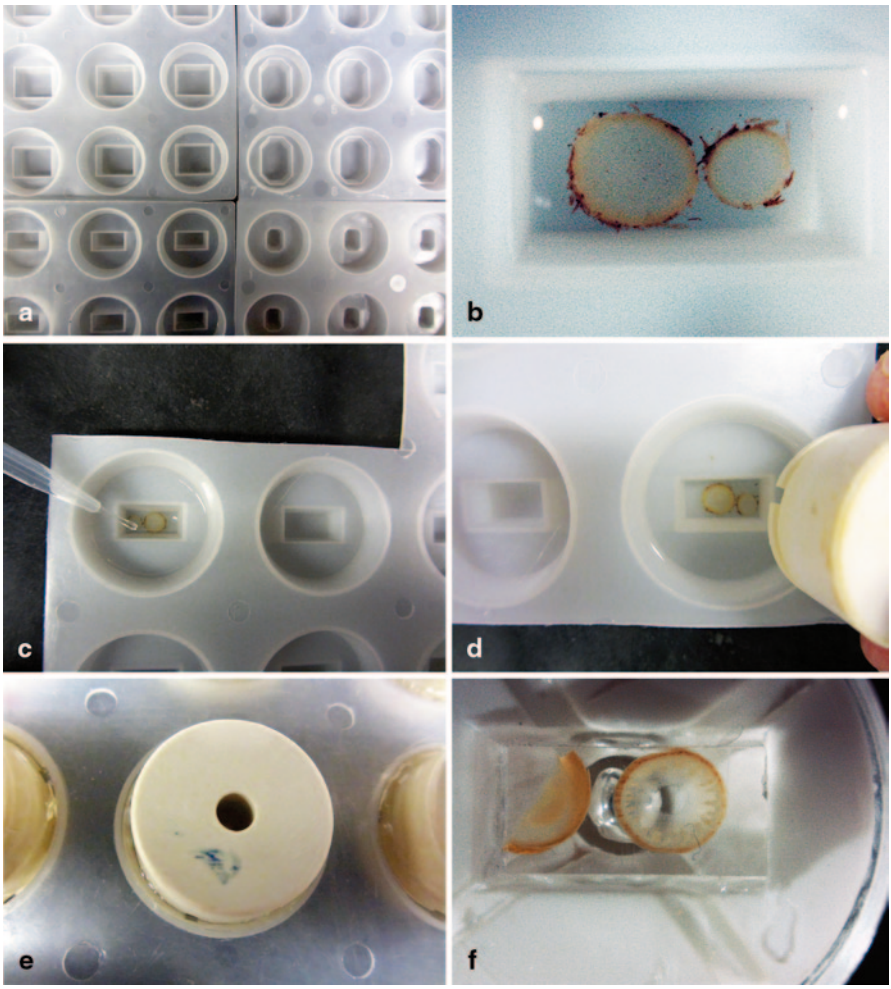

Fig. 4.2 Embedding procedures. **a** Different sizes of embedding mold. **b** Tissue pieces are transferred into a molding cup with infiltration solution. **c** Once the tissues are properly arranged, the infiltration solution is removed using a disposable pipette and the specimens are rinsed once with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

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

4.4.2 Sectioning

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

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

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

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

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

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

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

4.5 Notes

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

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

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

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

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

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

4.6 Concluding Remarks

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

References

1. Newman SB, Borysko E, Swerdlow M (1949) New sectioning techniques for light and electron microscopy. Science 110:66–68
2. Litwin JA (1985) Light microscopic histochemistry on plastic sections. Prog Histochem Cytochem 16:1–84
3. Rosenberg M, Bartl P, Lesko J (1960) Water-soluble methacrylate as an embedding medium for the preparation of ultrathin sections. J Ultrast Res 4:298–303

4. Feder N, O'Brien TP (1968) Plant microtechnique: some principles and new methods. Am J Bot 55:123–142
5. Yeung EC, Chan CKW (2015) Glycol methacrylate: the art of embedding and serial sectioning. Botany 93:1–8
6. Bennett HS, Wyrick AD, Lee SW, McNiel HJ Jr (1976) Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technol 51:71–97
7. Gerrits PO, Smid L (1983) A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. J Microscopy 132:81–85
8. Gerrits PO, Horobin RW (1996) Glycol methacrylate embedding for light microscopy: basic principles and trouble-shooting. J Histotech 19:297–311
9. Lackie S, Yeung EC (1996) Zygotic embryo development in *Daucus carota*. Can J Bot 74:990–998
10. Glauert AM, Lewis PR (1998) Biological specimen preparation for transmission electron microscopy. Princeton University press, New Jersey
11. Lee Y-I, Hsu S-T, Yeung EC (2013) Orchid protocorm-like bodies are somatic embryos. Amer J Bot 100:2121–2131
12. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi, Melbourne
13. Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, New York
14. Horobin RW (1982) Histochemistry. Gustav Fischer, Stuttgart
15. Reid N (1975) Ultramicrotomy. North-Holland Publishing Co., Amsterdam
16. Hand NM (2002) Plastic embedding media and techniques. In: Bancroft JD, Gamble M (eds) Theory and practice of histological techniques. Churchill Livingstone, London, pp 663–677
17. Quester R, Knifka J, Schroder R (2002) Optimization of glycol methacrylate embedding of large specimens in neurological research. Study of rat skull-brain specimens after implantation of polyester meshes. J Neurosci Meth 113:15–26
18. Yeung EC, Law SK (1987) Serial sectioning techniques for a modified LKB Historesin. Stain Technol 62:147–153
19. Yeung EC (1984) Histological and histochemical staining methods. In: Vasal IK (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic, New York, pp 689–697
20. Yeung EC, Saxena PK (2005) Histological techniques. In: Jain SM, Gupta PK (eds) Protocols for somatic embryogenesis in woody plants. Springer, Dordrecht, pp 517–538
21. Soukup A (2014) Selected simple methods of plant cell wall histochemistry and staining for light microscopy. Meth Mol Biol 1080:25–52
22. Pappas PW (1971) The use of a chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technol 46:121–124
23. Gerrits PO, Eppinger B (1995) Effects of storing initiated 2-hydroxyethyl methacrylate solutions for embedding tissues for light microscopy: some practical implications. Biotech Histochem 70:155–163
24. Goodbody KC, Lloyd CW (1994) Immunofluorescence techniques for analysis of the cytoskeleton. In: Harris N, Oparka KJ (eds) Plant cell biology. IRL Press, Oxford, pp 221–243
25. Gerrits PO, Horobin RW, Stokroos I (1992) The effects of glycol methacrylate as a dehydrating agent on the dimensional changes of liver tissue. J Microscopy 165:273–280

Chapter 5
Epoxy Resins for Light and Transmission Electron Microscopy

Michael John Sumner

5.1 Introduction

Epoxy resins were first introduced as an embedding medium for electron microscopy in 1956 by Glauert et al. [1], and by Richardson et al. [2] in 1960 for light microscopy. The liquid resin mixture contains three main ingredients: (A) an epoxy resin (an aliphatic or aromatic epoxide), (B) a hardening agent which when heated forms a solid cross-linked polymer that is rigid enough to produce ultrathin sections (between 50 and 100 nm) and is stable under the transmitted electron beam and (C) an accelerator. The three most commonly used resins are Araldite, Epon and ERL. Using the recipes of Luft, [3], Epon 812 has been the most widely used epoxy embedding medium for electron microscopy [4]. Epon 812 was discontinued by the manufacture Shell in 1984. There are a number of Epon substitutes [4] now available from a variety of EM supply companies. These resin mixtures section easily, provide good specimen contrast when stained with heavy metals, and have a relatively low viscosity as compared to Araldite. This allows the Epon resin mixture to penetrate the tissue faster than Araldite, which has a much higher viscosity. According to Ellis [5], the various Epon substitutes on the market today are not identical to the original Epon 812 produced by Shell, and users should include in publications the precise mixture and chemistry of the Epon substitute used. In 1969, Spurr [6] introduced a new epoxy resin, vinylcyclohexene dioxide (VCD) (ERL 4206 of Union Carbide—now Dow Chemical), having a much lower viscosity than Epon 812 and its substitutes, that facilitates rapid infiltration into tissues. ERL 4206 and its hardener, nonenyl succinic anhydride (NSA) alone produce a very hard block. Spurr countered this problem by adding a reactive flexibilizer, diglycidyl ether of polypropylene glycol (DER 736), to the embedding medium. The hardness of the resin block is controlled by varying the amount of DER 736.

M. J. Sumner (✉)
Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
e-mail: Michael.Sumner@umanitoba.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_5

ERL 4206 is no longer available and has been substituted with ERL 4221. ERL 4221 is somewhat more viscous than ERL 4206; however, the properties of the new Spurr's resin medium, using ERL 4221, is very similar to the original formulation. Glauert and Lewis [7] recommend using benzyldimethylamine (BDMA) as the accelerator in the Spurr's epoxy medium in place of the original Spurr's accelerator, dimethylaminoethanol (DMAE). DMAE is widely used as the accelerator in the Spurr's embedding medium and continues to produce satisfactory results in our lab. The low viscosity of the Spurr's embedding medium makes it ideal for the infiltration of plant tissue which, unlike animal tissue, has the complexity of hard extracellular cell walls, starch grains, calcium oxalate crystals and a waxy cuticle that are difficult to infiltrate using traditional Epon and Araldite embedding media. A new epoxy resin has been recently introduced by Electron Microscopy Sciences (Ultra Bed Low Viscosity Epoxy catalogue # 14310) and Polysciences (Embed-it catalogue # 64-86-8). These new very low viscosity resin kits are reported to be similar to their Spurr ERL 4221 low viscosity embedding kits and are less hazardous *(see Note 3)*. This chapter focuses on the standard protocols used in the preparation of Spurr's epoxy embedded plant tissue for transmission electron microscopy as well as the protocols used to section and stain epoxy embedded tissue for light microscopy.

5.1.1 Tissue Preparation for Transmission Electron Microscopy [7–11]

The high-resolution transmission electron microscope (TEM) is but one of the many tools available to biologists in the quest to understand the structure and function of the living cell. Start your anatomical investigation with a thorough examination of the anatomical literature specific to the subject matter, followed by an in-depth examination of the relevant tissues of the living organism using techniques presented in Chaps. 1–4 of this book. Only after a thorough understanding of the three-dimensional structure and organization of the relevant tissues is achieved should one proceed to the use of the TEM in a research project; of course, its use is dependent on the research question being asked.

In general, the preparation of tissue for TEM involves six fundamental steps: fixation, dehydration, infiltration, embedding, sectioning and staining. The process starts with the careful dissection of small (less than 1 mm) segments of hydrated living tissue with a sharp razor blade and ends with nonliving dehydrated tissue that has been infiltrated and embedded in an epoxy resin matrix. Thin (1–2 μm) survey sections are affixed to coated glass slides, stained and viewed with a light microscope. Ultrathin (60–80 nm) sections are subsequently cut from the same block, picked up on copper or nickel grids, stained and viewed with a TEM to localize the subcellular structures that we hope are representative of the once living tissue. However careful and precise we are during the preparation process, artifacts are an unavoidable consequence at all steps of the process [12].

5.2 Materials

5.2.1 Major Equipment

1. TEM
2. Compound light microscope
3. Ultramicrotome
4. Glass knife maker
5. Diamond knife for ultrathin sectioning *(see Note 1)*.

5.2.2 General Laboratory Equipment and Supplies

1. Glass vials (10–15 mL), vial holder and small identification labels inserted into the vial
2. Double-sided razor blades and high quality dissecting kit
3. Top loading balance for measuring Spurr's resin components
4. Disposable plastic beakers and pipettes
5. Rotary mixer to hold the glass vials during fixation, dehydration and infiltration
6. Aluminum embedding (weighing) dishes (44 mm)
7. Fine-bladed piercing saw for cutting out 2–3 mm pieces of embedded tissue
8. Plastic Beem® capsules containing blank polymerized epoxy resin stubs
9. Fine carpenter's file for precise flattening (90° to the long axis) and removal of the pyramidal end of the blank epoxy resin stub
10. Five minute epoxy glue to glue the pieces of embedded tissue to the end of the epoxy resin stub (termed the tissue block)
11. Rotary tools (e.g. Dremel) can be used for careful removal of excess resin from around tissue block (optional)
12. Silver tape and dental wax to make glass knife boat, clean boated triangle glass knives for thin survey sectioning
13. Ten cc syringes fitted with 0.45 µm filters for distilled water and stains
14. Eyelash attached to wood stick by dental wax for manipulating ultrathin sections floating on the surface of the diamond knife boat
15. Standard and locking fine forceps (# 5)
16. Drawn glass microprobe, loop or shaved wooden spatula stick to pick up thin sections from the glass knife boat
17. Copper and nickel TEM grids *(see Note 2)*
18. Petri dishes with filter paper to store each type of cleaned TEM grid
19. Petri dish with a numbered rubber pad for keeping track of stained grid sections
20. Stained grid storage containers
21. Microscope slides, number 1.5 coverslips
22. Slide warmer
23. Glass strips for making knives.

5.2.3 Embedding Kits, Chemicals and Stains

1. Spurr low viscosity embedding kit (EMS catalogue # 14300) (Ted Pella catalogue # 18108) (Polysciences catalogue # 01916-1) *(see Note 3)*
2. 2–4 % glutaraldehyde primary fixative in buffer *(see Note 4)*
3. 1–2 % osmium tetroxide (OsO_4) secondary fixative in buffer
4. Ethanol or acetone for dehydration, propylene oxide for infiltration (optional when using Spurr's resin)
5. Filtered distilled water
6. Light microscope stains and related reagents: crystal violet, toluidine blue O (TBO), borax to stain for thin survey sections,aniline blue black, calcofluor, aniline blue stains, periodic acid, Schiff's reagent, sodium metabisulphite, DNPH (2, 4-dinitrophenol-hydrazine), gelatin, glycerol, chromium potassium sulphate for coating frosted microscope slides, non-permanent mounting media (70 % sucrose)
7. Chloroform for stretching thin and ultrathin sections
8. TEM stains and related reagents: uranyl acetate and lead citrate stains for ultrathin sections, thiocarbohydrazide, silver proteinate, acetic acid, potassium ferricyanide, calcium chloride and cacodylate buffer.

5.3 Methods

5.3.1 Chemical Fixation for Conventional TEM

A comprehensive discussion of fixation is found in Chap. 2 of this book. A standard protocol is to fix tissue in 3 % glutaraldehyde in 0.05 M phosphate or cacodylate buffer (pH 6.8–7.2) for 2 h at room temperature, followed by 24 h at 4 °C in the same fixative, then post-fixed for 4 h in 2 % OsO_4 in 0.05 M phosphate or cacodylate buffer (pH 6.8–7.2, *see Note 5*).

5.3.2 Dehydration (see Note 6)

Tissues are dehydrated in a graded ethanol series 30, 50, 70, (20–30 min each), 95 (two changes 20–30 min each), 100 % (three changes 30 min each) followed by successive changes of ethanol:propylene oxide (3:1; 1:1; 1:3; for 30 min each), and three changes of 100 % propylene oxide (30 min each). The tissues are constantly rotated during all stages of dehydration. Be careful while decanting solutions during dehydration; tissue samples *must not be allowed to dry out*.

5.3.3 Infiltration

1. Spurr's resin medium [6, 8, 10] *(see Note 3)*

	EMS (mL)	Polysciences (g)
ERL—4221	18	4.10
D.E.R.736	14	1.43
NSA	48	5.90
DMAE	0.6	0.1

2. In our lab, tissues are typically infiltrated with a mixture of propylene oxide and Spurr's low viscosity epoxy resin 3:1; 1:1; for 2 h each; 1:3 for 48 h, followed by daily changes of 100 % fresh Spurr's resin for 3–7 days depending on the type of plant tissue being infiltrated.
3. Tissues containing abundant starch and particularly thick cell walls should be infiltrated for a week. Infiltration is a key step in the process and the precise time needed for complete infiltration can only be determined by trial and error. The tissues are constantly rotated during all stages of infiltration.

5.3.4 Embedding

1. Following infiltration, the tissues are embedded in 100 % Spurr's resin in shallow 44 mm diameter aluminum dishes, separated from each other using the shaved tip of a wooden stick and polymerized at 70 °C under partial vacuum for 8–12 h.
2. Observe the embedded tissue (black due to OsO_4 fixation) within the disk of epoxy resin using a stereomicroscope and a transmitted light source. Draw a rectangular outline around the tissue using an extra fine black felt pen and note the orientation of the tissue within the resin.
3. Secure the resin disk in a vice and slowly cut the outlined cube of embedded tissue from the resin disc using a fine-bladed piercing saw. Using fast curing epoxy adhesive such as the 5-minute epoxy glue, glue the cube to a cylindrical Beem resin stub with the long axis of the tissue precisely parallel to the flat stub face for cutting longitudinal sections or with the long axis of the tissue precisely perpendicular to the flat stub face for cutting transverse sections *(see Note 7)*.

5.3.5 Sectioning

5.3.5.1 Thin Sectioning for Light Microscopy

1. Start by reading the microtome instruction manual to gain a thorough understanding of how the machine operates.

2. The block of epoxy embedded tissue being initially used for light microscopy is typically trimmed to a rhomboid shaped surface of 1 mm^2 or less. Because of the small size of the block face, microtomes used for thin sectioning should be fitted with a binocular magnification system similar to that found on an ultramicrotome.
3. Reset the microtome advance mechanism and retract the course and fine stage manipulators.
4. Insert and firmly secure the block into the chuck. Be sure the lower block face is parallel to the knife-edge.
5. Clamp a "wet" triangular glass knife onto the stage and initially set the knife angle to 3°. Adjust the water level in the knife boat to wet the knife-edge.
6. Carefully advance the stage knife toward the block using the course and fine adjustments. When the knife-edge is about 0.5 mm from the block face, carefully advance the stage toward the block using the fine adjustment while simultaneously advancing the block toward the knife at a rate of 2 μm per cycle until a small portion of the block face is initially cut. Reset the microtome to 1 μm and continue to cycle the block until the entire block face is being cut with each cycle.
7. If the face of the block has been previously rough trimmed to remove damaged wound tissue at the initial cut line, you can begin to collect sections with a drawn glass microprobe (or a wire loop or a wooden spatula) and transfer them to drops of filtered distilled water on clean Haupt's coated microscope slides *(see Note 8)*. Allow the slide to dry on a slide warmer at 85 °C. Stain the 1–2 μm survey sections with crystal violet [13] or TBO [10].
8. Once the target zone is reached, stop, retract the microtome stage and carefully remove the block from the microtome to be retrimmed for ultamicrotomy using a diamond knife.

5.3.5.2 Ultrathin Sectioning for Transmission Electron Microscopy

1. Start by reading the ultramicrotome instruction manual to gain a thorough understanding of how the machine operates.
2. Novice users should practice and become proficient at ultrathin sectioning with a "wet" triangular glass knife prior to sectioning valuable research material with a clean diamond knife.
3. Examine the most recent stained light microscope survey sections to locate the centre and extent of the target zone.
4. Using a new clean fine razor blade, carefully retrim the block from Sect. 5.3.5.1 to a size that is as small as possible (maximum 0.5 mm) and still includes the target zone. Be sure the top and bottom faces of the block face are trimmed precisely parallel to each other to allow for a straight ribbon of ultrathin sections to be cut using the ultramicrotome. Trim the sides to maintain the rhomboid shape of the block face. Clean all trim debris from the block.

5. Insert the trimmed block into the ultramicrotome chuck, making sure that no more than 5 mm of the block extends beyond the chuck to minimize vibrations that produce ultrathin sections with chatter [12].
6. Insert a clean diamond knife *(see Note 1)* into the ultramicrotome stage holder and set the knife angle according to the manufacturer's instructions. Fill the boat with water from a clean filtered (0.45 μm) syringe. Slowly raise the water level in the boat (slightly convex) to wet the knife-edge. Withdraw a small amount of water until a slightly flat to concave water level is achieved. With proper adjustment of the illumination from the ultramicrotome stereomicroscope system, the knife-edge, the surface of the water and the face of the block should become visible. From this point onward, all observations will be made through the microscope.
7. Carefully advance the stage knife toward the block face using the coarse and fine adjustments of the stage. Manually cycle the block until it is level with the knife-edge. Use the stage fine adjustment to advance the knife toward the block until the shadow of the knife appears on the illuminated block face.
8. Manually cycle the ultramicrotome to advance the block face toward the knife-edge at 1 μm intervals until the block face shadow of the knife-edge is very thin. Using the chuck fine adjustment, rotate the block until the lower edge of the block face is perfectly parallel with the knife-edge.
9. Switch the ultramicrotome to automatic with a cutting speed of about 2–3 mm/s and a section thickness of 200 nm (purple interference colour). Continue cycling until the block face first contacts the knife-edge. Stop the ultramicrotome.
10. Set the section thickness to between 80 nm (pale gold) to 60 nm (silver) and begin to collect ribbons of sections. Dip the end of an applicator stick in chloroform or xylene and pass the stick over the sections for 5 s. The vapours of the solvent will stretch the sections. Periodically add water to the boat to maintain the surface illumination and section interference colours. Gold coloured sections are fine for low magnification electron microscopy while grey to silver are better for higher magnification work. It is advisable to cut and collect both for future use.
11. Using the tip of the eyelash applicator stick, move the ribbons toward the centre of the boat and arrange in clusters of 2–3 ribbons per cluster.
12. Pick up the edge of a dry clean grid *(see Note 2)* with a pair of fine (# 5) locking forceps. Bend the edge of the grid so that the locking forceps are at a 45° angle relative to the grid surface. With a steady hand lower the grid (dull side down) over a grid cluster. The cluster will be attracted to the grid surface along with a drop of boat water.
13. Place the wet grid (section side up) on a filter paper in a glass petri dish. Cover and let dry. Repeat the process for each section cluster.

5.3.6 Staining

5.3.6.1 Staining Epoxy Sections for Light Microscopy *(see Note 9)*

Unlike glycol methacrylate (GMA), a water permeable plastic, the epoxy resins are relatively water impermeable. Traditional staining techniques developed for fresh, paraffin-embedded and GMA-embedded tissue rely heavily on dyes dissolved in water or aqueous buffers. The majority of these staining techniques have proven to be ineffectual when applied to epoxy-embedded plant tissue. Limited success has been achieved using cationic dyes (e.g. TBO, methylene blue, azure II) at high pH (10).

Crystal Violet [13] *(see Note 10)*

1. Superior contrast is obtained by staining epoxy sections, on a slide warmer at 85 °C, with 2 % ethanolic crystal violet in 0.05 M ammonium oxalate buffer at pH 6.7 for 0.5–1.0 min. Use a filtered (0.45 μm) syringe and apply drops of stain directly on the sections on the slide.
2. Rinse in running distilled water for 5 min. Use Coplin jars.
3. Decolourize in 95 % EtOH if necessary
4. Mount in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*

TBO [10] *(see Note 12)*

1. Stain tissue with 1 % TBO in 1 % aqueous borax at 50–85 °C on a slide warmer for 1–5 min. Use a filtered (0.45 μm) syringe and apply drops of stain directly on the sections on the slide.
2. Rinse in running distilled water for 5 min. Use Coplin jars.
3. Mount in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*.

Aniline Blue Black [14] *(see Note 13)*

1. Sections are stained in 1 % aniline blue black in 7 % acetic acid for 15 min at 60 °C. Use Coplin jars in a water bath.
2. Rinse in 7 % acetic acid to remove excess stain. Use Coplin jars.
3. Mount in glycerol containing 5 % acetic acid using size 1.5 coverslips.

Periodic Acid—Schiff Reaction (PAS) [10] *(see Note 14)*

1. Block aldehydes using a saturated solution of DNPH (2,4-dinitrophenol-hydrazine) in 15 % acetic acid. Use Coplin jars.
2. Wash in running distilled H_2O for 10 min.
3. 1 % aqueous periodic acid for 30 min.
4. Wash in running distilled H_2O for 5 min.
5. Schiff's reagent 35–45 min.
6. 0.5 % sodium metabisulphite in 1 % dilution of concentrated HCl (optional) 2 × 2 min.
7. Wash in running distilled H_2O 10 min.
8. Counter stain (optional) with TBO, crystal violet or aniline blue black.
9. Mount crystal violet and TBO stained sections in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*.
10. Mount aniline blue-black stained sections in glycerol containing 5 % acetic acid using size 1.5 coverslips.

5.3.6.2 Staining for Fluorescence Microscopy

1. Sections from material fixed in glutaraldehyde -OsO_4 are immersed in 1 % periodic acid or 10 % hydrogen peroxide for 30 min to bleach osmium from the tissues.
2. Using a diamond point pencil on the bottom of the glass slide, outline the clusters of epoxy sections adhering to the Haupt's adhesive on the upper surface of the slide. Prior to staining epoxy-embedded material with fluorochromes (e.g. calcofluor, aniline blue) the resin must be removed from the sections *(see Note 15)*

Calcofluor [15] *(see Note 16)*

Epoxy sections are stained in 0.01 % aqueous calcofluor white M2R for 5 min, rinsed in running water, mounted in 70 % sucrose and viewed with ultraviolet (UV) light using a UV excitation filter (330–380 nm), dichroic mirror DM400 and barrier filter transmitting above 420 nm.

Aniline Blue [10] *(see Note 17)*

1. Epoxy sections are stained for 5 min in 0.05 % aniline blue in 0.01 M phosphate buffer at pH 8.5, mounted in the buffered stain and illuminated with UV light using a UV excitation filter (330–380 nm), dichroic mirror DM400 and barrier filter transmitting above 420 nm.

5.3.6.3 Staining for Transmission Electron Microscopy *(see Note 18)*

Conventional Staining of Aldehyde/OsO_4 Fixed Tissues with Uranyl Acetate Followed by Lead Citrate [7–11]

A. Uranyl Acetate *(see Note 19)*

1. Add 3.75 g uranyl acetate to 50 mL of 50% methanol.
2. Stain grids with a filtered(0.45 µm) syringe of a saturated solution of uranyl acetate in 50% methanol at room temperature for 5–25 min.
3. Cover sections during staining to block light induced precipitates.
4. Rinse 2x in 50% methanol; 2x filtered degassed H_2O.
5. An optional procedure is en bloc staining with uranyl acetate prior to dehydration [10, 11].

B. Lead citrate [16] (alternative methods *see* [17, 18])

1. Add 0.02 g lead citrate to 10 mL of degassed distilled water in centrifuge tube.
2. Add 0.1 mL of 10 N NaOH, seal and shake to dissolve.
3. Stain grids with a solution of lead citrate for 5–10 min. Centrifuge before use.
4. Staining must be done in a CO_2 free environment to prevent the formation of lead carbonate precipitates *(see Note 20)*.
5. Place drops of stain on squares of Parafilm, dental wax or plastic petri dishes.
6. Rinse in degassed filtered water.
7. Dry.
8. Excellent contrast with sections that have been fixed with OsO_4.

Periodic Acid—Thiocarbohydrazide—Silver Proteinate (PA-TCH-SP) [19] *(see Note 21)*

1. Collect ultrathin sections of tissue, fixed in glutaraldehyde—OsO_4 (*see* Sect. 5.3.1), on clean dry nickel grids *(see Note 2)*.
2. Float grids section-face down on 1% aqueous periodic acid for 30 min in a high humidity chamber, followed by three changes of 10 min each in distilled water.
3. Grids are transferred to 0.2% thiocarbohydrazide (TCH) in 20% aqueous acetic acid for 5 h followed by successive rinses of 10, 5 and 1% aqueous acetic acid (20 min each) and three rinses of distilled water (30 min each).
4. The grids are then immersed in 1% aqueous silver proteinate for 30 min in the dark followed by three successive rinses of 1 h each in distilled water.

OsO_4 and Potassium Ferricyanide (OsFeCN) [20] *(see Note 22)*

1. Tissue is fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) with 5 mM calcium chloride for 2 h at room temperature followed by 24 h at 4 °C in the same fixative.

2. Following a wash in 0.05 M caycodylate buffer (pH 6.8) with 5 mM calcium chloride, the tissue is post-fixed in a mixture of 2 % OsO_4 and 0.8 % potassium ferricyanide in 0.05 M cacodylate buffer with 5 mM calcium chloride for 4 h at room temperature.
3. The methods of dehydration, infiltration, embedding, orientation, sectioning and staining are the conventional TEM methods described previously.
4. The OsFeCN method enhances overall membrane contrast and in particular the ER and nuclear envelope.

5.4 Notes

1. We highly recommend investing in a diamond knife for TEM studies. "Wet" triangular glass knives will cut ultrathin sections; however, the longevity of the glass knife-edge is short and unpredictable. Weeks of valuable time in the preparation of tissue for TEM are potentially wasted if the sharp edge of the glass knife becomes dull as one enters the target zone. Retracting the knife stage, inserting a new glass knife in the knife holder and precisely realigning the knife to the block face is very difficult. Resuming sectioning with the new glass knife generally results in the loss of some if not all of the target zone tissue.
2. TEM grids (analogous to the glass slide in light microscopy) are round (3.05 mm) typically copper or nickel mesh supports, which hold the stained ultrathin sections of tissue. Grids come in a wide variety of mesh shapes and sizes [8]. In our experience, hexagonal and rectangular (75 × 300) mesh grids allow for adequate support of the epoxy sections while maximizing the area of tissue that can be exposed to the electron beam and thus be visible in the TEM. Prior to use, grids should be cleaned first in acetone then in ethanol in a small beaker using a periodic swirling motion. The ethanol is decanted, the beaker (containing the ethanol wet grids) is inverted over a filter paper and placed in a 60 °C oven to dry. The clean dry grids are stored in a clean dust free petri dish.
3. All epoxy resin components are skin irritants and potential carcinogens. ERL 4206 and ERL 4221 in particular are toxic and hazardous. Wear gloves and work in a fume hood over paper towels to absorb any spilled epoxy resin medium liquids. Collect all contaminated vials, paper towels, plastic beakers, etc. within the fume hood in a covered container. Polymerize prior to disposal.
4. Use only electron microscopy-grade glutaraldehyde (available in sealed 25–70 % ampoules).
5. Depending on the research question being asked, there are numerous protocols in the literature for fixing tissues for electron microscopy [4, 7–11, 21].
6. ERL 4221 resin is miscible in ethanol or acetone and does not require a transitional solvent such as propylene oxide following dehydration [4]. Many microscopists still use propylene oxide following ethanol dehydration as a transition solvent with ERL 4221 infiltration and to further facilitate complete dehydration.

7. This step is critical for obtaining precise longitudinal and transverse sections. The orientation of the block of embedded tissue in the microtome/ultramicrotome chuck as well as the glass or diamond knife can be changed by altering the adjustment mechanisms for the microtome chuck and knife holder. However, we have found it preferable to obtain the desired orientation of the tissue during the post-embedding process that then only requires minimal adjustments to centre the trimmed tissue block relative to the knife-edge.
8. Clean microscope slides 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 Haupt's adhesive solution and placed vertically in a dust-free area to dry. Haupt's adhesive [22]: Dissolve 1 g of gelatin in 100 mL of distilled water at 90 °C. Cool to 30 °C add 15 mL of glycerine, stir and filter. Finally the adhesive is stirred and filtered and stored at 4 °C. As a preservative, 2 g of phenol may be added. Another excellent adhesive [23] may be prepared by dissolving 5 g of gelatin in 1000 mL of warm distilled water, and then adding 0.5 g of chromium potassium sulphate (*see* Chap. 4).
9. Prior to staining material fixed in glutaraldehyde—OsO_4 the sections should be immersed in 1% periodic acid (as part of the PAS reaction) or 10% hydrogen peroxide for 30 min to bleach osmium from the tissues.
10. The use of crystal violet (a cationic dye) to stain plant tissues embedded in epoxy resins has not been previously reported, though it is commonly used in bacteriology as part of the Gram staining procedure, and in animal histology to metachromatically stain amyloid. We routinely use crystal violet to stain epoxy survey sections in our lab and find the results superior to other cationic stains (e.g. TBO, methylene blue, azure II at high pH).

    Crystal violet stain recipe:
    Solution A: 2 g crystal violet (certified) in 20 mL 95% EtOH
    Solution B: 0.8 g ammonium oxalate in 80 mL distilled H_2O
    Mix solutions A and B, store for 24 h, filter and store at room temperature in a dark bottle.

11. After viewing with the compound light microscope, wash the slides in Coplin jars using running distilled water to remove the sucrose and coverslip. Store dry.
12. TBO is a cationic metachromatic dye which will stain certain chemical components of tissues a colour different from that of the dye in solution. TBO generally binds to macromolecules containing large numbers of polyanionic binding sites (e.g. carboxyl, sulphate and phosphate groups). The colour shift is from blue to red with the strongest metachromatic reaction (i.e. reddest) being for polymers containing sulphated groups; polymers containing carboxyl groups exhibit a slightly weaker reaction (i.e. pink); and polymers containing phosphate groups exhibit the weakest metachromatic reaction (purple). Sulphated polysaccharides (e.g. fucoidan) and carboxylated polysaccharides (e.g. pectic acids, alginic acids) will stain red to dark pink, RNA will stain purple and DNA will stain blue. By what is termed negative metachromasia, lignin and some

polyphenols stain green [24]. The high pH TBO used to stain epoxy sections shows a much weaker metachromatic reaction when compared to using the stain on fresh sections (*see* Chap. 1) or GMA sections (*see* Chap. 4). Crystal violet also exhibits weak metachromatic reactions similar to high pH TBO.

13. Aniline blue black, an anionic dye, is used to localize protein. The cytosol of healthy cells stain light blue while that of degenerating cells, presumably rich in hydrolytic proteins, stain dark blue. Mitochondria, plastids and regions of nuclei stain light to dark blue. It provides excellent contrast when used as a counter stain following the PAS reaction.
14. The PAS reaction is used to localize insoluble carbohydrates. The mechanism of this two-step histochemical procedure is well documented [25]. Periodic acid (PA) oxidizes vicinal 1,2 glycol groups (hydroxyl groups at the 2nd and 3rd carbon) in carbohydrates to aldehydes, which react with the Schiff's reagent to produce a magenta colour at the reaction site. Glucans with 1–4 linkages, such as pectic compounds, hemicellulose, starch and the oligosaccharide side chains of glycoproteins, are PAS positive. Cellulose, as it occurs in cell walls, is reported to be PAS negative, due perhaps to steric hindrance of available vicinal glycol groups [10]. Compounds will be PAS negative where the hydroxyl groups are not attached to vicinal carbon atoms or where one of the vicinal carbons is involved in linkages (Beta 1–3 glucans, e.g. callose). Certain compounds, other than carbohydrates, will also exhibit a PAS positive reaction. These include the amino acids serine, threonine and hydroxylysine, which have vicinal hydroxy-amino groups that can be oxidized to dialdehydes. Certain lipids will also react positively [26]. Lignin will combine with Schiff's reagent and give a positive reaction without prior periodic acid oxidation [10].
15. Prior to staining Spurr's epoxy-embedded material with fluorochromes, the resin must be removed from the sections using a modification of the procedure of Lane and Europa [27]. Sections mounted on Haupt's gelatin-coated slides are immersed in a saturated solution of potassium hydroxide in 95% ethanol for 2 min, rinsed in three changes of 95% ethanol, followed by 5 min in running water. The resin is removed to facilitate access of the fluorochrome to the tissue and to eliminate the problem of background autofluorescence caused by the resin.
16. Calcofluor has been used as a general stain for plant cell walls [28] and in the study of the site of cellulose synthesis and microfibril assembly [29]. Calcofluor has some specificity for mixed linkage β-glucans [30] and it has been shown to bind to cellulose and chitin fibrils [31].
17. The fluorochrome in aniline blue is thought to be specific for the β1–3 glucan, callose [22], though Smith and McCully [32] question the absolute specificity, suggesting it may also bind to other cell wall polysaccharides. They have shown that aniline blue specificity for callose is enhanced by prior treatment of the tissues with TBO (to block carboxylated polysaccharides) and the PAS procedure (to block polysaccharides with vicinal glycol groups).
18. The most common way to stain TEM sections is when they are mounted on grids [10]. The grid is floated section-down on a drop of stain in a petri dish

on a sheet of dental wax or Parafilm for the appropriate period of time. The stained grid is picked up with locking forceps and thoroughly washed by running drops of filtered distilled water over the sections for 30–60 s. Alternatively, the stained grid is dipped in filtered distilled water in a beaker (10–15 times in each of three fresh changes of distilled water). Air-dry and store in grid boxes. TEM stains contain heavy metals and as such are poisonous. Dispose of using appropriate safety procedures.

19. Uranyl acetate solution is photolabile (store foil wrapped bottles), radioactive and poisonous. It provides good contrast for structures rich in nucleic acids; some proteins, some cell walls.
20. A simple CO_2-free staining chamber is a petri dish containing a reservoir of NaOH solution [recommended by 10] or NaOH pellets that traps CO_2. Place 3–4 drops of lead citrate on the strips of fresh dental wax or Parafilm and quickly float a grid section-down on each drop of stain. Immediately place the cover on the chamber. Alternatively, we routinely use a glass chamber with an outlet and an inlet connected by a hose to a tank of nitrogen gas.
21. The PA-TCH-SP procedure, used to localize insoluble carbohydrates, is a modification of the PAS procedure. PA is used to selectively oxidize vicinal glycol or glycol-amino groups to aldehydes. The aldehydes are then condensed with thiocarbohydrazide (TCH) to produce thiocarbohydrazones, which are strong reducing agents [26]. Subsequent staining with silver proteinate (SP) results in the deposition of reduced silver over the reaction site. Compounds that are PA-TCH-SP positive or negative correspond to those described previously for the PAS reaction. The control most commonly used in this study was the substitution of water for periodic acid. Additional controls were the blockage of aldehydes with aqueous 0.1 % sodium borohydride and the omission of TCH or SP from the staining procedure [26].
22. The OsFeCN post-fixation procedure is used to stain the endoplasmic reticulum (ER) and nuclear envelope. The initial use of this procedure in plant ultrastructure was by Hepler [20] in a study of the distribution of ER in dividing cells of barley. The chemical basis of the staining reaction is unknown; however, White et al. [33] suggested that metal-containing proteins on membranes chelate the cyano-bridged osmium-iron complexes resulting in the deposition of these electron-opaque complexes on the membrane sites. The removal of calcium chloride from the fixatives or the addition of phosphate buffer, which would precipitate the calcium, abolishes the selective staining of the ER and nuclear envelope [20]. We have found that the OsFeCN procedure does not ubiquitously stain all ER. Frequently, in contiguous cells, with equal access to the stain, the ER cisternae of one cell were stained positively, while the ER cisternae of the adjacent cell connected via a common plasmodesma were unstained. There must be a difference in the chemical content of the stained and unstained ER at the time of fixation; however, until the mechanism of staining is understood, the value of the OsFeCN method will be to discern the distribution of ER in the cell and to enhance overall membrane contrast (Figs. 5.1, 5.2, 5.3).

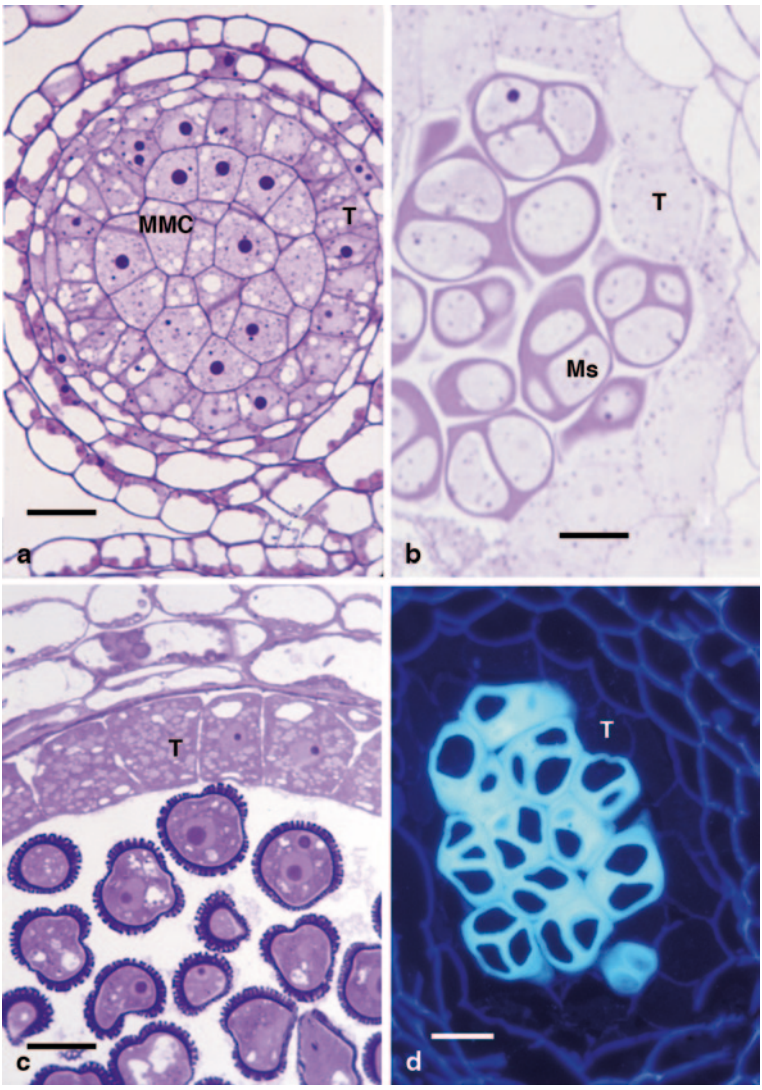

Fig. 5.1 Light and fluorescence micrographs of various stages of pollen development in Saskatoon. **a** Premeiotic anther showing microspore mother cells *(MMC)*, tapetum *(T)* and four outer anther wall layers. Stained with crystal violet. Scale bar = 14.5 µm. **b** and **d** Mature tetrads containing microspores *(Ms)* surrounded by tapetum *(T)*. **b** Stained with PAS for insoluble carbohydrates. Scale bar = 9.0 µm. **d** stained with aniline *blue* for callose and viewed with UV light. Scale bar = 4.5 µm. **c** Anther loculus containing two-celled pollen grains. Tapetum *(T)*. Stained with crystal violet. Scale bar = 15 µm

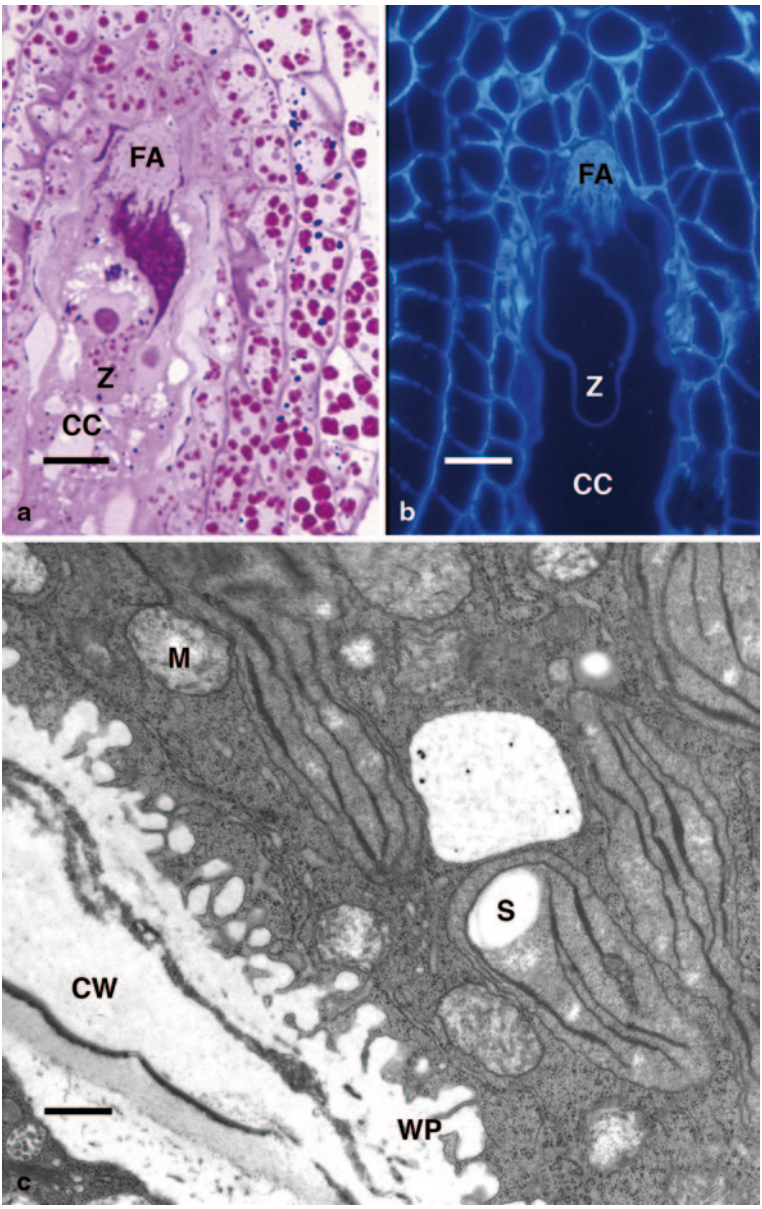

Fig. 5.2 Light, fluorescence, and TEM micrographs of a young seed of Canola. **a** The zygote *(Z)* is contiguous with the embryo sac central cell *(CC)* and a degenerate synergid, containing a PAS positive filiform apparatus *(FA)*. The surrounding integument tissue is rich in PAS positive starch. Stained with PAS and crystal violet. Scale bar = 10.5 μm. **b** Fluorescence micrograph of a serial section to **a**. The section was stained with Calcofluor and viewed with UV light. Beta 1,4 glucan cell wall material of the zygote *(Z)*, the degenerate synergid filaform apparatus *(FA)* and the integument tissue fluoresce a *pale blue*. Scale bar = 10.5 μm. **c** The lateral cell walls *(CW)* of the central cell adjacent to the egg apparatus, zygote and young embryo contain numerous electron-transparent cell wall projections *(WP)* and central cell chloroplast starch *(S)* and mitochondria *(M)*. Stained with uranyl acetate and lead citrate. Scale bar = 0.45 μm

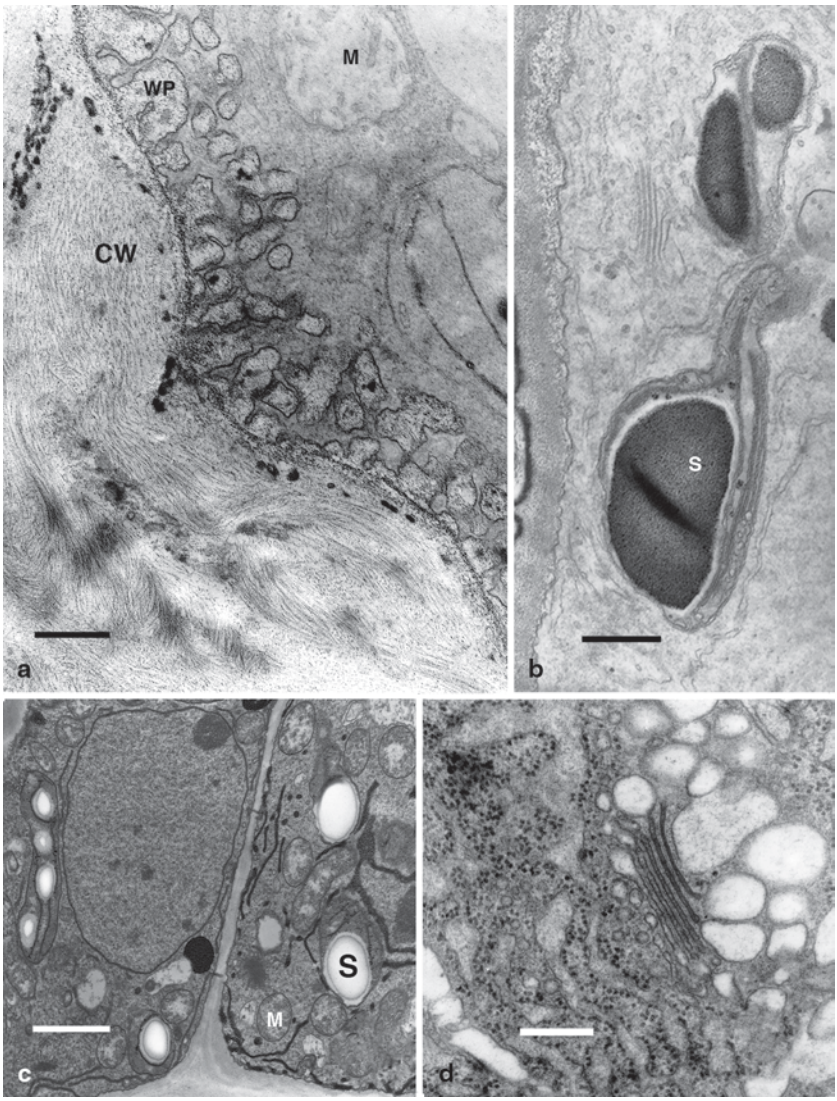

Fig. 5.3 TEM micrographs of a young seed of Canola. **a** There are deposits of electron-opaque silver over the central cell wall *(CW)* and wall projections *(WP)*. Mitochondrion *(M)*. Stained with PA-TCH-SP for insoluble carbohydrates. Scale bar = 0.4 µm. **b** There are deposits of electron-opaque silver over integument cell starch *(S)* and cell wall. Stained with PA-TCH-SP. Scale bar = 0.65 µm. **c** Two integument cells stained by OsFeCN resulting in electron-opaque deposits within the ER cisternae and enhanced staining of the nuclear envelope. Starch *(S)*, Mitochondrion *(M)*. Scale bar = 2.2 µm. **d** Median section through a synergid cell Golgi apparatus. Stained with uranyl acetate and lead citrate. Scale bar = 0.27 µm

5.5 Concluding Remarks

One does not become accomplished at the use of anatomical techniques overnight, and in particular, the many techniques related to light and electron microscopy. This chapter is a brief overview and a starting point for learning the basic techniques of light and electron microscopy. The techniques are challenging but doable if one follows the protocols carefully and accurately. There are no short cuts. A final comment from O'Brien and McCully [10] "There are three general working attitudes necessary to become a good microtomist: patience; organization; intolerance of dirt and grease. Knives, water, slides, grids, forceps and working surfaces are potential sources of contamination. Fingers are a source of grease and the atmosphere is a source of dust". The traditional use of TEM in plant anatomy has been to provide a high-resolution method of describing the cellular contents of cells. Improvements in fixation techniques, the use of immunocytochemistry and advances in all aspects of genetics, molecular and cell biology, biochemistry and physiology has provided a renaissance in the use of TEM to elucidate the structure and function of the living cell.

References

1. Glauert AM, Rogers GE, Glauert RH (1956) A new embedding medium for electron microscopy. Nature 178:803
2. Richardson KC, Jarett L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313–323
3. Luft JH (1961) Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414
4. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, Cambridge
5. Ellis AE (2014) No more Epon 812: this product does not exist today. Microscopy Today 22:50–53
6. Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43
7. Glauert AM, Lewis PR (1998) Biological specimen preparation for transmission electron microscopy. Princeton University Press, Princeton
8. Bozzola JJ, Russell LD (1999) Electron microscopy, 2nd edn. Jones and Bartlett, Sudbury
9. Russell SD, Preston L, Strout G (2014) Principles and techniques of transmission electron microscopy. Samuel Roberts Noble Microscopy Laboratory, University of Oklahoma online resource. http://www.ou.edu/research/electron/bmz5364/. Accessed 13 July 2015
10. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi, Melbourne
11. Hayat MA (1986) Basic techniques for transmission electron microscopy. Academic, London
12. Chang RFE, Klomparens KL (eds) (1988) Artifacts in biological electron microscopy. Plenum, New York
13. Gephart P, Murray RGE, Costilow RN, Wood WA et al (1981) Manual of methods for general bacteriology (Amer Soc Microbiol). ASM Press, Washington, D.C.
14. Fisher DB (1968) Protein staining of ribboned epon sections for light microscopy. Histochemie 16:92–96

15. Fulcher RG, Wong SI (1980) Inside cereals—a fluorescence microchemical view. In: Inglett GE, Munch L (eds) Cereals for food and beverages—recent progress in cereal chemistry. Academic, New York, pp 1–26
16. Venable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. J Cell Biol 25:407–408
17. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212
18. Hanaichi T, Sato T, Hoshin M, Mizuno N (1986) A stable lead stain by modification of Sato's method. Proc XIth Int Cong Electron Microscopy. Kyoto, pp 2181–2182 (English corrected)
19. Thiery JP (1967) Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J Microsc 6:987–1017
20. Hepler PK (1981) The structure of the endoplasmic reticulum revealed by osmium tetroxide-potassium ferricyanide staining. Eur J Cell Biol 26:102–110
21. Hayat MA (1981) Fixation for electron microscopy. Academic, New York
22. Jensen WA (1962) Botanical histochemistry: principles and practice. Freeman, San Francisco
23. Pappas PW (1971) The use of a chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technol 46:121–124
24. O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
25. Hale AJ (1957) The histochemistry of polysaccharides. Int Rev Cytol 6:193–263
26. Roland JC (1978) General preparation and staining of thin sections. In: Hall JL (ed) Electron microscopy and cytochemistry of plant cells. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 1–62
27. Lane BP, Europa DL (1965) Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. J Histochem Cytochem 13:579–582
28. Hughes J, McCully ME (1975) The use of an optical brightener in the study of plant structure. Stain Technol 50:319–329
29. Schnepf E, Deichgraber G, Herth W (1982) Development of cell wall appendages in *Acanthosphaera zachariasi* (Chlorococcales): kinetics, site of cellulose synthesis and microfibril assembly, and barb formation. Protoplasma 110:203–214
30. Wood PJ, Fulcher RG (1978) Interactions of some dyes with cereal beta glucans. Cereal Chem 55:952–966
31. Herth W, Schnepf E (1980) The fluorochrome, calcofluor white, binds oriented structural polysaccharide fibrils. Protoplasma 105:129–133
32. Smith MM, McCully ME (1978) A critical evaluation of the specificity of aniline blue induced fluorescence. Protoplasma 95:229–254
33. White DL, Mazurkeiwicz JE, Barrnett RJ (1979) A chemical mechanism for tissue staining by osmium tetroxide-ferrocyanide mixtures. J Histochem Cytochem 27:1084–1091

Chapter 6
LR White Acrylic Resin

Edward C. Yeung and Bing Quan Huang

6.1 Introduction

A better understanding of resin chemistry and improvements in formulation designs lead to development of acrylate embedding media with many useful features, which are suitable to the study of histology and cell biology of organisms. The use of acrylate resins with cross-linking ability provides structural stability upon polymerization, which allows sections to be viewed with an electron microscope. Furthermore, the ability of acrylate resins to polymerize at low temperatures and the hydrophilic nature of the resins improves immunolabeling of antigens on-sections. These features further strengthen their usefulness and applications, especially when compared to conventional epoxy resins. The commercialization of Lowicryl and LR White resins by Polysciences and the London Resin Company, respectively, in the early 1980s, further popularized the use of acrylic resins and made them an indispensable family of embedding media for light and electron microscopy.

Through their careful studies, Newman, Hobot, and collaborators [1–5] provide basic information and protocols in the use of the LR White resin, especially on immunological applications. Today, LR White resin is routinely used as the embedding medium for the immunogold detection method of antigens at the ultrastructural level. Recent successes in correlative imaging of fluorescent proteins in plant materials [6] further highlight the usefulness of this resin in the study of plant cell biology. The objective of this chapter is to provide an overview on the use of the LR White resin. Sample protocols for light and electron microscope preparations are also included. Many reports on the use of this resin in the study of plant cell biology

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

B. Q. Huang
Center for Basic Research in Digestive Disease, Mayo Clinic, Rochester, Minnesota, USA

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_6

are available in literature (e.g., [7–17]). In addition, many suppliers also provide information and protocols of this product on their websites. Readers are urged to consult these and other references for additional information.

6.1.1 General Characteristic of LR White Resin

LR White resin has a very low viscosity (8 cps) and is miscible with a low amount of water, approximately 12 % by volume [2, 4]. These features allow the resin to readily infiltrate into dense tissue, much easier than the majority of epoxy resins. Since the resin is miscible with water, a partial dehydration scheme can be used in sample processing [1]. This method can minimize the extraction of macromolecules during the dehydration and infiltration processes, especially those that are fixed with a low percentage of glutaraldehyde for immunolabeling.

The key feature that makes LR White resin one of the key embedding media for microscopy is that the polymerized resin does not interfere with the staining procedure and the resin is hydrophilic, which allows the penetration of immunoreagents [4, 5] and the accessibility of antibodies to antigens. The relative ease of staining LR White embedding tissues, especially for immunogold labeling, makes this resin indispensable for the ultrastructural detection of antigens for different types of biological specimens. This is in contrast to the majority of epoxy resins which can react and copolymerize with tissue elements and antigenic sites [18], making the detection of antigens difficult.

For light microscopy studies, many staining recipes used for glycol methacrylate sections can be applied to LR White sections. However, large LR White resin blocks are difficult to section and the sections are difficult to flatten on glass slides. This is due to the cross-linking of acrylic monomers upon polymerization. Therefore, for the study of large tissue blocks, it is advisable to use the Technovit resins instead, if ultrastructural information is not needed (*see* Chap. 4). Similarly, although light microscope immunodetection techniques are available for LR White-embedded tissues, for large specimens, the use of Technovit 8100 may be more beneficial.

6.1.2 Commercial Preparations

LR White resin is sold in different forms. There are catalyzed and non-catalyzed forms, as well as hard, medium, and soft grades. In addition, the use of LR Gold, which is formulated for low-temperature polymerization, can be considered if polymerization at low temperatures is required.

LR White is currently supplied in either catalyzed or un-catalyzed form. The advantage of the catalyzed form is that it is ready to use upon purchase, as the catalyst, benzoyl peroxide, is included in the monomers prior to shipping. However, once added, polymerization begins, albeit very slow, when stored at 4 °C. When purchasing the catalyzed form, it is important to realize that the shelf life may be shorter

than the time indicated. Since it is not possible to know the storage conditions prior to receiving the resin, it is advisable to use this form of resin in a short period of time before the expiration date. In the un-catalyzed form, the catalyst has to be added and dissolved completely prior to use. Once prepared, the resin has approximately 12 months of shelf life, if stored properly. To prepare one's own catalyzed LR White, it is imperative to follow the recommendations by the manufacturer. It is advisable to test new batches of the resin prior to regular use.

There are three grades of LR White available: hard, medium, and soft. The hard grade appears to be the most popular among investigators. The different grades are composed of different percentages of the same acrylate monomers, which result in slight differences in physical properties. It is important to note that the method of resin infiltration and the temperature of polymerization can also alter the physical characteristics of the resin blocks [19]. Hence, it is preferable to use the same grade of LR White resin in order to obtain consistent results. Preliminary testing is the best way to determine which grade is best suited for one's studies.

LR Gold is used as an embedding medium for low-temperature infiltration and polymerization. The resin is formulated using benzil as the catalyst and this formulation is designed to be polymerized using blue light at temperatures down to −20°C. LR Gold can also be polymerized under ultraviolet (UV) excitation if benzoin methyl ether is used as the catalyst. This medium can be used in conjunction with freeze substitution procedures, which is beneficial to antigen preservation. LR Gold appears to be better suited for in situ hybridization protocols as the resin appears to be less cross-linked compared to LR White [11]. If resin infiltration is required at an even lower temperature, Lowicry K4M, HM20 acrylic resin should be used instead of LR Gold, as polymerization can occur below −20 °C [5, 20]

6.2 Practical Comments on the Use of LR White

6.2.1 Fixation, Processing, and Embedding

The approach to fixation and processing depends on the purpose of the experiment. For general histological studies, the standard paraformaldehyde and glutaraldehyde combination for routine light microscopy can be used (*see* Chaps. 2 and 4). If ultrastructural information is needed, the tissue is then post-fixed with osmium tetroxide (OsO_4), which is common to epoxy embedding procedures (*see* Chap. 5). For OsO_4 post-fixation, the resin has to be polymerized using heat since OsO_4 will interfere with the UV-activated low-temperature polymerization. Because the tissue is well fixed, dehydration can be done in a graded fashion, up to 100% ethanol prior to infiltration with the embedding medium. Unlike infiltration procedures for certain epoxy resins, a transitional fluid is not required with LR White.

For immunological studies and immunogold procedures, the samples are often fixed with 3–4% paraformaldehyde and a low concentration of glutaraldehyde (0.05–0.2%) [5]. Post-fixation with OsO_4 is generally not recommended; instead, the samples can be treated with phosphotungstic acid during ethanol dehydration, prior to the infiltration of resin [21], in order to improve the contrast of the specimen. Tannic acid has also been recommended as an additive prior to dehydration which appears to increase membrane contrast and improves ultrastructure and antigenicity of the tissues [19]. Other fixatives such as periodate-lysine-paraformaldehyde [22] should be tested, as excellent results have been reported in the literature (*see* Chap. 2).

For immunohistochemical studies, since the tissue is mildly fixed, in order to avoid extraction of macromolecules, the tissues can be partially dehydrated up to 70% alcohol and then infiltrated with a mixture of 70% ethanol and LR White resin [1, 2, 5]. Newman et al. [1] demonstrated that partial dehydration improves membrane configuration and reduces extraction of cytoplasmic components in animal and human tissues. The decision to use the partial dehydration method depends on the type of tissue processed. If resin infiltration begins at the 70% step of dehydration, it is imperative that the concentration of ethanol be accurate. A higher amount of water in the ethanol solution will render the resin milky and unusable. In environments with a high humidity, it is advisable to dehydrate the samples to 85–90% ethanol prior to resin infiltration. This is to ensure that the resin is miscible with the residual water present, avoiding mistakes. For plant cells, since a majority of cells are vacuolated and have a high water content, it is advisable to carry out a complete dehydration to ensure proper polymerization of the resin. If symptoms of extraction and poor membrane imaging are present using the regular dehydration and infiltration process, partial dehydration should be tested.

Although OsO_4 post-fixation is usually not carried out for immunostaining, successes in immunogold staining for cell wall epitopes have been reported [12]. It appears that the wall epitopes are not altered by OsO_4; hence, it does not interfere with the subsequent immunostaining process.

Vacuum extraction of air from plant samples is necessary. As discussed in Chap. 4, air is often present in plant tissues, and this interferes with the penetration of fixatives, dehydrating solvents and the embedding medium. Furthermore, oxygen prevents proper polymerization of acrylate resins. It is important to note that vacuuming can extract solvent and resin components from the embedding medium; therefore, the solvent/resin needs to be replaced with a fresh one after each vacuuming step.

6.2.2 Embedding and Resin Polymerization

Correctly embedded and sectioned specimens will result in proper morphology and interpretation. Tissue orientation is a key step during embedding. Similar to glycol methacrylate, the difficulty in embedding with LR White resin is that it

has a low viscosity and proper sample orientation within the embedding mould is problematic. If certain orientation is preferred, the specimens need to be excised carefully with the preferred orientation in mind, at the time of fixation. This will facilitate tissue orientation during embedding. To overcome the technical problem of orientation, the specimen can be embedded in a flat embedding mold and remounted in a desirable orientation after polymerization as detailed by Palmieri and Kiss [23].

For acrylic resins, oxygen will interfere with the generation of free radicles required for the polymerization process [24, 25]. If air is not excluded completely, the resin blocks will be soft and sticky, and thus difficult to section. A simple addition of dental wax can serve as an oxygen barrier during the thermal polymerization process [26]. For flatbed embedding, custom molds can be prepared using hand-cut gaskets on microscope slides to form a chamber and covered with a protective film [23] such as Aclar or food wrap film. It is critical to avoid air bubble formation in the chamber or gelatin capsule when laying out the film on the surface of the resin.

When heat is used to polymerize LR White resin blocks, gelatin capsules and flat bottom polypropylene capsules are preferred. These capsules are more heat stable and air does not readily diffuse through their walls, when compared to the commonly used polyethylene Beem® capsules. Beem® capsules can be used if embedding is carried out at room temperature using an accelerator. For UV polymerization, gelatin capsules are preferred as they are transparent to UV light.

LR White can be polymerized at different temperatures depending on the catalyst used. A detailed discussion is presented by Hand [24]. Heat is usually the easiest method for polymerization. Between 50 and 60 °C, benzoyl peroxide begins to breakdown, generating free radicles and initiating the polymerization process [24]. Newman and Hobot [2] found that sections from resin blocks polymerized at 50 °C favored antigen uptake. The standard recommended temperature is 60 °C for approximately 24 h. The majority of data sheets provided by the suppliers recommend that LR White resin be polymerized for 24–48 h between 50 and 65 °C. Rapid polymerization schedules have also been reported, for example, 2–2.5 h at 55 °C in the study of pteridophyte spermatogenous cells [15] and in the study of chloroplast development [14]. Recently, a rapid infiltration and embedding method for morphological and immunological analysis was used to greatly shorten the time of processing by centrifugation through increasing resin concentrations of LR White, followed by polymerization at 100 °C for 1.5–2 h [27]. The rapid polymerization appears to have no adverse effects on the outcome of staining. Again, the best way to determine the optimal temperature for heat curing is by systematic testing of different polymerization temperatures.

LR White resin can also be cold cured at 0 °C with an accelerator. Curing at low temperature prevents heat damage to the specimens, especially the sensitive antigens [3, 4]. The addition of an accelerator, such as dimethyl toluidine [24], allows for polymerization to occur between 0 °C and room temperature. The addition of a carefully measured amount of an accelerator will initiate polymerization in a few

minutes, which greatly shortens the time of polymerization. Using this method, it is preferable to polymerize the samples at 0 °C as heat is generated during polymerization. It is advisable to use a heat sink when an accelerator is used. In addition to chemically activated polymerization, UV light coupled with a UV-sensitive activator can also be used. As detailed by Satiat-Jeimatire and Hawes [16], after fixation at room temperature, the samples were dehydrated using an ethanol series together with a concomitant lowering of temperature to −20 °C. The samples were then infiltrated with LR White resin in which 0.5–1 % benzoin methyl ether had been added. The resin was polymerized with UV light at −20 °C for 24 h and the temperature was subsequently raised to room temperature for curing for another 12–16 h. This method ensures minimal damage to the tissue due to heat of polymerization. UV polymerization can be carried out using a dedicated cryo chamber which is available commercially. It is important to note that if OsO_4 is used for post-fixation, the size of the specimen should not be larger than 1 mm^3. The "darkened" specimen is not compatible with UV polymerization as the dark color can lead to local heat accumulation which can damage the tissues.

LR Gold resin is designed specifically for low-temperature embedding. After fixation at 4 °C, dehydration is gradually lowered to −20 °C and LR Gold resin is infiltrated into the tissue at −20 °C. It can be polymerized using blue light with benzil or with UV light if the UV catalyst, benzoin methyl ether is used.

LR White resin can also be polymerized using microwave energy. In recent years, the development of microwave fixation and processing method has greatly shortened the processing time of specimens. Microwave protocols are now available for embedding using LR White resin (e.g., [7, 9, 28]). The improved microwave instrument design with attachments, such as the PELCO ColdSport accessory, prevents the generation of hot spots ensuring proper polymerization of resins. Successes on immunostaining have also been reported using microwave-processing schemes ([9], also *see* Chap. 2).

6.2.3 Sectioning and Staining

LR White resin blocks are more brittle compared to epoxy blocks. Hence block trimming requires care. For gelatin/Beem capsule blocks, perform rough trimming to remove excess resin around the specimen using sharp double-edged razor blades. To generate the final block face with a suitable size and shape, trim the block face slowly using glass knives; this is to avoid damaging the embedded specimen, especially if ultrastructural information is intended. For semi-thin and thin sectioning techniques, consult [29] and *see* Chap. 5 for further details.

We prefer using glass knives fitted with a "boat," especially for cutting large semi-thin sections. The sections will float on the surface of water and can be readily picked up using toothpicks or similar tools. During sectioning, due to the hydrophilic nature of LR White resin, water tends to adhere to the block face. If water

appears on the block face, it should be dried using a filter paper before cutting the next section. One can minimize this problem by lowering the water level in the boat just below the knife edge. Chloroform vapor can be used to flatten the sections on the surface of water, if necessary. The sections can be picked up using a toothpick and transferred onto drops of water placed on silane-coated slides and dried using a slide warmer. For thin sections, the sections are picked up using clean 200–300 mesh nickel grids.

Staining of semi-thin sections for general histological observation can be done using acidic and basic dyes [4]. At the light microscope level, the silver enhancement method can be used after immunogold staining to improve contrast [4, 30]. For routine ultrastructural observation, the traditional uranyl acetate and lead citrate staining method for epoxy sections can be applied [12, 19]. Immunogold staining protocols at the ultrastructural level are readily found in the literature [e.g., 7, 11]. Post-staining after immunogold observation at the ultrastructural level is not always necessary. In general, there is sufficient contrast between the background plastic and the cells, allowing for the detection of gold particles on sections. If additional contrast is necessary, uranyl acetate and lead citrate method can be used [12].

6.3 Materials

6.3.1 Laboratory Equipment and Embedding Supplies

1. Rotary and ultramicrotomes (different suppliers).
2. Triangular knife makers (different suppliers).
3. UV polymerization chamber, for example, Pelco UVC3 cryo chamber from Ted Pella, Inc.
4. Vacuum chamber/oven and vacuum pump.
5. Glass strips for making knives (different suppliers such as Electron Microscopy Sciences, Hatfield, PA (EMS)).
6. Knife boat making supplies such as disposable plastic boats, tape, and clear nail polish (EMS).
7. Gelatin capsules and holders, polypropylene capsules, Beem ®capsule, polytetrafluoroethylene (PTFE) flat embedding moulds, and Aclar ®film (Ted Pella, Inc., Redding, CA).
8. Rotary mixer (Pelco R1 Single Speed Rotator).
9. Oven incubators.
10. Slide warmer.
11. General laboratory supplies such as dissecting tools, razor blades, vials, beakers, flasks, measuring cylinder, forceps, slides, coverslips, and tooth picks.

6.3.2 Embedding Kits and Chemicals

1. LR White embedding kits (different suppliers such as EMS, PolySciences, and Ted Pella) *(see Note 1)*.
2. Various catalysts for LR White resin can be obtained from Ted Pella, Inc., that is, LR White accelerator for cold cure (18185), a UV catalyst - benzoin methyl ether (18186), and benzoyl peroxide which is supplied with the LR White embedding kit.
3. Twenty-five percent glutaraldehyde solution, EM grade (EMS, cat. no. 16400).
4. Pararformaldehyde powder (Sigma-Aldrich P6148).
5. Osmium tetroxide.
6. Uranyl acetate.
7. Lead citrate.
8. Chemicals for preparing various buffers and common laboratory reagents: hydrochloric acid, sodium hydroxide, sodium phosphate, lysine, sodium iodate, chloroform, and ethanol.

6.3.3 Preparation of Embedding Medium, Fixatives, and Solutions

1. Pre-catalyzed LR White resin is used directly. If un-catalyzed resin is purchased, prepare the catalyzed resin according to the supplier's instruction *(see Note 2)*.
2. Fixative solution for primary fixation: paraformaldehyde stock solution (16%): 16 g paraformaldehyde powder is dissolved in 60 mL dH_2O and heated to 65 °C with continual stirring, add a few drops of 2 N NaOH to aid in depolymerizing the powder. Once dissolved, bring to a final volume of 100 mL with dH_2O, filter before use *(see Note 3)*.
3. Osmium tetroxide (4%) stock solution: break the ampule containing OsO_4 crystal and dissolve crystal in distilled water. To prepare a 2% osmium tetroxide fixative, mix 1 part of 4% OsO_4 with 1 part of 0.2 M phosphate buffer (or 0.1 M cacodylate buffer) *(see Note 4)*.
4. A general fixative for light microscopy: 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer, pH 7.0. To make 100 mL of fixative, combine 0.2 M phosphate buffer (25 mL), 25% glutaraldehyde (10 mL), 16% paraformaldehyde (10 mL), dH_2O to make 100 mL.
5. A routinely used fixative for immunohistochemistry is 4% paraformaldehyde and 0.1–0.2% glutaraldehyde in phosphate buffer, pH 6.9. Prepare fresh just before use.
6. Periodate-lysine-paraformaldehyde fixative (10 mM $NaIO_4$, 75 mM lysine, 37.5 mM phosphate, and 2% paraformaldehyde, pH 6.2) [22]. Dissolve 1.83 g of lysine hydrochloride in 50 mL distilled H_2O, adjust the pH to 7.4 by adding 0.1 M Na_2HPO_4 (approximately 5 mL) and bring to 100 mL with 0.1 M $NaPO_4$, pH 7.4. Prepare an 8% paraformaldehyde stock solution in distilled H_2O, similar

to Step 2. Just prior to use, combine 75 mL of 0.1 M lysine with 25 mL of 8% paraformaldehyde stock solution and add 214 mg $NaIO_4$. The final pH is approximately 6.2 *(see Note 5)*.
7. An ethanol dehydration series.

6.4 Protocol

6.4.1 A General Protocol for High-Resolution Light Microscopy

1. Excise or collect plant material of interest, cut the tissues with a specific plane of orientation in mind using a sharp double-edged razor blade. For light microscopy studies, the tissue slices can be large (approximately 5×5 mm^2) but the thickness should not exceed 2 mm. Preferably, the tissues are excised in a buffer to prevent from drying and should be transferred immediately to a vial containing fixative.
2. Fix the tissues by immersion fixation at room temperature using the general fixative. After stabilizing the tissues for an hour, evacuate air from the specimens to allow for proper infiltration of fixative and dehydration solutions. Continue to fix the tissues at room temperature for 4 h or overnight at 4 °C.
3. After fixation, gently wash the tissues with buffer several times before commencing the dehydration steps using an ethanol series (30, 50, 70, 80, 95, 2×100%), 30–60 min each *(see Note 6)*. Dehydration is preferably carried out at 4 °C to minimize extraction of macromolecules. To ensure air is no longer present within the tissues, perform an additional vacuuming step after the first change of 100% ethanol.
4. Commence infiltration with 1:2; 1:1; 2:1 (resin: ethanol; v:v) and 2×100% LR White resin. The time depends on the size of the sample. The speed can be hastened if the samples are kept on a rotary mixer at 4 °C. Vacuum the sample after the first change of pure LR White resin.
5. Prior to embedding, dry the embedding capsules and molds at 50 °C for several hours to remove moisture *(see Note 7)*.
6. Place the capsules in a holder. Fill the capsule with fresh resin and transfer the samples to the capsules *(see Note 8)*. If the samples are in thin discs, allow each disc to rest at the bottom of the capsule. Orient the sample using a toothpick. Fill the other half of the capsule and quickly fit both together to avoid trapping of air. For flat-bed embedding, the PTFE molds can be used. Each mold should first be partially filled with resin before applying the samples. Orient the sample and slightly overfill the cavity with resin, apply an appropriate Aclar film or food wrap film covering the cavity.
7. Polymerize the capsules/molds in an oven at 60 °C for 24 h.

8. When using the gelatin or polypropylene capsules, trapping of a small air bubble is unavoidable. This renders the upper part of the resin block slightly softer than the bottom half. After polymerization, we recommend removing the upper part of the capsule using a jewel saw and gluing the bottom half to an epoxy cylinder or aluminum mount using epoxy glue. This provides a secure, stable resin block for subsequent sectioning.
9. Trim the side of the blocks using a new double-edged razor blade under a stereomicroscope. For detailed trimming and sectioning techniques, *see* [29].
10. Once excess plastic and tissues have been removed, secure the specimen block to a microtome. The block face is gently trimmed and sectioned using "wet" glass knives, that is, triangular glass knives fitted with a boat with water *(see Note 9)*. Ensure the water meniscus is slightly lower than the knife edge *(see Note 10)*. Section at a slow speed to avoid compression of sections. The sections can be lifted from the water surface using a toothpick and transferred to a drop of water on silane-coated slides.
11. Allow the sections to dry using a slide warmer and stain accordingly.

6.4.2 A General Protocol for TEM and Immunogold Detection of Carbohydrate Epitopes

For general ultrastructural observation, the procedures for fixation are similar to the epoxy embedding methods (*see* Chap. 5). Since OsO_4 is used for post-fixation, the tissue pieces have to be kept small (1 mm^3) as the rate of OsO_4 penetration into the tissue is slow. A recent publication details a protocol for TEM studies [12]. This method is also suitable for the detection of cell wall epitopes.

1. Excise the tissues carefully with a size approximately 1 mm^3 into the primary fixative and fix the tissues for 4 h at room temperature or overnight at 4 °C. Rinse the tissues with three changes of cold buffer, 15 min each, and post-fix using a 1–2 % OsO_4 solution for approximately 4 h at 4 °C. This step must be carried out inside a fume hood.
2. After post-fixation, rinse the tissues with three changes of cold buffer, 15 min each.
3. Dehydrate the samples through a graded ethanol series (30, 50, 70, 80, 90, 2 × 100 %) at 4 °C, 15–30 min each. If needed, sample dehydration can be stopped at the 70 % step and samples stored in 70 % ethanol overnight in a refrigerator. To ensure that air is not present within the samples, they can be subjected to one more vacuuming step after the first change of 100 % ethanol.
4. Infiltration, polymerization, and subsequent processing are carried out similar to steps 4–10 in Sect. 6.4.1.
5. For preliminary examination, "thick" sections are stained using staining solution such as 0.1 % toluidine blue O in water. Once the desirable part of the tissue is identified, the block can be retrimmed and thin sectioned, preferably with a diamond knife. Thin sections are collected using nickel grids, especially if the immunogold procedure is to be followed.

6.4.3 A General Protocol for Immunogold Staining of Protein Epitopes Using Chemical Fixation

Chemically fixed specimens followed by LR White embedding are suitable for the detection of protein epitopes using the immunogold staining method. As discussed in Chap. 2, freshly prepared paraformaldehyde solution should be used as the main fixing agent. When viewed using an electron microscope, a small amount (0.1–0.2 %) of glutaraldehyde can be added to improve the cytological features of cells and tissues.

1. Select and cut tissues into small pieces, that is, 1 mm^3 in size. Fix tissues using a fixative containing 4 % paraformaldehyde and 0.2 % glutaraldehyde in a buffer solution.
2. The tissues are processed similar to steps 2–4 as detailed in Sect. 6.4.2.
3. As an alternative to heat polymerization, an LR White accelerator for cold cure can be used to polymerize the resin between 0 °C and room temperature. Carefully calculate the amount of accelerator needed and mix with the resin. Polymerization begins immediately, hence one has to work quickly *(see Note 11)*. If a UV polymerization chamber is available, a UV catalyst can be added and allow the resin to polymerize at −20 °C for 1–2 days. If the resin is still soft, continue to polymerize for 1 more day at room temperature.
4. Once the resin blocks have polymerized, they can be sectioned as in step 5 in Sect. 6.4.2.
5. For immunogold staining procedure, *see* Chap. 14.

6.5 Notes

1. Be sure to read the instructions from the supplier regarding handling and storage procedures upon receiving the product. Use the resin prior to the expiration date. If the resin becomes viscous before the expiration date, stop using it. Dispose the resin properly as a hazardous waste according to official regulations. Although the acrylic resins are not considered toxic, they are irritants and can cause skin dermatitis. One should take proper precautions in handling the resin, such as wearing protective gloves.
2. In preparing one's own catalyst, follow the manufacturer's instructions. Catalyze an entire bottle by dissolving the benzyl peroxide provided. Dissolving the catalyst takes at least 2 h with constant stirring using a stirrer at room temperature, or 24 h by regular shaking. Do not attempt to use the heat to speed up the process. Let the catalyzed resin rest overnight at 4 °C in the refrigerator. Re-shake the bottle for an hour the next day ensuring all of the catalyst has dissolved. Note the date of preparation. Before regular use, test the quality of the catalyzed resin by polymerizing at 60 °C to ensure that it performs as expected.

3. Commercial formaldehyde contains a stabilizer such as methanol to reduce the polymerization of formaldehyde molecules. In order to ensure the proper concentration of formaldehyde in a fixation protocol, the generation of formaldehyde from paraformaldehyde is preferred. It is important to take necessary precautions in preparing the solution. For details, *see* Chap. 2.
4. Osmium tetroxide crystals dissolve slowly in water. The solution needs to be prepared ahead of time. Furthermore, one needs to take proper precautions in preparing and handling of the solution. Osmium tetroxide crystals sublimate into vapor from a solid state. It is imperative that one should prepare and handle the solution in a fume hood. The solution should be stored in double-sealed dark containers.
5. The periodate-lysine-paraformaldehyde fixative can be a useful alternative to the traditional paraformaldehyde fixative, *see* [22]. Different modifications of the original formulation can be found in the literature.
6. Ethanol is the commonly used solvent in a dehydration series. Acetone dehydration is not recommended as it is a free radicle scavenger and can interfere with the polymerization process. If acetone is used for freeze substitution, replace with pure ethanol and wash the tissue several times, followed by LR White infiltration. Alternatively, rinse directly with LR White for a few times until acetone is completely replaced.
7. In order to reduce the moisture content of the polypropylene and polyethylene capsules, it is a good practice to heat-dry them before use, at 50 °C for several hours in an oven.
8. Since oxygen interferes with the polymerization process, it is advisable to degas the embedding capsules together with the resin using a vacuum system just prior to embedding. The walls of the embedding capsule can be porous and may trap air. Degassing for a short time can reduce stickiness of the resulting resin blocks.
9. When compared with Technovit resins, large LR White resin blocks are difficult to section. This is due to the cross-linking nature of the polymerized blocks. Although different types of knives can be used for sectioning, the triangular glass knives are still the best type of knives for sectioning LR White resin blocks. One can prepare knife boat using ready-made plastic trough or tape (*see* [29]). Glass strips of different thicknesses (up to 1 cm) are currently available; hence, large tissue blocks can still be sectioned. Floating sections on water as they come off the knife edge reduce friction and the sections can be picked up using a toothpick or similar tools.
10. Wetting of the block face prevents sections from floating on the water trough and slows sectioning. Besides lowering the water level, one needs to check the clearance angle and speed of sectioning to minimize the wetting of block face [29]. We prefer “wet” sectioning because better-quality sections can be obtained and sections can be picked up readily and transferred to slides.
11. For chemical polymerization at low temperature, an accelerator is necessary. Polymerization occurs rapidly. However, heat will be generated during the polymerization process, albeit for a short period of time. In order to prevent

heat damage, a heat sink using an aluminum block can be used. A homemade heat sink can be made by drilling holes of appropriate sizes in an aluminum block. One can also obtain cooling chambers from suppliers such as EMS.

6.6 Concluding Remarks

LR White resin is a versatile embedding medium and is relatively easy to use. However, there are many variables that can influence the outcome of the experiment. The age of the resin, the temperature of polymerization, and the rate of polymerization can alter the properties of the resin blocks, affecting sectioning and staining qualities. For immunolabeling studies, the nature of the antigen and the fixing agents used in fixation can also influence the outcome of the immunostaining procedure. One needs to have a proper understanding of the entire embedding process, especially the chemical nature of the resin, to properly interpret one's results. It is necessary to optimize the procedures prior to embarking on a detailed investigation.

References

1. Newman GR, Jasani B, Williams ED (1983) A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. Histochem J 15:543–555
2. Newman GR, Hobot JA (1987) Modern acrylics for post-embedding immunostaining techniques. J Histochem Cytochem 33:971–981
3. Newman GR (1987) Use and abuse of LR White. Histochem J 19:118–120
4. Newman GR, Hobot JA (1993) Resin microscopy and on-section immunocytochemistry. Springer-Verlag, Berlin
5. Newman GR, Hobot JA (1999) Resins for combined light and electron microscopy: a half century of development. Histochem J 31:495–505
6. Bell K, Mitchell S, Paultre D, Posch M, Oparka K (2013) Correlative imaging of fluorescent proteins in resin-embedded plant material. Plant Physiol 161:1595–1603
7. Kuo J (ed) (2014) Electron microscopy, methods and protocols, 3rd edn. Humana Press, New York
8. Lee KJD, Knox JP (2014) Resin embedding, sectioning, and immunocytichemical analyses of plant cell walls in hard tissues. Meth Mol Biol 1080:41–52
9. Webster P (2014) Microwave-assisted processing and embedding for transmission electron microscopy. Meth Mol Biol 1117:21–37
10. Giannoutsou F, Sotiriou P, Apostolakos P, Galatis B (2013) Early local differentiation of the cell wall matrix defines the contact sites in lobed mesophyll cells of *Zea mays*. Ann Bot 112:1067–1801
11. Vaughan K (2013) Immunocytochemistry of plant cells. Springer, New York
12. Wilson SM, Bacic A (2012) Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling pf carbohydrate and protein epitopes. Nat Protoc 7:1716–1727
13. Marttila S, Santén K (2007) Practical aspects of immunomicroscopy on plant material. In: Mendez-Vilas A, Diaz J (eds) Modern research and educational topics in microscopy. Formatex, Badajoz, pp 1015–1021

14. Pettigrew WT, Vaughn KC (1998) Physiological, structural, and immunological characterization of leaf and chloroplast development in cotton. Protoplasma 202:23–37
15. Hoffman JC, Vaughn KC, Joshi HC (1994) Structural and immunocytochemical characterization of microtubule organizing centers in pteridophyte spermatogenous cells. Protoplasma 179:46–60
16. Satiat-Jeunemaitre B, Hawes C (1992) Redistribution of a golgi glycoprotein in plant cells treated with brefeldine A. J Cell Sci 103:1153–1166
17. VandenBosch KA (1991) Immunogold labelling. In: Hall JL, Hawes C (eds) Electron microscopy of plant cells. Academic Press, London, pp 181–218
18. Mollenhauser HH (1993) Artifacts caused by dehydration and epoxy embedding in transmission electron microscopy. Microscopy Res Tech 26:486–512
19. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications. Cambridge University Press, Cambridge
20. Maunsbach AB (2006) Immunoelectron microscopy with Lowicryl resins. In: Celis JE (ed) Cell biology, a laboratory handbook, vol 1, 3rd edn. Elsevier Academic Press, Burlington, pp 289–297
21. Sakai Y, Hosaka M, Hira Y, Watanabe T (2005) Addition of phosphotungstic acid to ethanol for dehydration improves both the ultrastructure and antigenicity of pituitary tissue embedded in LR White acrylic resin. Arch Histol Cytol 68:337–347
22. McLean W, Nakane PF (1974) Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
23. Palmieri M, Kiss JZ (2005) A novel technique for flat-embedding cryofixed plant specimens in LR White Resin. Microscopy Res Tech 68:80–84
24. Hand NM (2002) Plastic embedding media and techniques. In: Bancroft JD, Gamble M (eds) Theory and practice of histological techniques. Churchill Livingston, London, pp 663–677
25. Gerrits PO, Horobin RW (1996) Glycol methacrylate embedding for light microscopy: basic principles and trouble-shooting. J Histotech 19:297–311
26. Bowling AJ, Vaughn KC (2008) A simple technique to minimize heat damage to specimens during thermal polymerization of LR White in plastic and gelatin capsules. J Microsc 231:186–189
27. McDonald KL (2014) Rapid embedding methods into epoxy and LR white resins for morphological and immunological analysis of cryofixed biological specimens. Microsc Microanal 20:152–163
28. Hillmer S, Joachim S, Robinson DG (1991) Rapid polymerization of LR-White for immunocytochemistry. Histochemistry 95:315–318
29. Reid N (1975) Ultramicrotomy. American Elsevier Pub, Co., New York
30. Lackie PM (1996) Immunogold silver staining for light microscopy. Histochem Cell Biol 106:9–17

Chapter 7
High-Pressure Freezing and Freeze Substitution of In Vivo and In Vitro Cultured Plant Samples

Jose M. Seguí-Simarro

7.1 Introduction

The development of improved specimen preparation methods for viewing cells and subcellular elements has been a major concern to electron microscopists ever since Ernst Ruska introduced the first biological specimen into the electron beam of his original microscope [1]. From this very first moment, most of the electron microscopy studies have been based on cells and tissues fixed by means of chemical cross-linking agents, then dehydrated, and finally embedded in a resin. After thin sectioning, the embedded samples can be viewed in an electron microscope. Specimens prepared in this manner allow for the production of clear images of the subcellular architecture, and provide fundamental insights into the functional organization of organelles and cytoskeletal elements. Our current understanding of the ultrastructural organization of cells and tissues is based mainly on the traditional methods of specimen preparation.

In recent years, for fine details, especially for membrane-bound cell organelles and compartments, chemical fixation is judged to be inadequate (*see* Chap. 2). The poor structural preservation of membranous elements reflects the inability of aldehydes, the primary fixing agents, to stabilize the lipid components of the membranes. Different fixing agents possess different abilities in stabilizing or reacting to different biological molecules (*see* Chap. 2). For example, glutaraldehyde does not cross-link nucleic acids or carbohydrate molecules [3]. Chemical fixatives may promote vesiculation in membranous organelles such as the endoplasmic reticulum or chloroplasts. They may also induce the fusion of different membranous elements, such as Golgi-derived vesicles or cell plate intermediates [3]. Furthermore, these fixatives penetrate the cells by diffusion and require seconds to minutes to immobilize the internal structures of cells. This makes the study of dynamic cellular events

J. M. Seguí-Simarro (✉)
COMAV-Universitat Politècnica de València, CPI, Camino de vera, s/n. 46022, València, Spain
e-mail: seguisim@btc.upv.es

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_7

difficult as they occur in seconds [2, 3]. Additionally, once the fixatives penetrate inside the cell, they need time to cross-link the molecules and to generate a tridimensional rigid matrix. The unfixed cytoplasmic molecules may move around from their original locations during fixation and subsequent steps of sample processing. As a result, chemical fixatives have the potential to generate ultrastructural disorders, that is, artifacts [3, 4].

The alternative is to use physical fixation methods (*see* Chap. 2). This consists of the combined use of high-pressure freezing and freeze substitution (HPF/FS). HPF freezes non-fixed tissues to −196 °C in 5–7 ms. The rapid freezing prevents the ice crystal formation and avoids damage to cells and tissues [3]. After HPF, the samples can be freeze substituted, replacing the frozen water by an organic solvent. During freeze substitution (FS), the frozen specimens are maintained at −80 or −90 °C for several days in a solution of dry acetone and additives (contrasting or postfixative agents). Since acetone is in excess, water molecules are first dissolved in it and then replaced by the acetone. In parallel, contrasting and postfixing agents penetrate the specimen and bind to their specific targets. At the end of this process, the samples are completely dehydrated and stabilized, ready to be warmed and infiltrated with the embedding resin.

Today, chemical fixation methods are being progressively replaced by low temperature-based fixation technology. However, this replacement is slow, principally due to the high cost of the equipment necessary for HPF and FS. This certainly precludes a wider adoption of these Resolutive techniques. Very few laboratories around the world have enough financial resources to acquire these equipment for their exclusive use. At present, the equipment for HPF and FS is generally purchased by electron microscopy services at universities and research centers, maintained by trained personnel, and offered to the research community on a pay-per-use basis. However, users are required to process their own samples according to specific protocols of their particular specimens.

Being a model plant, *Arabidopsis* has been widely used as the experimental system to study different aspects of plant growth and development. Several protocols of HPF/FS are available to study different *Arabidopsis* tissues in the literature [5–9]. In recent years, this methodology has progressively extended to other experimental materials, including onion seedlings, BY-2 suspension-cultured cells, maize endosperm cells, and barley pollen grains [10, 11]. In this chapter, I provide a HPF/FS-based methodology for *Brassica napus* zygotic embryos and isolated microspore cultures by which microspores are induced to enter embryogenesis, developing as microspore-derived embryos [12]. I describe two different procedures for samples to be used for ultrastructural analysis, where the main goal is to preserve the ultrastructure, and for samples destined to perform in situ localization studies, where preservation of antigen reactivity is a priority. These protocols can potentially be extended, with few modifications, to other microspore embryogenesis systems, as well as in vitro plant cell cultures in general.

7.2 Materials

7.2.1 Plant Materials

1. *B. napus* microspore cultures prepared as previously described [13] (*see Note 1*), containing embryogenic microspores (Fig. 7.1a) and microspore-derived embryos (Fig. 7.1b, c).
2. *B. napus* zygotic embryos (Fig. 7.2) are excised from siliques at different developmental stages. Excise the entire inflorescences and developing siliques from plants and keep them in a glass with water and melting ice.

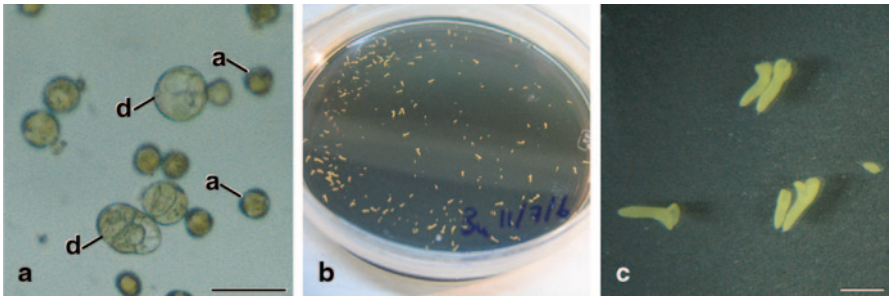

Fig. 7.1 *Brassica napus* microspore cultures. **a** *B. napus* dividing microspores (d) together with nondividing, arrested microspores (a). **b** 6-cm plate where some microspores have transformed into heart-shaped, torpedo, and cotyledonary embryos. A *black* plastic circle was placed below the plate to provide a better contrast to microspore-derived embryos. **c** Details of some *B. napus* microspore-derived embryos. Bars: **a** 50 µm; **c** 100 µm

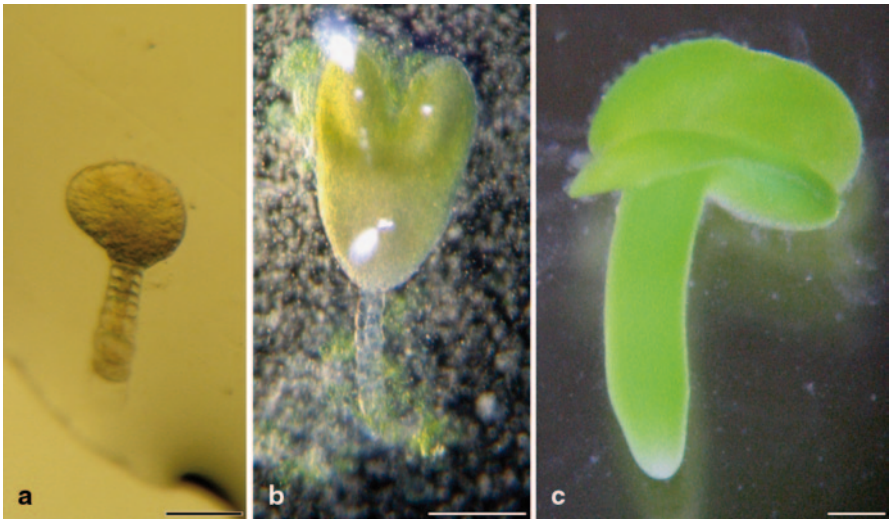

Fig. 7.2 *B. napus* zygotic embryos excised from the ovule at different developmental stages. **a** Globular embryo. **b** Torpedo embryo. **c** Cotyledonary embryo. Bars: **a** 50 µm; **b, c** 200 µm

7.2.2 High-Pressure Freezing

1. High-pressure freezer, BAL-TEC HPM 010 (Technotrade, Manchester, NH) or Leica HPM100 (Leica Microsystems, Vienna, Austria).
2. Specimen carriers: 3 mm type A (Prod No. 16770141) and type B (Prod No. 16770142) Leica aluminum Sample Carriers (Leica Microsystems, Vienna, Austria) or type A (Prod. No. 39200) and type B (Prod No. 39201) Pelco aluminum Freezer Hats (Ted Pella, Redding, CA).
3. Spectra-Por in vivo microdialysis hollow fibers (Spectrum Labs, Breda, The Netherlands; Prod No. 132295).
4. Other equipment and supplies: liquid nitrogen, a dissecting microscope, sterile lancet and toothpicks, Whatman filter paper, conical centrifuge tubes (15 or 50 ml), centrifuge with adapters for 15 or 50 ml conical tubes (Eppendorf 5804 R from Eppendorf, Hamburg, Germany, or similar), and vacuum filtration funnel and 0.45 μm pore size nitrocellulose filter membranes.
5. Cryoprotectants: sucrose solutions or culture medium (*see Note 2*) and cultured *Saccharomyces cerevisiae* (*see Note 3*).

7.2.3 Freeze Substitution

1. Leica EM AFS2 automatic FS system (Leica Microsystems, Vienna, Austria).
2. FS mixture for ultrastructural analysis: 2% osmium tetroxide ($OsO_{4)}$ in anhydrous acetone (*see Note 4*).
3. FS mixture for in situ localization studies: 0.25% glutaraldehyde and 0.1% uranyl acetate in anhydrous acetone (*see Note 5*).
4. Other equipment and supplies: cryovials, aluminum block with holes of the diameter of the cryovials used, and extruded polystyrene foam box.

7.2.4 Embedding

1. Embed-812 kit or low viscosity Spurr's kit (Electron Microscopy Sciences, Hatfield, PA-EMS).
2. Lowicryl HM20 resin kit (EMS).

7.2.5 Sectioning and Section Staining

1. Ultramicrotome (Leica UC6, from Leica Microsystems, Vienna, Austria or similar).
2. Formvar and carbon-coated, copper or nickel EM grids (EMS).

3. Diamond knife (Ultra 35° from Diatome, Biel, Switzerland, or similar).
4. A 2% (w/v) uranyl acetate solution in 50% ethanol, or a 4% aqueous uranyl acetate solution, both at pH 4–5.
5. Reynolds' lead citrate solution [14].

7.3 Methods

7.3.1 Processing for Ultrastructural Analysis

7.3.1.1 Cryofixation of Microspores

1. Prepare one type A (to carry the microspores) and one type B (to cover the microspores) sample carriers for each freezing round. To facilitate releasing the specimen from the carriers under liquid nitrogen or at later processing stages, sample carriers may be previously coated with a release agent such as 1-hexadecene or 1% lecithin in chloroform as described in McDonald K et al (2010)'s work "Tips and tricks" for HPF of model systems [15].
2. Prepare a sufficient number of cryovials with FS mixture (2% OsO_4 in dry acetone) ahead of time. At the beginning of the cryofixation session, remove their caps to have them ready to use, and keep them frozen by fully submerging them in a liquid nitrogen pool.
3. Concentrate the microspores at the desired culture stage. Depending on the equipment available, two main procedures can be used to concentrate the microspores: centrifugation and filtration. For the centrifugation method, pipet suspended microspores from the culture medium into 15 or 50 ml conical centrifuge tubes and pellet the suspension at low speed (800 rpm for the Eppendorf 5804 R centrifuge). Discard most of the supernatant; do not allow the microspores to desiccate. For the filtration method, set up the vacuum filtration funnel with a 0.45 μm pore size membrane, pour the medium with suspended microspores into the funnel and apply a gentle vacuum. Once the microspores have concentrated into a dense paste over the membrane, turn off the vacuum. Do not allow microspores to dry.
4. Rapidly transfer the microspores to the sample carriers. For this, the simplest procedure is to collect samples with a spatula (for filtered microspores) or with a pipet (for microspore pellets) and apply them to an A-type sample carrier, paying attention to prevent both carrier overload and the formation of air bubbles. Air would collapse during pressurization and could likely damage the sample (*see Note 6*).
5. Moisten the base of a type B sample carrier with a thin layer of culture medium and place it on top of the type A carrier (see Fig. 7.3a).

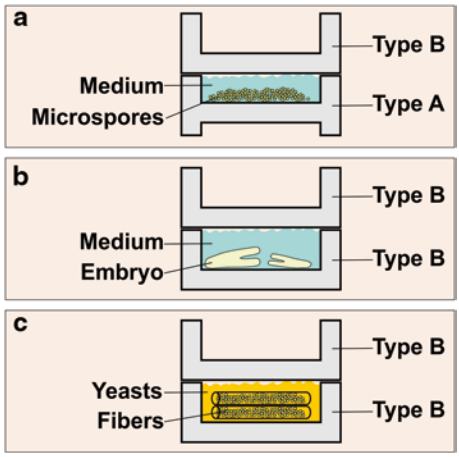

Fig. 7.3 The different sample carrier combinations used to cryofix cultured microspores for ultrastructural analysis (**a**), zygotic and microspore-derived embryos (**b**), and cultured microspores for in situ localization studies (**c**). See text for further details

6. Pick up the sandwiched sample and load it into a sample holder immediately (*see Note 6*).
7. Cryofix the sample with the HPF machine according to the manufacturer's directions. The Leica HPM100 system will automatically transfer the carriers to a small Dewar with liquid nitrogen.
8. Transfer the carriers with cryofixed samples from the small Dewar to cryovials containing the FS mixture kept in liquid nitrogen. During transfer, always work under liquid nitrogen to avoid thawing.
9. Be sure to write down the identification information (letters, numbers, or color-coded inserts) of the cryovial used for each sample. Close the cryovials once filled with samples.

7.3.1.2 Cryofixation of Microspore-derived Embryos

1. Prepare two type B sample carriers for each freezing round.
2. Prepare a sufficient number of cryovials with FS mixture, remove their caps and keep them ready to use in a liquid nitrogen pool.
3. For small (globular) microspore-derived embryos, pick up the embryos from the culture dish, under a dissecting microscope, using a pipette. Transfer the embryos together with a small amount of culture medium to a type-B sample carrier, remove excess medium with a small piece of Whatman filter paper. Repeat this step several times, in order to load 4–5 small embryos into the carrier. Avoid overloading the carrier with medium and the trapping of air bubbles within the carrier.

For larger (heart-shaped, torpedo, or cotyledonary) microspore-derived embryos, pick embryos up with a toothpick or a lancet, place them into a type-B sample holder, and fill the carrier with NLN-13 culture medium to remove air pockets.

Depending on their size, some cotyledonary embryos may not fit into the carrier. In this case, the user may cut them in two halves, discarding one or processing both halves. Alternatively, when using the Leica HPM100 machine, a 6-mm kit can be used to accommodate larger size samples (*see Note 7*).

1. Once the carrier is filled, moisten the flat side of the second type B holder with a thin layer of culture medium, and put it on top of the bottom one (*see* Fig. 7.3b).
2. Follow the steps 6–9 of Sect. 7.3.1.1.

7.3.1.3 Cryofixation of Zygotic Embryos

1. Prepare two type B sample carriers for each freezing round.
2. Prepare a sufficient number of cryovials with FS mixture, remove their caps and keep them ready to use in a liquid nitrogen pool.
3. On a glass plate, add some drops of the 150 mM sucrose solution to make a pool of solution.
4. Excise the developing seeds from the siliques and immerse them into the sucrose solution.
5. Under the dissecting microscope, excise the embryo from the seed (*see Note 6*). For very young embryos (e.g., quadrant, octant) it may be more convenient to process the entire seed and then look for the embryo in the processed sample.
6. Pick embryos up with a toothpick or a lancet, place them into a type B sample carrier, and fill the carrier with 150 mM sucrose solution to remove air pockets. Depending on their size, large embryos may not fit into the carrier. In this case, the user may cut them in two halves, discarding one or processing both halves. Alternatively, when using the Leica HPM100 machine, a 6-mm kit can be used to accommodate larger size samples (*see Note 7*).
7. Once the carrier is filled, moisten the flat side of the second type B with a thin layer of sucrose solution, and put it on top of the bottom one, according to the scheme of Fig. 7.3b.
8. Follow the steps 6–9 of Sect. 7.3.1.1.

7.3.1.4 Freeze Substitution

1. *Day 0*. After HPF, place the aluminum cooling block into a polystyrene box and add liquid nitrogen to cool the block down to −196 °C. Then, add the sample-containing cryovials to the cooled aluminum block. Keep the box in a −80 °C freezer for 2 days. Alternatively, if a −80 °C freezer is not available, the box may be filled with dry ice (to keep the sample temperature at −80 °C) and stored in a −20 °C freezer for 5 days.
2. *Day 5*. Take the aluminum block with samples out of the box and transfer it to a −20 °C freezer for 24 h.

3. *Day 6*. Transfer the aluminum block with samples to a 4 °C refrigerator for 24 h.
4. *Day 7*. Transfer the samples to room temperature and wait for at least 30 min. Further steps will be performed at room temperature.
5. Prepare Embed-812 or Spurr resin according to manufacturer's specifications, but without accelerator (BDMA for Embed-812 and DMAE for Spurr), in order to keep resin fluidity longer.
6. Carefully detach the samples from carriers by smoothly shaking the carrier while holding it with a forceps immersed in the solution. For sticky samples, a toothpick can be used to smoothly scratch them. In any case, it is essential to keep the samples always submerged in the solution within the cryovial, to prevent acetone evaporation. If this cannot be guaranteed, just skip this step and wait until full resin embedding.
7. Carefully pipet out the OsO_4 solution, avoiding touching the bottom of the vial where the samples are located. Wash the samples with acetone for 30 min. For highly hydrated samples that require exhaustive water removal, an additional dehydration step with propylene oxide may be considered at this point (*see Note 8*). Wash samples with acetone twice more for 30 min each. See OsO_4 safety issues in *Note 4*.
8. Transfer samples to 2 % resin in acetone for 2 h.
9. Transfer samples to 5 % resin in acetone for 2 h.
10. Transfer samples to 10 % resin in acetone overnight.
11. *Day 8*. Transfer samples to 25 % resin in acetone for 8 h. From now on, samples should be kept in a rotary shaker at its lowest speed.
12. Transfer samples to 50 % resin in acetone overnight.
13. *Day 9*. Transfer samples to 75 % resin in acetone for 8 h.
14. Transfer samples to 100 % resin overnight.
15. *Day 10*. Prepare Embed-812 or Spurr resin according to manufacturer's specifications, including accelerator (BDMA for Embed-812 and DMAE for Spurr).
16. Transfer samples to freshly prepared 100 % resin (with accelerator) for 8 h.
17. Transfer samples to 100 % resin, overnight.
18. *Day 11*. Transfer samples to 100 % resin for 2 h.
19. Prepare new resin for encapsulation.
20. Transfer samples to selected capsules/molds and fill with resin.
21. Polymerize the resin at 70 °C for 20 h.
22. Label the polymerized blocks and store them at room temperature permanently. They are now ready for sectioning, staining, and observation.

7.3.1.5 Sectioning and Staining

1. Prior to sectioning, prepare coated slot or meshed copper grids (*see Note 9*).
2. Trim the block to expose the sample and prepare a trapezoidal sectioning front of approximately 1 mm^2.
3. Obtain semi-thin sections (0.5–2 μm) with a glass knife for preliminary observation under the light microscope.

4. Observe the semi-thin sections with phase contrast optics to check that the tissue is not damaged. Alternatively, if phase contrast optics is not available, sections can be stained with general stains such as toluidine blue O and observed in bright field.
5. Obtain ultrathin sections (~80 nm) with a diamond knife. Ultrathin sections can be identified by their silver interference color when floating in the knife boat.
6. Pick sections up by touching them with the coated side of the grid.
7. Contrast the sections with uranyl acetate and lead citrate, and examine with a transmission electron microscope. Examples of specimens processed using this protocol can be found in Figs. 7.4 and 7.5c, d.

7.3.2 Processing for In Situ Localization Studies

In essence, processing of samples for in situ localization studies (immunolocalization, in situ hybridization, etc.) is similar to processing for ultrastructural analysis. However, there is a critical difference that influences the way samples are handled: The need for preservation of antigen reactivity. For this, OsO_4 must be avoided, since it alters the structural conformation of proteins, which strongly affects their cross reactivity. But most importantly, the entire processing (including resin polymerization) must be performed under a strict control of temperature, always between −90 °C (for dehydration) and −50 °C (for resin infiltration). This is why it is advisable to use automated processing devices such as the Leica EM AFS2 system for FS, low temperature embedding, and polymerization. However, the use of this

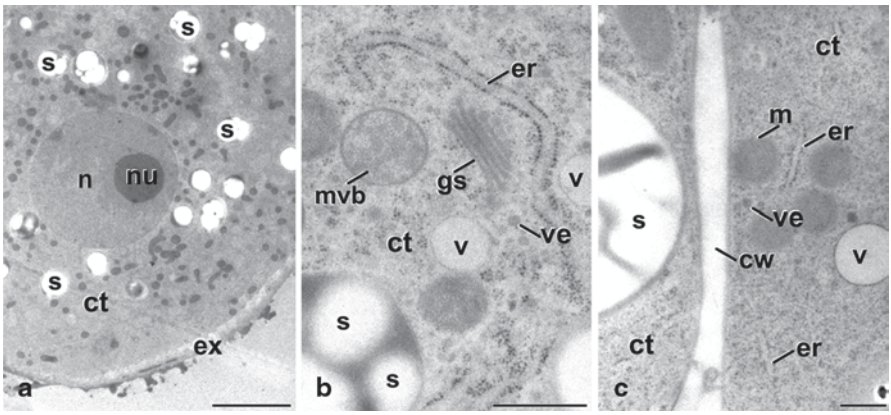

Fig. 7.4 Examples of different cells processed by HPF/FS. **a** Overview of a cultured pollen-like microspore. **b** Detail of the cytoplasm of a cultured embryogenic microspore. **c** Detail of two cells of a heart-shaped microspore-derived embryo. *ct* cytoplasm, *cw* cell wall, *er* endoplasmic reticulum, *ex* exine wall, *gs* Golgi stack, *m* mitochondrion, *mvb* multivesicular body, *n* nucleus, *nu* nucleolus, *s* starch, *v* vacuole, *ve* vesicle. Bars: 500 nm

system imposes some technical modifications (*see Note 10*) with respect to the protocol for ultrastructural analysis, as described below.

7.3.2.1 Cryofixation of Microspores

1. Prepare type A and B sample carriers and cryovials as explained in steps 1 and 2 of Sect. 7.3.1.1. However, the FS mixture for this type of processing consists of 0.25 % glutaraldehyde and 0.1 % uranyl acetate in dry acetone, prepared as described in *Note 5*.
2. Concentrate the microspores at the desired culture stage by filtration or centrifugation as described in step 3 of Sect. 7.3.1.1.
3. Apply the end of a capillary hollow fiber to the pelleted microspores, and let them move into the fiber by capillary action (*see Note 11*).
4. In a glass slide, make a dense pool of culture medium mixed with *Saccharomyces* cells.
5. Immerse the microspore-containing fibers into the pool, cut into small pieces. If suspected that during further processing there may be a significant loss of cells through the open fiber ends, try to seal them according to the method described in McDonald K et al (2010)'s work "Tips and tricks" for HPF of model systems [15]. Load the fibers into a type B sample carrier (*see Note 6*).
6. Fill the carrier with *Saccharomyces* cells to eliminate air around the fibers.
7. Moisten the base of a type B sample carrier with a thin layer of culture medium, and place it on top of the bottom carrier, according to the scheme of Fig. 7.3c.
8. Follow the steps 6–9 of Sect. 7.3.1.1.

7.3.2.2 Cryofixation of Microspore-derived and Zygotic Embryos

For cryofixation of microspore-derived and zygotic embryos, follow all the steps of Sects 3.1.2 and 3.1.3, respectively. The only difference is that for in situ localization studies, the FS mixture consists of 0.25 % glutaraldehyde and 0.1 % uranyl acetate in dry acetone, prepared as described in *Note 5*.

7.3.2.3 Freeze Substitution

For FS using the AFS2 machine, sample-containing carriers may be kept in cryovials with FS mixture, or transferred to the different kits provided with the AFS2 system (perforated capsules, flat-bottomed perforated wells, etc.) to ensure an efficient fluid exchange.

1. *Day 0*. Switch on the AFS2 machine, load/introduce, and execute a program to:
    1. Maintain temperature constant at −90 °C for 5 days.
    2. Then, allow for a slow increase of temperature from −90 to −50 °C in 20 h (slope of 2 °C/h).

3. Finally, keep temperature constant at −50 °C indefinitely.
2. Introduce the samples into the refrigerated chamber and allow for incubation in the substitution mixture for 5 days.
3. *Day 5*. Wash the samples three times (30 min each) with anhydrous acetone previously cooled to −50 °C.
4. In the meanwhile, prepare resin(Lowicryl HM20 resin) according to manufacturer's specifications.
5. Transfer the samples to 25 % resin in acetone for 6–8 h.
6. Transfer the samples to 50 % resin in acetone overnight.
7. *Day 6*. Transfer the samples to 75 % resin in acetone for 6–8 h.
8. Transfer the samples to 100 % resin. Incubate overnight.
9. *Day 7*. Separate the samples from the carriers if they are still attached, and remove the sample carriers.
10. Wash the samples three times (1 h each) with freshly made resin.
11. Transfer the samples to the corresponding capsule or flat-embedding system for polymerization *(see Note 12)*.
12. Kill the program in use. Load/introduce and execute a program to:
13. Maintain temperature at −50 °C for 3 days.
14. Increase from −50 to 0 °C in 10 h (slope of 10 °C/h).
15. Maintain temperature at 0 °C for 10 h.
16. Increase from 0 to 22 °C in 2 h (slope of 11 °C/h).
17. Maintain temperature constant at 22 °C for 26 h.
18. Connect the UV lamp to the AFS2 machine and start polymerization.
19. *Day 12*. Switch the AFS 2 system off, and pick up the samples (*see Note 13*).

7.3.2.4 Sectioning and Staining

1. Prior to sectioning, prepare coated (*see Note 9*) slot or meshed nickel or gold grids (*see Note 14*).
2. Follow steps 2–6 of Sect. 7.3.1.5.
3. Perform the in situ localization experiments. Different protocols can be found in classical general electron microscopy textbooks (for example, see references [16, 17]) or in specialized book chapters [9, 18], and technical papers [19–21].
4. Contrast the sections with uranyl acetate and lead citrate and observe them with a transmission electron microscope.

7.4 Notes

1. It is advised to prepare at least four 6-cm culture dishes for each of the stages to be collected and processed, in order to have enough material to work with. It is also advised to check the cultures under an inverted microscope before processing them, to verify that the microspores responded to embryogenesis induction.

2. Cryoprotectants are substances that help to avoid the growth of intracellular ice crystals, which is the main drawback of HPF—the formation of ice crystals. Cryoprotectants include, among others, dextran, mannitol, hexadecene, or sucrose. When using sucrose as cryoprotectant, it is usual to expose the plant material to sucrose prior to tissue dissection [5, 10], and then to immerse the dissected tissue into the freshly prepared sucrose aqueous solution (typically 100–150 mM; see references [5, 9, 10]). However, *B. napus* isolated microspore cultures are usually carried out in NLN-13 medium [22], which contains 130 g/l sucrose. This sucrose concentration is considerably higher than those typically used as cryoprotectants. Therefore, *B. napus* microspores can be cryoprotected by their own culture medium. Furthermore, since they are exposed to such a high sucrose concentration from the onset of microspore culture, there is no need for previous exposure to sucrose. This would also apply to other microspore culture systems with sucrose concentrations similar or higher than 150 mM. However, microspore culture in certain species requires the use of lower sucrose concentrations or no sucrose at all, in order to achieve an efficient embryogenesis induction. In these cases, alternative cryoprotectants should be considered.
3. A detailed protocol on growth and maintenance of *Saccharomyces cerevisiae* can be found in Two-hybrid systems, vol 177. Methods in molecular biology, edited by MacDonald P [23].
4. Preparation of 2% (w/v) OsO_4 in acetone: Break an ampoule of 1 g of OsO_4 (Electron Microscopy Sciences, Hatfield, PA) in 50 ml anhydrous acetone. Remove the broken ampoule, dissolve the OsO_4 and aliquot into cryovials (1–1.5 ml per vial). Add a color-coded insert into the cap or a number/letter in the white marking area to identify each cryovial. Do not use permanent markers, as marks can be dissolved by acetone. Instead, mark vials with a pencil. Freeze in liquid nitrogen and store frozen until use. OsO_4 is a highly volatile and toxic chemical with a high oxidizing power. Thus, it is advised to use lab coat, lab goggles, double nitrile gloves, and work in a certified chemical hood with minimal equipment or obstructions to ensure good containment of vapors. Working surfaces should be protected with plastic-backed absorbent pads to ensure containment of any spills. Ensure that the safety shower and eye wash are operational and access is unblocked.
5. Preparation of 0.25% glutaraldehyde and 0.1% uranyl acetate in anhydrous acetone: Dilute a 10% glutaraldehyde solution in acetone (Electron Microscopy Sciences, Hatfield, PA) and prepare a 1% solution of uranyl acetate (powder) in anhydrous acetone to reach the desired final concentrations. Aliquot and add a color-coded insert into the cap or a number/letter in the white marking area, to identify each cryovial. Do not use permanent markers, as marks can be dissolved by acetone. Instead, mark vials with a pencil. Freeze in liquid nitrogen and store frozen until use. Glutaraldehyde and uranyl acetate are toxic substances that must be handled with extreme care. Follow the advice given in *Note 4* for OsO_4 handling.

6. It is essential to perform these steps as quickly as possible, in order to prevent sample desiccation. Thus, previous training with disposable samples is advised.
7. The Leica HPM100 includes a unique 6-mm-diameter carrier system which allows for cryofixation of larger sample areas. For this, special 6 mm sample carriers, together with the corresponding carrier adaptors, are available.
8. For particularly hydrated samples, or if it is suspected that the samples may still have some water after acetone dehydration, an additional dehydration step with propylene oxide may be added. It consists of incubating the sample for 5–10 min with a mixture of acetone—propylene oxide (1:1), and then with 100 % propylene oxide for 15 min, and finally washing three times with acetone, 15 min each. It must be noted that propylene oxide is highly volatile and very aggressive with tissues. Thus, it must be used only when necessary (when acetone alone is not sufficient for exhaustive dehydration), and for a short time. As for safety issues, those of OsO_4 apply (*see Note 4*).
9. Coating grids with a film help to support sections during examination. In addition to plastic films (formvar, collodion, parlodion, etc.), grids can also be coated with a carbon layer that provides additional strength and stability when exposed to the electron beam. Carbon coating is especially recommended when in situ localization experiments are to be performed, since grids will be exposed to frequent handling and successive incubations and washings. Detailed procedures for grid coating can be found in classical transmission electron microscopy handbooks (for example, see references [16, 17]). It must be noted that a successful grid coating is highly dependent on the relative humidity of the place where grids are to be coated. When working in a city close to the sea, where humidity is usually high, successful coating can be especially difficult. In this case or when only few grids are occasionally needed, it may be more convenient to order coated grids from specialized distributors. The same applies to plastic film solutions, which can be made by the user or purchased as solutions in 1,2-dichloroethane.
10. The typical Leica EM AFS2-based protocol relies on the use of perforated capsules or flat-bottomed wells inserted in the corresponding aluminum container which allows for an efficient fluid exchange. For larger tissue pieces, such as embryos or embryo pieces, the holes of the perforated capsules are small enough to keep the samples safe during the fluid exchange. However, loss through the capsule holes can be a major problem when processing small samples such as microspores. Therefore, additional or alternative steps should be considered in order to prevent such a loss. In this section, I describe the use of capillary fibers, adapted from the method described in references [11] and [15]. Other authors have previously described other methods, potentially applicable to microspore cultures, such as the use of 20 % dextran for cultured tobacco BY-2 cells [10]. In our hands, dextran also works well for microspore cultures. It is therefore advisable as well. Dextran not only acts as a cryoprotectant but also holds cells together throughout the FS and resin embedding steps.
11. In reference [11], the authors developed an improved protocol to high-pressure freeze barley immature pollen cultures. They used capillary fibers filled with a mixture of barley immature pollen and cyanobacteria. After cutting the fibers

into pieces, they put them into the sample carriers, and used yeast (*Arxula adeninivorans*) paste to eliminate air spaces in the carrier. In our experience with *B. napus* microspore cultures, this useful method can be simplified by skipping the use of cyanobacteria and replacing *A. adeninivorans* by a more commonly used yeast, *S. cerevisiae*.

12. Prior to polymerization, it is advised to thoroughly clean any possible resin spill in the chamber. Otherwise, it will polymerize in the chamber and will be very difficult to remove.
13. Freshly polymerized HM20 resin may have a pale pink color. It will disappear after few days exposed to direct sunlight, close to a window. This will also provide additional curing and hardening.
14. Copper grids are usually reactive to some of the reagents used in in situ localization protocols (immunocytochemistry and in situ hybridization). This is why the use of grids made of nonreactive metals such as nickel or gold is advised.

7.5 Technical Comments

7.5.1 Comparison Between Chemical Fixation and Freeze Substitution

Figure 7.5a and b show a *B. napus* microspore preserved by chemical fixation. Compared to well-frozen HPF/FS-fixed microspores (Fig. 7.5d, e), chemically fixed specimens show many electron-light vesicle-like structures dispersed through the cytoplasm (asterisks in Fig. 7.5d). They may represent the aldehyde-induced fusion of small Golgi-derived vesicles or the fragmentation of larger membranous structures such as the endoplasmic reticulum, Golgi cisternae, or the vacuole. It is difficult to determine what they actually represent. In addition, membranes show the undulations typical for aldehyde-fixed cells. In contrast, membranes of Fig. 7.5c, d are all straight, well defined, and the cytoplasm is devoid of electron-light vesicle-like structures. It is evident that they are artifacts created by chemical fixatives.

7.5.2 The Danger of Ice Crystals

As mentioned in *Note 2*, the main drawback of HPF is the formation of ice crystals. Ice crystals can be easily identified in a poorly preserved sample (Fig. 7.5e, f). Crystals may be due to poor cryofixation or to recrystallization during FS.

As for cryofixation, the possible causes of ice damage may be due to: (1) Presence of air bubbles or pockets in the sample carrier. To solve this, it is important to ensure that all carriers are filled with cryoprotectant. (2) Bad choice of cryoprotectant. Each tissue may require a different cryoprotectant. When fixing a tissue for the first time with no previous information available, it is advisable to try different

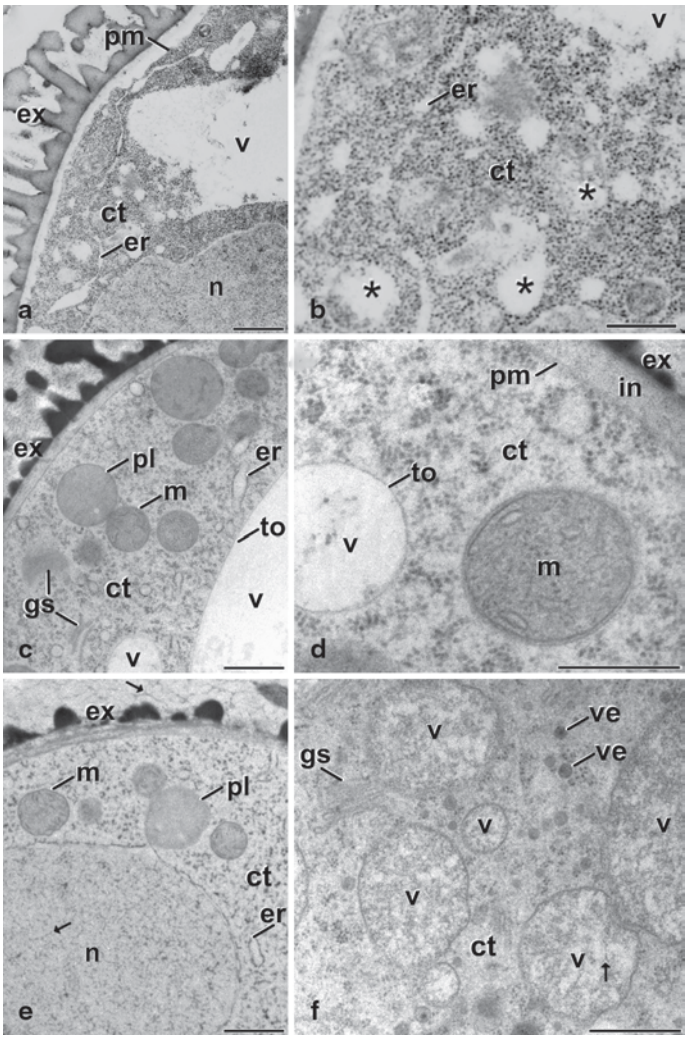

Fig. 7.5 Comparison between chemically fixed, well-frozen and ice-damaged cells. **a** and **b** show a chemically fixed *B. napus* microspore. Note the waviness of membranes such as the plasma membrane (*pm*) or the endoplasmic reticulum (*er*). In **b**, many vesicle-like elements can be observed (*asterisks*). However, it is difficult to figure out whether they are vesicles as those shown in **f** or fragmented vacuole pieces. **c** and **d** show well-preserved cryofixed microspores where membranous elements such as the plasma membrane, tonoplast (*t*), Golgi stacks (*gs*) and endoplasmic reticulum (*er*) are straight and well defined. Note the clear vacuolar lumen (*v*) and the excellent contrast and preservation of the mitochondrial cristae and double membrane in **d. e** shows an ice-damaged microspore. Note the waviness of membranes, and the presence of fibrillar strands, indicative of ice damage, in the cytoplasm (*ct*), nucleus (*n*), and in the extracellular space (*thin arrows*). **f** shows an ice-damaged microspore cytoplasm where selective ice damage is clearly observed. The cytoplasm is fairly well preserved, but the vacuolar lumen has clear evidence of ice crystals. *ex* exine, *in* intine, *m* mitochondrion, *pl* proplastid, *ve* vesicle. Bars: **a, c, e** 1 μm; **b, d, f** 500 nm

cryoprotectants (*see Note 2*). (3) Specimen too thick. The specific thermal properties of particular specimens may preclude a fast and efficient dissipation of heat, even when all other parameters are correct. In this case, thinner specimens may help to avoid ice damage. (4) Poor machine performance. High-pressure freezers need some time (minutes) to recover operating pressure after each freezing shot. In older machines, waiting time has to be empirically determined. If waiting time is insufficient, freezing may not be fast enough. In new machines, the system blocks the possibility of new shots (a message of "Wait for pressure" is shown in the screen) while it is reaching operating pressure. Once reached, a message of "Ready for freezing" is shown. After each shot, old and new machines display the time needed to raise pressure to 2100 bar and freeze. If the time is within the range of a few (5–8) ms, freezing is most likely correct. Otherwise, the frozen specimen should be discarded and the shot repeated.

As for recrystallization, it occurs when the temperature of well-frozen, hydrated samples rises above −80°C. At this temperature, vitreous (amorphous) ice may become crystal ice. For this reason, it is strongly recommended to keep the hydrated samples always at a safe temperature. Depending on the sample, recrystallization may even occur around −80°C. This is why Leica recommends maintaining the samples at or below −85 °C during FS. Once water has been removed, there is no risk of ice damage, and temperature can then be raised.

Finally, it is important to bear in mind that ice crystal formation may not affect all cells of a sample in a similar manner. It is possible to observe tissues with good and ice-damaged cells together. It is even possible to observe cells with a well-frozen cytoplasm and a damaged nucleus, or with a damaged cytoplasm and well-preserved organelles (plastids and mitochondria), or with a well-preserved cytoplasm but with ice-containing vacuoles (Fig. 7.5f). Thus, it is advised to carefully revise the specimens before using them for further studies.

7.5.3 Safety Precautions when Working with Liquid Nitrogen

Inappropriate handling of liquid nitrogen may be dangerous. Liquid nitrogen is extremely cold (−196 °C). Direct contact with it, as well as with any object cooled by it, will surely cause severe frost bite and burns. In addition, working with liquid nitrogen in a closed or poorly ventilated room may cause accumulation of nitrogen gas, which is not toxic but reduces the oxygen content in the air available for breathing. Since nitrogen gas is odorless and tasteless, breathing in a nitrogen-rich and oxygen-depleted atmosphere may produce immediate fainting and deep unconsciousness without any previous symptoms.

To prevent these potential risks, always bear in mind the following precautions:

(1) Ensure that the room has good ventilation at all times. (2) When filling, transferring, or immersing objects at room temperature in liquid nitrogen, protect from nitrogen boiling. (3) Wear a face shield or safety glasses (open above and below, not goggles). (4) Wear cryo-gloves with very long sleeves. (5) Prevent penetration

of liquid nitrogen into or under protective clothing. It will vaporize immediately, causing freezing injuries. Try to keep the sleeves over the top of the safety gloves. When wearing boots, make sure that the leg of the pants is covering the upper part of the boot.

Acknowledgements I want to acknowledge Dr. Patricia Corral-Martínez and Mrs. Verónica Parra-Vega for providing some of the images used in this manuscript. Thanks are also due to the staff of the Electron Microscopy Service of Universitat Politècnica de València.

References

1. Ruska E (1980) The early development of electron lenses and electron microscopy. Hirzel Verlag, Stuttgart
2. Craig S, Staehelin LA (1988) High-pressure freezing of intact plant tissues—Evaluation and characterization of novel features of the endoplasmic reticulum and associated membrane systems. Eur J Cell Biol 46:80–93
3. Gilkey JC, Staehelin LA (1986) Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J Electron Microsc Tech 3:177–210
4. McDonald KL, Auer M (2006) High-pressure freezing, cellular tomography, and structural cell biology. Biotechniques 41:137–143
5. Seguí-Simarro JM, Austin JR, White EA, Staehelin LA (2004) Electron tomographic analysis of somatic cell plate formation in meristematic cells of arabidopsis preserved by high-pressure freezing. Plant Cell 16:836–856
6. Otegui M, Austin JR (2007) Visualization of membrane-Cytoskeletal Interactions during Plant Cytokinesis. In: McIntosh JR (ed) Cellular electron microscopy. Methods Cell Biol 79:221–240
7. Kang BH, Nielsen E, Preuss ML, Mastronarde D, Staehelin LA (2011) Electron tomography of RabA4b- and PI-4Kbeta1-labeled trans Golgi network compartments in Arabidopsis. Traffic 12:313–329
8. Otegui MS, Staehelin LA (2004) Electron tomographic analysis of post-meiotic cytokinesis during pollen development in *Arabidopsis thaliana*. Planta 218:501–515
9. Kang B-H (2010) Electron microscopy and high-pressure freezing of Arabidopsis. Methods Cell Biol 96:259–283
10. Karahara I, Kang B-H (2014) High-pressure freezing and low-temperature processing of plant tissue samples for electron microscopy. In: Žárský V, Cvrčková F (eds) Plant cell morphogenesis, vol 1080. Methods in molecular biology. Humana Press, New York, pp 147–157
11. Daghma DS, Kumlehn J, Melzer M (2011) The use of cyanobacteria as filler in nitrocellulose capillaries improves ultrastructural preservation of immature barley pollen upon high pressure freezing. J Microsc 244:79–84
12. Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134:1–12
13. Corral-Martínez P, Parra-Vega V, Seguí-Simarro JM (2013) Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. J Exp Bot 64:3061–3075
14. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212
15. McDonald K, Schwarz H, Müller-Reichert T, Webb R et al (2010) "Tips and tricks" for high-pressure freezing of model systems. Methods Cell Biol 96:671–693

16. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, Cambridge
17. Robards AW, Wilson AJ (1993) Procedures in electron microscopy. Wiley, Chichester
18. Merighi A (1992) Post-embedding electron microscopic immunocytochemistry. In: Polak JM, Priestley JV (eds) Electron microscopic immunocytochemistry. Principles and practice. Oxford University Press, Oxford, pp 51–85
19. Wilson SM, Bacic A (2012) Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. Nat Protoc 7:1716–1727
20. Thiry M, Thiry-Blaise L (1989) In situ hybridization at the electron microscope level: an improved method for precise localization of ribosomal DNA and RNA. Eur J Cell Biol 50:235–243
21. Thiry M, Thiry-Blaise L (1991) Locating transcribed and non-transcribed rDNA spacer sequences within the nucleolus by in situ hybridization and immunoelectron microscopy. Nucl Acids Res 19:11–15
22. Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105:427–434
23. Bergman L (2001) Growth and maintenance of yeast. In: MacDonald P (ed) Two-hybrid systems, vol 177. Methods in molecular biology. Humana Press, Totowa, pp 9–14

Chapter 8
Three-Dimensional Imaging for Electron Microscopy of Plastic-Embedded Plant Specimens

Jose M. Seguí-Simarro

8.1 Introduction

Electron microscopy of plastic resin-embedded specimens is the most used approach to observe minute (nanometer-sized) details of plant cell ultrastructure, due to its high resolution and to the convenience of observing specimens at room temperature. Observation of plastic-embedded plant specimens by transmission electron microscopy (TEM) has been remarkably useful to understand the structure and function of many subcellular elements, such as microtubules, vesicles, Golgi stacks, mitochondria, plastids, the cell plate, or the cell wall. However, the reliability of classical TEM studies has been traditionally limited by two main factors: the artifacts induced by the use of chemical fixatives (*see* Chap. 2 of this book) and the lack of three-dimensional (3-D) information derived from the two-dimensional (2-D) nature of electron microscopy observations.

It is obvious that cells and subcellular elements are 3-D objects. Usually, thin TEM sections are 60–100 nm thick, and provide results in the form of 2-D projections (micrographs) of the volume contained in the section thickness. This may often lead to misinterpretations of the actual size and shape of a structure. A micrograph showing a circular profile could be a good example to illustrate this point. The circular profile might correspond to a sectioned sphere or to a sectioned cylinder. It is impossible to find the right option just with the data provided by the micrograph. Assuming that the real shape of the object is spherical, the diameter might be that of the circular profile or it might not. Indeed, the odds of sectioning a sphere through its equatorial plane are low. The most likely situation is that the real diameter of the circular profile is shorter than the diameter of the sphere where the profile comes from. These examples reflect the distorted view of the 3-D reality that 2-D images may provide. Thus, to avoid such misinterpretations, it is essential to use 3-D approaches when performing fine ultrastructural studies.

J. M. Seguí-Simarro (✉)
COMAV-Universitat Politècnica de València, CPI, Camino de vera, s/n., 46022 València, Spain
e-mail: seguisim@btc.upv.es

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_8

The first 3-D approach applied to TEM was stereology [1]. Stereology can be defined as a set of numerical techniques, based on geometry and statistics. Stereology allows for a 3-D interpretation of 2-D cross-sectional views of cells or tissues. In addition to the qualitative information, stereology provides quantitative information about 3-D objects extracted from measurements made on 2-D sections of these objects. The basic requisites to apply classic stereology to TEM are: (1) a set of randomly picked, representative samples, (2) a minimal set of sample micrographs, (3) a computer, and (4) a profound knowledge of the stereological techniques to be applied. Despite this technical simplicity, stereology is not a widely used technique nowadays. Two main facts count against the use of stereology: (1) in general, young cell biologists are not instructed in the knowledge and use of these techniques; and (2) current technology allows for more precise, accurate, and graphical alternatives to obtain quantitative and qualitative 3-D information. The two alternatives are serial section reconstruction and electron tomography.

8.1.1 Serial Section Reconstruction

The classical way to produce serial section reconstructions is through TEM imaging. TEM serial section reconstruction typically includes the following steps (Fig. 8.1):

1. ultramicrotomy (serial thin sectioning) of the resin-embedded specimen.
2. collection of the serial sections made from the specimen.
3. acquisition of TEM micrographs of each serial section.
4. generation and alignment of an image stack made with all the serial micrographs.
5. 3-D modeling of the objects included in the stack.

This approach has been employed successfully to reconstruct the 3-D organization of whole cells or mitotic spindles, nuclear compartments, and cytoplasmic organelles such as mitochondria, plastids, or vacuoles in many types of yeast and plant cells [2–9]. This technique produces 3-D reconstructions with a resolution in the X and Y-axis (5–10 nm) much higher than in the Z-axis (80–100 nm). However, the main drawback is the long time needed to generate the final model. Such a slow process is mainly due to the time-consuming nature of the two principal steps: serial imaging and modeling.

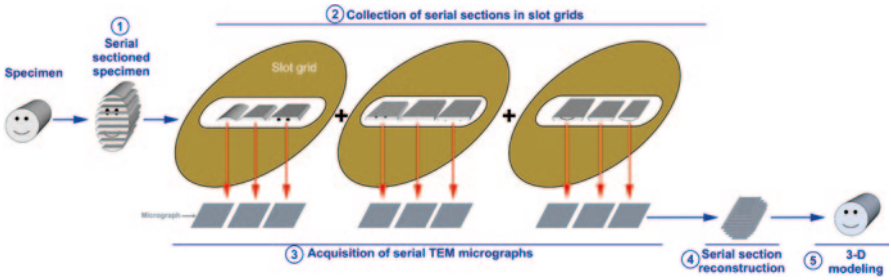

Fig. 8.1 Scheme of the different steps for 3-D reconstruction from TEM serial sections. See text for further details

Modeling is a laborious process that implies tracing the observed structures in serial slices, in a process called hand-segmentation. This way, it is possible to produce 3-D reconstructions of all or some of the individual objects present in the reconstructed volume. In models, the total volume can be analyzed either as a whole, or after subdivision into sub-volumes. A careful analysis of the models may yield not only detailed insights into the subcellular structures, but also significant quantitative information about the structures, including their number, density, surface area, and volume. Fortunately, the availability of 3-D modeling software such as IMOD [10] has greatly reduced the time needed to produce a model compared to the building of physical models (the classical alternative), although it is still time and effort-consuming.

With respect to serial imaging, the emergence of new electron microscopes is reducing considerably the time needed to obtain the stack of serial images. Currently, there is an alternative to the laborious preparation of physical serial sections: the use of a field emission scanning electron microscope (FESEM) equipped with a focused ion beam (FIB). A FESEM is a scanning electron microscope (SEM) equipped with a field emission (FE) gun instead of the tungsten hairpin or LaB_6 filaments typical of conventional SEMs. This electron source allows for enhanced performance, reliability, and durability. In practice, the FESEM provides surface images with resolution much higher than a conventional SEM. Indeed, the resolution of a FESEM can be similar to that of a conventional TEM, which makes it possible to work at high magnification and acquire high-resolution images of the specimen surface, exactly as in TEM. The FIB is used to precisely mill specific regions of the specimen surface with a predefined thickness. Depending on the FIB type, the milled surface can be as thin as 20 nm, and even 5 nm in some cases [11]. When combined, a FESEM-FIB is an excellent tool to generate high-resolution serial images from the surface of the sample. FESEM-FIB serial section reconstruction would typically include the following steps (Fig. 8.2):

1. Automated acquisition of FESEM-FIB micrographs: successive rounds of surface milling and image acquisition. Each of these rounds will produce a serial image of the sample at different depths along the Z-axis.
2. Generation and alignment of an image stack made from all the serial images.
3. 3-D modeling of the objects included in the stack.

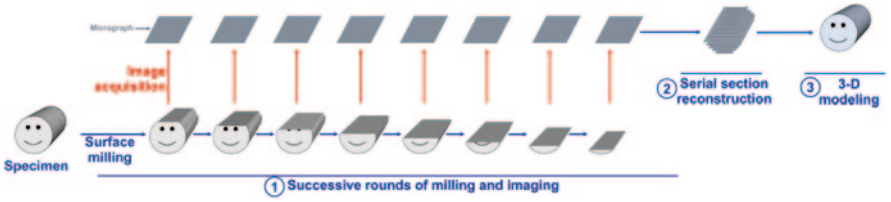

Fig. 8.2 Scheme of the different steps for 3-D reconstruction from FESEM-FIB serial sections. See text for further details

This approach has three main advantages compared to TEM serial sectioning: (1) the resolution of the Z-axis is higher, (2) areas much larger than with TEM serial sectioning can be imaged [12, 13], and (3) the laborious step of serial ultramicrotomy is entirely avoided. However, the FESEM-FIB approach is limited by two main factors: the high cost of this equipment, not available in all electron microscopy services, and its destructive nature. Since serial imaging is based on surface imaging and then removal (milling) of the imaged surface, it is not possible to apply post-embedding techniques such as in situ localization (immunogold labeling, in situ hybridization) or reexamination of areas of interest at a higher magnification, which is sometimes very helpful.

8.1.2 Electron Tomography

Despite the advantages of serial sectioning, it may not be the ideal approach for certain fine studies. Whereas the resolution in the X and Y-axes of the sections is ~4 nm, the resolution in the Z-axis is reduced to the thickness of the physical section (100 nm), or to 20–40 nm if a conventional FESEM-FIB is available. This limited Z-resolution of non-isodiametric 3-D reconstructions may be sufficient for studies dealing with the reconstruction of organelles (such as vacuoles, plastids, or mitochondria) or whole cells, as mentioned above. However, this is an important limitation when trying to determine the fine 3-D architecture of small subcellular elements such as microtubules, vesicles, endoplasmic reticulum, multi-vesicular bodies, Golgi cisternae, or even macromolecular complexes. In these cases, electron tomography is the solution. For electron tomography, two approaches are available. On the one hand, a high-performance FESEM-FIB may reach a Z-resolution of ~4–5 nm, producing isodiametric volume elements (voxels) of 5 × 5 × 5 nm (for the X, Y, and Z-axes). This way, FESEM-FIB serial sectioning will produce 5 nm thick slices of the specimen. Once stacked, these slices will have the same resolution in the three axes. Therefore, they will constitute a tomogram. However, this equipment is only available in very few labs worldwide.

On the other hand, a more popular (although still limited) option is the use of dual-axis electron tomography [14, 15]. Dual-axis electron tomography consists of the computer-assisted integration of data obtained by imaging a sample at different tilt angles in a TEM (Fig. 8.3) [10, 15, 16]. When combined into a tomogram, these datasets allow for the virtual reconstruction of cell volumes. Thus, a tomogram can be defined as a 3-D block of data that is represented as an array of voxels [17]. Thick-sectioned samples (150–400 nm thick) are viewed in a high (HVEM) or intermediate voltage electron microscope (IVEM), and two orthogonal stacks of images are taken by tilting the section at defined angles (usually from +60° to −60°, every 1–1.5°). Each of the individual tilted images constitutes the back projection of the whole specimen at a defined angle (Steps 1 and 5 in Fig. 8.3), thus providing unique information about the actual 3-D arrangement of the different objects within the section. Once transferred to the computer (steps 2 and 6 in Fig. 8.3), for each orthogonal stack all the tilted sections are aligned and then integrated into a single-axis, low-resolution tomogram that integrates the complementary information

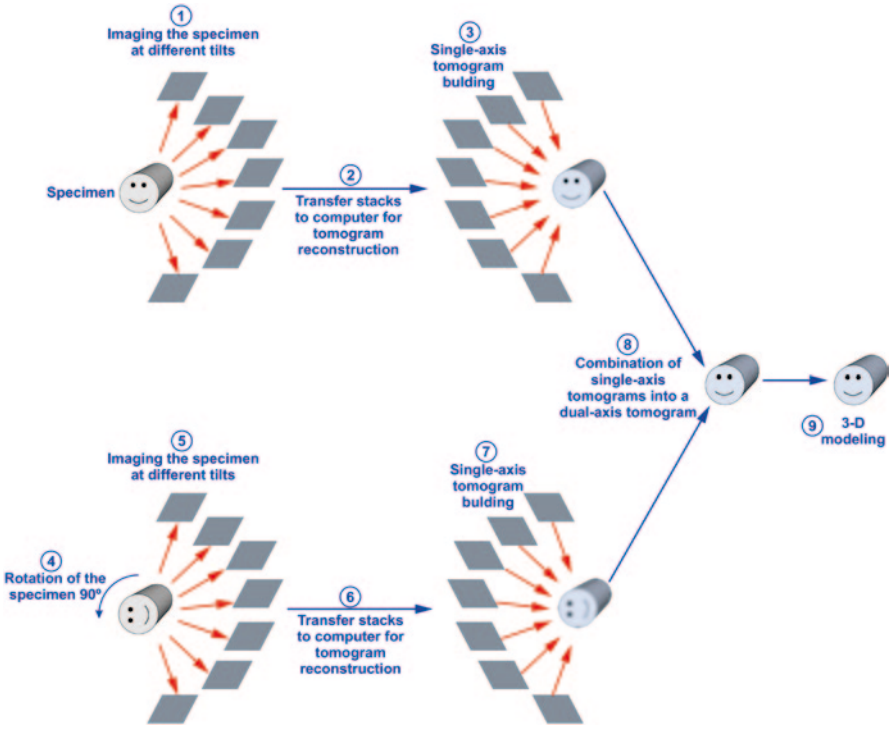

Fig. 8.3 Scheme of the different steps for 3-D reconstruction through dual-axis electron tomography from TEM tilted sections. See text for further details

coming from the different pictures (steps 3 and 7 in Fig. 8.3). Each single-axis tomogram is well resolved in the axis where the specimen was tilted along, but provides little information about its orthogonal counterpart. This is the reason why two individual, orthogonal stacks (rotated 90°, step 4 in Fig. 8.3) are recorded. The two single-axis tomograms are then combined into a final dual-axis tomogram (step 8 in Fig. 8.3), which integrates the information coming from the two axes, yielding ~2–4 nm thick tomographic slices [10, 15, 16]. As seen, electron tomography is the most powerful technique for fine 3-D reconstruction. However, it requires access to very expensive equipment such as a high-performance FESEM-FIB or an HVEM or IVEM. Thus, it may not be available for all labs.

As seen, each of the three electron microscopy-based 3-D approaches (TEM serial section reconstruction, FESEM-FIB serial section reconstruction, and dual-axis electron tomography) has specific advantages and limitations (summarized in Table 8.1). Depending on the study planned and the resources available, one option may be more advantageous than others. Thus, in this chapter, I provide an introduction to the three approaches. The aim of this chapter is not to give a detailed hands-on protocol for these approaches, which would require more than one chapter for each. Instead, the aim of this chapter is to provide an overview of what these techniques are useful for, when to choose one of them, what materials and equipment are needed in each case, and what are the main technical steps in

Table 8.1 Comparison of the three methods for 3-D reconstruction described in this chapter

	TEM serial sectioning	FESEM-FIB serial sectioning	Electron tomography
Useful for	Organelles, cell regions, entire cells		Molecular complexes, small subcellular structures, organelles
Microscope used	*Conventional TEM*	*FESEM-FIB*	*IVEM or HVEM*
Advantages	Microscope availability, no need for special electron microscopes	No need for ultramicrotomy	High 3-D resolution
		Larger areas can be imaged	True isodiametric reconstructions
		Z-resolution higher than in TEM serial sectioning	
		Isodiametric reconstructions (tomograms) can be obtained with high-performance FESEM-FIB	
Limitations	Non-isodiametric 3-D reconstructions with limited Z-axis resolution	Very reduced availability of FESEM-FIB	Reduced availability of IVEM or HVEM
	High dependence on serial ultramicrotomy (time consuming)	Post-embedding techniques and specimen reexamination are not possible	Tomogram reconstruction may be a complex process
		With conventional FESEM-FIB, non-isodiametric 3-D reconstructions with lower Z-axis resolution are obtained	

each case. For detailed protocols including every single step, the reader is referred to the different reviews, book chapters, and Web sites cited hereby.

8.2 Materials

Plastic resin-embedded specimens are used for all the methods described in this chapter. It is strongly recommended to fix the tissue by high-pressure freezing and then to dehydrate it by freeze substitution. The resins used for embedding should be suitable for thin sectioning (Embed 812, Spurr, etc.). Detailed protocols for high-pressure freezing, freeze substitution, and resin embedding can be found in Chap. 7 of this book.

8.2.1 Materials for TEM Serial Sectioning

1. Formvar and carbon-coated *(see Note 1)* copper EM grids (Electron Microscopy Sciences, Hatfield, PA). Although meshed grids may be used, slot grids are advised *(see Note 2)*.
2. Ultramicrotome (Leica UC6, from Leica Microsystems, Vienna, Austria, or similar).
3. Diamond knife (Ultra 35° from Diatome, Biel, Switzerland, or similar).
4. 2 % (w/v) uranyl acetate solution in 50 % ethanol, or 4 % aqueous uranyl acetate, both at pH 4–5.
5. Reynolds' lead citrate solution [18].
6. Conventional TEM.
7. Computer equipped with the IMOD software package *(see Note 3)*.

8.2.2 Materials for FESEM-FIB Serial Sectioning

1. FESEM-FIB microscope (The Auriga Compact model from Carl Zeiss, or similar).
2. Computer equipped with the IMOD software package *(see Note 3)*.

8.2.3 Materials for Dual-Axis Electron Tomography

1. See items listed for Sect. 8.2.1.
2. Sputter coater (Denton vacuum DV5 or similar).
3. 10 nm colloidal gold solution (Prod. No. 5703-20 from Ted Pella, Redding, CA, or similar).
4. HVEM or IVEM (FEI Tecnai F30 or similar) equipped with Serial EM.

8.3 Methods

8.3.1 TEM Serial Sectioning

1. Prior to sectioning, prepare coated slot grids *(see Note 1)*.
2. Trim the resin block to expose the sample and prepare a trapezoidal sectioning front of approximately 1 mm^2.
3. Obtain semi-thin sections (0.5–2 μm) with a glass knife for preliminary observation under the light microscope.

4. Observe the semi-thin sections with phase contrast optics to check that the tissue is not damaged. Alternatively, sections can be stained with general stains such as toluidine blue O and observed in bright field.
5. Obtain ribbons of ultrathin sections (~80 nm) with a diamond knife (see Note 4). Ultrathin sections can be identified by their silver interference color when floating in the knife boat. To obtain good (straight) ribbons, it is essential that the top and bottom edges of the trapezoidal block face are parallel. Otherwise, the ribbons will be curved and may not fit into the grid slot. Thus, pay special attention to produce parallel top and bottom edges when hand trimming the block sides. For inexperienced users, previous training with empty blocks (containing only resin, no sample) is advised.
6. Pick ribbons up by touching them with the coated side of the slot grid. The grid should be held with TEM forceps. Pay special attention to ensure that the ribbon is positioned within the slot. For users with trembling hands, holding the working hand with the other hand may help to reduce shaking. Again, previous training with empty section ribbons is advised for inexperienced users.
7. Contrast the sections with uranyl acetate and lead citrate.
8. Once in the TEM, take a micrograph of each of the sections that contain the object to be reconstructed. Make sure that your images are saved in TIF format. To avoid confusions, it is advised to assign images the same name followed by consecutive numbers for the consecutive images as they are acquired. For example: sample_01.tif, sample_02.tif, sample_03.tif… to the first, second, third… image.
9. Once all the serial micrographs are taken, transfer them to the computer. Create a folder for the images in the home directory selected as the working directory during IMOD installation *(see Note 5)*.
10. Within the image folder, check the image collection to ensure that the order of images is correct.
11. If needed, balance the brightness and contrast of all or some images.
12. Create a stack and align it. This can be done by launching the Etomo user interface and following the instructions described in the Etomo tutorial available at http://bio3d.colorado.edu/imod/doc/etomoTutorial.html. Otherwise, for a step-by-step procedure, follow steps 13–21 (next).
13. Convert the TIF image collection into an image stack (a.st file). For this, launch the tif2mrc program. From the command shell, type:
    tif2mrc *.tif *.st *(see Note 6)*
    Once the stack file (.st) is created, the TIF image collection is no longer needed. It can be deleted or stored elsewhere.
14. Pre-align the stack with the xfalign program. From the command shell, type:
    xfalign -pr XXX.st XXX.xf *(see Note 6)*
    where XXX is the name assigned to the stack file as well as to all the accessory files derived from it. The xfalign program will produce a transform file (.xf) that includes all the transformations made between two consecutive sections.
15. Check the alignment by launching the Midas graphic user interface program from the command shell *(see Note 7)*.

16. If alignment is almost good, finish alignment manually with Midas. Save the transform file as XXX.xf.
17. If alignment is still too rough, manually pre-align the stack with Midas *(see Note 7)*, save the transform file as XXX.prexf, and launch again xfalign with the –i option *(see Note 6)* to search for transformations starting from the transforms in the XXX.prexf file, as follows:
    xfalign -i XXX.st XXX.prexf
18. Convert the transformations between sections (the.xf file) into alignment transforms (a.xg file), to be applied to each section to obtain a single consistent set of alignments in the final stack. From the command shell, type:
    xf2xg –n 0 XXX.xf *(see Note 6)*
19. Generate the final, aligned stack (.ali) with the newstack program. From the command shell, type:
    newstack –xg XXX.xg –fl 2 XXX.st XXX.ali *(see Note 6)*
20. Check the aligned stack with the 3dmod graphic user interface program *(see Note 8)* from the command shell.
21. Start modeling with 3dmod *(see Note 9)*. Figure 8.4 shows an example of a 3-D reconstruction done by TEM serial sectioning and modeled with 3dmod.

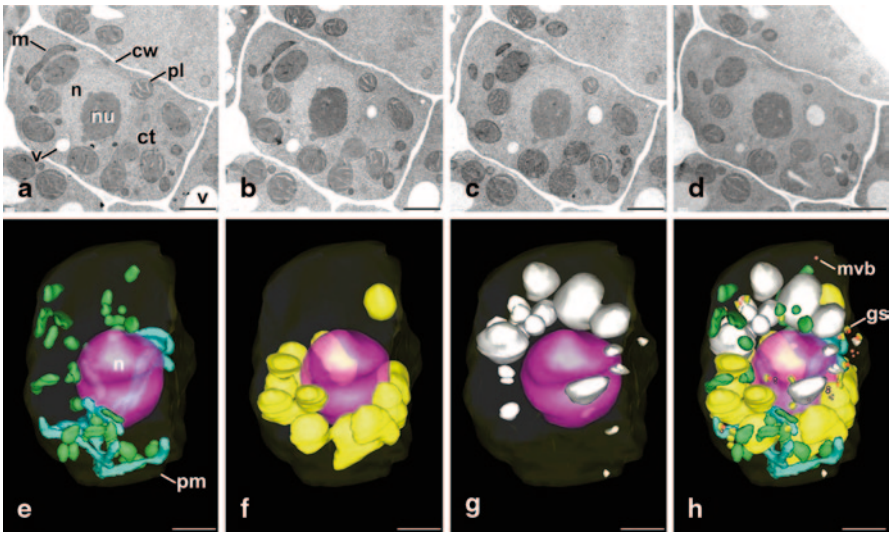

Fig. 8.4 TEM serial section reconstruction. **a–d** Examples of TEM serial micrographs from four consecutive sections of an *Arabidopsis* shoot apical meristem cell. Note the changes in shape along the four sections of the labeled mitochondrion (*m*) and plastid (*pl*). **e–h** 3-D models of the entire cell showing the plasma membrane (*pm*) and the nucleus (*n*) as references, as well as different organelles. **e** The mitochondrial population characteristic of these cells, comprising individual (*green*) and network-forming mitochondria (*blue*). **f** Plastids (*yellow*). **g** Vacuoles (*white*). **h** Mitochondria, plastids, vacuoles, Golgi stacks (*gs*), and multi-vesicular bodies (*mvb*). *ct* cytoplasm, *cw* cell wall, *nu* nucleolus, *v* vacuole. Bars: **a–d**: 2 µm; **e–h**: 1 µm

8.3.2 FESEM-FIB Serial Sectioning

1. Trim the resin block to expose the sample and prepare a rectangular sectioning front. As opposed to TEM serial sectioning, the front size can be larger than 1 mm^2 if needed. It is important to generate a flat front, without irregularities, to obtain high-quality images.
2. In the FESEM-FIB, mill the resin block with the FIB to create a window (Fig. 8.5a, b) that exposes the object (a group of cells, a single cell, a cell region, or an organelle) to be reconstructed *(see Note 10)*.
3. Take a micrograph of the exposed sample object (the block front). Make sure that your images are saved in TIF format.
4. Mill the front with the FIB at the desired milling thickness.
5. Repeat step 3 as many times as needed to obtain all the serial images necessary for the reconstruction. It is advised to assign all images the same name followed by a consecutive number as explained in step 8 of Sect. 8.3.1.
6. Follow steps 9–21 of Sect. 8.3.1. Note that raw FESEM-FIB images have an appearance different from those of conventional TEM (Fig. 8.5c). They look like a negative of TEM images, where electron light regions (cytoplasm, cell wall) have a dark look and electron dense regions (osmium-contrasted membranes, chromatin, nucleolus) appear light. In order to transform them to a more conventional format, image contrast can be inverted with conventional image

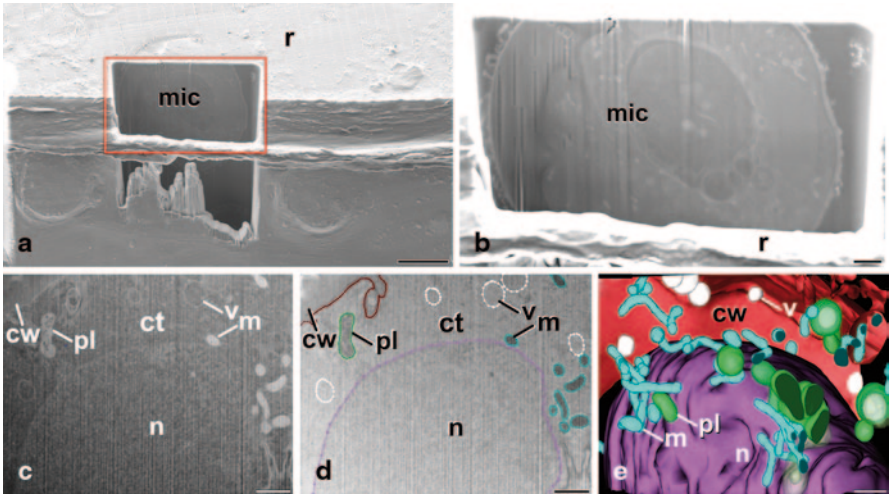

Fig. 8.5 FESEM-FIB serial section reconstruction. **a** Microspore (mic) specimen embedded in plastic resin (*r*). A window (*red box*) has been opened with the FIB to expose the cytoplasm of the selected specimen. **b** Enlarged view of the window boxed in A. **c** An example of raw FESEM serial image. **d** Contrast-inverted version of the image shown in **c**, to make it similar in appearance to TEM images. Colored profiles correspond to the different objects modeled in 3dmod. **e** 3-D model reconstructed with 3dmod by meshing all the profiles modeled in serial sections. Images adapted from [25]. *ct* cytoplasm, *cw* cell wall, *m* mitochondrion, *pl* plastid, *v* vacuole. Bars: **a**: 2 μm; **b**: 2 μm; **c–e**: 1 μm

editing software or with the "reverse contrast" option in Midas. Figure 8.5d and e show examples of a processed (contrast inverted and modeled) section, and a 3-D model reconstructed with 3dmod.

8.3.3 Dual-Axis Electron Tomography

1. Prior to sectioning, prepare 0.7% formvar and carbon-coated slot copper or gold grids *(see Notes 1, 11)*. Prior to formvar coating, deposit drops of 0.7% formvar over the grids placed on a filter paper, to increase the adherence of the formvar film to the grids (especially for gold grids). Let the drops air dry.
2. Trim the resin block to expose the sample and prepare a trapezoidal sectioning front of approximately 1 mm2.
3. Obtain semi-thin sections (0.5–2 μm) with a glass knife for preliminary observation under the light microscope.
4. Observe the semi-thin sections with phase contrast optics to check that the tissue is not damaged. Alternatively, sections can be stained with general stains such as toluidine blue O and observed in bright field.
5. Obtain ribbons of few (4–5) sections, 200–400 nm thick with a diamond knife (see Note 4).
6. Pick ribbons up by touching them with the coated side of the slot grid. Pay special attention to ensure that the ribbon is positioned within the slot.
7. Contrast the sections with uranyl acetate and lead citrate, as usual *(see Note 12)*.
8. Holding the grid with a forceps, apply a drop of 10 nm colloidal gold *(see Note 13)* to one side of the grid, and incubate for 5 min. Remove the excess of liquid, turn the grid upside down and apply a second drop to the other side of the grid. After 5 min, remove the excess of liquid and air dry the grid.
9. Apply additional layers of carbon to both sides of the grid with a sputter coater, available in most electron microscopy facilities *(see Note 14)*.
10. Observe the sections with a conventional TEM at low magnification (1000–2000×), to map the cells of interest for tomography.
11. Once in the HVEM or IVEM, launch the user interface of the microscope, the image acquisition software (Digital Micrograph for FEI microscopes), and also Serial EM *(see Note 15)*.
12. Insert the grids into the HVEM or the IVEM and search for the cells to be reconstructed with the help of the maps taken with the conventional TEM *(see Note 16)*.
13. Adjust focus, brightness, and the rest of required parameters, and start the acquisition of the first image stack *(see Note 17)*. Serial EM will produce a single-axis stack (.st extension) with all the images acquired at different tilts. To avoid confusion, it is advised to name the first stack as XXXa.st, where XXX is the name of the final tomogram, and "a" is the first single-axis stack.
14. Once the acquisition of the first stack is done, take the grid out of the microscope, rotate it 90° clockwise and repeat steps 12–13 to acquire the second stack, which will be named XXXb.st.

15. Once the second stack is acquired, transfer the two stacks to the computer for tomogram generation.
16. Check both stacks in 3dmod.
17. Align each stack, build the two single-axis tomograms, and combine them into the final, dual-axis tomogram. For this, launch the Etomo user interface and follow the instructions described in the Etomo tutorial available at http://bio3d.colorado.edu/imod/doc/etomoTutorial.html. A typical tomographic slice should look like that shown in Fig. 8.6a.
18. To study larger volumes, several dual-axis tomograms can be built from serial thick sections and then combined into a serial tomogram. Using this type of scaling it is possible to produce high-resolution tomographic reconstructions of large cell volumes. For this, follow steps 1–18 to build each dual-axis tomogram, and then follow the instructions of the tutorial for joining serial section tomograms with Etomo, available at http://bio3d.colorado.edu/imod/doc/JoinTutorial.html.
19. Start modeling with 3dmod. Figure 8.6b and 8.6c show examples of 3dmod-modeled 3-D reconstructions from dual-axis tomograms. To illustrate the possibilities of this technique to visualize small macromolecular complexes, Fig. 8.6d and 8.6e show the identification of a clathrin-coated bud emerging from the cell plate, and the modeling of the corresponding triskelions.

8.4 Notes

1. Coating of grids is necessary to add a continuous and electron transparent surface to support sections. In addition to plastic films (Formvar, collodion, parlodion, etc.), grids may also be coated with a carbon layer to provide them with additional strength and stability when exposed to the electron beam. Carbon coating is especially recommended when in situ localization experiments are to be performed, since grids will be subjected to frequent handling and successive incubations and washings. Detailed procedures for grid coating can be found in classical transmission electron microscopy handbooks (for example, see Refs. [19, 20]). It must be noted that successful grid coating is highly dependent on the relative humidity of the place where grids are to be coated. When working in a city close to the sea, where humidity is usually high, successful coating can be especially difficult. In this case or when only few grids are occasionally needed, it may be more convenient to order coated grids from specialized distributors. This also applies to plastic film solutions, which may be user-made or purchased as solutions in 1, 2-dichloroethane.
2. Usually, thin sections are collected on meshed grids, since mesh contributes to the stability of sections. However, when collecting serial sections, it is possible that in some of them, the object to be reconstructed becomes hidden by the mesh. To prevent this, the use of slot grids is advised. Slot grids have a

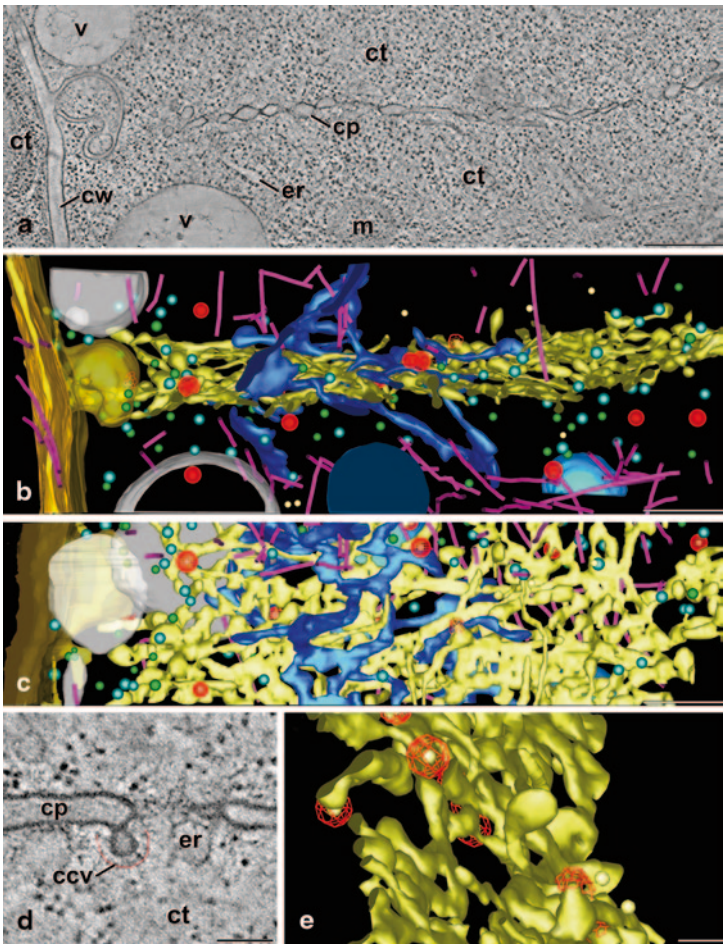

Fig. 8.6 Dual-axis electron tomography. **a** Representative 2 nm-thick electron tomographic slice of maturing cell plate at the tubular network stage [26] of an *Arabidopsis* shoot apical meristem cell. **b** 3-D model constructed from the tomogram containing the slice shown in **a**. **c** 90° rotation through the X-axis of the model shown in **b**. **d** Enlarged view of a tomographic volume, oriented with the Slicer tool to observe a clathrin-coated bud (*red line*) emerging from the cell plate (*cp*, *yellow* in models). **e** The corresponding model volume, oriented for a better visualization of the forming clathrin-coated buds and the already formed clathrin-coated vesicles (*ccv*, *red spheres* in models). Note the disposition of the modeled triskelions (*red rods*) of the clathrin coat. *ct* cytoplasm, *er* endoplasmic reticulum (*dark blue* in models), *m* mitochondrion (*light blue* in models), *v* vacuole (*white* in models). The *purple rods* are microtubules and the *light blue* and *green small spheres* are Golgi-derived vesicles. Bars: **a–c**: 500 nm; **d–e**: 100 nm

single central hole, with no mesh. Although the carbon coat, the plastic film, and the sections would be more unstable in these grids, the entire section area will be exposed for TEM observation. Obviously, handling grids with care is advised.

3. As stated on the Web site of the Boulder Laboratory for 3-D Electron Microscopy of Cells (the research facility that developed IMOD), IMOD is "a set of image processing, modeling, and display programs used for tomographic reconstruction and for 3-D reconstruction of EM serial sections and optical sections. The package contains tools for assembling and aligning data within multiple types and sizes of image stacks, viewing 3-D data from any orientation, and modeling and display of the image files." IMOD can be downloaded from: http://bio3d.colorado.edu/imod/. Release packages are available for LINUX, Macintosh, and Windows-based computers. For each operating system, specific information about hardware and operating system requirements are detailed on the Web site. Complete instructions for full installation and use, as well as tutorials are also available on the Web site. Once installed, each of the techniques described in this chapter will require the use of specific modules. These modules can be launched through a command shell-based user interface. For users not experienced with the use of Linux-based command shells, a previous basic training in Linux is advised.
4. In blocks where the front is difficult to trim, rough section edges may make ribbon formation difficult. In this case, ribbon formation may be facilitated by applying a thin layer of diluted rubber cement to the base of the trapezoid, as described in [21]. This way, adherence of the newly cut sections to the growing ribbon will be easier.
5. For specific directions on IMOD installation, use, and troubleshooting check: http://bio3d.colorado.edu/imod/doc/guide.html.
6. To review the features, usage, options, or syntax of specific programs of the IMOD package, check: http://bio3d.colorado.edu/imod/doc/program_listing.html.
7. Midas is a graphical user interface for manual alignment of serial sections by translation, rotation, and stretching, using mouse movements, controls, or hotkeys. Manual alignment is done by comparing two consecutive sections, viewing them alternately in quick succession, or in a two-color overlay mode. For more information, check the Midas description at http://bio3d.colorado.edu/imod/doc/man/midas.html.
8. 3dmod is a model editing and image display program that can display 3-D graphic data sets in many views simultaneously, can model these data sets, and can display models and graphic data in 3-D through different views that include a slice through the 3-D volume, a projection of a sub-volume, and orthogonal views with contour overlays. For more information, check the 3dmod description at http://bio3d.colorado.edu/imod/doc/man/3dmod.html.
9. The wealth of modeling options and possibilities that 3dmod offers for modeling is impossible to compile in this chapter. Therefore, before using 3dmod for the first time, it is strongly recommended to visit an experienced research group or to carefully read the *Introduction to 3dmod* online manual available at http://bio3d.colorado.edu/imod/doc/3dmodguide.html.
10. As for all electron microscopes, each FESEM-FIB has its own working protocols. Before using it, the advice and instruction of the technician in charge of it is advised.

11. For dual-axis electron tomography, the use of slot grids is mandatory, since specimen sections must be tilted and imaged from +60° to −60°. At such high tilts, a meshed grid would preclude visualization of the sections.
12. If usual contrasting times are insufficient, try extended times or solutions of uranyl acetate in 70% methanol.
13. For a fine alignment of the tilt series, it is essential to have an external, invariable reference of the tilt and drift that each image may have with respect to the rest of the stack. For this reason, gold particles (fiducial markers) are dispersed over both sides of the section. Gold particles have a defined size, shape, and electron density. This means that they will be easily identified in each of the tilt images, since their position in the tilted images will only depend on the tilt angle. This is why they are used as fiducial markers. In the computer-assisted alignment step, the position of each gold particle in each tilted image will be tracked to build a fiducial model. Then, this fiducial model will be used as a reference for fine alignment of the entire stack.
14. After carbon coating, if holes or cracks are observed in the formvar film, apply an additional formvar layer to one side of the grid. If the film is bent but not broken, apply a new film layer at the side of the grid where the bent film appears concave.
15. Serial EM is a program to acquire different data from FEI and JEOL electron microscopes, including, among others, automated tilt series acquisition for electron tomography. Automation of the process is based on an approach that predicts the specimen position during the tilt series from the position at previous tilts [22, 23].
16. Before starting, the HVEM or IVEM must be set up with the optimal parameters for this technique and all the required software, including Serial EM. Adjusting the optimal conditions for electron tomography requires a good knowledge of the microscope and the microscope user interface. Usually, this is done by technical personnel of the electron microscopy facilities, not by users. Thus, it is recommended to go to experienced facilities to perform this type of techniques.
17. In old microscopes, the acquisition of stack images was done "manually." The user was required to adjust the sample tilt to the highest angle (usually +60°), and then move the tilt down stepwise, at 1.5–2° steps, until reaching the lowest angle (usually −60°). At each of these steps, the user acquired a micrograph. In general, a stack used to be acquired in 4–8 h. At present, modern microscopes and software developments such as Serial EM allow for automation of these tasks, making electron tomography much easier and faster. Now, once the region of interest is focused and all the parameters adjusted, tilting, repositioning, refocusing, and image acquisition is done automatically. This way, the user is present only to check that the whole process is running well. Usually, no further interaction is required and a single-axis stack can be obtained in 1.5–2 h. For example, a single stack of 2000 × 2000 pixel images, taken every 1°, can be acquired with Serial EM in about 20–25 min with a four-port readout CCD camera [24].

8.5 Specific Comments About Dual-Axis Electron Tomography

1. Dual-axis electron tomography is a complex process where a constant interaction between the user and the IMOD software package is required. To help users worldwide to use IMOD and its different options, the Boulder Laboratory for 3-D Electron Microscopy of Cells has extensive guides for general and particular procedures, as described in **Notes 5** and **6**. In addition, there is a specific Youtube channel (https://www.youtube.com/user/BL3DEMC) with demonstrations, tutorials, and lectures about the use of IMOD and Serial EM.
2. Many different variables (before, during, or after image acquisition, tomogram reconstruction, and modeling) may influence the final quality of the dual-axis tomogram and the corresponding model. This makes possible that for each particular case (a particular sample imaged in a particular microscope and reconstructed in a particular computer), different problems, questions, and doubts may arise. To help solve these specific issues, the Boulder Laboratory for 3-D Electron Microscopy of Cells has a discussion listserver specifically devoted to IMOD users. The IMOD discussion listserver provides a forum for users and developers to pose questions or to expose problems, offer solutions to others' problems, or make suggestions about improvements. There is also an IMOD mailing list for notices about new versions and IMOD trainings. For more information and instructions on how to subscribe to both lists, visit http://bio3d.colorado.edu/imod/joinlist.html. Similarly, there is also a Serial EM discussion list (see http://bio3d.colorado.edu/SerialEM).

Acknowledgments I want to thank the help of Mrs. Verónica Parra-Vega in the FESEM-FIB reconstructions. Thanks are also due to the staff of the Electron Microscopy Service of Universitat Politècnica de València (Valencia, Spain), and to the staff of the Boulder Laboratory for 3-D Electron Microscopy of Cells (Boulder, CO, USA).

References

1. Williams M (1977) Stereological techniques. In: Glauert AM (ed) Practical methods in electron microscopy. North Holland/American Elsevier, Amsterdam
2. Medina FJ, Risueño MC, Moreno Díaz de la Espina S (1983) 3-D reconstruction and morphometry of the fibrillar centres in plant cells in relation to the nucleolar activity. Biol Cell 48:31–38
3. Lockhausen J, Kristen U, Menhardt W, Dallas WJ (1990) Three-dimensional reconstruction of a plant dyctiosome from series of ultrathin sections using computer image processing. J Microsc 158:197–205
4. Ding R, McDonald KL, McIntosh JR (1993) 3-dimensional reconstruction and analysis of mitotic spindles from the yeast *Schizosaccharomyces pombe*. J Cell Biol 120:141–151
5. Bereiter-Hahn J, Voth M (1994) Dynamics of mitochondria in living cells: shape changes, dislocations, fusion and fission of mitochondria. Microsc Res Tech 27:198–219

6. Winey M, Mamay CL, Otoole ET, Mastronarde DN, et al (1995) 3-dimensional ultrastructural analysis of the *Saccharomyces cerevisiae* mitotic spindle. J Cell Biol 129:1061–1615
7. Seguí-Simarro JM, Coronado MJ, Staehelin LA (2008) The mitochondrial cycle of *Arabidopsis* shoot apical meristem and leaf primordium meristematic cells is defined by a perinuclear tentaculate/cage-like mitochondrion. Plant Physiol 148:1380–1393
8. Seguí-Simarro JM, Staehelin LA (2006) Cell cycle-dependent changes in Golgi stacks, vacuoles, clathrin-coated vesicles and multivesicular bodies in meristematic cells of *Arabidopsis thaliana*: a quantitative and spatial analysis. Planta 223:223–236
9. Seguí-Simarro JM, Staehelin LA (2009) Mitochondrial reticulation in shoot apical meristem cells of *Arabidopsis* provides a mechanism for homogenization of mtDNA prior to gamete formation. Plant Signal Behav 4:168–171
10. Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-dimensional image data using IMOD. J Struct Biol 116:71–76
11. Cantoni M, Genoud C, Hébert C, Knott G (2010) Large volume, isotropic, 3D imaging of cell structure on the nanometer scale. Microsc Anal 24:13–16
12. Heymann JAW, Hayles M, Gestmann I, Giannuzzi LA, et al (2006) Site-specific 3D imaging of cells and tissues with a dual beam microscope. J Struct Biol 155:63–73
13. Merchán-Pérez A, Rodriguez J-R, Alonso-Nanclares L, Schertel A, DeFelipe J (2009) Counting synapses using FIB/SEM microscopy: a true revolution for ultrastructural volume reconstruction. Front Neuroanat 3:18
14. McDonald KL, Auer M (2006) High-pressure freezing, cellular tomography, and structural cell biology. Biotechniques 41:137–143
15. McIntosh R, Nicastro D, Mastronarde D (2005) New views of cells in 3D: an introduction to electron tomography. Trends Cell Biol 15:43–51
16. Mastronarde DN (1997) Dual-axis tomography: an approach with alignment methods that preserve resolution. J Struct Biol 120:343–352
17. Seguí-Simarro JM, Otegui MS, Austin JR, Staehelin LA (2008) Plant cytokinesis—Insights gained from electron tomography studies. In: Verma DPS, Hong Z (eds) Cell division control in plants, vol 9. Plant cell monographs. Springer, Berlin, pp 251–287
18. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212
19. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, Cambridge
20. Robards AW, Wilson AJ (1993) Procedures in electron microscopy. John Wiley & Sons, Chichester
21. Kang B-H (2010) Electron microscopy and high-pressure freezing of *Arabidopsis*. In: Thomas M-R (ed) Methods in cell biology, vol 96. Academic Press, New York, pp 259–283
22. Mastronarde DN (2005) Automated electron microscope tomography using robust prediction of specimen movements. J Struct Biol 152:36–51
23. Mastronarde DN (2003) SerialEM: a program for automated tilt series acquisition on Tecnai microscopes using prediction of specimen position. Microsc Microanal 9(Suppl. 2):1182CD
24. The Boulder Laboratory for 3-D Electron Microscopy of Cells (2014) http://bio3d.colorado.edu/SerialEM/index.html. 7 July 2015
25. Parra-Vega V (2015) Basic and applied aspects of embryogenesis induction in pepper and rapeseedmicrospores. PhD Thesis. Universitat Politècnica de València, Valencia, Spain
26. Seguí-Simarro JM, Austin JR, White EA, Staehelin LA (2004) Electron tomographic analysis of somatic cell plate formation in meristematic cells of arabidopsis preserved by high-pressure freezing. Plant Cell 16:836–856

Chapter 9
Fluorescent Staining of Living Plant Cells

Sarah Schoor, Shiu-Cheung Lung, Dustin Sigurdson and Simon D. X. Chuong

9.1 Introduction

Fluorescence is the luminescence emitted when photons are absorbed and released by molecules known as fluorochromes (fluorophores); as a consequence of energy transference, the wavelengths of the fluorescent light are longer. Fluorescence imaging has many benefits, one of which being the high degree of sensitivity. Fluorochromes can be excited repeatedly, thus, a single molecule can absorb and release thousands of detectable photons. As a result of the amplified signal, fluorochromes allow for highly specific cellular and organellar differentiation with limited background. Additionally, fluorochrome detection is nonlethal and does not require chemical fixation, allowing for noninvasive in vivo and real-time imaging. This chapter highlights common fluorochromes, particularly fluorescent dyes, and how they can be applied to plant cell imaging.

9.1.1 Autofluorescence Imaging in Living Plant Tissues

When examining plant cells using fluorescence microscopy, one can excite fluorochromes already present or introduce them. The first option makes use of autofluorescence, wherein endogenous molecules absorb light in the UV and visible light spectra. The most abundant example of autofluorescence is chlorophyll fluorescence. While the majority of light energy absorbed by chlorophyll is allocated to photosynthesis, some of the absorbed energy can be dissipated as heat

S. D. X. Chuong (✉) · S. Schoor · S.-C. Lung · D. Sigurdson
Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada
e-mail: schuong@uwaterloo.ca

S.-C. Lung
School of Biological Sciences, University of Hong Kong, Hong Kong SAR, China

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_9

or luminescence (fluorescence). Even with only 1–2 % of absorbed light being diverted to chlorophyll fluorescence, the sensitive nature of fluorescence imaging allows for easy chlorophyll detection [1]. As chlorophyll molecules are embedded in the thylakoids of chloroplasts, red fluorescence emitted by chlorophyll (Table 9.1) allows for dye-free imaging of the entire plastid (Fig. 9.1). Other plant structures that can be imaged by autofluorescence include: cuticles of aerial organs (cutin [2]), pollen exine wall (sporopollenin [3]), secondary cell walls (lignin [4]), periderm (suberin [5]), and vacuoles (anthocyanin [6], flavonol [7]).

9.1.2 Exogenous Fluorochrome Application in Living Plant Tissues

If a cellular structure cannot be visualized through autofluorescence alone, exogenous fluorochromes are introduced. Synthetic organic dyes were the first fluorochromes applied to biological research. Over the past 100 years, derivatives of the original dyes have been further modified and novel probes developed. Fluorescent dyes are still commonly used for observing plant cells today, as they are convenient and easily applied to both living and chemically fixed tissues. The majority of fluorescent dyes are small (~0.5 nm) organic molecules [8] that can passively enter the cell or be taken up via active transport. Due to their small size, common fluorescent dyes such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) can also be conjugated to other biomolecules such as antibodies, without interfering in their biological functions. Cross-linking fluorescent dyes to protein-based probes allows for highly specific detection, however, exogenous protein application in living plant cells is problematic. Although semipermeable, plant primary cell walls only allow molecules and proteins up to 60–120 kDa in size to enter [9, 10], making them impermeable to antibodies exceeding this size limit. Thus, to increase the access of antibody, cell walls can be enzymatically removed and the released protoplasts studied [11].

For live-cell imaging, the expression of biological fluorochromes has become a valuable and powerful alternative to immunofluorescence. In 1994, green fluorescent protein (GFP) was cloned from jellyfish (*Aequorea victoria*) and used as a fluorescent reporter [12]. Since its early use, derivatives of the original GFP and many other fluorescent proteins have been developed for biological expression systems. Biological fluorochromes can be introduced to a large variety of species and expressed alone or fused to a protein of interest allowing protein function to be investigated [13]. Despite their numerous benefits, biological fluorochromes have limitations; most notably, stable transformation mediated by *Agrobacterium tumefaciens* can be time consuming and some plant species remain recalcitrant to this method. Additionally, expressing large amounts of light-emitting proteins can induce artificial responses or cell toxicity. Finally in protein fusion studies, the size of the fluorescent protein can also affect biological functions, ultimately altering the behavior of the protein [14].

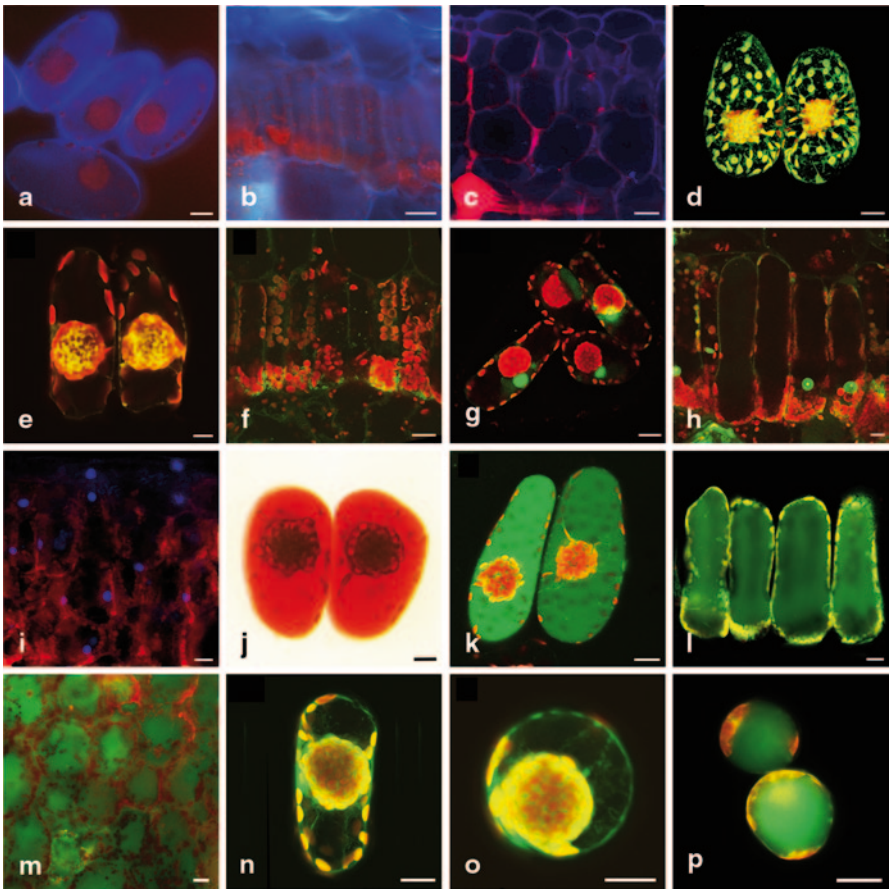

Fig. 9.1 Mesophyll cells and protoplasts of *Arabidopsis thaliana*, *Bienertia sinuspersici, Suaeda aralocaspica, Kalanchoe blossfeldiana*, and *Kalanchoe daigremontiana* stained with various fluorescent dyes to visualize cellular structures such as cell walls, ER, mitochondria, nuclei, and vacuoles. (**a**–**c**) Cell walls in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. daigremontiana* stained with fluorescent brightener 28 (*FB28*). **d** ER in *B. sinuspersici* mesophyll cells stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). (**e**, **f**) Mitochondria in mesophyll cells of *B. sinuspersici*, and *S. aralocaspica* stained with rhodamine 123 (*R123*). (**g**–**i**) Nuclei in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. daigremontiana* stained with acridine orange and 4′,6-diamidino-2-phenylindole (*DAPI*). (**j**–**m**) Vacuoles in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. blossfeldiana* stained with neutral red and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA). (**n**–**p**) Viability of mesophyll cells and protoplasts of *B. sinuspersici* and *A. thaliana* stained with FDA. Fluorescence is shown in false colors with green for acridine orange, R123, carboxy-DCFDA, $DiOC_6$ and FDA, blue for DAPI and FB28, and red for the autofluorescence of chloroplasts. Overlapping signals between green and red appear yellow. Panels **a**–**c**, **i**, **m**, and **p** represent epifluorescence micrographs. Panel **j** represents brightfield micrograph. Panels **d**–**h**, **k**, **l**, **n**, and **o** represent confocal micrographs. Scale bars = 25 μm

9.1.3 Fluorescent Staining of Plant Structures and Organelles

When selecting a fluorescent dye, the spectral properties of the fluorochrome should be closely examined. One major concern is ensuring that a fluorescent dye's emission spectrum does not overlap with that of another. Maximum wavelengths of absorption and emission are typical terms used to refer to a fluorochrome, however emission spectra can be very broad [8]. To avoid fluorochrome "bleed-through," it is important to ensure that the microscope is equipped with appropriate optical filters or light sources and the dyes in use exhibit distinct emission spectra. Special care needs to be taken in photosynthetic tissues as the strong chlorophyll fluorescence often masks photon emissions from other fluorochromes [15]. As chlorophyll fluoresces at >600 nm, dyes emitting red light are particularly affected, but fluorescence of all color spectra can be reduced as a result of autofluorescence. To increase fluorescence signals, ethanol treatment can be used to lower chlorophyll levels, however such treatment impairs cellular metabolism and causes cell damage. As an alternative to pigment removal, non-photosynthetic cells can be studied and/or only dyes with strong fluorescence utilized. Here we describe several common fluorescent stains that are routinely used to facilitate microscopic observation of plant structures.

9.1.3.1 Cell Wall

Primary cell walls are mainly composed of cellulose, hemicellulose, and pectin. Unlike the heavily lignified secondary walls of wood tissues which produce endogenous fluorescence, primary cell walls do not typically autofluoresce. For live-cell imaging of primary walls, *calcofluor white* (Fluorescent Brightener 28) is one of the most widely used fluorescent dyes. Calcofluor white binds to β-linked cellulose [16] where it exhibits a broad excitation and emission spectra; calcofluor white has an excitation maximum of 410 nm (Table 9.1) but any filter set with UV or violet/blue excitation wavelengths will generate strong blue fluorescence (Fig. 9.1a, b, and c). Due to the dye's emission spectrum, calcofluor white pairs nicely with other fluorescent dyes that emit 500 nm and above. Finally, as calcofluor white binds to the outer cell wall, membrane permeability is not an issue and the dye is readily taken up by tissues and individual cells (Table 9.2).

9.1.3.2 Endoplasmic Reticulum (ER)

In 1984, 40 years after the endoplasmic reticulum was first discovered by transmission electron microscopy, fluorescence imaging of the membrane network was achieved using *3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$*; [17]). DiOC$_6$ had been previously characterized as a mitochondrial dye [17], but higher concentrations of the fluorochrome resulted in ER localization [18]. Unlike earlier attempts using

Table 9.1 Fluorescent dyes commonly used to visualize plant organelles and structures

Cellular structure	Fluorescent dye	Excitation (nm)	Emission (nm)
Cell wall	Calcofluor white	410	455
Chloroplast	Chlorophyll autofluorescence	488	685(740)
ER	3,3′Dihexyloxacarbocyanine iodide ($DiOC_6$)	482	504
Mitochondria	$DiOC_6$	482	504
	Rhodamine 123	511	534
Nucleus	Acridine orange (dsDNA)	502	525
	Acridine orange (ssDNA, RNA)	460	650
	4′, 6-diamidino-2-phenylindole (DAPI)	350	470
Vacuole	Acridine orange	475	590
	5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA)	504	529
	Neutral red	495	540
Viability	Fluorescein diacetate (FDA)	494	518

ER endoplasmic reticulum

immunofluorescence, which required treatments with organic solvents and detergents to increase antibody permeability, $DiOC_6$ stained both living and glutaraldehyde-fixed cells without damaging the ER membrane (Fig. 9.1d; [18]). Although immunofluorescence and fluorescent proteins have since been further developed, $DiOC_6$ is still commonly used. $DiOC_6$ is a potential membrane-sensitive, cationic fluorescent dye. It is the dye's hydrophilic groups that allow it to strongly accumulate in intracellular membranes where it absorbs blue light and emits a strong green fluorescence signal (Table 9.1). $DiOC_6$ only works with living cells but they can quickly become damaged from photodynamic toxicity; to prevent cell death, it is recommended to limit excitation times. Finally, $DiOC_6$ is a strong dye but it is not very photo-stable thus anti-fade reagents may be added to the mounting medium.

9.1.3.3 Mitochondria

Detection of mitochondria exploits the electron transport system and their accumulation of positively charged redox dyes inside the inner membrane. Similar to $DiOC_6$, *Rhodamine 123 (R123)* is another potential membrane-sensitive, cationic green fluorescent dye (Table 9.1) that is readily sequestered by active mitochondria (Fig. 9.1e, f; [19]). As a result of electric potential across the mitochondrial

membranes, R123 selectively accumulates in the mitochondria. Moreover, under recommended concentrations and incubation times, stained cells often exhibit no accumulation of the dye in other subcellular compartments. However, it has been reported that exposure of plant cells to R123 over long periods of time results in vacuolar accumulation [20]. Although R123 has no cytotoxic effect, the dye and hence fluorescence intensity are easily lost once the mitochondria experience a loss of membrane potential and thus the dye requires living cells with functional mitochondria [20].

9.1.3.4 Nucleus

The porous nuclear envelope readily allows fluorescent dyes to passively enter the nucleus. Fluorochromes for visualizing nuclei bind to and sometimes alter nucleic acids, making long-term exposure toxic to living tissues. One commonly used nuclear dye is *acridine orange*, a cationic dye that binds to both DNA and RNA via electrostatic attractions [21]. When bound to double-stranded DNA, acridine orange has an excitation and emission maxima of 502 and 525 nm (green), respectively (Fig. 9.1g, h), but it has an excitation of 460 nm and emission of 650 nm (red) when bound to the phosphate groups of single-stranded DNA or RNA [21]. Acridine orange is readily taken up by both living and fixed cells (Table 9.2), and its detection of both RNA and DNA makes it ideal for cell cycle studies [21]. A potential drawback to using acridine orange is signal overlap with other fluorochromes with similar emission spectrum.

4',6-diamidino-2-phenylindole (DAPI) is a classic nuclear stain for fluorescence microscopy in both living and fixed cells [22]. DAPI strongly binds to the minor groove of double-stranded DNA, particularly adenine- and thymine-rich regions, and absorbs light in the UV spectrum (Table 9.1); despite excitation maximum being in the UV spectrum, DAPI will readily absorb violet light. Once excited, DAPI emits blue or cyan fluorescence making it ideal for pairing with other fluorochromes that emit longer wavelengths (Fig. 9.1i). Additionally, when bound to DNA, fluorescence emitted from DAPI is 20 times greater than that of unbound, greatly reducing background signals [23]. Thus, its selectivity for DNA and high cell permeability allow for efficient staining of nuclei with limited cytosolic fluorescence.

9.1.3.5 Vacuole

Accumulation of pigments in the vacuole often provides some autofluorescent activity [6, 7], however the levels of these metabolites are dependent on the cell type and the stage of development. An alternative method for vacuole detection exploits the organelle's acidity and subsequent accumulation of basic dye such as *acridine*

Table 9.2 Working concentrations and incubation times of fluorescent dyes for living plant cells

Fluorescent dye	Organelle	Solvent	Final (μg/mL)	Incubation (min)	Treatment pH
Acridine orange	Nucleus	DMSO	10	30	7
	Vacuole		1		7.4–8.5
Calcofluor white	Cell wall	Water	1	5	7
DAPI	Nucleus	Water	10	15	7
Carboxy-DCFDA	Vacuole	DMSO	1.25	20	5.5
$DiOC_6$	ER	Ethanol	5	5	7.4
	Mitochon-dria		0.25		
FDA	Nucleus/ vacuole	Acetone	10	5	7.6
Neutral red	Vacuole	Water	1–10	15	7.2–8.0
Rhodamine 123	Mitochon-dria	Methanol	10	30	7

DAPI 4′, 6-diamidino-2-phenylindole, *FDA* fluorescein diacetate, *DMSO* dimethyl sulfoxide, *ER* endoplasmic reticulum

orange and *neutral red* [24]. Proton motive force, which drives the transport of protons from the cytosol to the vacuole, regulates the cytoplasmic pH and facilitates the movement of solutes into and out of the vacuole. As a consequence of protonation, the vacuole becomes acidic (pH 5–5.5) and acts as an ion trap for membrane-permeable bases. Therefore, vacuolar dyes are often used as pH indicators. As discussed in Sect. 1.3.4, acridine orange is a nuclear stain; however, when applied under basic conditions (Table 9.2), the dye also accumulates in the vacuole [25] and emits red fluorescence (Table 9.1). Neutral red is used for both its strong red fluorescence (Table 9.1; [26]) and its distinctive red color that can be observed under bright-field microscope (Fig. 9.1j). Since neutral red requires active transport into the vacuole, only living cells may accumulate the dye. However, the working concentrations of neutral red vary depending on the tissue types and plant species under investigation. For example, low concentrations are used to stain *Arabidopsis* cell suspensions, 0.1 mg/L [27, 28], *Brassica* protoplasts, 1 mg/L [29], and *Arabidopsis* roots, 1 mg/L [30], whereas high concentrations are required for onion parenchyma cells, 4 mg/L [31] and whole carrots seedlings and embryos, 20 mg/L [25].

5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (*carboxy-DCFDA*) is another group of vacuolar stains that rely on intracellular esterases to release negatively charged carboxyfluorescein fluorochromes. Carboxy-DCFDA is nonfluorescent until the acetate groups of the fluorochrome are released by intracellular esterases [32]. Once cleaved, carboxy-DCFDA becomes 5-(and-6)-carboxy-2′-dichlorofluorescein, a fluorochrome that absorbs blue light and emits green fluorescence (Table 9.1, Fig. 9.1k, l, and m). Carboxy-DCFDA is membrane-permeable and the fluorescein derivatives are strongly sequestered in the vacuole using anionic transporters (Table 9.2).

9.1.4 Fluorescent Staining to Assess Cell Viability

Viability, or the ability of an organism or a cell to maintain itself, can be an important factor in experimental procedures. Fluorescent dye such as R123 requires mitochondrial activity [19] and can be used as indicators of cell viability; however, *fluorescein diacetate* (FDA) is the most routinely used for assessing cell viability. FDA is a cell-permeant dye that exhibits no fluorescence until intracellular esterases release fluorescein [33]. The strong green fluorescence of fluorescein (Table 9.1) is used as an indicator of both enzymatic activity and membrane permeability, as intact membranes allow fluorochrome accumulation. Once cleaved, fluorescein localizes to the cytosol, nucleus, and vacuole of the living cell (Fig. 9.1n, o, and p).

9.2 Materials

9.2.1 Equipment

Major equipment: confocal microscope (optional) or compound microscope equipped with light source capable of delivering excitation wavelengths near UV and blue range, pH meter, and centrifuge with swing-bucket rotor (optional).

9.2.2 Supplies for Tissue Preparation

Laboratory supplies: double-edge stainless steel razor blade (Electron Microscopy Sciences 72000), mortar and pestle, Pasteur pipettes, microcentrifuge tube (1.5 mL), nylon mesh (40 μm; Spectrum Laboratories Inc. 146502), staining dishes, fine-tipped forceps, glass slides and cover slips, depression slides, and number 1 Whatman filter paper.

9.2.3 Chemical Reagents

1. Solvents (acetone, dimethyl sulfoxide (DMSO), methanol, ethanol, or deionized water).
2. Chemicals for buffer preparations: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma H4034); 2-(*N*-morpholino) ethanesulfonic acid (MES; BioShop MES503); 1,4-piperazinediethanesulfonic acid (PIPES; Sigma P6757); dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$; BioShop SPD579); monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$; BioShop SPM400); sodium chloride (NaCl; BioShop SOD002); ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic

acid (EGTA; BioShop EGT202); magnesium sulfate (MgSO4; Fisher 964443); D-mannitol (Fisher 937002); and potassium hydroxide (KOH; BioShop PHY202).
3. Fluorescent dyes: acridine orange (Sigma A6014), carboxy-DCFDA (Invitrogen C369), DAPI (Sigma D9564), $DiOC_6$ (Sigma 318426), FDA (Sigma F7378), Fluorescent brightener 28 (Sigma F3543), Neutral red (Sigma 4638), and R123 (Sigma R8004).

9.2.4 Solution Preparation

1. 0.1 M HEPES buffer, pH 7–8.2: For 100 mL, dissolve 2.38 g of HEPES in 90 mL of deionized water, adjust the pH to the desired value with 1 M KOH and then adjust the volume to 100 mL with deionized water.
2. 0.1 M MES buffer, pH 5.5: For 100 mL, dissolve 2.13 g of MES in 90 mL of deionized water, adjust the pH to 5.5 with 1 M KOH and then add deionized water to 100 mL.
3. 0.1 M PIPES buffer, pH 7–7.5: For 100 mL, dissolve 3.02 g of PIPES in 90 mL of deionized water, add 1 M KOH until the desired pH is obtained and then adjust the volume to 100 mL with deionized water.
4. 0.1 M sodium phosphate buffer, pH 7–7.8: (a) Prepare the 0.1 M solution of $NaH_2PO_4 \cdot H_2O$ (monobasic) by dissolving 1.38 g in 100 mL of deionized water. (b) Prepare the 0.1 M solution of $Na_2HPO_4 \cdot 7H_2O$ (dibasic) by dissolving 2.68 g in 100 mL of deionized water. For 100 mL, the desired pH can be obtained by mixing the monobasic and dibasic solutions as follow:

pH	Monobasic (mL)	Dibasic (mL)
7.0	39	61
7.1	33	67
7.2	28	72
7.3	23	77
7.4	19	81
7.5	16	84
7.6	13	87
7.7	10.5	89.5
7.8	8.5	91.5

5. Stabilization buffer: For 100 mL, dissolve 0.877 g NaCl (final 150 mM), 0.38 g EGTA (final 10 mM), 0.06 g $MgSO_4$ (final 5 mM), 5.47 g D-mannitol (final 300 mM) in 0.1 M HEPES, pH 7. Store solution at 4 °C to reduce potential contamination.
6. Staining solution: The fluorescent dye solutions are prepared as 1000-fold stock solutions; for example, dissolve 100 mg of DAPI in 10 mL deionized water for a

concentration of 10 mg/mL (please refer to Table 9.2 for appropriate concentrations and solvents). To remove precipitate, filter stock solution with Whatman paper (No. 1) and store in the dark, ≤4 °C. To obtain working concentration of dye, mix 1 µL of 1000-fold stock solution with 999 µL buffer (Table 9.2).

9.3 Methods

9.3.1 Fluorescent Staining of B. sinuspersici Chlorenchyma Cells

1. Excise four mature leaves (>2 cm) and gently crush in 2 mL of stabilization buffer (*see Note 1*) using a mortar and pestle.
2. With a Pasteur pipette, transfer the cell homogenate onto a 40-µm nylon mesh filter. Discard broken cells of the flow-through and collect intact cells in 1.5-mL centrifuge tube by washing the mesh with 1 mL stabilization buffer.
3. Add appropriate amount of fluorescent dye to cells (Table 9.2). Gently mix by inverting the tube twice.
4. Incubate cells in the dark for 5–30 min at room temperature.
5. After cells have settled to the bottom of the tube, discard the supernatant and wash with 1 mL of fresh stabilization buffer. Gently mix and allow cells to settle for 10 min (*see Note 2*).
6. Repeat step 5.
7. After washing cells twice in stabilization buffer (*see Note 3*), resuspend cells in 50 µl of stabilization buffer.
8. Using a Pasteur pipette, transfer the stained cells in stabilization buffer to a depression slide (*see Note 4*) and place a glass cover slip over the sample.
9. Image cells using compound or confocal microscope immediately after staining (*see Note 5*).

9.3.2 Fluorescent Staining of Free-Hand Leaf Sections of K. blossfeldiana, K. daigremontiana, or S. aralocaspica

1. Cut freshly excised leaf (4–5 cm long) into 0.1–0.2 mm thick sections using a double-edged razor blade (*see Note 6*).
2. Place leaf sections into staining dish containing the buffer with an appropriate pH (Table 9.2) and incubate for 5 min (*see Note 7*).
3. Move sections to a well containing fluorescent dye diluted in appropriate buffer (Table 9.2). Incubate in the dark for 5–30 min at room temperature.

4. Wash leaf sections twice in fresh buffer without the dye for 10 min each (*see Note 3*).
5. Mount leaf sections on a depression slide, cover samples with fresh buffer, and place a glass cover slip on top.
6. Observe leaf sections using either a compound or confocal microscope, image sections within an hour of preparation (*see Note 8*).

9.4 Notes

1. HEPES can be replaced with PIPES, sodium phosphate, or MES buffer but consult Table 9.2 for the appropriate pH. Some plant species may also prefer sucrose as an osmoticum in place of mannitol.
2. As an alternative to allowing cells to naturally settle to the bottom of the tube, cells may be pelleted at 100 g for 2 min using a swing-bucket rotor. The use of fixed-angle rotor generally results in higher number of damaged cells.
3. Fluorochrome detection can be obscured by high background fluorescence if unbound dye is not sufficiently removed. By replacing staining solution, excess stain is diluted and background fluorescence is greatly reduced. If background is still too high, additional wash steps can be performed or the concentration of dye used can be lowered.
4. When working with non-fixed cells, one need to be careful not to crush cells by applying cover slip on a conventional glass slide. A depression slide is recommended because it provides the minimum space of 0.1 mm needed between the slide and the cover slip.
5. Observation and imaging should be done as soon as possible to ensure fluorescent dye is retained and cell death is minimized. Since fluorescent dyes are prone to photobleaching, minimize time of exposure of stained cells to the excitation wavelength.
6. Ideally, tissue sections should be 2–3 cell layers thick so that the dye has proper access and that the cellular detail can be revealed. For plant tissues that are not strong enough for free-hand sectioning, other support material such as Styrofoam or carrot root can be used. Alternatively, a vibratome may be used if the desired thickness is difficult to obtain using free-hand sectioning. To avoid crushed and damaged cells, leaf sections must be cut with a fresh razor blade.
7. Typically buffer alone is sufficient for staining of leaf sections. If an osmoticum is required, then 0.3 M mannitol or sucrose can be added to buffer.
8. If the uptake of dye is low, infiltration can be improved by: increasing dye concentration, adding 0.01 % (v/v) DMSO or 0.1 % (v/v) Triton X-100 to fluorescent dye incubation buffer and/or vacuum-infiltrate leaf sections with fluorescent dye for 5–15 min. If membrane permeability is required for fluorescent dye uptake, one must include controls with just the DMSO or detergent alone to ensure the signal observed is not an artifact due to cell damage.

9.5 General Comments

This chapter is intended to serve as a general guideline for staining of living plant cells or tissue sections and that there may be some variation with the overall results for other plant species. Thus, in order to obtain reliable and reproducible results, parameters such as osmolarity, buffer pH, dye concentration, incubation time, and wash procedure need to be empirically determined and optimized. For example, the optimum time of staining varies for different plant tissues and should be tested individually. Too long staining time will lead to over-staining and nonspecific staining whereas too short will result in under-staining. Another factor affecting the staining specificity of cellular structures is the pH of the buffer. In particular, pH sensitive dyes such as carboxy-DCFDA which has a low pKa and can be loaded into cells under acidic condition. It is extremely useful for monitoring pH changes in acidic organelles such as vacuoles. When optimizing a fluorescent staining protocol, it is of great importance to include appropriate controls. The preparation and observation of unstained samples alongside that of your stained specimen can be crucial in differentiating true signal from that of background autofluorescence or artifacts.

Acknowledgments This research was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Waterloo Start-Up Fund to SDXC.

References

1. Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51:659–668
2. Fernández S, Osorio S, Heredia A (1999) Monitoring and visualizing plant cuticles by confocal laser scanning microscopy. Plant Physiol 37:789–794
3. Singh J, Devi S (1992) Development of fern sporangia: a fluorescence microscopy study. Biotech Histochem 67:261–264
4. Donaldson LA, Radotic K (2013) Fluorescence lifetime imaging of lignin autofluorescence in normal and compression wood. J Microsc 251:178–187
5. Biggs AR (1985) Detection of impervious tissue in tree bark with selective histochemistry and fluorescence microscopy. Stain Technol 60:299–304
6. Poustka F, Irani NG, Feller A, Lu Y et al (2007) A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-vacuole protein-sorting route in Arabidopsis and contributes to the formation of vacuolar inclusions. Plant Physiol 145:1323–1335
7. Palevitz BA, O'Kane DJ, Kobres RE, Raikhel NV (1981) Vacuole movements and changes in morphology in differentiating cells as revealed by epifluorescence, video and electron microscopy. Protoplasma 109:23–55
8. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T (2008) Quantum dots versus organic dyes as fluorescent labels. Nat Methods 5:763–775
9. Tepfer M, Taylor IE (1981) The permeability of plant cell walls as measured by gel filtration chromatography. Science 213:761–763

10. Baron-Epel O, Charyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta 175:389–395
11. Brière C, Barthou H, Petitprez M (2004) A new tool for plant cell biology: In vivo antibody uptake in plant protoplasts. Plant Cell Rep 12:878–884
12. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805
13. Tsien RY (1998) The green fluorescent protein. Ann Rev Biochem 67:509–544
14. Brandizzi F, Fricker M, Hawes C (2002) A greener world: the revolution in plant bioimaging. Nature 3:520–530
15. Held MA, Boulaflous A, Brandizzi F (2008) Advances in fluorescent protein-based imaging for the analysis of plant endomembranes. Plant Physiol 147:1469–1481
16. Herth W, Schnepf E (1980) The fluorochrome, calcofluor white, binds oriented to structural polysaccharide fibrils. Protoplasma 105:129–133
17. Matzke MA, Matzke AJM (1986) Visualization of mitochondria and nuclei in living plant cells by the use of a potential-sensitive fluorescent dye. Plant Cell Environ 9:73–77
18. Terasaki M, Song J, Wong JR, Weiss MJ, Che LB (1984) Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. Cell 38:101–108
19. Johnson LV, Walsh ML, Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci USA 77:990–994
20. Wu FS (1987) Localization of mitochondria in plant cells by vital staining with rhodamine 123. Planta 171:346–357
21. Traganos F, Darzynkiewicz Z, Sharpless T, Melamed MR (1977) Simultaneous staining of ribonucleic acids in unfixed cells using acridine orange in flow cytofluorometric system. J Histochem Cytochem 25:45–56
22. Kapusicinski J (1995) DAPI: a DNA-specific fluorescent probe. Biotech Histochem 70:220–233
23. Barcellona ML, Cardiel G, Gratton E (1990) Time-resolved fluorescence of DAPI in solution and bound to polydeoxynucleotides. Biochem Biophys Res Commun 170:270–280
24. Timmers ACJ, Tirlapur UK, Schel JHN (1995) Vacuolar accumulation of acridine orange and neutral red in zygotic and somatic embryos of carrot (*Daucus carota* L.). Protoplasma 188:236–244
25. Moore A, Donahue CJ, Bauer KD, Mather JP (1998) Simultaneous measurement of cell cycle and apoptotic cell death. Methods Cell Biol 57:265–278
26. Verbelen JP, Tao W (1998) Mobile arrays of vacuole ripples are common in plant cells. Plant Cell Rep 17:917–920
27. El-Maarouf-Bouteau H, Moreau E, Errakhi R, Sallé G (2008) A diffusible signal from germinating *Orobanche ramose* elicits early defense responses in suspension-cultured *Arabidopsis thaliana*. Plant Signal Behav 3:189–193
28. Lee EK, Jin YW, Park JH, Yoo YM et al (2010) Cultured cambial meristematic cells as a source of plant natural products. Nature Biotech 28:1213–1217
29. Chen S, Halkier BA (2000) Characterization of glucosinolate uptake by leaf protoplasts of *Brassica napus*. J Biol Chem 275:22955–22960
30. Dubrovsky JG, Guttenberger M, Saralegui A, Napsucialy-Mendivil S, Voigt B, Baluska F, Menzel D (2006) Neutral red as a probe for confocal laser scanning microscopy studies of plant roots. Ann Bot 97:1127–1138
31. Gonzalez ME, Jernstedt JA, Slaughter DC, Barrett DM (2010) Microscopic quantification of cell integrity in raw and processed onion parenchyma cells. J Food Sci 75:E402–E408
32. Inselman AL, Gathman AC, Lilly WW (1999) Two fluorescent markers identify the vacuolar system of *Schizophyllum commune*. Curr Microbiol 38:295–299
33. Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining the viability of cultured cells. Stain Technol 47:189–194

Chapter 10
Improved Methods for Clearing and Staining of Plant Samples

Alexander Lux, Marek Vaculík and Ján Kováč

10.1 Introduction

In recent years, the rapid development of high-tech methods, such as various kinds of electron microscopy (EM), confocal laser scanning microscopy (CLSM), variable angle epifluorescence microscopy (VAEM) and many others, have greatly advanced the visualization of plant tissues and cell structures (e.g. [1]). Despite optimization of these techniques, free-hand sectioning of living plant tissues is still an adequate and valuable method for rapid and inexpensive microscopic observation of internal structures (also see Chap. 1).

For many years plant anatomists have tried to improve free-hand sectioning techniques with the aim to obtain better quality images. Towards this end, various clearing techniques of plant samples have been described. Different clearing methods can be used to emphasize different cellular components within cells, including cell organelles and inclusions; or alternatively, to make the tissues more translucent in order to accentuate cell walls and theirs components. Pigments and phenolic compounds, which deteriorate the observation at a microscopic level, need to be removed from the cells. For this purpose, common chemicals and reagents, such as ethanol, methanol or commercially available bleaching agents, usually based on chlorine (e.g. sodium hypochlorite) are used. In the nineteenth century several scientists like Harting, Schleiden, Schacht or Warington introduced various chemicals, for example, calcium chloride and glycerol for mounting and preserving tissue samples. Later it was found that some of these substances can also be used for clearing purposes and can therefore be combined with dyes to stain particular tissue components at the same time. For example, Herr [2] retrieved new uses of calcium chloride for clearing and staining of lignified tissues by simultaneous staining with acid phloroglucinol or toluidine blue O. One of the most common clearing

A. Lux (✉) · M. Vaculík · J. Kováč
Department of Plant Physiology, Faculty of Natural Sciences,
Comenius University in Bratislava, Mlynska dolina B2, Bratislava 84215, Slovak Republic
e-mail: lux@fns.uniba.sk

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_10

solutions was introduced by Herr more than 40 years ago. This so called "4 and ½ clearing fluid" consists of lactic acid, chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1, v:v). Later the 4 and ½ clearing fluid was modified by the addition of benzyl benzoate to BB 4 and ½ clearing fluid [3, 4]. In general clearing methods have been categorized into three groups according to their strength: (1) relatively mild and non-destructive methods using lactic acid or lactophenols, (2) medium-strength methods using lactic acid or lactophenol together with weak potassium hydroxide (1 % KOH) and autoclaving and (3) strong clearing methods using higher strengths of KOH (5 %) with simultaneous autoclaving and tissue bleaching [5]. Similarly, KOH has been shown to be an efficient clearing component that allows better visualization of veins in leaves and also in experiments with pre-staining with safranin in ethanol followed by clearing with warm KOH [6]. However, the use of KOH or inorganic acids seems to be harsh for some fine tissues and cell structures, therefore many other reagents such as hydrogen peroxide, chloral hydrate, lactic acid, phenol and their combinations have been proposed [7–10].

Recently Visikol™ a new non-toxic clearing medium, with high refractive index (1.445), has been introduced, replacing the commonly used chloral hydrate [11]. Clearing can also be combined with fixation procedures as suggested for gymnosperm needle sections using 2 × polyvinyl alcohol-lactic acid-glycerol (2 × PVLG) [12]. A promising clearing reagent for thick sections and whole-mount samples was described by Dubrovsky et al. [13], using sodium iodide. This compound has a high refractive index and it preserves most cellular contents. It is a non-toxic alternative to chloral hydrate; however, it is not suitable for clearing of highly pigmented and dense tissues [13, 14].

The techniques of free-hand sectioning, clearing and staining of plant tissues, especially roots, are routinely used in our laboratories. The original protocol for fluorescent staining of lamellar and non-lamellar suberin in root endodermis and exodermis using berberine hemisulphate or fluorol yellow 088 in polyethylene glycol–glycerol has been described by Brundrett et al. [15, 16]. However, free-hand sections of various plant organs are often blurred by cell inclusions such as starch, resulting in poor quality UV or bright-field images. Moreover, we have successfully modified these protocols by clearing using lactic acid saturated with chloral hydrate as well as establishing some new protocols to study developmental changes involving endodermal and exodermal cells and root vasculature along the root axis [10, 17]. These methods as well as additional clearing and staining tips, including clearing and staining of whole-mount samples on specially modified slides are summarized in this chapter.

10.2 Materials

10.2.1 Hand Sectioning

This method requires simple laboratory supplies, that is, double-edged razor blades, glass Pasteur pipettes, rubber stoppers, aluminium foil, Parafilm, microscope slides, cover glasses, Petri dishes, tissue paper, brushes, oven, a stereomicroscope and a

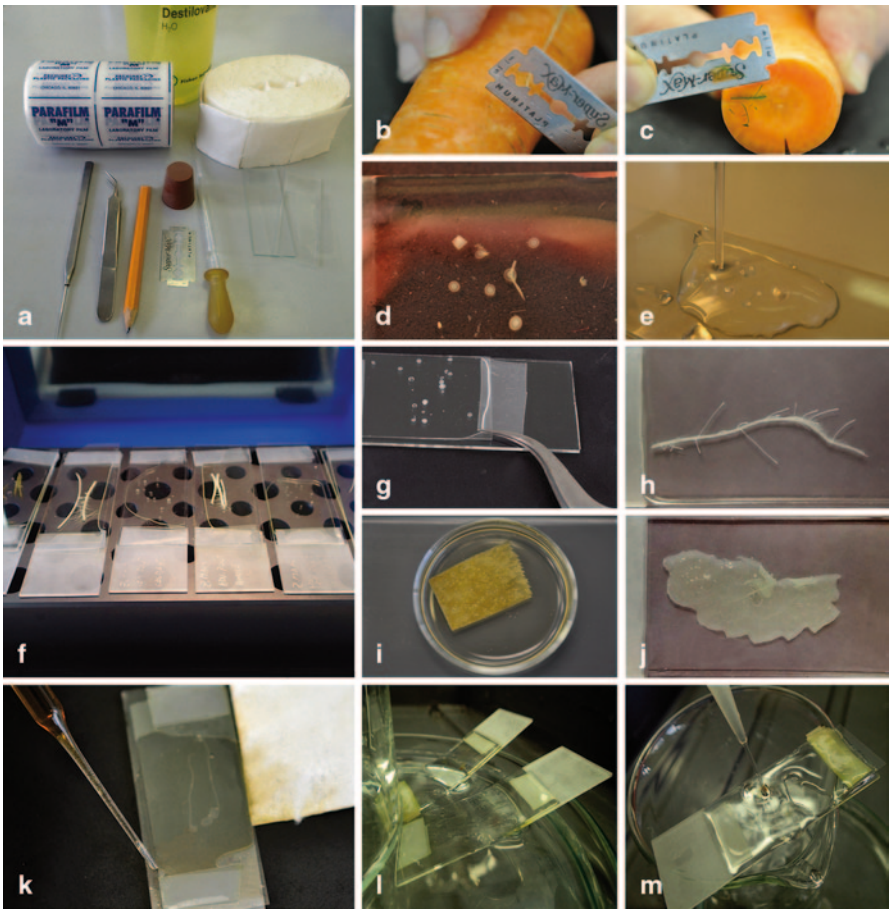

Fig. 10.1 **a** Material required for sample preparation, cutting and clearing. **b** The use of carrot as a support for preparation of free-hand sections of a root. Double-edged razor blade is used for cutting and sections are transferred from the blade to solution on microscope slide. **c** The use of carrot for preparation of sections of a leaf. The leaf blade is inserted into the incision of the carrot and *thin* sections are cut together with the supporting carrot tissue. **d** Free-hand cross sections of a root transferred to the clearing solution on a microscope slide. **e** Sucking and washing the clearing solution from the slide by use of a glass pipette. **f** Microscope slides with clearing solutions and plant samples in microchambers on the hot plate during clearing. **g** Manipulation with covering glass during preparation of a microchamber. **h**, **i**, **j** Whole root (**h**), leaf segment in Petri dish (**i**) and in microchamber (**j**) after clearing. **k** The use of spacers between microscope slide and cover glass and exchange of solutions by adding drops by pipette on one side and absorbing the solution by tissue paper on the other side of the slide. **l**, **m** Exchange of solutions and washing of samples covered by cover glass on microscope slide by inserting half of inclined microscope slide in distilled water (**l**) and subsequently washing the samples and cover glass by stream of distilled water (**m**)

light microscope (Fig. 10.1a). Fresh storage roots such as carrot can be used to "embed" the soft root tissue for hand sectioning (Fig. 10.1b, c).

10.2.2 Chemicals and Stains

1. Chemicals: lactic acid, chloral hydrate *(see Note 1)*, potassium hydroxide, ethanol, methanol, sodium hypochlorite or commercial bleach, glycerol, ferric chloride, hydrochloric acid and agarose.
2. Stains: aniline blue, berberine hemisulphate (Sigma), fluorol yellow 088 (Sigma), phloroglucinol, safranin, toluidine blue O, iodine and potassium iodide *(see Note 2)*.

10.2.3 Solutions

10.2.3.1 Clearing Solutions

1. Lactic acid 85–92 % in water.
2. Lactic acid saturated with chloral hydrate.

10.2.3.2 Clearing and Fluorescence Staining Solutions—"2 in 1"

1. Prepare, at room temperature, a 0.1 % (w/v) berberine hemisulphate in lactic acid *(see Note 3)*.
2. Prepare a 0.01 % (w/v) solution of fluorol yellow 088 in lactic acid by heating at 70 °C for 1 h in the fume hood *(see Note 3)*.

10.2.3.3 Staining Solutions

1. Toluidine blue O (Merck): prepare a 0.1 % (w/v) solution in distilled water.
2. Aniline blue (Sigma-Aldrich): prepare a 0.5 % (w/v) solution in distilled water.
3. Safranin O (Sigma-Aldrich): prepare a 0.5 % (w/v) solution in 50 % ethanol.
4. Prepare a solution of 2 % phloroglucinol in 96 % ethanol.
5. Prepare a 25 % HCl (hydrochloric acid) solution from concentrated HCl.
6. Lugol's solution (used as an indicator for the presence of starches). The solution consists of 5 % (w/v) iodine and 10 % (w/v) potassium iodide mixed in distilled water.

10.2.3.4 Mounting Solution

1. Dissolve 0.1 % (w/v) $FeCl_3$ in 100 mL of 50 % glycerol for stabilization of fluorescent dyes.
2. 50 % glycerol for bright field observations.

10.3 Methods

10.3.1 Preparation of Plant Samples

Grow plants for experiments or use suitable plant materials for demonstration of individual tissues, such as onion roots for endodermis and exodermis, thin *Arabidopsis*, *Brassica juncaea* roots or thin lateral roots of cereals for whole-mount samples, leaf samples, young stems, etc.

10.3.2 Methanol Fixation

If fresh material cannot be processed immediately, for example, due to lack of time and/or presence of numerous samples to investigate, the samples can be stored for several weeks or months in methanol (> 70 %) at 8 °C in a refrigerator. Methanol, in contrast to ethanol, does not cause plasmolysis.

10.3.3 Hand Sectioning

1. Add a drop of distilled water on a microscope slide.
2. Put fresh, well-hydrated plant sample on the surface of wet carrot root (Fig. 10.1b) or inside the carrot incision (Fig. 10.1c) and cut thin sections with smooth stokes and transfer them from the blade immediately into the drop of water on a microscope slide inserting the blade with section to the drop (Fig. 10.1d). Alternatively a rubber stopper can be used as a support for sectioning of firm material. However, in our laboratory, carrot provides better results.
3. Remove excess water from the microscope slide using a tissue paper or a glass Pasteur pipette (Fig. 10.1e); be careful not to leave sections dry *(see Note 4)*.

10.3.4 Hand Sectioning of Very Thin Roots (see [17])

1. Fresh or methanol-fixed material can be stained with toluidine blue O en bloc, otherwise the thin organs are difficult to detect during the following manipulation.
2. Embed the samples in 6 % agarose in a box made of aluminium foil, shaped according to the size of the specimen. Prepare thin hand sections of agarose box with embedded material. Sections are transferred by inserting the blade with section to the drop of water or using stainless needle.
3. Clear and stain with fluorescent dyes, as in Subheading 10.3.6 *(see Note 3)*.

10.3.5 Clearing

1. Add several drops of clearing solution to the sections on microscope slide *(see Note 5)*. Alternatively use the whole thin roots for whole-mount clearing.
2. Place the microscope slide with sections into covered Petri dish and place them in an incubator oven at 60 °C for 1 h.
3. Wash the sections by adding drops of distilled water directly to the clearing solution with sections on a microscope slide and carefully remove the mixed solution by a glass Pasteur pipette; repeat washing several times until the clearing solution is washed out (Fig. 10.1f).

10.3.6 Clearing and Fluorescence Staining of Sections in One Step Using "2 in 1" Solution

1. Add several drops of clearing and fluorescence staining solution ("2 in 1") to the sections prepared as in Subheading 3.3 on microscope slide. Adequate solution might be selected according to the structure or cell components that should be visualized (*see Note 2*).
2. Place the microscope slide with sections into a covered Petri dish and place them in an incubator at 70 °C for 1 h in the dark.
3. Wash the sections by adding drops of distilled water and carefully removing the mixed solution by glass Pasteur pipette; repeat washing several times until the clearing and staining solution is removed (Fig. 10.1e).

10.3.7 Clearing and Post-Staining of Sections

1. Clear and wash the sections as in Subheading 10.3.5
2. Add drops of staining solutions to the sections on a microscope slide and stain; the time of staining depends on the stain used and specimens should be judged individually after observation of sections in a microscope *(see Note 6)*.
3. Wash the sections directly on the slide and carefully remove the mixed solution using a glass Pasteur pipette; repeat washing several times until the staining solution is removed (Fig. 10.1e). Differences between cleared and non-cleared cross sections of different plant objects can be seen in Figs. 10.2, 10.3.

10.3.8 Clearing and Post-Staining of Whole Objects

This method is similar to step 10.3.7 and it is suitable for clearing and post-staining of whole plant organs or their parts like leaves, seedlings or fine roots, without cutting. In case of leaves or green plant parts, incubate samples before clearing

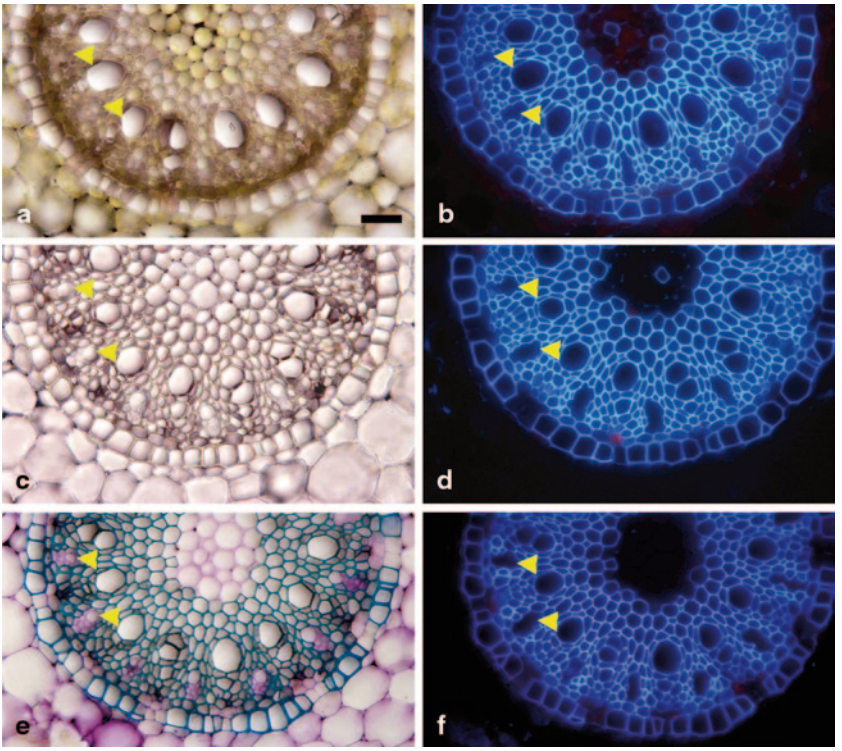

Fig. 10.2 Free-hand cross sections of roots (aerial roots of orchid *Oncidium* sp.) with and without clearing viewed with bright-field or fluorescence microscopy. **a**, **c**, **e** Samples in white light and **b**, **d**, **f** under UV light , without clearing (**a**, **b**), after clearing in lactic acid saturated with chloral hydrate (**c, d**), and with clearing as in (**c**) and (**d**), but after staining with toluidine blue O (**e** and **f**). Note that phloem poles (indicated with *yellow arrowheads*) are visible under bright field after clearing and staining (**c** and e) and undistinguishable in fresh sections (**a**). Scale bar = 50 µm. Reproduced with permission from [10]

in methanol, ethanol or a bleaching solution that dissolves pigments *(see Note 7)* (Fig. 10.1f–m).

1. Add sample to small glass container in a sufficient volume of clearing solution (Fig. 10.1i).
2. Incubate glass container in gradually increasing temperature up to 60 °C and stain at these conditions for at least 60 min. After incubation slowly cool down the samples to laboratory temperature.
3. Transfer samples from container to a microscope slide (Fig. 10.1j). Add post-staining solution and stain; the time of staining depends on the stain used and the specimen should be judged individually after observation in a microscope.
4. Wash the samples by adding drops of distilled water directly to the staining solution with whole objects on a microscope slide and carefully remove the mixed solution by glass Pasteur pipette; repeat washing several times until the staining solution is washed out and cover with cover glass, if necessary use spacers (Fig. 10.1g).

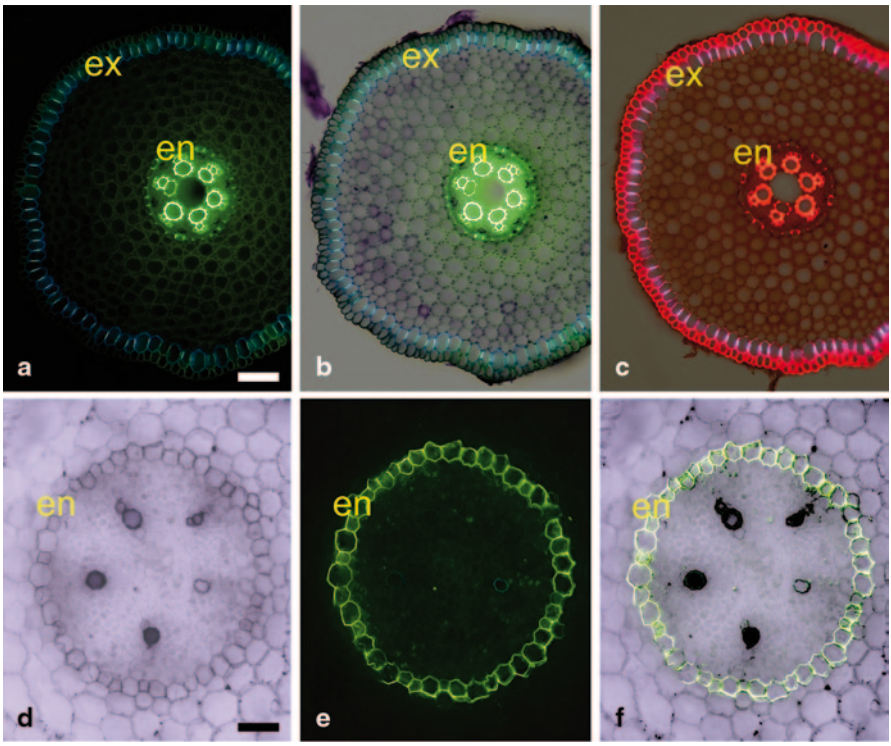

Fig. 10.3 Free-hand cross sections of roots with and without clearing, stained with berberine hemisulphate or fluorol yellow 088. **a**–**c** roots of onion; **d**–**f** roots of melon. **a**, **b** The sections cleared in lactic acid with berberine, post-stained in an aqueous solution of berberine and with toluidine blue, in UV light (**a**) and overlapped micrographs in UV light and in white light (**b**). **c** The section cleared and stained with lactic acid containing berberine and post-stained with safranin, overlapped picture of photos in white light and UV light. **d**–**f** The same section cleared and stained with lactic acid containing fluorol yellow 088, in white light (**d**), in UV light (**e**) and the overlapped image of two previous photos (**f**). Note the contrast of lamellar suberin stained with fluorol yellow 088 and the regular, non-collapsed endodermal cells. *Ex* exodermis, *en* endodermis. Scale bars: A = 100 µm (for **a**–**c**); D = 50 µm (for **d**–**i**). Reproduced with permission from [10]

10.3.9 Clearing and Post-Staining of Fine or Delicate Objects in Glass Microchambers

1. Add the sample to a drop of clearing solution on a microscope slide and cover with cover glass (Fig. 10.1h). Use spacers and prepare the microchamber (Fig. 10.1g) *(see Notes 8, 9)*.
2. Incubate the microscope slide with samples in gradually increasing temperature up to 60 °C and stain at these conditions for at least 60 min (Fig. 10.1f).
3. After incubation slowly cool down the samples to room temperature. Do not remove the cover glass from the microscope slide.

4. Immerse half of the microscope slide with the samples into distilled water for 5–10 min (Fig. 10.1l).
5. Gently wash both cover glass and slide with drops of distilled water to remove the remaining clearing and/or clearing and staining solution from samples and cover glass (Fig. 10.1m).
6. Add the post-staining solution (e.g. phloroglucinol and HCl) between the microscope slide and the cover glass using a pipette. Apply the staining solution to one side of the microchamber and use tissue paper to draw the staining solution through the specimen to the other side of the microchamber (Fig. 10.1k). This allows the specimen to be stained without removing it from the slide *(see Note 10)*.
7. Wash staining solution if necessary (step 4). Differences between cleared and non-cleared whole-mount objects can be seen on Fig. 10.4.

10.3.10 Mounting

1. Put a few drops of 50% glycerol to the sections after clearing and washing or alternatively put solution of $FeCl_3$ dissolved in 50% glycerol to the cleared and stained sections for observation in UV light.
2. Cover sections in mounting solution by a cover glass.

10.4 Notes

1. Disadvantage of chloral hydrate is toxicity and because of its status as a regulated substance, chloral hydrate can be difficult to obtain. In many cases the use of lactic acid alone, applied on samples for prolonged time and at elevated temperature (70 °C), can be sufficient to obtain good results. Elevated temperature during clearing is especially important for the whole-mount samples. The experiments with heated lactic acid should be performed in fume hood or in well aerated space.
2. These dyes can be used for different staining purposes. Aniline blue is mostly used for visualization of callose [15], berberine hemisulphate for components of Casparian bands [15], fluorol yellow 088 for suberin [16], phloroglucinol for lignins [e.g. 14], and safranin and toluidine blue O for cell wall components [1].
3. Stability of berberine hemisulphate and fluorol yellow 088 solutions after dissolving in lactic acid is limited; it is recommended to prepare fresh solutions before application.
4. We prefer clearing, staining and washing of sections directly on glass slides. The use of small baskets for handling of sections, as recommended by Brundrett

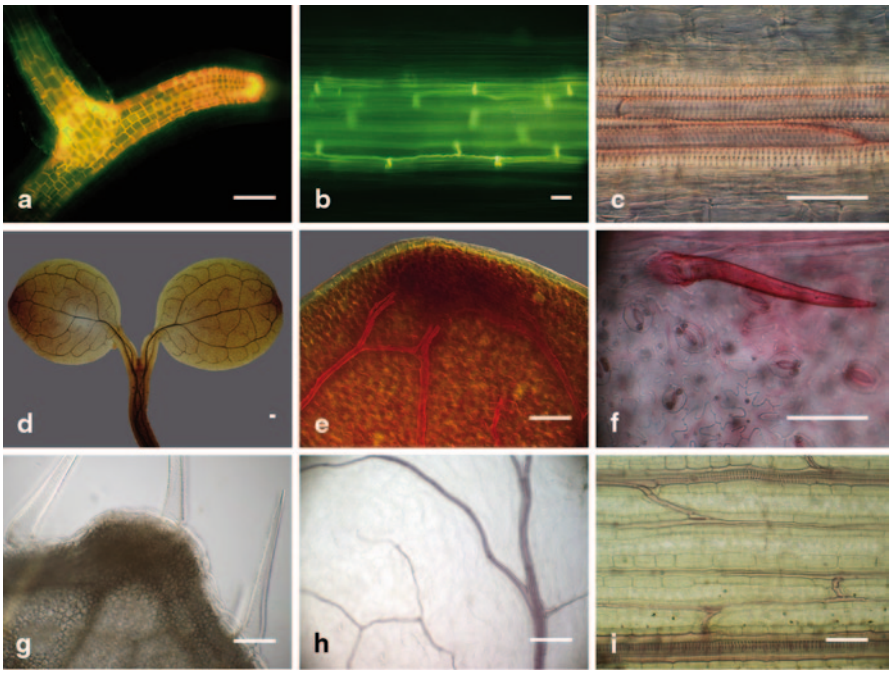

Fig. 10.4 **a** Whole-mount root of *Noccaea caerulescens*, cleared and stained in lactic acid with fluorol yellow for visualization of suberin lamellae developed in endodermal cells. **b** Whole mount of *Noccaea caerulescens* hypocotyl, cleared and stained as in (**a**) for visualization of suberin lamellae in endodermal cells (different colors of suberin lamellae in comparison with (**a**) are caused by different microscope settings. **c** Detail of the xylem vessels in root of *Brassica juncaea*. Samples were fixed in methanol, cleared in lactic acid and stained with phloroglucinol and HCl for visualization of lignin. **d** Cotyledons of *Noccaea caerulescens* seedling cleared in lactic acid and stained with Lugol. **e** Detail of apical part of cotyledon of *Noccaea caerulescens* cleared and stained as in (**d**). **f** Leaf epidermal cells of *Sambucus nigra* bleached and cleared in sodium hypochlorite and stained with Sudan III. Note staining of trichome and ventral cell walls of stomata. **g** Leaf apical part of *Brassica juncaea* without clearing. Fixed in methanol, stained with phloroglucinol and HCl. Note obscure tracheary elements. **h** Tracheary elements in leaf of *Brassica juncaea.* Fixed in methanol, cleared in lactic acid, stained with phloroglucinol and HCl. Compare with uncleared sample (**g**). **i** Veins in leaf of *Zea mays*. Fixed in methanol, cleared with lactic acid, and stained with phloroglucinol and HCl. Scale bars = 50 µm.

et al. [15], is practical for exchange of solutes and washing; however, for soft cleared samples it often results in their damage and/or lost during handling. In case of direct treatment of sections on glass slides checking of samples during the processing is possible with a stero- or light microscope. For exchange of solutes we prefer the use of glass Pasteur pipettes as the samples can be seen readily. Removing excess solution with tissue paper is more difficult as samples are often absorbed along with the solution.

5. For clearing solutions, *see* Sect. 10.2.3.1

6. For example, for visualization of lamellar suberin by fluorol yellow 088, stain the sections at least 30 min at laboratory temperature and whole-mount samples at least 60 min at 60 °C in the dark; for visualization of lignins by phloroglucionol, stain the sections for 2 min and whole-mount samples at least 5 min at laboratory temperature.
7. For example, chlorophylls and other pigments can be effectively removed by various bleaching reagents. In general young and soft leaf tissues can be bleached for several hours (e.g. young cotyledons of Arabidopsis can be bleached by ethanol or acetone for 24 h at laboratory temperature), bleaching of older mature tissues often requires longer time or more complicated processing [5].
8. For whole-mount samples of thin roots, staining of suberin in endodermal layer is clearly visible after application of clearing and staining procedure. Thicker roots are not suitable for this procedure.
9. Samples after clearing are soft and can be deformed and damaged when covered by cover glass. To prevent this it is possible to use spacers located at the edges of cover glasses (made, e.g. from small cuts of Parafilm).
10. For phloroglucinol- HCl staining, remove the water around the samples on the microscope slide using tissue paper or a glass Pasteur pipette. Leave a small amount of water around the samples. Add phloroglucinol until the whole space between spacers is filled. Wait 1–2 min. Remove phloroglucinol and add HCl [18].

10.5 Interpretation and Conclusion

Free-hand sections require patience, practice and experience, even though the advantage is observation of samples without artificial changes caused by fixation, dehydration and embedded material. If the number of samples is high and does not allow us to process them immediately (e.g. fresh material cannot be cut, stained and observed immediately after finishing an experiment), fixation and storage in methanol is an alternative solution. For hand sections, always use new double-edged razor blades and transfer the sections to a drop of water on microscope slides immediately. Clearing and staining of root sections with fluorescent dyes such as berberine hemisulphate or fluorol yellow 088 dissolved in lactic acid gives usually better results than previously described histochemical analysis with stains dissolved in polyethylene glycol–glycerol. Cells remain intact, do not collapse and the contrast is better retained. In conclusion, it can be stated that cleared sections offer better results and better micrographs of organ and tissue structure and provide good quality image even for students. Clearing of whole-mount samples, as thin roots or leaves, allows unique direct observation of inner structures without cutting. Examples are suberized endodermal walls along the root axis (Fig. 10.2) or lignified xylem elements inside roots or leaf blades (Figs. 10.3, 10.4).

Acknowledgments The work was supported by Slovak Research and Development Agency under the contract Nr. APVV-0140-10, APVV SK-CN-0016-12, APVV SK-FR-2013-0029, VEGA 1/0817/12 and was a part of COST FA 0905 Action.

References

1. Žárský V, Cvrčková F (eds) (2014) Plant cell morphogenesis, vol 1080. Springer, New York (Methods Mol Biol)
2. Herr JM Jr (1993) Clearing techniques for the study of vascular plant tissues in whole structures and thick sections. In: Golman CA, Hauta PL, O`Donnell MA, Andrews SE, van der Heiden R (eds) Tested studies for laboratory teaching, vol 5. Proceedings of the 5th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), pp 63–84
3. Herr JM Jr (1971) A new clearing-squash technique for the investigation of vascular plants morphology. Am J Bot 58:785–790
4. Herr JM Jr (1974) A clearing squash technique for the study of the ovule and megagametophyte development in angiosperms. In: Radford AE, Dickinson C, Massey JR, Bell CR (eds) Vascular plant systematics. Harper and Row, New York, pp 230–235
5. O`Brien TP (1974) Autoclaving as an aid in the clearing of plant specimens. Stain Tech 49:175–176
6. Kurth E (1978) A modified method for clearing leaves. Stain Tech 53:291–293
7. Stebbins GL Jr (1938) A bleaching and clearing method for plant tissues. Science 87:21–22
8. Peterson CA, Fletcher RA (1973) Lactic acid clearing and fluorescent staining for demonstration of sieve tubes in minor veins of leaves. Biotech Histochem 48:23–27
9. Beeckman T, Engler G (1994) An easy technique for the clearing of histochemically stained plant tissue. Plant Mol Biol Rep 12:37–42
10. Lux A, Morita S, Abe J, Ito K (2005) An improved method for clearing and staining free-hand sections and whole-mount samples. Ann Bot 96:989–996
11. Villani TS, Koroch AR, Simon JE (2013) An improved clearing and mounting solution to replace chloral hydrate in microscopic applications. Applic Plant Sci 1:1300016
12. Eo J-K (2012) A simple technique for cross-sectioning Gymnosperm needle leaves using microtome. Turk J Bot 36:213–216
13. Dubrovsky JG, Soukup A, Napsucialy-Mendivil S, Jeknić Z, Ivanchenko MG (2009) The lateral root initiation index: an integrative measure of primordium formation. Ann Bot 103:807–817
14. Soukup A, Tylová E (2014) Essential methods of plant sample preparation for light microscopy. In: Žárský V, Cvrčková F (eds) Plant cell morphogenesis. Methods in molecular biology, vol 1080. Springer, New York, pp 1–23
15. Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. Protoplasma 146:133–142
16. Brundrett MC, Kendrick B, Peterson CA (1991) Efficient lipid staining in plant material with Sudan red 7B or Fluorol yellow 088 in polyethylene glycol-glycerol. Biotech Histochem 66:111–116
17. Zelko I, Lux A, Sterckeman T, Martinka M, Kollárová K, Lišková D (2012) An easy method for cutting and fluorescent staining of thin roots. Ann Bot 110:475–478
18. Kisser J, Steininger A (1950) Versuche zur Haltbarmachung der Phloroglucin–Salzsäure–Reaktion. Mikroskopie 5:39–41

Part II
Microtechniques Related to Cell and Molecular Biology Studies

Chapter 11
Whole-Mount Immunofluorescence Staining of Plant Cells and Tissues

Masaki Shimamura

11.1 Introduction

Fluorescence microscopy allows sensitive detection of fluorescent substances within cells and tissues. Through the use of appropriate fluorescent stains, it is possible to identify and visualize the distribution of organelles and macromolecules at the cellular level. Many fluorescent stains and protein expression tags are available to detect various organelles and inclusions [1–5, also *see* Chap. 9]. Certain fluorescent compounds such as fluorescein isothiocyanate can covalently link to antibodies allowing for the detection of antigens within cells.

Many protocols are currently available in the literature detailing a variety of fluorescent staining methods. In this chapter, a whole-mount immunofluorescent staining method of cells and tissues is detailed. Immunostaining using fluorescence-labeled antibodies remains an important method in cell biology due to its sensitivity in antigen detection. Cytoskeletal elements, that is microtubules and actin filaments, are readily detected using the immunostaining procedures [6, 7]. Traditionally, immunostaining for plant cells and tissues is most commonly applied to paraffin, polyester wax, or plastic resin sections. The results provide two-dimensional information on the distribution of target molecules at the cellular level. In order to prepare for immunostaining, the tissues need to be embedded and sectioned. These processes are time-consuming and require a dedicated microtome and related equipment. As an alternative, direct preparations of plant cells or tissues for immunofluorescent staining without embedding and serial sectioning have been developed [6, 7]. The whole-mount preparation in combination with observation using a confocal laser microscope is a powerful method that significantly shortens the time needed for specimen preparation and allows the three-dimensional viewing of antigens at

M. Shimamura (✉)
Department of Biological Science, Graduate School of Science,
Hiroshima University, Higashi-Hiroshima, Japan
e-mail: mshima@hiroshima-u.ac.jp

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_11

the cellular level [8]. This technique greatly augments the conventional immunostaining procedures.

Compared to other organisms, there are challenges in applying immunofluorescence technique on whole-mount preparations of plant cells or tissues [9]. For example, air-filled intercellular space, cell walls, and vacuoles can interfere with fixation and processing of tissues (*see* Chap. 2). The cell wall also acts as a critical barrier to antibodies. The autofluorescent compounds in the cell wall and cytoplasm can interfere with the detection of antigens by fluorescent antibodies and probes. In this protocol, subsequent to fixation, the cell wall which prevents the access of antibody is partially digested enzymatically, and the autofluorescence which prevents the detection of the fluorescent labels is removed by chemical detergent and cold methanol treatments. Unlike spherical protoplasts made from living tissues, the shape of each cell and the distribution of cellular contents are retained. Our protocol is applicable to sperm cells, spores, pollen, small ovules, suspension culture cells [10–13], as well as whole mount of vegetative tissues and embryos [13–15] in diverse plant species (Fig. 11.1). Immunofluorescence staining of cytoskeletal proteins presented here is also applicable to other target proteins for which antibodies are available.

11.2 Technical Comments

Although the basic principle for the whole-mount immunofluorescence staining is relatively simple, there are many challenges involved. The following sections draw attention to key processing steps of the protocol. Common technical issues and their solution(s) are listed in Table 11.1.

11.2.1 Preparation and Handling of Plant Materials

Plant cells and tissues can be processed readily without the need of dissection if the size is small, for example, pollen, ovules, protonema, leaves of bryophytes, etc. For large parenchymatous plant tissues, proper dissection of tissue into appropriate size is necessary prior to fixation. One of the key factors in determining the appropriate size of a specimen used for a whole-mount preparation is the time required for enzyme digestion after fixation. If the size is too large, a prolonged digestion can yield a mixed population of cells with different degrees of wall digestion and cell separation. Hence, the final tissue size needs to be determined by trial and error in order to obtain optimal results in both fixation and maximum cell separation, and yet maintaining the appropriate shape of individual cells. During the course of fixation, it is advisable to extract air from the specimen to ensure proper infiltration of fixative and digestive enzymes. This can be achieved using a water aspirator or a vacuum system (*see* Chaps. 2 and 3).

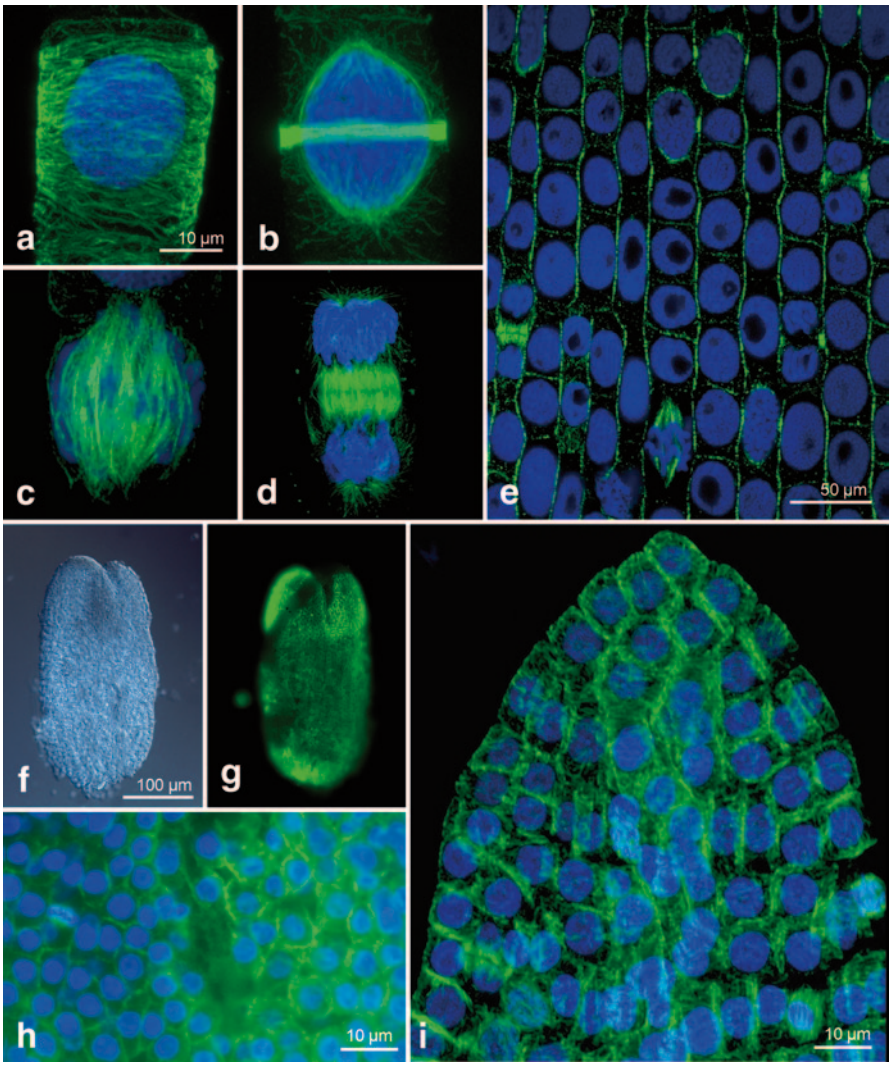

Fig. 11.1 Whole-mount immunofluorescent staining of microtubules (*green*) and nuclei (*blue*) in various plant cells and tissues. **a–d** Whole mounts of isolated cells of onion root tip imaged by CLSM. **a** Cortical microtubules in interphase. **b** Preprophase band of microtubules and prophase spindle. **c** Spindle in metaphase. **d** Phragmoplast in telophase. **e** A whole-mount onion root tip imaged by CLSM (a single optical section). **f–h** Whole mount of an embryo of *Antirrhinum majus* (garden snapdragon) imaged using a conventional fluorescence microscope. **f** The overall image of an embryo in differential interference contrast view. **g** Staining of microtubule and nuclei. **h** An enlarged view of a part of the same preparation as in **g**. **i** A whole-mount image of *Physcomitrella patens* leaf

Table 11.1 Troubleshooting

Problem	Cause	Remedy
No or low signal	Antibody concentration is too low or incubation time of immunostaining is too short	Increase the concentration of primary and/or secondary antibodies
		Increase antibody incubation time, e.g., overnight at 4 °C
	Modifying the epitope where primary antibody recognizes	Reduce the duration of fixation
		Omit glutaraldehyde, methanol, and ethanol treatments
		Try other primary antibody
	The target protein is not present in the tissue	Run positive control (e.g., Western blotting)
	Loss of activity of antibody due to improper storage	Store antibodies properly and avoid repeated freeze and thaw cycles
	Inadequate cell wall digestion and/or permeabilization	Increase duration of cell wall digestion and/or permeabilization
	Antibody concentration is too high or incubation time of immunostaining is too long	Reduce the concentration of primary and/or secondary antibodies Reduce antibody incubation time
	Samples dried out during immunostaining	Avoid complete drying of tissues
Nonspecific staining	Inadequate cell wall digestion and/or permeabilization	Increase duration of cell wall digestion and/or permeabilization
Remaining autofluorescence derived from chlorophyll or lipids in cytoplasm		Try cold methanol treatment before antibody staining
		Try 100 % ethanol treatment after immunostaining
Deformation of cells and tissue	Inadequate fixation	Increase duration of fixation
	Mechanical pressure between glass slide and coverslip	Attaching the samples to coverslips and covering by an A–G film
		Add a spacer between glass slides and coverslips

11.2.2 Buffer and Fixation

For immunostaining, paraformaldehyde (PFA) is commonly used as a fixing agent [16, 17; also *see* Chap. 2]. Although glutaraldehyde is considered to be a better fixing agent than PFA for histological studies, it is seldom used as a fixing agent for immunohistochemical staining of protein antigens (*see* Chaps. 2 and 14). This is primarily due to the fact that it is a more efficient cross-linker than PFA [18]. Excessive cross-linking alters protein configuration, thus preventing the recognition

between the antibody and the antigen. Furthermore, glutaraldehyde can generate autofluorescence and interfere with the observation [19].

Different buffers such as phosphate-buffered saline (PBS) can be used in preparing fixatives. For the fixation of microtubules, various microtubule-stabilization buffers are available [9, 20]. Such buffers might also be suitable for other antigens, as microtubules associate with various subcellular structures.

11.2.3 Enzymatic Digestion of Cell Wall and Chemical Permeabilization of Plasma Membrane

Plant cells are surrounded by cell walls which need to be removed at least partially before incubation in antibodies. In vegetative tissue of land plants, the major cell wall polymers are cellulose, hemicellulose, and pectin. These components can be digested with an enzyme mixture consisting of cellulase, hemicellulase, and pectinase. The reaction time, the amount of each enzyme, and the osmotic condition should be optimized according to the plant material used (*see Note 1*). Instead of preparing one's own mixture of enzymes mentioned above, commercial enzyme mixtures are also available (*see Note 2*). It is important to note that some enzymes show significant batch-to-batch variations with respect to their digestive activity. Therefore, adjustment of incubation time is recommended for each batch of enzymes used [14]. Ethylene glycol tetraacetic acid (EGTA) in the microtubule-stabilization buffer may enhance wall disruption by the chelation of calcium ions.

To estimate the processing time of wall digestion, one needs to check the progress of the treatment. For monitoring enzyme treatment of free cells, such as pollen, sperms, and suspension cell cultures, one needs to examine the materials at regular intervals using a phase contrast or a differential interference contrast microscope with long working distance objectives [9]. Digestion is considered complete when the thickness of cell wall has been reduced and difficult to discern. In this process, there is no need for full digestion of the cell wall.

For tissues, a small amount of sample should be removed every 5–10 min during processing and examined with a microscope. Place a coverslip on the sample and squash it gently. Wall digestion is completed when the majority of cells are dispersed readily as single cells or short files of cells (Fig. 11.2). If most of the cells are easily crushed and take on a round appearance, cell wall digestion is excessive. The time of digestion needs to be reduced.

Certain antigens may be sensitive to proteases derived from broken plant cells and tissues as well as contaminants from the commercial wall-digesting enzymes. Specific protease inhibitors (e.g., phenylmethanesulfonylfluoride and leupeptin) [9, 21] or commercially available protease inhibitor cocktails can be added to the enzyme solution.

To facilitate the entry of antibodies, enzymatic removal of the cell wall is followed by permeabilization of the plasma membrane using chemical detergents and/or alcohols. The autofluorescence derived from chlorophyll and lipidic polymers

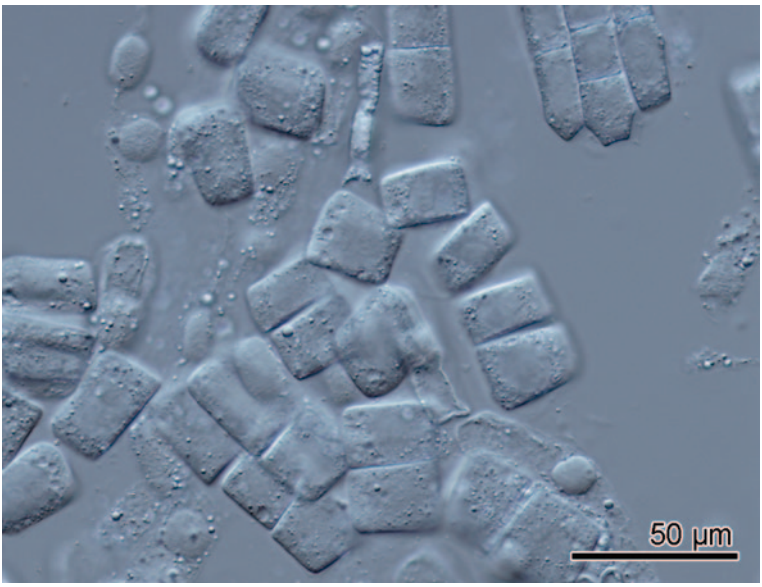

Fig. 11.2 Isolated cells from a root tip by partial enzymatic removal of cell walls. Each cell still maintains its intact shape

is also reduced through this process. Nonionic chemical detergents, such as Triton X-100 and IGEPAL CA-630 (formerly known as Nonidet P-40), are commonly used for permeabilization of the plasma membrane. The optimal concentration and reaction time for permeabilization also vary depending on the experimental material. The delicate meristematic cells such as those of the root apical meristem may be easily permeabilized by a cold methanol treatment for 10 min without the need of chemical permeabilization [22]. On the other hand, reproductive cells and whole-mount vegetative tissues usually tolerate chemical permeabilization well. They often require 30 min or more treatment time with high concentrations of detergent (1% or more) [14, 15, 23]. Persistent autofluorescence derived from chloroplasts and lipidic polymers might be eliminated by the combination of chemical permeabilization and methanol or ethanol treatment [10, 24].

11.2.4 Attaching Cells and Tissues to Glass Slide or Coverslip

The glass slide serves as a carrier of samples through subsequent steps in the immunostaining procedure. The cells and tissues need to adhere to the coated surface of the glass slides. Many types of coated glass slides are commercially available. Although poly-L-lysine (PLL) and aminosilane (APS)-coated slides are commonly used, we prefer Matsunami adhesive silane (MAS)-coated glass slides (Matsunami Glass Inc, Japan). In these slides, chemically stable amino groups positively charge

the glass surface, resulting in improved adhesion of cells compared to PLL and APS coating.

The other alternative is to prepare Mayer's egg albumen-coated slides [25] (*see* Sect. 11.4.7.1 for more detail). Although it has the disadvantage of being stained by many histological stains, egg albumin exhibits minimal background in immunofluorescent staining [9].

To obtain a higher resolution, coverslips can be used in place of glass slides as a sample carrier. The advantage of using coverslips over glass slides is that the samples are situated closer to the objective lens [9]. Adhered samples can be covered with a thin agarose-gelatin film [26], preventing the loss and distortion of the samples during processing (*see* Sect. 11.4.7.2 for more detail).

11.2.5 Antibodies and Indirect Immunofluorescence Technique

There are two main methods of immunofluorescent staining, direct and indirect immunofluorescence [18]. In direct immunofluorescence, fluorochrome is directly conjugated to the primary antibody against the target protein. On the other hand, the procedure of indirect immunostaining comprises two successive immune reactions. The antigen is first detected by the primary antibodies and then the primary antibody is recognized by secondary antibodies conjugated to a fluorochrome [27]. Indirect immunofluorescence has greater sensitivity than direct immunofluorescence, because more than one secondary antibody can attach to each primary antibody. Two or more primary antibodies can be applied simultaneously or successively to detect the localization of multiple antigens. When choosing secondary antibodies, consideration must be given to their cross-reactivity. Secondary antibodies conjugated with various fluorochromes having different fluorescent characteristics are commercially available. At the outset, using green fluorescence excited by blue light is an optimum choice to avoid excessive autofluorescence.

One of the most important considerations in immunofluorescence techniques is the specificity and affinity of primary antibody against an antigen [9]. The simple way to check the specificity of antibody is by immunoblot (Western blot). Negative controls should also be added to increase confidence in the antibody specificity. Commonly used negative controls include: (i) the use of pre-immune serum instead of primary antibodies, (ii) pre-absorption of primary antibodies with the antigen, (iii) omitting the first antibody, and (iv) the use of mutants which lack the objective antigen, if available.

11.2.6 Microscopy

Fluorescent images are usually obtained using a conventional fluorescence microscope with appropriate excitation/emission filter sets or a confocal laser scanning microscope (CLSM). The image quality is greatly improved by using CLSM. The key feature of CLSM is its ability to acquire in-focus images from selected depths

(optical sections). The signals collected can be computer reconstructed using an appropriate software [28] to generate the three-dimensional distribution of the organelle or antigen of interest.

11.3 Materials

11.3.1 Equipment and Supplies

Fluorescent microscope or CLSM equipped for UV and blue excitation, light microscope, stereomicroscope, magnetic stirrer with hot plate, incubator (37 °C), refrigerator and freezer, reciprocal shaker, MAS-coated glass slides (Matsunami Glass Inc., Japan) or other adhesive-coated slides, coverslips (18 × 18 or 24 × 24 mm), fine-tipped tweezers, razor blades (breakable double-edged razor blades made from carbon steel are recommended), glass vials (screw capped, 10–20 mL), Coplin staining Jars, humid chamber (plastic box containing a wet paper towel), filter paper (cut into small pieces), and nail polish.

11.3.2 Chemicals

Cellulase Onozuka RS (Cat. #16420, Yakult Honsha Co.), pectolyase Y-23 (Cat. #63–0501 Kyowa Chemical Products), protease inhibitor cocktail (Cat. #11697498001, Roche) or equivalents including serine and cysteine protease inhibitors, monoclonal anti-α-tubulin produced in mouse (Cat. #T5168 Sigma-Aldrich), Alexa Fluor 488 Goat anti-mouse IgG antibody (Cat. #A-11001 or #A-11029, Invitrogen), 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology), dimethyl sulfoxide (DMSO), Triton X-100, IGEPAL CA-630 (formerly known as Nonidet P-40, Sigma-Aldrich), bovine serum albumin (BSA) IgG and protease free, gelatin (biochemistry grade), PFA, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), EGTA, tris(hydroxymethyl)aminomethane (Tris), *p*-phenylenediamine, glycerol, magnesium sulphate ($MgSO_4 \cdot 7\ H_2O$), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), methanol, and sodium azide (*see Note 3*).

11.3.3 Preparation of Solutions

1. Microtubule stabilizing buffer (PME buffer)**:** prepare a buffer solution with twice the concentration of the desired final concentration. The 2X PME buffer contains the following components: 100 mM PIPES, 10 mM EGTA ,and 2 mM $MgSO_4 \cdot 7\ H_2O$ (adjust to pH 6.8 using NaOH). PME buffer can be stored at 4 °C.

2. PFA 8% stock solution: add 8 g of PFA to 45 mL of pre-warmed (60–70 °C) distilled water and stir the mixture slowly on a hot plate using a magnetic stir bar (maintain the temperature at 60–70 °C). Add a few drops (about 50–100 μL) of 1 N NaOH and stir continuously until all PFA is dissolved. After the solution cools, add 2 mL DMSO and dilute to 100 mL with distilled water. Check the pH with pH test paper, adjust the pH to 7 using 1 N NaOH. The PFA solution should be prepared inside a fumehood just before use. To prepare a 4% PFA fixative, add an equal volume of 2X PME to the 8% PFA stock solution.
3. PBS buffer: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 (adjust to pH 7.4 using KOH). PBS containing sodium azide (0.2 g/L) can be stored for several months at 4 °C.
4. Blocking solution: for preparing a 10% BSA stock solution, dissolve 1 g of BSA in 10 mL of distilled water; store frozen at −20 °C.
5. Detergent solutions: for preparing 10% (w/v) Triton X-100 stock solution, dissolve 10 g of Triton X-100 in 80 mL of distilled water by stirring. Triton X-100 dissolves quickly by warming the mixture. Add distilled water to make 100 mL and gently stir until well mixed. A 10% IGEPAL CA-630 stock solution is prepared using the same procedure. The stock solutions can be stored at 4 °C.
6. Enzyme stock solution for cell wall digestion: dissolve 2% (w/v) Cellulase Onozuka RS, 0.5% (w/v) Pectolyase Y-23 in 2x PME buffer by stirring. Some residues in the enzyme mixture solution need to be removed by centrifugation or filtration by syringe filters (0.4 μm). The stock solution of enzyme mixture is dispensed in 5 mL portions and stored at −20 °C.
7. To prepare the wall-digesting enzyme solution, mix 5 mL of the enzyme stock solution, 1 mL of 10% BSA stock, 0.1 mL of 10% IGEPAL CA-630 stock, 0.5 mL of protease inhibitor cocktail stock, and 0.1 g glucose. Adjust the final volume to 10 mL by adding distilled water. The solution should be prepared fresh just before use.
8. Membrane permeabilization buffer contains 0.1% Triton X-100 and 1% BSA in PME buffer.
9. Primary antibody: the primary antibody is diluted in a PBS buffer containing 1% BSA (*see Note 4*). In this protocol, for immunostaining of microtubules, monoclonal anti-α-tubulin antibody produced in mouse is used at 1:1000 dilution.
10. Fluorophore-labeled secondary antibody: the secondary antibody is diluted in PBS buffer containing 1% BSA. For the detection of the primary antibody mentioned above, goat anti-mouse IgG (H+L), Alexa 488 Fluor-conjugated antibody (Invitrogen) is used at 1:500 dilution; store the antibody in the dark at 4 °C.
11. Cold methanol: prior to the experiment, place methanol into a Coplin Jar with a cover or screw cap and cool at −30 °C in a freezer.
12. DAPI solution for nuclear staining: to make a 1 mg/mL stock solution, dissolve 1 mg of DAPI in 1 mL of distilled water or DMSO. Prepare a 1 μg/L working solution by diluting 1 μL of the stock solution in 999 μL of PBS. The stock and working solutions can be stored for long time at 4 °C in the dark.

13. The mounting medium is composed of 50% glycerol, 0.1 M Tris (pH 9.0), and 1 g/L *p*-phenylenediamine. Commercially available antifade mounting medium, such as SlowFade (Invitrogen) and VECTASHIELD (Vector Laboratories), can be also used.

11.4 Methods

The root tip of seedling is a useful experimental material for immunofluorescence staining, because the cells have low autofluorescence and are easily permeabilized by the antibodies. As a practical example, a method for microtubule staining in onion (*Allium cepa*) root-tip cells based on previous studies [20, 21] is shown below. With some modifications, the protocol is applicable to various tissues of diverse plant species.

11.4.1 Preparation and Fixation of the Samples

1. Germinate onion seeds in the dark at 18–25 °C for 3–6 days on filter papers moistened with distilled water.
2. Place seedlings on a glass slide and excise the root tips with a sharp razor blade transversely at about 2 mm from the tip. Perform the dissection in PME buffer under a stereomicroscope (*see Note 5*).
3. Transfer the root tips into a small vial containing 5–10 mL of fixative, using a pipet with large bore tip (made by cutting off the end of a pipet tip). Place the capped vial on a reciprocal shaker at room temperature for 90 min. If samples are still floating within 10 min, the vial with the cap loosened is placed into a desiccator connected to a water aspirator or a vacuum pump; evacuate samples for a short time to remove air.
4. After 90 min, remove the fixative from the vial with a fine-tip pipette and discard into a liquid waste bottle containing formaldehyde. About 10 mL of PME buffer is immediately added to the vial and the root tips are gently washed by placing the vial on a reciprocal shaker. After 5 min, remove the PME buffer. Repeat this washing process two or three times.

11.4.2 Enzymatic Removal of the Cell Wall and Chemical Permeabilization of the Plasma Membrane

1. After washing in PME buffer, incubate the tissues in the wall-digesting enzyme solution for 15–30 min (*see Note 6*) on a reciprocal shaker at room temperature (*see Note 7*).

2. For checking the results of the enzymatic treatment, remove a small amount of sample from the vial and place it on a slide. Place a coverslip and squash gently with a finger. Confirm that the cells are dispersed singly or in short files under a microscope (Fig. 11.2).
3. Remove the enzyme solution and replace it with the permeabilization buffer containing 0.1 % Triton X-100 and 1 % BSA in PME buffer. After two quick rinses, incubate the tissues in the permeabilization buffer for 10 min on a reciprocal shaker to permeabilize the plasma membrane at room temperature.

11.4.3 Attaching Samples to Glass Slides

1. Remove the permeabilization buffer from the vial and wash the samples in PME buffer two times. Remove the root tips with a large-bore pipet tip from the vial and place one or two root tips on MAS-coated glass slides (or other type of coated slides). Orient the root tips carefully using a fine tweezers.
2. Place a coverslip on the samples and squash it gently with a finger.
3. Remove the coverslip by lifting it up carefully using tweezers. Be sure not to shift the position of the coverslip. Large tissue fragments remaining on the slide can be removed using the tweezers under a stereomicroscope. Some single cells should remain on the slide.
4. Dry the slide slightly at room temperature for about 5 min to ensure that cells adhere to the slide. Be sure not to dry the slide completely as it can cause deformation of cell shape and cytoplasmic content. Check that most of the isolated cells are immobilized on the glass slides without coverslip under a microscope.

11.4.4 Cold Methanol Treatment

1. After partial drying, transfer the slides to a Coplin jar containing 100 % cold methanol for 10 min at −30 °C (*see Note 10*).
2. After the treatment, transfer the slides immediately to another Coplin jar containing PBS buffer at room temperature.

11.4.5 Immunostaining

1. Remove excess PBS from the slides by blotting using small pieces of filter paper. Apply 1 % BSA blocking solution to the slides and incubate in a moist chamber for 30 min at room temperature.
2. Remove excess blocking solution and apply 30–50 μL of primary antibody diluted with 1 % BSA blocking solution; incubate the slides in a moist chamber for 60 min at 37 °C or, alternatively, overnight at 4 °C.

3. After incubation, remove the primary antibody from the slides. Glass slides are placed into a Coplin jar containing PBS buffer for 10 min to remove the primary antibody.
4. Remove excess PBS and apply 30–50 μL of the secondary antibody appropriately diluted in PBS containing 1 % BSA. Incubate the slides in a moist chamber for 60 min at 37 °C in the dark.
5. After incubation, remove the secondary antibody from the slides and wash them in a Coplin jar containing PBS buffer for 10 min.

11.4.6 Counterstaining and Mounting

1. To visualize nuclei, apply the DAPI working solution onto the slides and incubate in a moist chamber for 10 min at room temperature.
2. Remove the excess DAPI solution and add a drop of mounting medium onto the slides. Cover the sample with a coverslip; remove excess mounting medium with a piece of filter paper. Seal the coverslip by applying a nail polish along its edges.
3. Fluorescent signals are imaged on a fluorescence microscope or a laser scanning microscope with appropriate excitation/emission combination of DAPI (ultraviolet excitation and blue emission) and Alexa 488 (blue excitation and green emission).

11.4.7 Techniques for Improving the Adhesion of Cells and Tissues

11.4.7.1 Egg Albumin Adhesive

The egg albumin adhesive is suitable for attaching relatively large specimens. For preparing egg albumin coating, mix thoroughly 50 mL of fresh egg white and 50 mL of glycerol, using a magnetic stirrer. Add 1 mg of sodium azide as a preservative. The mixture can be stored for several weeks at 4 °C. Apply a small drop of adhesive coating on a clean slide or coverslip and spread evenly with a clean finger until a thin film remains. The coated slides or coverslips are stored in a dust-free box at room temperature and must be used in a few days. Long storage is not suitable because the adhesive strength will decrease due to drying.

11.4.7.2 Preparing an Agarose-Gelatin Film

Covering the coverslip with agarose-gelatin film ensures that fewer cells will be lost during processing. It is also effective in preventing the deformation and breakage of whole-mounted tissue. Although the covering with agarose-gelatin film can be applied at any step in the procedure, the duration time of fixation, cell wall digestion, and washing might need to be increased.

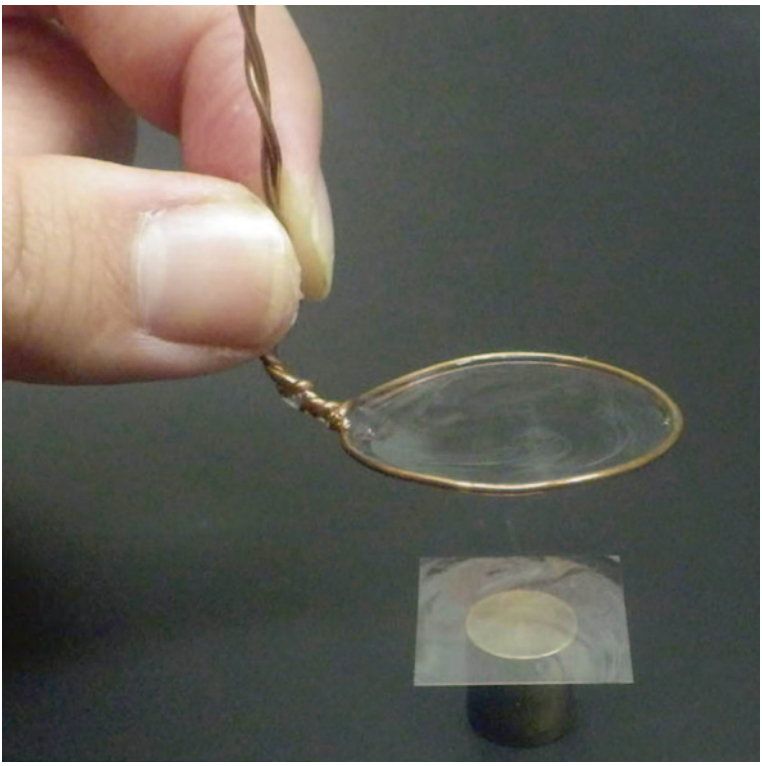

Fig. 11.3 Handling of the agarose-gelatin film for covering the samples on coverslip

1. Prepare a solution of 0.75 % agar and 0.75 % gelatin in distilled water. Glucose can be added as an osmoticum. Vegetative cells and tissues require about 1 % glucose, whereas sporocytes and pollen may require up to 10 % or more.
2. Heat the agarose-gelatin solution in a water bath until it becomes clear.
3. Place a small drop of fixative containing fixed samples onto each coverslip coated with albumin adhesive as indicated in Sect. 11.4.7.1.
4. Immerse the wire loop into agarose-gelatin solution and raise it slowly so that a film will form inside of the loop.
5. Cover the materials with agarose-gelatin film. The coverslips should be elevated in some way so that the loop with a film can pass over it (Fig. 11.3) (*see Note 11*).
6. The coverslip is placed into a small Petri dish with the film surface upwards for subsequent processing.

11.5 Notes

1. To prevent rupture and deformation of cells, osmolality of the enzyme solution should be adjusted, according to the plant material used, by the addition of glucose or mannitol. The vegetative cells and tissues typically require about 1 %

glucose or mannitol, whereas reproductive cells such as sporocytes of bryophytes and pollen of flowering plants typically require 10% or more [9].

2. For whole mounts of the tissue and embryos of flowering plants, a commercially available enzyme mixture Driselase (Sigma) is preferably used instead of preparing the mixture of individual enzymes. Treatment with high concentration of DMSO and IGEPAL CA-630 subsequent to driselase treatment enhances the permeabilization of antibodies. Incubation time of immunostaining should be prolonged according to the size of materials (e.g., several hours or overnight) [14, 15]. For bryophytes, a mixture of cellulase, pectolyase, and β-glucuronidase is used for wall digestion [9]. Addition of β-glucuronidase derived from garden snail (Sigma-Aldrich) accelerates the digestion of thick cell walls. The permeabilization buffer for bryophytes contains 1% or more Triton X-100. Persistent autofluorescence from oil bodies in the cytoplasm of liverwort cells is removed by ethanol treatment after immunostaining [24].
3. Wear gloves when handling toxic reagents such as PFA, DMSO, protease inhibitors, and sodium azide. Use a fume hood for solution preparation whenever possible.
4. When an antibody is used for the first time, the concentration must be optimized experimentally. Repeated freeze/thaw cycles can denature an antibody. If diluted, antibodies are stored for a long time at 4 °C, the dilution buffer should contain 0.1% sodium azide.
5. Breakable razor blades made form carbon steel and a tweezers having sharp ends are useful for preparing the materials. When the edge becomes dull, the razor blades made from carbon steel can be broken obliquely to create a new cutting edge.
6. The reaction time of each step and composition and concentration of the enzyme mixture and permeabilization buffer should be changed according to plant materials.
7. When the room temperature is 20 °C or lower, materials should be incubated in an incubator or a water bath maintained at 25 °C.
8. The treatment with a detergent such as Triton X-100 is a critical step and the process needs to be properly controlled. A higher concentration or treatment time could damage the cell morphology.
9. Cell walls covered with thick cuticular waxes, typically occurring in epidermal tissue, are difficult to remove enzymatically. Mechanical disruption of cell walls using liquid nitrogen prior to the enzymatic digestion may increase accessibility of enzymes [29]. A method using methanol and xylene as organic solvents to remove cuticular waxes has been reported. However, the treatment may lead to problems with some antigens [15].
10. There are multiple influences of the methanol treatment. It can dissolve the autofluorescent materials from permeabilized cells that cannot be removed by the chemical permeabilization step. “Cold” methanol also increases the permeability of the cell wall and plasma membrane by physical destruction through freezing and/or dehydration of water. In some cases, it also improves antibody binding and specificity.

11. Agar-gelatin films can easily slip off of the coverslips; better adherence can be obtained by etching the border of the coverslips with commercially available glass etching cream (Armour Etch, Armour Products, Hawthorne, New Jersey). Paint a narrow border 2–3 mm around the edge of the coverslip. After etching, wash the coverslips in dishwashing detergent and wipe clean with 70% ethanol. Store the coverslips in a dust-free container until use.

References

1. Jordan EG, Zatsepina OV, Shaw PJ (1992) Widely dispersed DNA within plant and animal nucleoli visualized by 3-D fluorescence microscopy. Chromosoma 101:478–482
2. Terasaki M, Loew L, Lippincott-Schwart J, Zaal K (2001) Fluorescence staining of subcellular organelles: ER, Golgi Complex, and mitochondria. Curr Protocols in Cell Biol Unit 4.4. doi:10.1002/0471143030.cb0404s00
3. Nelson BK, Cai X, Nebenführ A (2007) A multi-color set of in vivo organelle markers for colocalization studies in Arabidopsis and other plants. Plant J 51:1126–1136
4. Brandizzi F, Fricker MD, Hawes C (2002) A greener world: the revolution in plant bioimaging. Nature Rev Mol Cell Biol 3:520–530
5. Segami S, Makino S, Miyake A, Asaoka M, Maeshima M (2014) Dynamics of vacuoles and H+-pyrophosphatase visualized by monomeric green fluorescent protein in Arabidopsis: Artifactual bulbs and native intravacuolar spherical structures. Plant Cell 26:3416–3434
6. Weber K, Osbom M, Franke WW, Seib E et al (1977) Identification of microtubular structures in diverse plant and animal cells by immunological cross-reaction revealed in immunofluorescence microscopy using antibody against tubulin from porcine brain. Cytobiologie 15:285–302
7. Franke WW, Seib E, Osborn M, Weber K et al (1977) Tubulin containing structures in the anastral mitotic apparatus of endosperm cells of the plant Leucojum aestivum as revealed by immunofluorescence microscopy. Cytobiologie 15:24–48
8. Hepler PK, Gunning BES (1998) Confocal fluorescence microscopy of plant cells. Protoplasma 201:121–157
9. Brown RC, Lemmon BE (1995) Methods in plant immunolight microscopy. Methods in Cell Biol 49:85–107
10. Shimamura M, Brown R, Lemmon BE et al (2004) g-Tubulin in basal land plants: characterization, localization, and implication in the evolution of acentriolar microtubule organizing centers. Plant Cell 16:45–59
11. Brown RC, Lemmon BE (2005) g-tubulin and microtubule organization during microsporogenesis in *Ginkgo biloba*. J Plant Res 118:121–128
12. Palevitz BA, Cresti M (1989) Cytoskeletal changes during generative cell division and sperm formation in *Tradescantia virginiana*. Protoplasma 150:54–71
13. Hasezawa S, Nagata T (1991) Dynamic organization of plant microtubules at the three distinct transition points during the cell cycle progression of synchronized tobacco BY-2 cells. Bot Acta 104:206–211
14. Friml J, Benková E, Mayer U, Palme K, Muster G (2003) Automated whole mount localisation techniques for plant seedlings. Plant J 34:115–124
15. Sauer M, Paciorek T, Benková E, Friml J (2006) Immunocytochemical techniques for whole mount *in situ* protein localization in plants. Nat Protoc 1:98–103
16. Eltoum I, Fredenburgh J, Myers R (2001) Introduction to the theory and practice of fixation of tissues. J Histotech 24:173–190

17. Eltoum I, Fredenburgh J, Myers RB, Grizzle WE (2001) Advanced concepts in fixation: 1. Effects of fixation on immunohistochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues. 2. Developmental methods of fixation. J Histotechnol 24:201–210
18. Kiernan JA (1999) Histological and histochemical methods: theory and practice, 3rd edn. Butterworth-Heinemann, Oxford
19. Collins JS, Goldsmith TH (1981) Spectral properties of fluorescence induced by glutaraldehyde fixation. J Histochem Cytochem 29:411–414
20. Marc J, Mineyuki Y, Palevitz BA (1989) The generation and consolidation of a radial array of cortical microtubules in developing guard cells of *Allium cepa* L. Planta 179:516–529
21. Mineyuki Y, Wick SM, Gunning BES (1988) Preprophase bands of microtubules and the cell cycle: Kinetics and experimental uncoupling of their formation from the nuclear cycle in onion root-tip cells. Planta 174:518–526
22. Wick SM, Seagull RW, Osborn M, Weber K, Gunning BES (1981) Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells. J Cell Biol 89:685–690
23. Brown RC, Lemmon BE (1988) Microtubules associated with simultaneous cytokinesis of coenocytic microsporocytes. Am J Bot 75:1848–1856
24. Brown RC, Lemmon BE, Shimamura M (2010) Diversity in meiotic spindle origin and determination of cytokinetic planes in sporogenesis of complex thalloid liverworts (Marchantiopsida). J Plant Res 123:589–605
25. Mayer P (1883) Einfache Methode zum Aufkleben mikroskopischer Schnitte. Mitt. Zool Stat Neapel 2:521–522
26. Molé-Bajer J, Bajer AS (1968) Studies of selected endosperm cells with the light and electron microscope: The technique. Cellule 67:257–265
27. Weller TH, Coons AH (1954) Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc Soc Exp Biol Med 86:789–794
28. Livingston TD, Tuong, Gadi S et al (2010) 3D volumes constructed from pixel-based images by digitally clearing plant and animal tissue. J Microscopy 240:122–129
29. Wasteneys GO, Willingale-Theune J, Menzel D (1997) Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. J Microscopy 188:51–61

Chapter 12
Protoplast Isolation and Staining

Shiu-Cheung Lung, Sarah Schoor, Dustin Sigurdson, Makoto Yanagisawa, Kelly Yeung, Mi Qi Liu and Simon D. X. Chuong

12.1 Introduction

Protoplasts are plant cells that have had their cell wall enzymatically or mechanically removed. Protoplasts were first isolated half a century ago by Cocking using a fungal cellulase on the root tips of tomato seedlings [1]; since this initial report, numerous protocols have been optimized for isolating protoplasts from various tissues of different plant species (Table 12.1). Protoplasts have become a versatile tool in the in vivo study of plant function and development. Protoplasts readily take up small molecules, facilitating feeding experiments and improving access of fluorescent dyes. In addition to improved ease of staining, fluorescence imaging protoplasts greatly reduces background signal, allowing for higher resolution imaging of subcellular structures (see Chap. 9). Protoplasts can also be manipulated to take up larger exogenous macromolecules such as DNA, RNA and proteins [2–6]. The ability of protoplasts to take up foreign DNA is particularly valuable in transient gene expression [3, 4, 7–11], subcellular protein localization [3, 4, 12–16; Fig. 12.3f, g and h], protein–protein interaction [13, 17, 18] and cell signalling studies [2]. Finally, the totipotent nature of protoplasts also renders them invaluable to plant geneticists as a practical breeding tool for introducing genetic varieties via interspecific and intergeneric protoplast fusion and regeneration [19–23]. In this chapter, a standard protocol for protoplast isolation based on Yoo et al. [14] with minor modifications will be described. Critical factors affecting protoplast yield and viability are also discussed, with the following species highlighted (Fig. 12.1):

S. D. X. Chuong (✉)
Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo N2L 3G1 Ontario, Canada
e-mail: schuong@uwaterloo.ca

S.-C. Lung · S. Schoor · D. Sigurdson · M. Yanagisawa · K. Yeung · M. Q. Liu
Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

S.-C. Lung
School of Biological Sciences, University of Hong Kong, Hong Kong SAR, China

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_12

	Plant species			
	Arabidopsis	*Kalanchoe*	*Bienertia*	*Lampranthus*
1. Vacuum infiltration	15 - 20 minutes	NA		15 - 20 minutes
2. Digestion of leaf segments to release protoplasts	1.5% cellulase, 0.4% macerozyme; 0.4 M mannitol; 3 hrs	1.5% cellulase, 0.5% macerozyme; 0.4 M mannitol; 1.5 hrs	1.5% cellulase; 0.7 M sucrose; 4 hrs	2% cellulase, 2% macerozyme; 0.8 M mannitol; 2.5 hrs
3. Filtration to remove cellular debris	100 μm nylon mesh		NA	
4. Centrifugation to collect protoplasts	100g, 2 minutes			
5. Isolation of healthy protoplasts in CS-sucrose	100g, 2 minutes		NA	

Fig. 12.1 Outline of the procedures used for isolation of protoplasts from various plant species. Species-specific adjustments are indicated at specific steps of the isolation of protoplasts from leaves of *Arabidopsis thaliana*, *Kalanchoe daigremontiana*, *B. sinuspersici* and *Lampranthus spectabilis*

Arabidopsis thaliana, *Bienertia sinuspersici, Kalanchoe daigremontiana* and *Lampranthus spectabilis*. Although protoplasts may be derived from a variety of plant tissues (Table 12.1), isolation of mesophyll protoplasts from leaves will be the main focus of this chapter.

12.1.1 Tissue Selection and Preparation

One of the most crucial factors affecting protoplast yield is the age and source of tissue. Protoplasts derived from distinct tissues often retain their tissue- or cell-specific activities or biological processes [16]. As leaf development varies by species, there is no universal age at which leaves can be sampled; however, it is usually best to avoid newly formed leaves that exhibit limited subcellular differentiation [4]. Conversely, the use of older leaves with waxy cuticle and thickened epidermal cell walls and/or high levels of secondary metabolite accumulation can also reduce protoplast yield [11, 24]. Accumulation of starch grains is one of the major factors that affect protoplast yield and viability. For example, it was shown that increasing starch levels was associated with lower protoplast viability in pea roots [25]. Similar observations have been documented in various species that starch granules are capable of disrupting protoplast integrity [26–28]. To reduce starch levels, plants can be maintained under dark conditions for 24–48 h prior to digestion. As starch levels vary in different tissue and species, pretreatment in the dark is not always required. For example, in this study we have found that a 48-h dark treatment had no significant effect on the isolation of protoplasts from *L. spectabilis,* while the same conditions greatly improved the yield and viability of protoplasts from leaves of *K. daigremontiana*.

As the leaves of terrestrial plants are protected by a waxy epidermis, the first step of a routine protoplast preparation is to expose the inner mesophyll cells to cell wall enzymes. To improve enzyme penetration, the epidermis of leaves can be peeled

Table 12.1 Examples of protoplasts isolated from various tissues of different plant species

Species	Tissues	Reference
Allium sativum	Callus	[30]
Arabidopsis thaliana	Cotyledon	[31]
A. thaliana	Leaf	[2]
A. thaliana	Suspension cultured cells	[3]
Artemisia judaica L. (Judean wormwood)	Leaf	[32]
Avena fatua (wild oat)	Leaf, aleurone layers	[17, 33]
Beta vulgaris L. (beet)	Callus	[34]
B. vulgaris L (beet)	Root	[35]
Bienertia sinuspersici	Leaf	[4]
Brachypodium distachyon	Leaf	[7]
Brassica napus (canola)	Hypocotyl	[8, 36]
Browollia speciosa (bush violet)	Cotyledon	[37]
Cucumis sativus (cucumber)	Leaf	[38]
Echinacea purpurea (purple coneflower)	Leaf	[32]
Echinops spinosissimus Turra (globe thistles)	Leaf	[39]
Euphorbia pulccherrima	Leaf	[12]
Gossypium hirsutum (cotton)	Cotyledon	[40]
Helianthus annuus (sunflower)	Cotyledon, hypocotyl	[41]
Hordeum vulgare (barley)	Leaf	[42]
Ipomoea batatas L. (sweet potato)	Stem, leaf	[9]
Jatropha curcas L.	In vitro leaf	[43]
Kalanchoe daigremontiana (Mother of Thousands)	Leaf	This study
Lactuca sativa (lettuce)	Leaf	[44]
Lampranthus spectabilis (Trailing Ice Plant)	Leaf	This study
Lupinus angustifolius (lupin)	Leaf	[45]
Manihot esculenta (cassava)	Leaf	[46]
Nicotiana tabaccum (tobacco)	Leaf	[27, 47]
N. tabaccum BY-2 cells	Suspension cultured cells	[3]
Oryza sativa (rice)	Stem and sheath	[13]
Panicum miliaceum (millet)	Leaf	[48]
Panicum virgatum (switchgrass)	Leaf	[10]
Phaseolus vulgaris L.	Cotyledons	[49]
Populas tremula x alba	Leaf	[11, 50]
Prunus dulcis (almond)	Suspension culture	[51]
Ricinus communis L.	In vitro leaf	[43]
Secale cereale L. (rye)	Leaf	[42]
Solanum lycopersicum (tomato)	Fruit, leaf and root	[1, 52]
Solanum tuberosum L. (potato)	Leaf	[53, 54]
Sorghum bicolor (sorghum)	Leaf	[55]
Triticum aestivum (wheat)	Leaf	[42]
Vitis vinifera (grape)	Leaf	[56]
Zea mays (corn)	Leaf	[2]

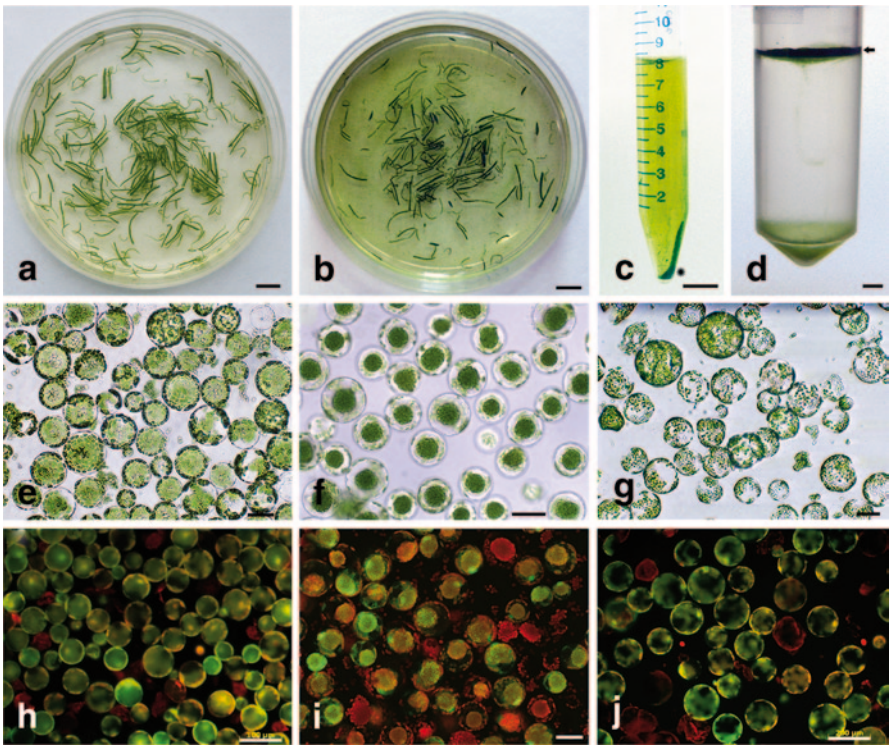

Fig. 12.2 Procedures used for isolation of mesophyll protoplasts from leaves of various dicotyledonous species. **a** Leaf sections at the beginning of cell wall removal treatment. **b** Leaf sections after 3 h in enzyme solution. **c** Protoplast pellet (*) obtained by centrifugation. **d** Healthy mesophyll protoplasts *(arrowhead)* in the floating layer of CS-sucrose buffer after centrifugation. Isolated healthy mesophyll protoplasts from **e** and **h** *Arabidopsis*; **f** and **i** *B. sinuspersici*; and **g** and **j** *K. daigremontiana* visualized under brightfield microscopy and under fluorescence microscopy after they have been stained with fluorescein diacetate (FDA) for viability assessment. Fluorescence is shown in false colours with *green* for FDA and *red* for chlorophyll autofluorescence. Overlapping signals appear as *yellow*. Scale bars **a**–**c** = 1 cm; **d** = 2 mm; **e**–**j** = 50 μm

off mechanically or removed by enzymatic digestion [4, 24, 27, 28]. Alternatively, a direct approach is to slice the leaf tissues into fine strips (Fig. 12.2a) and expose the mesophyll cells at the cut edges to the enzyme solution [14, 29]; we have found this method to be most effective for *K. daigremontiana* and *L. spectabilis*. If leaf sectioning is being performed, vacuum infiltration may be required to facilitate the penetration of the enzyme solution (Fig. 12.1). Although not all plant species require the vacuum infiltration step, such treatment can be useful in the removal of trapped air and exposure of cells in thicker tissues to the enzyme solution. Finally, mesophyll cells of succulent plant species such as *B. sinuspersici* can be directly released and exposed to the enzyme solution by gently pressing leaves with a mortar and pestle [4].

12.1.2 Cell Wall Digestion

The cell walls of plant tissues are composed of two layers: The pectin-rich middle lamella responsible for the cell–cell adhesion and the inner primary cell wall consisted mainly of cellulose microfibrils and hemicellulose that provides the structural support to the cell. Generally, the protoplast isolation procedure requires cell separation that involved treatment of plant tissue with pectinase to remove the pectin-rich matrix of the middle lamella followed by cellulase to remove the cellulosic walls. Currently, most protoplast isolation protocols routinely employ a mixture of enzymes such as cellulase and macerozyme, which consists of a mixture of pectinase and hemicellulase. The working concentrations of these enzymes vary depending on the plant species and how the tissue has been prepared (Fig. 12.1). For example, the loosely arranged spongy mesophyll cells of *Arabidopsis* require a lower enzyme concentration compared to that of the tightly packed, radially arranged mesophyll cells of *Lampranthus* (Fig. 12.1). Likewise, isolation of protoplasts from *Bienertia* leaves requires no pectinase treatment or inclusion of macerozyme in the enzyme solution as the mesophyll cells are loosely packed and can readily be released from the leaves by epidermal peel or using a mortar and pestle [4].

In addition to enzyme concentration, pH also greatly impacts the rate of digestion. As documented by Lung et al. [4], both macerozyme and cellulase are most active at a slightly acidic pH (5–5.5), however under normal conditions, the cytoplasmic pH of typical plant cells are kept at neutral; as a compromise to maintain the viability of the released protoplasts, digestion is generally performed at pH 5.7. When attempting to modify the protocol for other plant species, pH may need to be altered to ensure optimal protoplast release and viability. Furthermore, the cell wall normally provides a means of maintaining turgor pressure, thus removal of this structure can render protoplasts vulnerable to cell lysis. Thus, to counteract turgor pressure during the cell wall removal process, the presence of an osmotic solute (osmoticum) such as sorbitol, mannitol, glucose or sucrose is often required to maintain the osmotic potential. The optimum concentration and nature of an osmoticum, however, vary with different plant species (Fig. 12.1) and require empirical testing. Readers can also refer to a recent study that examined the effects of different osmotica on the yield and viability of protoplasts from a single-cell C_4 species, *B. sinuspersici*, which possesses a unique intracellular compartmentation of organelles in chlorenchyma cells [4].

The exposure time of tissue to enzyme solution also varies based on the species being studied (Fig. 12.1), but typically it should not exceed 4 h. When determining the optimal incubation time, it is not always necessary to wait for complete tissue digestion. For example, in *Arabidopsis*, portions of the undigested leaf segments are often remaining (Fig. 12.2b), when the optimal amounts of viable protoplasts have been released. To remove undigested tissue, the digest solution can be filtered using a 75–100 μm nylon mesh (Fig. 12.1). It is important to ensure the proper mesh size is used as removal of the cell wall renders the protoplasts fragile and susceptible to mechanical damage.

12.1.3 Isolation of Viable Protoplasts

The isolation of a pure population of non-stressed, healthy protoplasts is critical for maintaining reliability and reproducibility. Thus once the cell wall digestion is complete, the separation of viable and nonviable protoplasts becomes a crucial step. Depending on the osmolarity of the enzyme solution (Fig. 12.1), centrifugation will either pellet both living and dead protoplasts (Fig. 12.2c) or intact protoplasts will remain buoyant, forming a floating layer (Fig. 12.2d). With high-molecular weight solutes such as sucrose, concentrations as low as 0.4 M will allow intact protoplasts remain floating. On the other hand, no additional centrifugation steps are needed in the isolation of protoplasts from succulent plants such as *Bienertia* which requires a high concentration of osmoticum (0.7 M sucrose) [4]; Fig. 12.1). While the other solutes such as mannitol and sorbitol have nearly half the molecular weight than that of sucrose, higher concentrations of these osmotica will sufficiently increase fluid density. For example, *Arabidopsis* leaf sections are digested in 0.4 M mannitol where all protoplasts pellet, however *Lampranthus* protoplasts are released into 0.8 M mannitol, where only healthy protoplasts remain afloat at the top layer. If a floating layer is not obtained after the first centrifugation, then the pellet can be resuspended in a higher-density solution. Once the floating layer has been obtained, protoplast viability can then be assessed. Preliminary assessment of protoplast health can be performed using bright-field microscopy. Healthy protoplasts will be spherical and have uniform chloroplast distribution (Fig. 12.2e, f and g). Unhealthy protoplasts will exhibit chloroplast clumping, irregularity in shape and sometimes plasmolysis. For a more accurate assessment of protoplast viability, a small aliquot of the isolated protoplasts can be stained with FDA (Fig. 12.2h, i and j; see Chap. 9). If protoplasts that are for use in subsequent biochemical, cellular and molecular genetics studies to examine a variety of cell-specific/biological processes, viability rates of 80 % are recommended.

12.2 Materials

12.2.1 Equipment

Laboratory equipment: Controlled environmental chamber, desiccator, vacuum pump, incubators (55 and 70 °C), refrigerator, benchtop centrifuge equipped with swing-bucket rotor and a fluorescence microscope or confocal laser scanning microscope

12.2.2 Supplies for Protoplast Isolation

General supplies: Potting soil, 20:20:20 (N:P:K) fertilizers, *A. thaliana* (ecotype Col-0) seeds, Falcon tubes (15 and 50 mL), Petri dishes (90 mm), Nu-Base plate wax, flat-tip forceps, double-sided razor blades (Electron Microscopy Sciences 72000), nylon mesh (75 μm; Spectrum Scientific Inc 146490.), Pasteur pipettes, cover glasses, depression slides and hemocytometer (Neubauer).

12.2.3 Chemical Reagents

Bovine serum albumin (Sigma A7030), Cellulase "Onozuka" R-10 (Yakult Pharmaceutical Ind. Co. Ltd. L0012), Macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd. L0021), 2-(*N*-morpholino) ethanesulfonic acid (MES; BioShop MES503), D-mannitol (Sigma 63559), Sucrose (BioShop SUC507), Calcium chloride ($CaCl_2$; BioShop CCL444), Potassium chloride (KCl; BioShop POC888), Potassium hydroxide (KOH; BioShop PHY202)

12.2.4 Solution Preparation

1. 0.5 M MES-KOH stock solution, pH 5.7: For 500 mL, dissolve 10.65 g of MES in 400 mL of deionized water, adjust the pH to 5.7 with 1 M KOH and then add deionized water to 500 mL. Sterile by autoclaving.
2. 1 M $CaCl_2$ solution: For 500 mL, dissolve 73.51 g of $CaCl_2$ in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
3. 1 M KCl solution: For 500 mL, dissolve 37.27 g of KCl in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
4. 1 M NaCl solution: For 500 mL, dissolve 29.22 g of NaCl in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
5. Cell-stabilizing (CS)-mannitol buffer: For 50 mL, add 2 mL of 0.5 M MES-KOH, pH 5.7 (final 20 mM), 1 mL of 1 M KCl (final 20 mM) and 3.64 g of mannitol (final 400 mM). Adjust volume to 50 mL with deionized water and store at 4 °C for up to 2 weeks.
6. W5 buffer: For 50 mL, add 0.2 mL of 0.5 M MES-KOH, pH 5.7 (final 2 mM), 7.7 mL of 1 M NaCl (final 154 mM), 6.25 mL of 1 M $CaCl_2$ (final 125 mM) and 0.25 mL of 1 M KCl (final 5 mM). Adjust volume to 50 mL with water and store at 4 °C for up to 2 weeks.

7. CS-sucrose buffer: For 50 mL, add 2 mL of 0.5 M MES-KOH, pH 5.7 (final 20 mM), 1 mL of 1 M KCl (final 20 mM) and 6.84 g of sucrose (final 400 mM). Adjust volume to 50 mL with deionized water and store at 4 °C up to 2 weeks.
8. 10% (w/v) Bovine serum albumin (BSA): Dissolve 1 g of BSA in 10 mL of deionized water. Dispense in aliquots and store at −20 °C.
9. Enzyme solution: Incubate 10 mL of CS-mannitol buffer in a 15-mL falcon tube at 70 °C for 10 min. Cool it to below 55 °C before adding 150 mg of cellulase "Onozuka" R-10 (final 1.5% [w/v]) and 40 mg of macerozyme R-10 (final 0.4% [w/v]). Incubate at 55 °C for another 10 min to completely dissolve the cell wall enzymes. Cool the enzyme solution to room temperature before adding 100 µl of 10% BSA (final 0.1% [w/v]) and 100 µL of 1 M $CaCl_2$ (final 10 mM). Transfer the enzyme solution to a 90-mm Petri dish.

12.3 Methods

12.3.1 Isolation of Mesophyll Protoplasts from A. thaliana

1. Stratify *A. thaliana* seeds at 4 °C in the dark for at least 48 h.
2. Germinate the stratified seeds on potting soil covered with plastic domes during the first week in controlled environmental chambers with a day/night regime of 16/8 h at 22 °C with a photon flux density of approximately 150 µmol m^{-2} s^{-1}. Keep the plants well watered and fertilize with fertilizer regularly (*see Note 1*).
3. Harvest 20 well-expanded leaves from 3- to 4-week-old plants prior to bolting. Tissues should be processed immediately following harvesting in order to maintain cell turgor and ensure maximal healthy protoplast yield.
4. Prepare 0.5- to 1-mm strips from the middle region of a leaf using a fresh sharp double-sided razor blade on a dental wax pad (e.g. Nu-Base plate wax) (*see Note 2*).
5. Pick up the leaf strips using a pair of flat-tip forceps. Dip both sides of the strips in the enzyme solution before releasing them into the 10 mL enzyme solution in a Petri dish. (*see Note 3*)
6. Repeat steps 4 and 5 until all the leaves have been cut (*see Note 4*).
7. Vacuum infiltrate the leaf strips for 15 min in a desiccator connected to a vacuum pump (*see Note 5*).
8. Digest the leaf strips at room temperature in the dark for at least 3 h without shaking (*see Note 6*; Fig. 12.2a). Monitor the progress in cell wall digestion regularly by observing the release of protoplasts under a microscope (*see Note 7*).
9. Check the healthiness of the isolated protoplasts by their appearance under a microscope (*see Note 8*).

12.3.2 Purification of Healthy Protoplasts for Use in Cellular and Molecular Analyses

1. To remove cell debris, filter the enzyme–protoplast solution through a piece of 75-μm nylon mesh into a 50-mL falcon tube using a Pasteur pipette. Rinse the Petri dish gently by swirling with 10 mL of CS buffer and filter the content into the same falcon tube (*see Note 9*).
2. Equally divide the filtered protoplasts into two 15-mL falcon tubes and centrifuge at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (*see Note 10*; Fig. 12.2c).
3. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette.
4. If the presence of any unhealthy protoplasts and/or residual cell debris matters, protoplasts can be further purified on a sucrose medium as follows (optional). Otherwise, proceed to step 6. Resuspend each protoplast pellets in 10 mL of CS-sucrose buffer (*see Note 11*). Centrifuge at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (*see Note 12*; Fig. 12.2d).
5. Remove the pellets and the solution as much as possible without disturbing the floating layer which contains the healthy protoplasts using a Pasteur pipette. At this point, 10 μL of the healthy protoplasts can be used to determine the cell count using a hemocytometer (*see Note 13*).
6. Resuspend the protoplast pellets from step 3 or the floating layer of protoplasts from step 5 in 1 mL of W5 buffer by gentle swirling.
7. Centrifuge the protoplast suspension at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (see Note 14).
8. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette. The protoplast pellets can be used for various fluorescent staining procedures (see Chap. 9) or for transfection experiments as described by Lung et al. [4].
9. For staining, resuspend the protoplast pellets by gentle swirling in CS-mannitol buffer with an appropriate amount of dye (*see Note 15*). Incubate at room temperature for 15 min (*see Note 16*).
10. Centrifuge the protoplast suspension at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor.
11. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette.
12. Resuspend the protoplast pellets by gentle swirling in an appropriate volume of CS-mannitol buffer to achieve the best cell density for microscopy (*see Note 17*).
13. Transfer 50 μL of the stained protoplasts to a depression slide and place a coverslip. Examine and image the protoplasts using an epifluorescence microscope or a confocal microscope (*see Note 18*; Figs. 12.2 and 12.3).

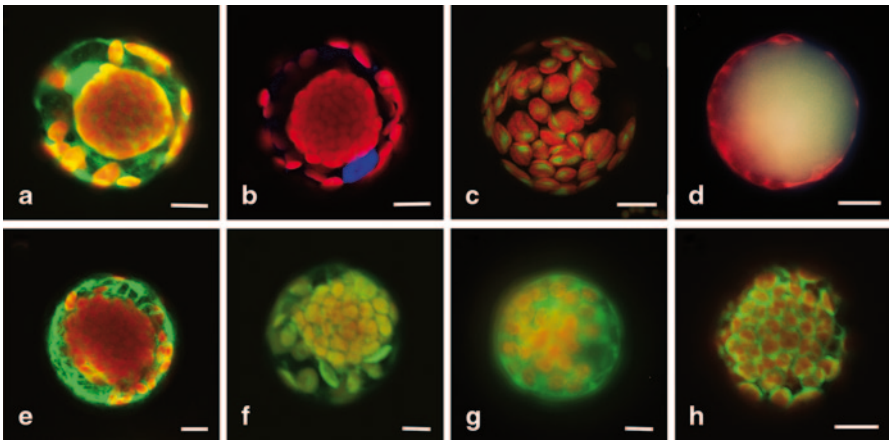

Fig. 12.3 Subcellular localization of compartment and organelles in mesophyll protoplasts stained with various fluorescent dyes **(a-d)** or transfected with chimeric constructs containing various organelle markers fused to green fluorescent protein (GFP; **e**–**h**). **a** Cytoplasm in *Bienertia sinuspersici* protoplast stained with fluorescein diacetate (FDA); **b** Nucleus in *B. sinuspersici* protoplast stained with 4′,6-diamidino-2-phenylindole (DAPI); **c** Mitochondria in *Arabidopsis* protoplast stained with rhodamine 123 (R123); **d** Vacuole in *Arabidopsis* protoplast stained with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetae (carboxy-DCFDA); **e** Transfected *B. sinuspersici* protoplast showing the actin filaments; **f** Transfected *B. sinuspersici* protoplast with GFP targeted to the stroma of chloroplasts; **g** Transfected *Arabidopsis* protoplast with cytosolic GFP expression; **h** Transfected *Arabidopsis* protoplast with GFP targeted to the chloroplast outer membrane. Fluorescence is shown in false colours with *green* for FDA, GFP and R123, *blue* for DAPI, *greenish-white* for carboxy-DCFDA and *red* for chlorophyll autofluorescence. Overlapping signals appear *yellow*. Panels a–c, e, f, h represent confocal micrographs. Panels d, g represent epifluorescence micrographs. Scale bars = 10 μm

12.4 Notes

1. The yield and health of isolated protoplasts critically depend on the source and status of starting plant materials. For reproducible results, plants should be maintained under standard conditions.
2. A sharp razor blade is necessary to avoid tissue crushing at the cutting edge. Do not use single-edge razor blade. Instead, use a double-edge razor blade which has been snapped in half before removing the paper covering. Move to an unused area of blade or change blade after cutting 4–5 leaves. When cutting, draw the blade over the leaf surface in a single, smooth motion. Uniform leaf strips are essential for the release of protoplasts and overall yield.
3. High-quality cellulase and macerozyme should always be used. Impurities in industrial-grade enzymes will severely affect the yield and viability of isolated protoplasts.
4. Scale up the volume of enzyme solution accordingly if more leaf materials are to be used. Incubation of leaf strips in an inadequate volume of enzyme solution will prolong the digestion time or result in incomplete cell wall digestion.

5. Make sure that air bubbles are being expelled out from the cutting edges of leaf strips during vacuum infiltration. Poor contact of the enzyme solution with the mesophyll cells will affect the protoplast yield.
6. Avoid unnecessary agitation during the digestion process as the isolated protoplasts become very fragile in the medium after the removal of their cell walls.
7. Toward the end of digestion period, the medium should turn green upon a gentle swirl of the Petri dish (Fig. 12.2b).
8. The protoplast–enzyme solution contains a mixture of healthy and unhealthy protoplasts. Healthy protoplasts are spherical with uniform chloroplast distribution whereas unhealthy protoplasts often are irregularly shaped with clumped chloroplasts.
9. Be gentle and avoid splashing when filtering the isolated protoplasts by running the cell suspension down the inner wall of the falcon tube. The diameters of mesophyll protoplasts from 3- to 4-week-old *A. thaliana* plants are approximately 30–50 μm in diameters. For filtering isolated protoplasts from other plant materials, the appropriate cut-off size of nylon mesh should be chosen to minimize cell damage.
10. Centrifugation using a swing-bucket rotor rather than a fixed-angle rotor can effectively minimize the loss of isolated protoplasts by collecting them at the bottom of the falcon tubes.
11. Resuspend the protoplast pellets by gentle swirling of the falcon tube. Do not resuspend by pipetting to minimize cell damage.
12. During centrifugation, the unhealthy and broken protoplasts will move toward the bottom of the tube forming a pellet whereas the healthy protoplasts will remain afloat in the upper layer of the medium.
13. Alternatively, isolated protoplasts of some plant species are extremely fragile and cannot survive the centrifugation step, allow them to settle at the bottom of the tube by gravity. Protoplasts will readily settle in W5 solution in 20–30 min.
14. Protoplast yield will vary depending on the species being used. Generally, a volume of 10-mL digest solution can yield up to 5×10^5 protoplasts from *K. daigremontiana*, *Bienertia* and *Lampranthus*, while a yield as high as 2×10^7 can be obtained from *Arabidopsis*.
15. Stock solutions of many fluorescent dyes are insoluble in aqueous solutions but readily soluble in organic solvents, such as acetone, ethanol, dimethyl sulfoxide and methanol. Negative controls without cytochemical stains should be set up by adding the same volume of organic solvents into the CS-mannitol buffer to confirm that the organic solvents do not affect the integrity and function of the organelles.
16. The optimum concentrations, incubation time and conditions may vary with different cytochemical stains.
17. The isolated protoplasts can be pelleted by low-speed (i.e., 100 g) centrifugation in CS-mannitol buffer without a significant loss of viability. If needed, this pelleting step can be routinely incorporated into any protoplast staining procedures to adjust the cell density for imaging or to wash the stained protoplasts further if the background fluorescence is too high.

18. A depression slide has a round, concave depression well at its centre so the isolated protoplasts will not be squashed and misshaped when a coverslip is applied and the aqueous medium will not be dried out as quickly as with flat microscopic slides.

12.5 General Comments

The expected results from a protoplast isolation procedure will vary depending on the plant species or tissues being used. The procedures described in this chapter are carried out on a small scale with the intention of increasing the scale when the method becomes successful and reproducible. Often, people encountered problems when scaling up their experiments and things did not turn out as expected. One of the sources of variability arises from the ratio of enzyme solution to tissue. It is likely that in order to release more protoplasts from more tissue, a larger volume of enzyme solution will be used. Moreover, the time needed to prepare all the plant material may be longer and so the time of digestion may differ too. In addition, the protoplast yield and health from other plant species may also vary with different concentrations and types of osmoticum in the enzyme solution, optimum time of digestion and concentrations of cell wall enzymes. These parameters should be tested empirically to determine the best conditions for the plant species of interest instead of those that are described for the representative plants in this chapter. In general, when attempting to follow a published protocol, it is always advisable to carry out small-scale trials to familiarize oneself with the procedure, have a feel for any dubious steps and determine whether improvements can be made. However, it is unwise to make minor variations on the reported steps unless one is very unhappy with the overall yield, since the authors probably have already spent some time making adjustments and reported what they considered the optimum procedure.

Acknowledgments This research was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Waterloo Start-Up Fund to SDXC.

References

1. Cocking EC (1960) A method for the isolation of plant protoplasts and vacuoles. Nature 187:962–963
2. Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127:1466–1475
3. Miao Y, Jiang L (2007) Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nat Protoc 2:2348–2353
4. Lung SC, Yanagisawa M, Chuong SDX (2011) Protoplast isolation and transient gene expression in the single-cell C_4 species, *Bienertia sinuspersici*. Plant Cell Rep 30:473–484

5. Gallie DR, Lucas WJ, Walbot V (1989) Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. Plant Cell 1:301–311
6. Cormeau J, Barthou H, Jauneau A, Petitprez M et al (2002) Cellular import of synthetic peptide using a cell-permeable sequence in plant protoplasts. Plant Physiol Biochem 40:1081–1086
7. Hong SY, Seo PJ, Cho SH, Park CM (2012) Preparation of leaf mesophyll protoplasts for transient gene expression in *Brachypodium distachyon*. J Plant Biol 55:390–397
8. Bergman P, Glimelius K (1993) Electroporation of rapeseed protoplasts: transient and stable transformation. Physiol Plant 88:604–611
9. Garcia R, Pimentel E, Somonte D, Mena J et al (1998) Isolation and transient transformation of stem and leaf protoplasts from sweet potato (*Ipomoea batatas* L). Asia Pac J Mol Biol 6:145–151
10. Mazarei M, Al-Ahmad H, Rudis MR, Stewart CN (2008) Protoplast isolation and transient gene expression in switchgrass, *Panicum virgatum* L. Biotech J 3:354–359
11. Guo J, Morrell-Falvey JL, Labbe JL, Muchero W et al (2012) Highly efficient isolation of *Populus* mesophyll protoplasts and its application in transient expression assays. PloS ONE 7:e44908
12. Pitzschke A, Persak H (2012) Poinsettia protoplasts: a simple, robust and efficient system for transient gene expression studies. Plant Methods 8:14
13. Chen S, Tao L, Zeng L, Vega-Sanchez M et al (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol 7:417–427
14. Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572
15. Eeckhaut T, Lakshmanan PS, Deryckere D, Bockstaele EV, Huylenbroeck JV (2013) Progress in plant protoplast research. Planta 238:991–1003
16. Faraco M, Di Sansebastiano GP, Spelt K, Koes RE, Quattrochio FM (2011) One protoplast is not the other. Plant Physiol 156 474–478
17. Galston AW, Altman A, Kaur-Sawhney R (1978) Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci Lett 11:69–79
18. Walter M, Chaban C, Schutze K, Batistic O et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40:428–438
19. Durieu P, Ochatt SJ (2000) Efficient intergeneric fusion of pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplast. J Exp Bot 5:1237–1242
20. Henn HJ, Wingender R, Schnabl H (1998) Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L and *Helianthus* species. Plant Cell Rep 18:220–234
21. Hu Q, Andersen SB, Dixelius C, Hansen LN (2002) Production of fertile intergeneric somatic hybrids between *Brassica napus* and *Sinapis arvensis* for the enrichment of the rapeseed gene pool. Plant Cell Rep 21:147–152
22. Kisaka H, Kisaka M, Kanno A, Kameya T (1998) Intergeneric somatic hybridization of rice (*Oryza sativa* L) and barley (*Hordeum vulgare* L) by protoplast fusion. Plant Cell Rep 17:362–367
23. Orczyk W, Przetakiewicz J, Nadolska-Orczyk A (2003) Somatic hybrids of *Solanum tuberosum*: application to genetics and breeding. Plant Cell Tissue Org Cult 74:1–13
24. Sutiojono E, Nonhebel HM, Kantharajah AS (1998) Factors affecting protoplast culture of *Cucumis melo* "Green Delica". Ann Bot 81:775–777
25. Crowder AJ, Landgren CR, Rockwood LL (1979) Cultivar differences in starch content and protoplast yields from root cortical explants of *Pisum sativum*. Physiol Plant 46:85–88
26. Chang MM, Loescher WH (1991) Effects of preconditioning and isolation conditions on potato (*Solanum tuberosum* L. cv. Russet Burbank) protoplast yield for shoot regeneration and electroporation. Plant Sci 73:103–109
27. Morris P, Thain JF (1980) Comparative studies of leaf tissue and isolated mesophyll protoplasts. J Exp Bot 31:83–95

28. Rose RJ (1980) Factors that influence the yield, stability in culture and cell wall regeneration of spinach mesophyll protoplasts. Aust J Plant Physiol 7:713–725
29. Davey MR, Anthony P, Power JB, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23:131–171
30. Hasegawa H, Sato M, Suzuki M (2002) Efficient plant regeneration from protoplasts isolated from long-term, shoot primordial-derived calluses of garlic (*Allium sativum*). J Plant Physiol 159:445–449
31. Dovzhenko A, Dal Bosco C, Meurer J, Koop HU (2003) Efficient regeneration from cotyledon protoplasts in *Arabidopsis thaliana*. Protoplasma 222:107–111
32. Pan ZG, Liu CZ, Murch SJ, El-Demerdash M, Saxena PK (2003) Plant regeneration from mesophyll protoplasts of the Egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Plant Sci 165:681–687
33. Hooley R (1982) Protoplasts isolated from aleurone layers of wild oat (*Avena fatua* L.) exhibit the classic response to gibberellic acid. Planta 154:29–40
34. Dovzhenko A, Koop HU (2003) Sugarbeet (*Beta vulgaris* L.): shoot regeneration from callus and callus protoplasts. Planta 217:374–381
35. Schmidt, R., Poole, R.J. (1980) Isolation of protoplasts and vacuoles from storage tissue of red beet. Plant Physiol 66:25–28
36. Barsby TL, Yarrow SA, Shepard JF (1988) A rapid and efficient alternative procedure for regeneration of plants from hypocotyl protoplasts of *Brassica napus*. Plant Cell Rep 5:101–103
37. Rybczyfiski JJ (1989) Plant regeneration and protoplast culture of *Browollia speciosa*. Plant Cell Rep 8:383–386
38. Coutts RHA, Wood KR. (1975) The isolation and culture of cucumber mesophyll protoplasts. Plant Sci Lett 4:189–193
39. Pan ZG, Liu CZ, Sobayed SMA, Saxena PK (2004) Plant regeneration from mesophyll protoplasts of *Echinacea purpurea*. Plant Cell Tissue Org Cult 77:251–255.
40. Firoozabady E, DeBoer DL (1986) Isolation, culture, and cell division in cotyledon protoplasts of cotton (*Gossypium hirsutum* and *G. barbadense*). Plant Cell Rep 5:127–131
41. Bohorova NE, Cocking EC, Power JB (1986) Isolation, culture and callus regeneration of protoplasts of wild and cultivated *Helianthus* species. Plant Cell Rep 5:256–258
42. Sarhan F, Cesar D (1988) High yield isolation of mesophyll protoplasts from wheat, barley and rye. Physiol Plant 72:337–342
43. Tudses N, Premjet S, Premjet D (2014) Optimal conditions for high-yield protoplast isolations of *Jatropha curcas* L and *Ricinus communis* L. Am-Eurasian J Agric Environ Sci 14:221–230
44. Engler DE, Grogan RG (1982) Isolation, culture and regeneration of lettuce leaf mesophyll protoplasts. Plant Sci Lett 2B:228–229
45. Sinha A, Wetten AC, Caligari PDS (2003) Optimization of protoplast production in white lupin. Biol Plant 47:21–25
46. Anthony P, Davey MR, Power JB, Lowe KC (1995) An improved protocol for the culture of cassava leaf protoplasts. Plant Cell Tissue Org Cult 42:299–302
47. Nagata T, Takebe I (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20
48. Edwards E, Lilley RM, Craig S, Hatch MD (1979) Isolation of intact and functional chloroplasts from mesophyll and bundle sheath protoplasts of the C_4 plant *Panicum miliaceum* Plant Physiol 63:821–827
49. Crepy L, Barros LM, Valente VRN (1986) Callus production from leaf protoplast of various cultivars of bean (*Phaseolus vulgaris* L.). Plant Cell Rep 5:124–126
50. Russell JA, Mccown BH (1986) Techniques for enhanced release of leaf protoplasts in populus. Plant Cell Rep 5:284–287
51. Wu SC, Kuniyuki AH (1985) Isolation and culture of almond protoplasts. Plant Sci 41:55–60
52. Gregory DW, Cocking EC (1965) The large-scale isolation of protoplasts from immature tomato fruit. J Cell Biol 24:143–146
53. Shepard JF, Totten RE (1977) Mesophyll cell protoplasts of potato. Plant Physiol 60:313–316

54. Binding H, Nehls R, Schieder O, Sopory SK, Wenzel G (1978) Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum*. Physiol Plant 43:52–54
55. Sairam RV, Seetharama N, Devi PS, Verma Al et al (1999). Culture and regeneration of mesophyll-derived protoplasts of sorghum (*Sorghum bicolour* L. Moench). Plant Cell Rep 18:972–977
56. Nishimuram, Hara-Nishimura I, Robinson SP (1984) Isolation of metabolically competent protoplasts from grapevine leave. Plant Sci Lett 37:171–175

Chapter 13
Guiding Principles for Live Cell Imaging of Plants Using Confocal Microscopy

Subramanian Sankaranarayanan and Marcus A. Samuel

13.1 Introduction

Confocal microscopy has revolutionized plant biology by enabling researchers to visualize and analyze complex processes in real time in a living cell. The confocal microscope works on the principle that when a diode laser beam is passed through the sample of interest, the reflected light is used to construct detailed images of optical sections through the tissue [1]. A laser is used to provide excitation light of high intensity and a dichroic mirror reflects the light from it. Two scanning mirrors, placed in front of the dichroic mirror allow the light to pass across the sample. The dichroic mirror then de-scans the emitted light from the sample and it passes through the pinhole and is measured by a detector. The detector is attached to a computer and is responsible for delivering the signals to construct the image. Confocal microscopy enables the collection of three-dimensional (3D) images from *z*-section series and image acquisition from thick specimens without any image degradation, and overcomes the limitations of conventional microscopy where microtome sectioning is employed to view cells in two-dimension (2D). Increased resolution of cellular components is achieved as a result of the removal of out-of-focus light by the pinhole, providing a cleaner background [2]. It also provides an increase in both maximum lateral and axial resolution in contrast to the conventional wide-field light microscope [3]. This helps in obtaining a detailed description of the cellular components even in thicker plant sections. A great advantage of live cell imaging is that it enables the use of visual markers such as green fluorescent protein (GFP) or its fluorescent variants. Optical imaging based on confocal laser scanning microscopy (CLSM) is advantageous as it minimizes interference caused by autofluorescence from plant cell walls, vacuolar contents, and chlorophylls [4]. The ability of the system to collect clear images in 3D plane has opened up wider application areas for confocal

M. A. Samuel (✉) · S. Sankaranarayanan
Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada
e-mail: msamuel@ucalgary.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_13

microscopy. Spinning-disc confocal microscope (SDCM) technology provides even more improved imaging in comparison to CLSM, enabling live cell imaging in real time with improved acquisition speed, minimal phototoxicity, reduced photobleaching and improved signal-to-noise ratio [5, 6]. But the use of SDCM in plant tissue is limited to non-photosynthetic tissue or tissue types with reduced autofluorescence. CLSM allows more efficient elimination of the autofluorescence signals than SDCM.

In addition to protein localization, confocal microscopy also facilitates the estimation of protein velocity, trajectory, distribution, and activity [4]. One of the important applications of confocal microscopy is in the field of biomedical sciences for imaging fixed or living tissues that are labeled with one or more fluorescent probes, and studying protein dynamics in living cells. In plants, live cell imaging has wider applications which include: determination of the 3D structure of the interphase microtubule array, visualization of cytoskeleton dynamics and cell wall enzymes in plant cells, study and visualization of auxin transport dynamics during plant development, quantification of cytoplasmic free calcium changes in plant cells in response to stimuli, studying symplastic phloem connections in intact *Arabidopsis* roots, and imaging of plant endomembranes [2, 4, 7–10]. Confocal microscopy also facilitates the usage of multiple labels and increases the utilization of genetically engineered fluorescent proteins (FPs) [3]. Confocal microscopes with spectral imaging capabilities can detect and separate overlapping spectra from multiple fluorochromes [4]. Rapid advancements in immunohistochemistry and development of novel fluorophores combined with confocal imaging have revolutionized imaging of live plant cells. Advanced dyes and conjugate (Alexa Fluor dyes) labeled antibodies are being used for tissue and subcellular organelle localization, while novel fluorophores like quantum dot nanocrystals are used to illuminate various biomolecules [11, 12]. Apart from the great advantages that confocal microscopy provides, there are some challenges encountered during live imaging of plant cells. The most common challenges are phototoxicity, photobleaching, and focal drift [13, 14]. Phototoxicity results in detrimental morphological changes leading to formation of spindle-shaped membrane structures, vacuolation, blebbing, and cell death during the process of image acquisition. Phototoxicity is attributed to the use of higher laser intensity and frequency during acquisition which leads to a rise in temperature or free radical accumulation that damages cellular membranes and lipids during excitation [13]. Photobleaching, on the other hand, is the loss of fluorescent signals over time. There are various factors that can lead to photobleaching, such as frequency of image acquisition, laser power, use of photosensitive fluorescent dyes, and the levels of the FPs expressed in the cell. Focal drift is also a major challenge in live cell imaging [13]. Time-lapse experiments often lead to axial fluctuations that arise as a result of gradual changes during focusing of the sample, leading to difficulty in maintaining the chosen focal plane over a time period [15]. Fluctuations in temperature, inefficient heating systems, intense laser intensity, vibrations in the microscopic instrument, and handling can result in focal drifts [13]. All these factors taken together pose major challenges in live cell imaging that can be overcome by taking the right precautions and making adjustments.

One of the major considerations in live cell imaging is the selection of the appropriate fusion protein. Tagging the protein of interest with the appropriate FP enables

its visualization in plant cells. There are several FPs available with different spectra of excitation and emission like blue, green, yellow, orange, and red. Apart from these, there are other variants such as enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP). Selection of the appropriate FP is based on a number of factors; for example, EYFP can be useful for short-term imaging as it absorbs 50% more light energy per molecule than the enhanced green fluorescent protein (EGFP), it is more prone to photobleaching than EGFP, making it less suitable for long-term time-lapse experiments [16, 17]. In the case of ECFP expressing plant cells, chlorophyll in photosynthetic tissues display higher autofluorescence at this excitation wavelength, and these shorter wavelengths used to excite ECFP are damaging to the tissues, hence making them less suitable for imaging in plants [16]. It is also important to make sure that the selected FP expresses efficiently without any toxic effects to the system, provides sufficient signal above the autofluorescence to be easily detected and imaged, has sufficient photostability, is insensitive to environmental effects and has minimal crosstalk in the excitation and emission channels when used in multiple-labeling experiments [18]. Therefore, it is essential that well-informed decisions be made to choose the right FP based on the tissue type to be observed and the conditions under which live imaging will be performed. There are numerous Web-based resources that provide comprehensive information on subcellular-targeted FP probes for plants that could be useful for FP selection [4, 18–22].

Once the right FP is chosen and expressed in plants, use of optimal laser power, avoiding higher frequency, and limiting multiple excitations would facilitate reducing the intensity of phototoxicity and photobleaching [13]. Use of dyes that require high ultraviolet (UV) excitation should be avoided to prevent phototoxicity in the living cells. Use of antioxidants such as ascorbic acid in the mounting medium can help in reducing the accumulation of free radicals, but it would be useful only when the mechanism of interest is not affected by it [13, 23]. The SDCM offers better protection against phototoxicity than the CLSM. Use of photostable fluorophores over organic dyes can prevent the loss of fluorescent signals caused by photobleaching [13]. Photobleaching is more frequently observed during live cell imaging of small fluorescent organelles like vesicles, compared to larger organelles like nucleus. Optimization can be carried out by adjusting the laser power and acquisition frequency to achieve stable imaging of these small organelles while taking care of photobleaching [13]. Focal drift can be minimized by the installation of an anti-vibration table and careful use of microscopic components [13]. Focal drift caused by the addition of reagents during imaging can be avoided by use of narrow-tipped pipettes. One approach that has been suggested to correct focal drift in long-term time-lapse confocal imaging is imaging the specimen at periodic time intervals to obtain stacks of images, and using software to calculate Pearson's correlation coefficient between each image in the *z*-stacks and the reference image in the stack that was selected at the beginning of the experiment. The maximal correlation coefficient of pixel intensities can be used to identify the image that corresponds to the focal plane of the reference image [15]. Some companies now provide hardware-based real-time focus correction systems to combat axial focus fluctuations caused by long-term time-lapse imaging. Installing these systems could be beneficial when imaging to chase a particular process occurring in a cell.

While carrying out imaging experiments with plants, one has to understand that plants can sense their external environment and respond to stimuli-like light, temperature, osmolality, humidity, gravity, and electric fields; they also have internal circadian rhythms that can affect gene expression [16]. Optimizing culture and imaging conditions are a must for successful live cell imaging of plants. Here, we have attempted to provide a detailed step-by-step protocol for live cell imaging of *Arabidopsis* roots expressing a YFP fusion protein.

13.2 Materials

13.2.1 Transgenic Arabidopsis Seeds

Transgenic *Arabidopsis* plants expressing the YFP fusion protein were generated by introducing a binary vector pCAMBIA1301 carrying the gene of interest as a C-terminal YFP fusion in *Agrobacterium tumefaciens* GV3101 by electroporation and then carrying out the plant transformation by floral dip method [24]. The harvested seeds from transformed plates were surface sterilized and spread on a Murashige and Skoog (MS) medium containing hygromycin to screen and select transgenic lines. Seeds harvested from the transgenic lines were used for this study.

13.2.2 Seed Sterilization Solution

For surface sterilization of *Arabidopsis* seeds, 70% ethanol is made by mixing 30% water with absolute ethanol to make the desired volume, and 30% commercial Javex bleach solution is made by mixing 70% water with the required volume of bleach solution.

13.2.3 Seed Germination and Growth Medium

The seed germination medium consists of 50% strength MS basal medium (contains MS salts and vitamins, with 1% sucrose [25]. The pH of the medium is adjusted to 5.7 and 5 g/L plant agar (PhytoTechnology Laboratories) is used as the gelling agent.

13.2.4 Equipment and Laboratory Supplies

A Leica TCS SP5 microscope was used for this study. A laminar flow hood, 1.5 mL microfuge tubes, microcentrifuge, forceps, sterile filter paper, Petri dish, Parafilm,

Kimwipes, 200–1000 μL pipettes, sterile tips, sterile culture tubes and beakers are the supplies needed for this experiment.

13.3 Methods

13.3.1 Arabidopsis Seed Sterilization and Germination

1. Perform all procedures in a laminar flow hood (*see Note 1*). Keep 20–100 transgenic *Arabidopsis* seeds in 1.5 mL microfuge tubes and wash for 1 min with 1 mL of 70 % ethanol. Remove ethanol with sterile pipette and wash three times with 1 mL sterile water (centrifuge between washes).
2. Incubate the seeds with 1 mL 30 % bleach solution for 10 min at room temperature and shake well. Remove the bleach solution with a pipette followed by three washes (5 min each) with 1 mL sterile water (centrifuge between washes).
3. Pipette seeds on a sterile filter paper, and let the seeds dry. Once the water has evaporated, place 10–20 seeds per Petri dish containing seed germination medium with 25 mg/L hygromycin using a sterile pair of forceps (*see Note 2*).
4. Cover the Petri dish, wrap with Parafilm and keep it in the dark at 4°C for 3 days, and then transfer to a culture room at 22–23 °C under a 16 h photoperiod with a light intensity of 102 μmol/m^2 for germination (*see Note 3*). The plates should be kept vertical, to allow germination and vertical growth of roots for 3–7 days (Fig. 13.1).

13.3.2 Imaging Procedure: Settings for the Confocal Microscope

1. We are using a Leica SP5 inverted confocal laser scanning microscope for this study. Read the user manual instructions before switching on the microscope (http://www.leica-microsystems.com).

Fig. 13.1 Seven-day-old *Arabidopsis* seedlings grown vertically on germination medium for the live cell imaging of plant roots

2. Switch on the workstation (computer switch) at the main switchboard and log on to the computer.
3. Turn on the microscope. Switch on the scanner on the main switchboard, followed by the laser. Turn on the laser power in the supply unit by activating the detachable-key switch on the main switchboard. You could also activate the external UV laser by activating the key switch on the front of the power supply.
4. Start the software by clicking on the icon on the desktop and wait till the initialization process is complete.
5. Set up the pinhole size to 1 airy unit (AU) to obtain the highest quality image versus brightness trade-off [26] (*see Notes 4* and *5*).
6. Set up the scan average level of image acquisition and make sure that the confocal microscope is well calibrated as per user manual instructions (*see Note 6*).
7. Ensure that the laser is warmed up for a sufficient time (allow at least 30 min) before imaging.
8. Select bit depth for your images. For most cases, 8-bit is recommened [26] (*see Note 7*).
9. Set the laser power and gain controls (*see Note 8*). Ensure that the pixels of interest are not over or underexposed.

13.3.3 Mounting and Observation

1. *Arabidopsis* seedlings as young as 3 days post-germination up to several weeks of age can be used for imaging roots [27]. It usually takes 3–4 days for roots to be ready for imaging. We have selected a 7-day-old seedling for live cell imaging.
2. Gently remove the YFP-expressing *Arabidopsis* seedlings from media using forceps, taking care not to damage the roots. Place it gently on a glass slide with water as the mounting medium (*see Note 9*). Carefully place a glass coverslip on top of the glass slide for microscopy. When using an inverted microscope, ensure that fixed specimens on the slides are inserted with the coverslip facing down. Mount the coverslip on the stage of the confocal microscope for observation (*see Notes 10–13*).
3. Select the objective under which you want to observe the specimen. In case of immersion objective, ensure that you apply adequate quantity of immersion medium (immersion oil, 80% glycerol or water) between the front lens of the objective and the specimen. In our experiment, we have selected the 40× oil immersion objective (*see Note 14*).
4. Select visual mode for observing the specimen through eyepieces and find a suitable position and focus on the specimen. In this case, you would look through the specimen and focus on a region of the plant root (*see Note 15*).
5. To obtain thin optical sections, it is essential to illuminate the specimen over the smallest possible area.

13.3.4 Image Acquisition

Considerations: It is useful to scan the liquid medium in a spectrometer (400 to 650 nm wavelength) to make sure that peaks are absent within the excitation and emission range (*see Note 16*). The YFP fusion proteins will be excited using 488 nm argon laser and emission collected at 500–535 nm. For rapid scanning without bleaching, set the scanner to 512 × 512 scan format. This means that the area to be scanned will be scanned with 512 lines with each line containing 512 pixels. Therefore, a zoomed-in area will be scanned with very high resolution with 512 × 512 scanning compared to a zoomed-out area scanned with 512 × 512 format. The scan speed will be set to 400 Hz to minimize exposure of the tissue to laser. It is imperative to do a λ scan (all wavelengths 400–700 nm) using the control (non-transgenic line) to establish the autofluorescence observed at the various wavelengths. If there is any autofluorescence observed at the YFP emission range (500–535 nm), standardize the lowest laser intensity and detector gain at which no autofluorescence will be observed in the control tissue. This has to be done using at least three controls prior to processing the transgenic lines. Following this, mount the transgenic line similarly and process the same way using the appropriate emission bandwidth. Make sure that the detector gain and laser intensity are never tuned above the level where autofluorescence was observed in the control tissue.

1. To begin data acquisition, one has to click the "Live" button. All the data acquired would be transferred to the video memory and displayed on the monitor continuously. You could capture the image and store it for later use once all the parameters are standardized and the image is of high quality.
2. It is possible to acquire a series of images (time series or *z*-stacks) in which the parameters are changed incrementally while scanning the individual images.
3. Use a "LiveDataMode" to store captured images automatically while changing setting parameters, manipulating the specimen, or bleaching sequences between individual scans.
4. Determine the intensity at which laser lines are used while scanning to obtain a noise-free image of the specimen and reduce specimen deterioration (*see Notes 17–20*).
5. Take several images of each sample to ensure reliability of results (*see Note 21*). We have observed nuclear and plasma membrane expression of the YFP-tagged protein in root hairs and exclusive nuclear localization in the root tip (Fig. 13.2a and b).
6. 6. It is advisable to reconfirm the expression pattern of your fusion protein with an organelle marker. For example, we have used FM4-64 dye, which localizes to the plasma membrane to ensure plasma membrane localization of our fusion protein by checking its co-localization with FM4-64 signal (*see Note 22*).
7. For FM4-64 staining, the transgenic *Arabidopsis* seedlings were taken out of the media and incubated in 20 μM FM4-64 styryl dye (Molecular Probes) for

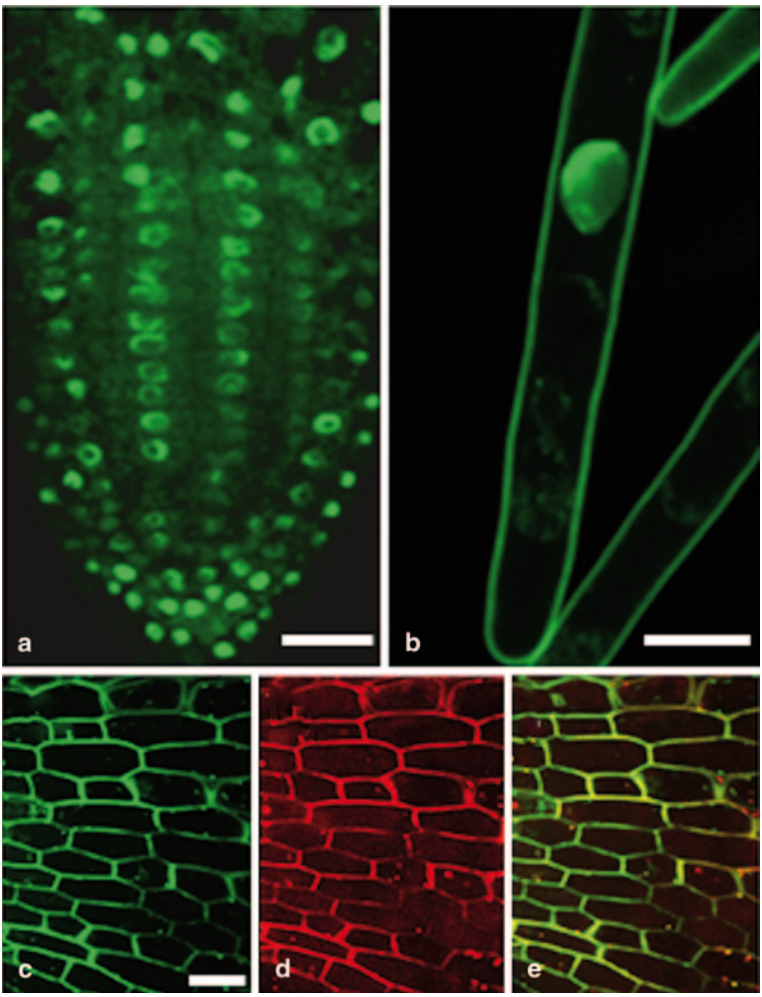

Fig. 13.2 Confocal images of plant root cells for the localization of YFP-tagged protein. Scale bars: **a** = 25 μm, **b** = 13 μm, and **c**–**e** = 38 μm

10 min. The seedlings were then taken out and quickly washed with distilled water before mounting on the slide. The maximum excitation wavelength for FM4-64 is 515 nm, argon ion laser at 488 or 514 nm should be used to image FM4-64 localization.

8. After imaging of the roots with the FM4-64 styryl dye, imaging for YFP localization is carried out in the same sample, in the same plane and a co-localization analysis of the saved images can be performed by using an ImageJ software [26]. A clear co-localization of our YFP signal with the FM4-64 signal was observed in our case (Fig. 13.2c, 13.2d, and 13.2e).

13.3.5 Finishing Up the Experiment

1. Take out the mounted slide from the microscope stage carefully.
2. Save all the image data and close the confocal software on the desktop.
3. Switch off the lasers in the supply unit and the external UV laser with the key switch.
4. Shut down the computer and turn off all the switches on the main board of the workstation that includes the scanner and laser.
5. Switch off the microscope and fluorescence lamps.
6. Perform a proper clean-up of the workstation and cover the microscope.

13.4 Notes

1. Perform all seed sterilization steps under sterile conditions in a laminar flow hood to reduce the chances of any fungal or bacterial contamination.
2. Prepare all tissue culture plates 1 day prior to use and keep them in the dark at room temperature for 24 h before use.
3. It is necessary to make sure that the plants used for imaging were grown in optimal growth conditions including light, temperature, osmolality, humidity, and nutrition [28]. In case the seedlings were raised in tissue culture media, it is essential to use the right pH for the media and make sure they were kept under controlled optimal growth conditions.
4. In confocal microscopy, a pinhole aperture is present in front of a signal detector. This helps in blocking the out-of-focus light producing a very sharp and clear image and enables generation of 3D images of the fluorescent biological specimens.
5. The process of collecting light from the focal planes of 3D objects by the pinhole is called optical sectioning [29]. The optical sections become thinner when the size of pinhole is reduced. Images of higher resolution can be achieved by using lenses with higher numerical aperture (NA) and a narrow pinhole diameter [3]. While it is important to choose a smaller pinhole, it is advisable not to close the pinhole too far.
6. Scan averaging and line averaging has the advantage of being resistant to noise effects, but it could increase photobleaching. So, optimization is essential to ensure image quality.
7. Bit depth determines the number of gray levels that can be differentiated in an image. Usually, 256 levels can be differentiated by 8-bit and 4096 levels for 12-bit [26].
8. High laser power can lead to photobleaching and phototoxicity in the living cells; keep laser power low.
9. Ensure that seedlings have rooted before taking them out of the germination medium. Do not exert too much pressure when covering the mounted seedling with the cover slip to avoid any damage to the roots.

10. While carrying out wet mounting of specimens, it is advisable to use the same medium used for the growth. However, culture media like MS [8], Schenk and Hildebrandt (HS), or Gamborg's B-5 may be unsuitable for specific imaging experiments even if they provide excellent osmotic balance [28]. It is advisable to use water-immersion lenses when using specimens mounted in less viscous mounting media.
11. The media selected should not alter the properties of fluorescent components or the refractive index. There is always a chance that the specimen would get heated and dehydrated if a liquid medium is not used.
12. There is a wide range of glassware, slides, and coverslips available in the market for live cell imaging. A glass component of 0.17 mm thickness, having an NA close to 1.5 is ideally recommended for live cell imaging [13].
13. One should also consider the cost, nature of experiment, and compatibility of microscope system while selecting the glassware for live cell imaging. Glass coverslips are cost effective for short-term imaging while glass-bottomed dishes are useful while carrying out long-term imaging (>20 h). Glass-bottomed dishes allow for easy handling without exposure of the tissues to the external environment [13]. When using an automated focus system, the compatibility of glassware with the microscope should be checked to avoid any optical problems.
14. Read the safety instructions in the user manual carefully and keep your eyes at a safe distance of at least 20 cm from the opening of the objective when you are using a laser-scanning microscope.
15. It is advisable to use fresh seedlings for imaging after every 30 min [7]
16. Impurities in the medium and added hormones or vitamins could give rise to these peaks and it is essential to eliminate them [28]. Presence of sugar or high molecular weight compounds in the medium can alter the refractive index and presence of agarose or other gelling agents can cause hindrance in generation of contrast like differential interference contrast (DIC), hence it would be good to avoid them, in the medium [28].
17. The signal-to-noise ratio is influenced by the speed of data capture in addition to the illumination intensity. Hence, it is essential to optimize the scanning speed to get high-quality images.
18. Make sure to close the shutter of the mercury lamp when you are not looking into the microscope as the specimen fluorescence could fade quickly.
19. The image quality in confocal microscope depends on whether the optical system in the image path is aligned with the illuminated pattern. Both axial and lateral alignment should be considered for getting optimal results.
20. Fluorophore saturation at the point(s) of illumination determines the maximum speed limit at which images can be obtained [30]. Illuminating an extended pattern in the specimen, such as an array of spots or a line can reduce fluorophore saturation [30].
21. Repeat the experiment a few times taking the biological variations into consideration; use different seedlings each time to have enough biological replicates.

22. FM4-64 dye can also be employed to monitor endocytic pathways in *Arabidopsis* root cells [31]. Take safety precautions while using FM4-64. FM4-64 should be protected from light and kept in a dry and cool place.

13.5 Summary

Live cell imaging has revolutionized the way in which cellular events are studied in a living organism. Recent advancements in fluorescent tools, development of new optical systems, fusion proteins, and biosensors enable us to understand the dynamics of cellular events. It is now possible to observe complex molecular interactions within a cell in greater detail with live cell imaging and superresolution microscopy. Successful live cell imaging in plants depends on optimization of microscopy settings including temperature control, optimal culture environment for plant growth, and use of the right fluorescent components. It is also essential to make attempts to overcome the problems of phototoxicity, photobleaching, and focal drift.

Acknowledgments MAS is supported by the Natural Sciences and Engineering Research Council of Canada funding. SS is supported by the Eyes High International Doctoral Scholarship and a Global Open Doctoral Scholarship from the University of Calgary. We thank Muhammad Jamshed for his technical assistance with the confocal microscope.

References

1. Misri R, Pande S, Khopkar U (2006) Confocal laser microscope. Indian J Dermatol Venereol Leprol 72:394–397
2. Wymer CL, Beven AF, Boudonck K, Lloyd CW (1999) Confocal microscopy of plant cells. Meth Mole Biol 122:103–130
3. Paddock SW, Eliceiri KW (2014) Laser scanning confocal microscopy: history, applications, and related optical sectioning techniques. Meth Mole Biol 1075:9–47
4. Held MA, Boulaflous A, Brandizzi F (2008) Advances in fluorescent protein-based imaging for the analysis of plant endomembranes. Plant Physiol 147:1469–1481
5. Oreopoulos J, Berman R, Browne M (2014) Spinning-disk confocal microscopy: present technology and future trends. Meth Cell Biol 123:153–175
6. Lam PY, Fischer RS, Shin WD, Waterman CM, Huttenlocher A (2014) Spinning disk confocal imaging of neutrophil migration in zebrafish. Meth Mole Biol 1124:219–233
7. Sampathkumar A, Wightman R (2015) Live cell imaging of the cytoskeleton and cell wall enzymes in plant cells. Meth Mole Biol 1242:133–141
8. Samuel MA, Tang W, Jamshed M, Northey J et al (2011) Proteomic analysis of *Brassica* stigmatic proteins following the self-incompatibility reaction reveals a role for microtubule dynamics during pollen responses. Mol Cell Proteomics 10(12):M 111 011338
9. Marcos D, Berleth T (2014) Dynamic auxin transport patterns preceding vein formation revealed by live-imaging of *Arabidopsis* leaf primordia. Front Plant Sci 5:235
10. Sampathkumar A, Krupinski P, Wightman R, Milani P et al (2014) Subcellular and supracellular mechanical stress prescribes cytoskeleton behavior in *Arabidopsis* cotyledon pavement cells. eLife 3:e01967

11. Muller F, Houben A, Barker PE, Xiao Y et al (2006) Quantum dots-a versatile tool in plant science? J Nanobiotechnol 4:5
12. Smith AM, Gao X, Nie S (2004) Quantum dot nanocrystals for *in vivo* molecular and cellular imaging. Photochem Photobiol 80:377–385
13. Tan TTT, Khaw C, Ng MML (2010) Challenges and recent advances in live cell bioimaging. In: Méndez-Vilas, A (ed), Microscopy: science, technology, application and education. Formatex Research Center, Spain, pp. 1495–1505
14. Frigault MM, Lacoste J, Swift JL, Brown CM (2009) Live-cell microscopy—tips and tools. J Cell Sci 122:753–767
15. Kreft M, Stenovec M, Zorec R (2005) Focus-drift correction in time-lapse confocal imaging. Ann New York Acad Sci 1048:321–330
16. Fang Y, Spector DL (2010) Live cell imaging of plants. Cold Spring Harb Protoc. doi:10.1101/pdb.top.68
17. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN et al (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nature biotechnol 22:1567–1572
18. Shaner NC, Steinbach PA, Tsien RY (2005) A guide to choosing fluorescent proteins. Nature methods 2:905–909
19. Mathur J, Radhamony R, Sinclair AM, Donoso A et al (2010) mEosFP-based green-to-red photoconvertible subcellular probes for plants. Plant Physiol 154:1573–1587
20. Mathur J (2007) The illuminated plant cell. Trends Plant Sci 12:506–513
21. Mano S, Miwa T, Nishikawa S, Mimura T, Nishimura M (2008) The plant organelles database (PODB): a collection of visualized plant organelles and protocols for plant organelle research. Nucleic Acids Res 36 (Database issue):D929–D937
22. Mano S, Miwa T, Nishikawa S, Mimura T, Nishimura M (2009) Seeing is believing: on the use of image databases for visually exploring plant organelle dynamics. Plant Cell Physiol 50:2000–2014
23. Knight MM, Roberts SR, Lee DA, Bader DL (2003) Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death. Am J Physiol Cell Physiol 284:C1083–C1089
24. Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
25. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473–497
26. French AP, Mills S, Swarup R, Bennett MJ, Pridmore TP (2008) Colocalization of fluorescent markers in confocal microscope images of plant cells. Nat Protoc 3:619–628
27. Haseloff J, Dormand EL, Brand AH (1999) Live imaging with green fluorescent protein. Meth Mole Biol 122:241–259
28. Shaw SL (2006) Imaging the live plant cell. Plant J 45:573–598.
29. Conchello JA, Lichtman JW (2005) Optical sectioning microscopy. Nat Method 2:920–931
30. White J (2014) Reflecting on confocal microscopy: a personal perspective. Meth Mole Biol 1075:1–7
31. Rigal A, Doyle SM, Robert S (2015) Live cell imaging of FM4-64, a tool for tracing the endocytic pathways in *Arabidopsis* root cells. Meth Mole Biol 1242:93–103

Chapter 14
Immunogold Labeling for Electron Microscopy: Strategy and Problem Solving

Fengli Guo and Bing Quan Huang

14.1 Introduction

Immunoelectron microscopy (IEM) is a very useful technique that demonstrates simultaneously the precise location of an antigen and its ultrastructural environment at nanometer resolution. Preservation of ultrastructure and antigenicity are two critical aspects in this technology. Although proper cellular structures can be preserved using traditional EM processing methods, very few antigens can survive after the conventional osmium tetroxide fixation and epoxy embedding [1]. In the 1970s, several breakthroughs occurred for IEM. The development of cryofixation–freeze substitution (FS) and the ultrathin cryosectioning techniques, together with the introduction of new embedding resins (i.e., Lowicryl and LR White resins) [2–6], enabled the preservation of antigens and their detection at the ultrastructural level. With the combination of colloidal gold conjugation techniques and improved cryomethods, IEM, especially in conjunction with immunogold labeling, has become a widely used technique for antigen detection [7–10].

In recent years, cryofixation has proven superior to conventional chemical fixation methods for morphological and antigen preservation. For most plant tissues, high-pressure freezing and freeze-substitution (HPF-FS) are necessary for optimal fixation with minimum freezing damage, especially for ultrastructural studies [11]. Samples processed by HPF-FS can be dehydrated and embedded in acrylic resins, or the user may proceed directly to ultrathin cryosectioning for immunolabeling (Fig. 14.1). Although conventional methods may work better than cryofixation for image contrast [9], the choice of sample processing and labeling method always depends on tissue type and antigen abundance, property, and distribution. A comparative study using different methods is necessary for the best result.

F. Guo (✉)
Stowers Institute for Medical Research, Kansas City, Missouri 64110, USA
e-mail: feg@stowers.org

B. Q. Huang
Center for Basic Research in Digestive Disease, Mayo Clinic, Rochester, Minnesota, USA

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_14

14.1.1 Choice of Labeling Methods

When performing immunolabeling there are four main methods of labeling, depending on the conditions of the samples (Fig. 14.1). These include: (a) pre-embedding labeling, (b) post-embedding labeling on chemically fixed sections, (c) post-embedding labeling on sections of cryofixed samples, and (d) immunolabeling on thawed ultracryo-sections. Each of these approaches has advantages and limitations. The method of choice depends on the user's experimental design, equipment, and materials available. However, one should have a good preliminary understanding of

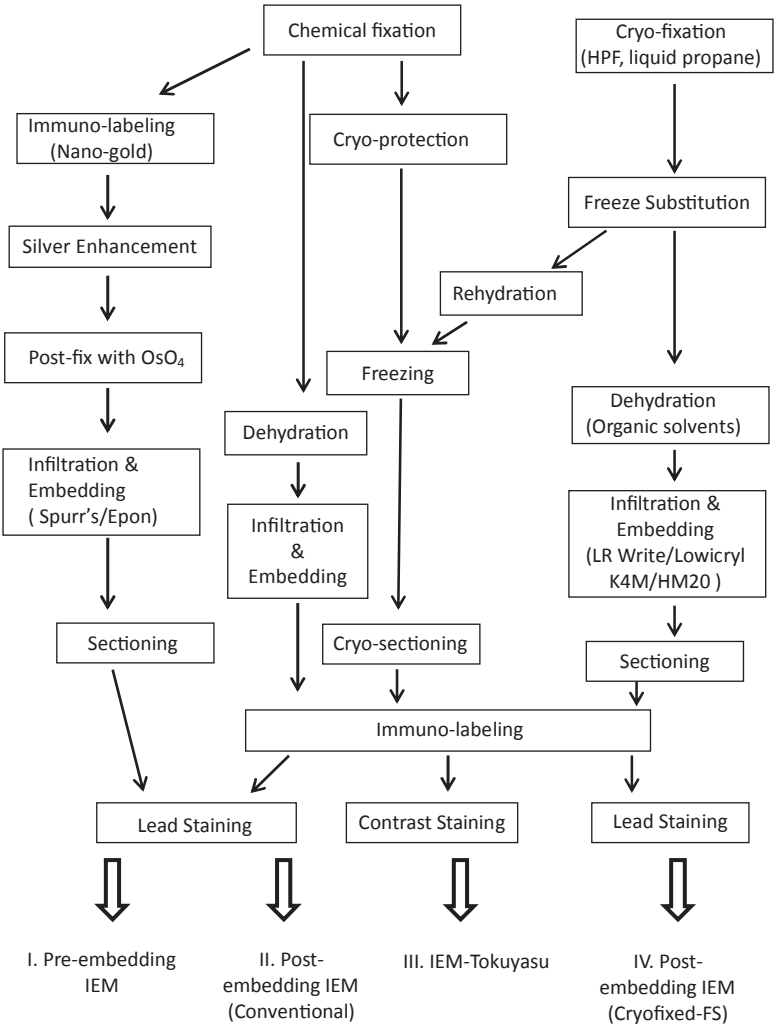

Fig. 14.1 IEM procedures for plant cells and tissues. Schematic representation of the main IEM approaches used for protein localization at ultrastructural level in plant cells

the experimental system before embarking on an ultrastructural investigation. The answers to the following questions may aid in the selection and design of a protocol. What is (are) the possible site(s) of localization? Is the antigen located inside the cytoplasm or nucleus, or on the cell surface? Is the antigen bounded on the cell membrane, or as a soluble protein in the cytoplasm? Can the antigen be located on some subcellular organelles? Most importantly, can the antigen be chemically fixed and resin embedded or can it be preserved and labeled by cryofixation methods only?

Pre-embed labeling dictates that the sample pieces or blocks are labeled before processing for ultrastructural studies. After mild chemical fixation, specimens are treated with detergent, i.e., Triton X-100, to permit the antibody to access the target antigen. Since specimens are subsequently post-fixed with osmium tetroxide and embedded in an epoxy resin, morphological preservation is superior to other labeling methods. However, as most plant cells have walls and other barriers that render cytoplasmic penetration to antibody very difficult, this technique has limited applications in plant cells [12] and is not applicable to most plant immunological studies at the EM level (Fig. 14.1, Method I). Therefore, we do not intend to describe and discuss this method further in this chapter. For details, readers are referred to relevant references [13, 14].

Post-embedding labeling is the labeling of plastic ultrathin sections after embedding the tissues in a resin (Figs. 14.2 and 14.3). Specimens are fixed and processed either by a chemical fixation method (Fig. 14.1, Method II) or by use of cryofixation and FS procedures (Fig. 14.1, Method IV), followed by embedding in a hydrophilic acrylic resin such as LR White or Lowicryl resin.

For Method II (Fig. 14.1), if the antigen is well preserved in paraformaldehyde (PFA) and can be labeled on paraffin-embedded sections by immunocytochemical

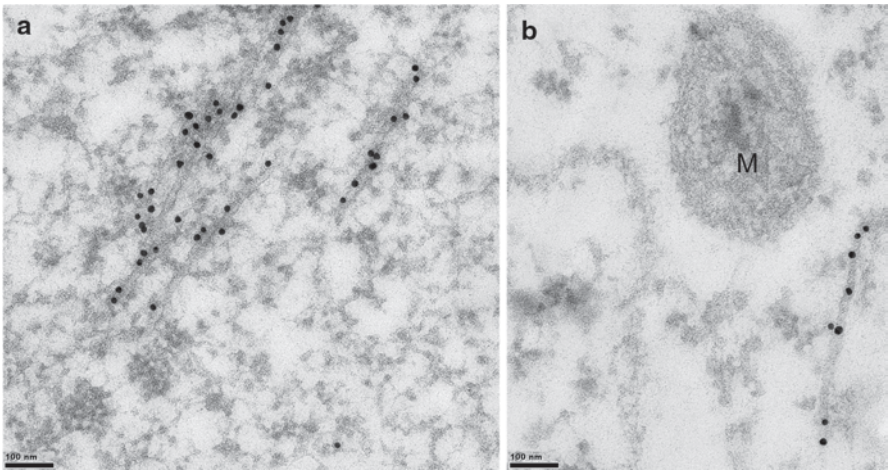

Fig. 14.2 Immunogold labeling of microtubules in the ovules of Chinese pine (*Pinus tabulaeformis*) [26, 27]. **a** Polyribosome-decorated microtubule bundles labeled with 10 nm gold particles in the egg cells. **b** A single microtubule in the sheath cells on the same section. *M* mitochondrion. Bar = 100 μm

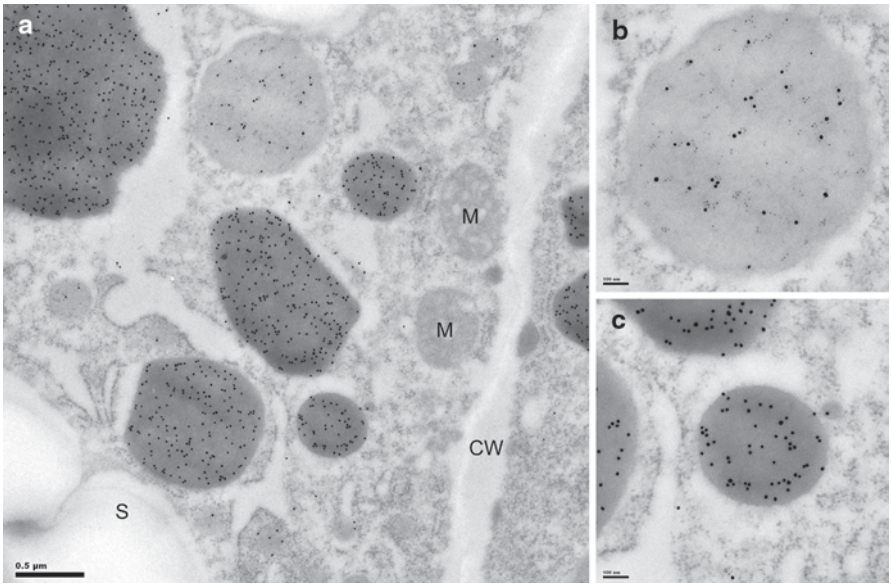

Fig. 14.3 Immunogold labeling of protein bodies in rice endosperm [28]. **a** A low magnification of portion of the endosperm area showing two types of gold-labeled protein bodies. *CW* cell wall, *M* mitochondrion, *S* starch granule. Bar = 500 nm. **b** Higher magnification of a protein body with double gold labeling. **c** Higher magnification of another protein body in the same cell with single gold labeling. Bar = 100 nm

staining or by immunofluorescence, the chemical fixation and LR White embedding methods are an option (Fig. 14.1, Method II). These methods are easily performed and do not require special equipment. Resin-embedded tissues are easy to section for immunolabeling, especially for larger tissue blocks. This method is suitable for processing most plant tissues, including root, stem, leaf, seed, and fruit. The main drawback with the post-embed labeling method is that only those antigens that are exposed on the surface of the section can be labeled by the antibody (Fig. 14.6), and therefore give less signal. The resin may also interfere with antigenicity and produce some background staining [15]. Furthermore, for plant cells with inherent features such as waxy cuticle, rigid cell wall, or large vacuoles, or for those undergoing rapid structural changes (e.g., fertilization), ultrastructure is often poorly preserved by chemical fixation and artifacts sometimes occur due to cell autolysis before complete fixation (*see* Chap. 2). Hence, cryofixation should be considered and is the preferred method.

Immunogold labeling of thawed ultrathin cryosections (also called Tokuyasu method, Figs. 14.1, 14.4, 14.5, Method III) is widely used to study cell biology of animal tissues but has limited applications for plant cell studies [16]. The main advantages are that antigens remain in a hydrated environment prior to immunolabeling, and that antigen accessibility is improved by avoiding resin embedding of samples. This approach greatly preserves the antigenicity of the specimen. Furthermore, visualization of cell membranes is excellent (Figs. 14.4 and 14.5) and

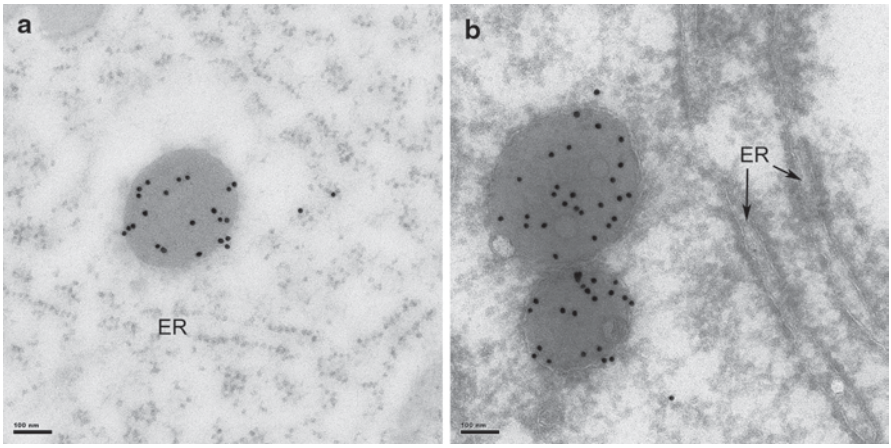

Fig. 14.4 Comparison of immunogold labeling of rice protein bodies using different methods. **a** Immunogold labeling on LR White-embedded sections. Bar = 100 nm. **b** Immunogold labeling on thawed ultracryo-sections (Tokuyasu). Bar = 100 nm

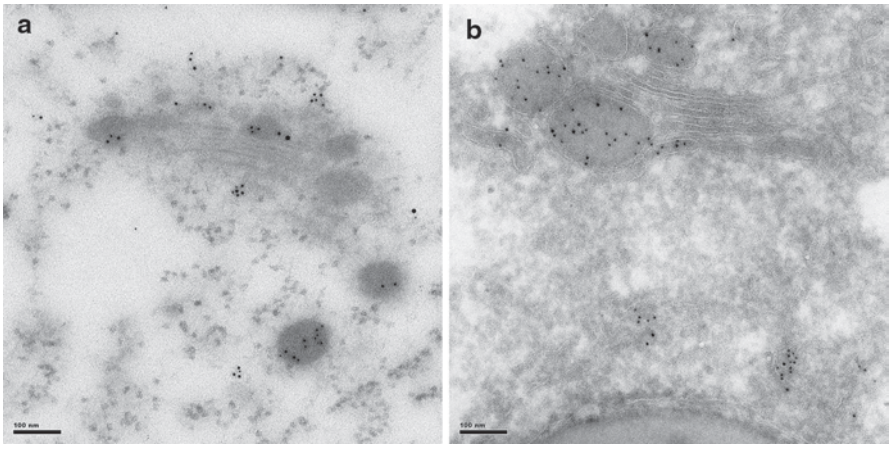

Fig. 14.5 Immunogold labeling of rice protein on Golgi apparatus of the endosperm cells. Bar = 100 nm. **a** LR White-embedded sections. **b** Immunogold labeling on thawed ultracryo-sections (Tokuyasu). Bar = 100 nm

multiple antigens can be visualized by the use of gold particles of different sizes. Therefore, it is the best option for immunolabeling of membrane-bound proteins and vesicles related to protein trafficking in both animal and plant cells [17, 18]. However, the drawback of this method, like conventional IEM (Method II), is its dependence on initial chemical fixation. For those antigens which cannot be properly fixed using aldehydes, the antigen may be lost during fixation and subsequent immunolabeling processing [15]. Moreover, ultrathin cryosectioning requires more specialized equipment and section handling is difficult. Obtaining good, large, and/

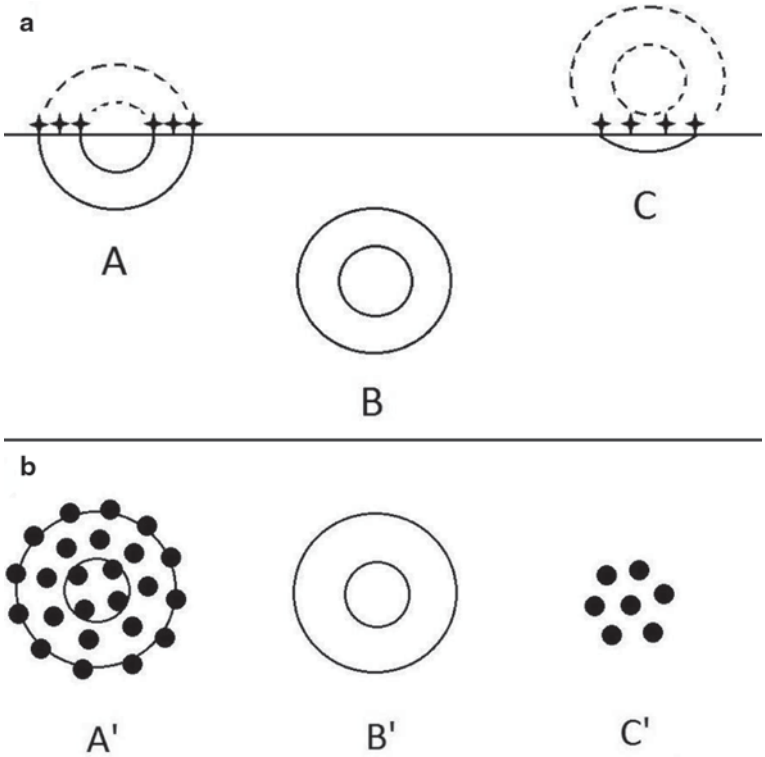

Fig. 14.6 Different labeling patterns of the same target structure on the cutting surface of resin-embedded sample. **a** Side view of a plastic section showing exposed structure and antigen (*). **b** Top view of the same section as showed in (**a**). The same target structure can be labeled differently depending on its location in the section

or serial sections is difficult but not impossible after some practice. This method is better than Method II for preserving cell and organelle membranes, and is suitable for protein trafficking or other cell biological studies.

In Method IV (Fig. 14.1), cryofixation by high-pressure freezing (HPF) or propane-jet freezing rapidly immobilizes and completely arrests cellular processes in seconds. The tissue is fixed by physical rather than chemical methods. This technique allows the preservation of not only the fine structure of the tissue and cells but also antigenicity for antibody recognition [7, 8]. In addition, FS and embedding at low temperature can minimize extraction of cell components by organic solvents and resins. It is particularly useful for those difficult-to-fix plant tissues that are poorly fixed by conventional chemical fixation. However, like conventional IEM, not all antigens can be immunolabeled after applying this method due to the inaccessibility of the resin section (Fig. 14.6) or the inactivation of some antigens by organic solvents and resins. To overcome this problem, cryofixation without chemical fixation and the cryosectioning technique are combined (Fig. 14.1) [15, 19]. After cryofixation and ultracryomicrotomy, sections are rehydrated and further processed

for immunogold labeling. In spite of the advantages of this combined approach, its application to plant cell studies is still limited due to the following reasons: (1) the equipment for HPF or propane-jet freezing is expensive; (2) sometimes the results for cryofixation are not consistent due to experimental conditions and operation; (3) only a limited size of tissue (up to 500 μm for HPF, 30–50 μm for propane-jet freezing) is well preserved and ice crystal damage frequently occurs; and (4) physical damage to tissues is common during sample handling and cryofixation.

14.2 General Technical Comments

14.2.1 First Things First

It is important to have a thorough knowledge of the specimen at the light microscopic (LM) level before embarking on an ultrastructural study (*see* Chaps. 1 and 2). This background knowledge ensures proper identification of cell types within the specimen. One needs to pretest the suitability of different fixation schemes (*see* Chap. 2). Before performing the IEM experiment, one should ascertain whether the same antibody has been successfully labeled on chemically fixed paraffin sections, fluorescence whole-mount preparations, or cryosections. If there is no information on the specific antibody that will be used for IEM, a preliminary labeling study using immunofluorescent staining is always necessary before immunogold labeling for EM. Samples must be fixed using the same conditions as for IEM. At the same time, negative controls using pre-immune sera/antibodies and positive controls should be run. Ideally, the specimens should have a strong fluorescent signal, with very low or absent background signal. Second, one should make a reference survey or perform experiments to check if the protein or antigen exists on the target component or membrane by biochemical studies (e.g., Western blot). Third, one should know the property of protein or antigen. For example, is it a membrane-bound protein or a cytoplasmic soluble protein? Is it abundant within the cell? All this information is required before choosing the best IEM method, and before determining whether modification to the protocol is necessary, such as increasing the sensitivity of the method by amplifying the weak immunolabeling signal.

14.2.2 Fixative and Fixation for Immunolabeling

Aldehydes are the most common chemical fixing agents for immunolabeling. Glutaraldehyde (GA) is a strong fixing agent commonly used for conventional EM. It can cross-link proteins irreversibly but penetration is relatively slow. Formaldehyde or PFA is a mild fixing agent, and fixation is partly reversible during buffer washing. Penetration of tissue is rapid due to smaller molecule size.

In most IEM cases, fixative choice for preservation of cell morphology and antigenicity represents a compromise. Mild chemical fixation is better for antigen preservation but limited for preservation of fine cellular structures. Therefore, the concentration of fixing agents used, especially for GA in a fixative mixture of GA and PFA, should be carefully considered. In general, GA can be used without sacrificing the efficiency of immunogold labeling. The concentration of GA needs to be experimentally determined. In general, the concentration of GA for IEM is between 0.005 and 0.5%.

We use a combination of 4% PFA and 0.01% GA for most of the experiments. For some sensitive antigens, 2–4% PFA only can be used for cryosections. To increase the effect of fixation, PFA should be freshly made before fixation (*see* Chap. 2). If the antigen is sensitive to GA and fixation by PFA alone gives poor results, periodate-lysine-paraformaldehyde (PLP) is an alternative fixative. This fixative mixture has been successfully used for animal cells [20]. It has less deleterious effects on epitopes and preserves the morphology of the cells. Fixation by PLP occurs through stabilization of carbohydrate moieties by periodate oxidation followed by lysine cross-linking of carbohydrate groups (*see* Chap. 2).

Before processing for immunogold labeling, one should evaluate fixation results by cutting and staining thick sections (stain with 1% Toluidine Blue O) and observing the cell morphology and profile at the LM level. If the result of fixation is suboptimal, one should stop the immunolabeling and reevaluate fixation conditions. Obtaining acceptable morphological preservation is the first important step toward high-quality immunogold labeling.

As previously detailed, if the use of chemical fixation cannot achieve optimal results with well-preserved antigenicity, one should use cryofixation–FS methods. The cryofixation–FS process can be combined with either plastic embedding or Tokuyasu methods to preserve the fine structure of the cell and antigenicity of difficult-to-fix tissues. FS with GA or even OsO_4 at low temperature not only preserves the ultrastructure well but also maintains antigenicity [21]. However, ice damage is a very common problem with cryofixation–FS, especially for larger-sized tissue. Therefore, one should section different parts of the tissue to evaluate the fixation result. In most cases, ice crystal damage to some portions of tissue is unavoidable. For details of cryofixation–FS for IEM, *see* Chap. 7.

14.2.3 Embedding Resins for Chemically Fixed and Cryofixed Tissues

Currently, commonly used embedding media for immunolabeling include hydrophilic polar resins (Lowicryl K4M, K11M, LR Gold, and LR White) and hydrophobic apolar resins (Lowicryl HM20 and HM23). These resins have low freezing points and viscosities (even at low temperature), and can be polymerized at low temperature. They demonstrate good cutting properties, with stability to the electron beam, and the plastic has low electron scatter properties for high-resolution imaging. In practice, the more hydrophobic HM20 has become widely used in immunohistochemistry and FS in EM tomography [1].

LR White is a low-viscosity, low-toxicity, and hydrophilic resin. Commercial products include soft, medium, and hard grade. Hard grade is normally suitable for biological materials, though medium grade is also sometimes used. Two different commercial products are available: catalyzed LR White (catalyst- or activator-added) and noncatalyst LR White (catalyst and resin are separated, and mixed before use). Storage of catalyst LR White is not recommended for long time periods, even in the refrigerator, since it can autopolymerize (*see* product manual). The "catalyzed" LR White can be easily polymerized with either heat (55–60 °C) or UV irradiation (365 nm) at low temperature (0–4 °C). A graded ethanol series is used for sample dehydration before embedding. Since LR White is compatible with a small amount of water, samples can be dehydrated up to 70 % ethanol and infiltration of resin can begin. Incomplete dehydration up to 70 % ethanol may reduce extraction of the organic solvent and retain antigenicity. For further discussion, *see* Chap. 6. Infiltration and embedding with LR White are easy for almost all plant tissues and suitable for both histological thick sections and EM ultrathin sections. Ultrathin sections are stable under electron beam for a long period of time during examination. Most cellular ultrastructure and organelles are detectable after proper counterstaining. Disadvantages of LR White include that oxygen interferes with the polymerization process, extraction of macromolecules and shrinkage of specimen are possible, and some delicate membrane-bounded vesicles can be damaged (Figs. 14.4 and 14.5).

14.2.4 Plastic LR White Sections vs. Frozen Sections

Ultrastructure preservation is another important issue in addition to the immunolabeling. LR White embedding can preserve most of the cellular structure, including microtubules and microfilaments (Fig. 14.2), but can cause damage to membranes and vesicles by some extraction during infiltration and embedding. Contrarily, the Tokuyasu method is best for visualizing the membrane system or membrane-bound organelles, although details of the cytoskeleton including microtubules and actin filaments can be lost (Figs. 14.4 and 14.5). Moreover, large cryosections are difficult to obtain because there is no resin support for the frozen tissue. During cryosectioning, the surface tension of sucrose tends to overstretch the sections and results in artifact formation. In this case, the use of a mixture of 2 % methyl cellulose and 2.3 M sucrose (at 1:1 ratio) as a pickup solution is recommended to obtain better-quality sections [22].

14.2.5 Primary Antibodies

The primary antibodies used in immunohistochemistry can be antiserum or purified monoclonal or polyclonal antibodies. In general, the use of purified antibodies is preferred for immunogold labeling. To evaluate if an antibody is suitable for IEM, the two most important factors to be considered are positive signal and specificity. For most cases, a pretest of the antibody at the LM level is necessary as mentioned

earlier, either with cultured cells or cryostat sections. This test enables not only the recognition of potentially useful antibodies but also assists in assessing both antibody dilution and whether further purification is necessary.

Antibody concentration used for EM should be much higher than that for LM because much less antigen is available or can be assessed on ultrathin sections. In practice, relatively high concentrations of antibody can give the highest signal-to-noise ratios. The optimal concentration is generally about a factor of 10 higher than the concentration that gives an optimal immunofluorescence (IF) signal [1]. However, unpurified antiserum usually gives a higher background than polyclonal and monoclonal antibodies, and therefore, a much higher dilution is necessary.

14.2.6 Secondary Gold-Conjugated Antibodies and Protein A-gold

IgG-gold secondary antibodies and protein A-gold (PAG) are two types of markers most commonly used for immunogold labeling. By using primary antibodies raised in different animals, secondary IgGs coupled with different sizes of gold particles are very convenient for double or triple labeling in one round of experiment. PAG can label most primary antibodies from different animal species. It tends to form less clusters and therefore presents a cleaner background. However, PAG is less stable; it needs to be diluted in very mild blocking buffer such as bovine serum albumin (BSA) and only stored up to half a year. Therefore, the use of expired PAG is not recommended. For double or multiple labeling, different sizes of PAG markers must be incubated separately (sequential labeling) with Protein A.

14.3 Chemical Fixation and LR White Embedding

This is the most common protocol for immunogold labeling. The samples are usually fixed using a buffered PFA fixation without an osmium tetroxide post-fixation step and subsequently embedded using LR White. Thin sections are then labeled using the immunogold protocol. In North America, chemicals and reagents can be obtained from Sigma-Aldrich; histological and immunostaining supplies can be purchased from Electron Microscopy Sciences, Hatfield, PA (EMS).

14.3.1 Materials

14.3.1.1 Reagents

1. EM-grade GA, 25 % [Electron Microscopy Sciences (EMS)]
2. PFA (Sigma-Aldrich); EM-grade 16 % formaldehyde aqueous solution (EMS)

3. LR White resin, medium grade (EMS) (*see Notes 1 and 3*)
4. Uranyl acetate (EMS)
5. BSA (Sigma-Aldrich or Jackson ImmunoResearch)
6. Gold-conjugated goat anti-rabbit IgG (H & L) 5 and 10 nm (EMS)
7. Milli-Q ultrapurified water
8. Ethyl alcohol, anhydrous, 200 mL (Ted Pella, Redding, CA)
9. Toluidine Blue O (TBO) (EMS)
10. Gold-conjugated goat anti-mouse IgG (H + L) 5 and 10 nm (EMS)

14.3.1.2 Solution Preparation

1. 2% Aqueous uranyl acetate (UA) solution: Add 1 g UA to 50 mL of Milli-Q water. Stir for 2 h and filter before use. Wrap the container in aluminum foil and store at 4 °C
2. 1% BSA solution for blocking: Add 1 g of BSA and 0.15 g glycine to 100 mL PBS. Prepare the blocking solution just before use. BSA powder should be stored at 4 °C
3. Ethanol series
4. Sodium phosphate buffer, 0.1 M, pH 7.4

14.3.1.3 Equipment and Laboratory Supplies

1. Ultracut R microtome (Leica Microsystems)
2. BioTwin transmission electron microscopy (TEM) (FEI)
3. Ultra 45° diamond knife (Diatome)
4. Copper or nickel grids, 100–200 mesh square (EMS)
5. Perfect Loop (EMS)
6. Gelatin capsules, size “0” (EMS)
7. General laboratory supplies: plastic transfer pipettes, forceps, oven, razor blades, toothpicks, lass microscope slides, and Parafilm

14.3.2 Method

14.3.2.1 Fixation

1. Dissect and cut fresh samples into small pieces (1–2 mm^3), while keeping samples wet in water, buffer, or fixative *(see Note 2)*.
2. Immerse samples in a fixative at room temperature for 5–6 h. Vacuum the samples, especially if they are floating *(see Notes 3–5)*

3. Remove the fixative and wash three times in 0.1 M sodium phosphate buffer (or PBS), pH 7.4.
4. Dehydrate samples using an ethanol series (10 min each step) *(see Notes 6 and 7)*.

14.3.2.2 Infiltration and Embedding

1. Prepare a 1:1 mixture of ethanol and LR White in a separate container and mix well by shaking. Remove ethanol and immerse samples in the mixture for 2 h.
2. Remove the mixture and replace with 100 % LR White for 2 h or leave overnight at 4 °C.
3. Transfer the samples into gelatin capsules and fill with fresh LR White. Make sure the samples sink to the bottom of the capsules, orient the samples with a needle if necessary. Cap the capsules with minimal trapped air, and polymerize at 60 °C overnight *(see Note 8)*.

14.3.2.3 Ultramicrotomy

1. After polymerization, the capsule can be peeled off with a razor blade. Trim the tip of the tissue block into a square or trapezoid shape.
2. Cut thick sections (0.5–1.0 μm) and check under a light microscope after brief staining with 1 % TBO *(see Note 9)*.
3. Cut thin sections of 50–70 nm in thickness, pick up and place sections on a copper or nickel grid *(see Notes 10 and 11)*. The grids can be kept at 4 °C before immunostaining.

14.3.2.4 Immunogold Labeling

All incubations are done on drops of solutions placed on a sheet of Parafilm.

1. Float the grids (with the section side down) on drops of the 1 % BSA blocking solution for 1 h at room temperature.
2. Transfer the grids onto drops of the primary antibody diluted with the same blocking solution and incubate for 1 h at room temperature or overnight at 4 °C.
3. Wash four times on drops of the blocking solution, 5 min each.
4. Incubate the grids on drops of the secondary antibody conjugated to gold particles, diluted with the blocking solution, for 1 h at room temperature or overnight at 4 °C.
5. Wash three times on drops of the blocking solution, 5 min each.
6. Wash three times on drops of PBS, 5 min each.
7. Transfer the grids on drops of GA fixative and fix for 5 min.

8. Wash in PBS two times, 5 min each.
9. Wash five times in distilled water, 2 min each.
10. Counterstain with 1–2 % aqueous UA for 4–6 min, or double stain with UA and lead citrate *(see Notes 12)*
11. Wash six times in distilled water, 2 min each.
12. Air-dry the grids for TEM examination.

14.4 Ultrathin Cryosectioning IEM (Tokuyasu Method)

Tokuyasu is currently the most sensitive method for immunogold labeling because no sample dehydration and resin embedding are necessary. Sample processing is faster with minimum reagent requirement. In brief, samples are mildly fixed, cryoprotected with sucrose (or sucrose/PVP mixture), frozen in liquid nitrogen and sectioned with a dry knife at −100 to −120 °C. Frozen sections can then be picked up with a drop of sucrose on a small metal loop. After a brief thawing at room temperature, the sections can be laid on a film coated grid for a subsequent immunogold labeling.

14.4.1 Material

14.4.1.1 Reagents

1. Sucrose (EMS)
2. Polyvinylpyrrolidone (PVP) (Amresco)
3. Methyl cellulose (EMS)
4. Sodium carbonate
5. Sodium azide
6. Oxalic acid
7. Other reagents, *see* section “Materials”

14.4.1.2 Solution Preparation

1. Prepare a 1 % methyl cellulose solution by adding 1 g methyl cellulose to 100 mL dH_2O in a beaker at 60–80 °C on a hot plate and stir for 1 h. Cool on ice. Transfer the beaker to a cold room or a refrigerator and continue to stir overnight. After overnight stirring, divide the solution evenly into 50 mL centrifuge tubes. Allow the solution to warm up slightly before centrifuging at 40,000 g on a centrifuge for 2 h. There should be a pellet. Aliquot the supernatant into plastic pipettes and store in a −20 °C freezer.

2. Prepare a 3 M sucrose solution by mixing 157.5 g sucrose with PBS until dissolved. Bring to a total volume of 200 mL. Add a few crystals of sodium azide as preservative and store aliquots in plastic pipettes in a −20 °C freezer.
3. Prepare a sucrose/PVP mixture (1.8 M sucrose/20 % PVP buffered with 0.055 M sodium carbonate). Mix 4 mL 1.1 M sodium carbonate in PBS (0.4664 g, bring to 4.0 mL with PBS) with 80 mL 2.3 M sucrose in PBS. Mix 10 mL buffered sucrose with 20 g PVP and then add the mixture to the remaining buffered sucrose and mix until dissolved. Cover lightly and leave at room temperature overnight to remove air bubbles or the solution can be sonicated or centrifuged to facilitate the removal of air bubbles. Aliquot into 1.5 mL plastic tubes and store in a −20 °C freezer.
4. Neutral uranyl acetate solution: Dissolve 1.89 g oxalic acid with 100 mL dH_2O in a beaker, Add 2 g UA and stir until dissolved. Filter mixture and adjust pH to 7.0 with NH_4OH. Aliquot into 1.5 mL microcentrifuge tubes and freeze with liquid nitrogen. Store at −20 °C or −80 °C.
5. 4 % UA solution: Add 4 g UA in dH_2O and bring to 100 mL. Stir until dissolved and filter.
6. Cryofix fixative (4 % PFA, 0.01 % GA in PBS).
7. 3–5 % Gelatin solution for embedding: Add gelatin to 1× PBS in a beaker on a hot plate (not more than 60 °C), stir until dissolved. Add a few sodium azide crystals to the gelatin solution and keep in a glass bottle in the refrigerator. Melt in microwave just before use.
8. 1 % BSA for Blocking: Add 1 g BSA and 0.15 g glycine to 100 mL 1× PBS and stir until dissolved.

14.4.1.3 Equipment and Laboratory Supplies

1. Leica Ultracut UCT ultramicrotome and ultracryo-unit.
2. Stereo microscope, for dissection and sample mounting.
3. Diamond knife for cryosectioning (Cryo Immuno and Trimmer) (Diatome).
4. Liquid nitrogen thermos flasks and liquid nitrogen tank for specimen storage.
5. Liquid nitrogen.
6. Metal specimen pins and forceps.
7. Formvar only or formvar/carbon-coated mesh grids (copper, nickel, or gold) *(see Note 11)*.
8. Wire loops (2.5–3.0 mm in diameter) for section retrieval.
9. Other laboratory supplies, i.e., Whatman filter paper, Parafilm, double-edged razor blade, cryo-storage tubes.

14.4.2 Method

14.4.2.1 Fixation

1. Organs or tissues can be fixed in the same way as described in LR White embedding method.
2. Cultured cells, pollens, protoplast, and isolated organelles need to be embedded in gelatin or agar before or after fixation *(see Note 13)*.

14.4.2.2 Infiltration with sucrose/PVP mix

1. Rinse the sample in PBS, two times, 5 min each.
2. Place in 2.3 M sucrose or sucrose/PVP mixture.
3. Leave overnight in a refrigerator at 4 °C.

14.4.2.3 Sample Freezing

1. Collect two pairs of forceps, cryo-storage tubes, clean specimen pins and liquid nitrogen.
2. Label the sample storage tubes with name and date.
3. Bore holes into the top half of the tube with a needle and place the tube and cap in liquid nitrogen.
4. Pour the samples in the sucrose/PVP solution into a petri dish.
5. Cut the sample into about 0.5 mm cubes.
6. Pick the sample pieces with a toothpick and put them on top of the cryo-pins *(see Note 14)*.
7. Freeze the sample pieces one at a time, one piece per cryo-pin, by immersion in liquid nitrogen.
8. Place the cryo-pins into the cryo-tube immersed in liquid nitrogen.
9. Place the cryo-tubes on a rack and store in liquid nitrogen quickly.

14.4.2.4 Ultracryo-Microtomy

1. Set up the ultramicrotome and cryo-attachment.
2. Connect the cryo-attachment to liquid nitrogen and precool the cryo-chamber to −90 °C.
3. Cut 200–300 nm thick sections with a dry glass knife.
4. Pick up sections using a metal wire loop with a drop of 2.3 M sucrose (*see Note 15*).

5. Thaw the sections in air and put them on a glass slide and cover the sections with a drop of sucrose solution on the slide.
6. Wash off the sucrose gently with water and stain with TBO for a few seconds.
7. Check the slide under a light microscope ensuring that the target cells or tissues are present.
8. Cool the ultramicrotome cryo-chamber to −120 °C.
9. Cut 50–70 nm thin sections at a lower speed, arrange the sections with a fine hair or eyelash.
10. Pick up sections the same way as in Step 4.
11. Thaw the sections in air for a few seconds and put on coated mesh grids *(see Note 16)*.

14.4.2.5 Immunogold Labeling of Thawed Cryosections

All incubations are done on drops of solutions placed on a sheet of Parafilm.

1. Rinse grids by floating on drops of PBS, three times for 5 min each (*see Note 17*).
2. Rinse in 1 % BSA three times for 5 min each.
3. Block with 1 % BSA (or with 1 % normal goat serum) for 30 min in a humid chamber.
4. Rinse in 1 % BSA three times for 5 min each.
5. Incubate with primary antibody for 60 min in a humid chamber at room temperature.
6. Rinse in BSA four times for 5 min each.
7. Incubate with gold-conjugated secondary antibody for 60 min in a humid chamber at room temperature.
8. Rinse in 1 % BSA three times for 5 min each.
9. Rinse in PBS three times for 5 min each.
10. Fix in 2.5 % GA for 5 min.
11. Rinse in PBS twice for 5 min each.
12. Rinse in dH_2O, six times for 1 min each.
13. Stain with neutral UA for 7 min.
14. Rinse in dH_2O, three times for 1 min each.
15. Stain with 4 % aqueous UA for 1 min.
16. Float grids on drops of cold methyl cellulose.
17. Pick up with loops, blot off excess methyl cellulose with damp filter paper, and dry on loops overnight.
18. Collect grids from loops and examine with a TEM.

14.5 Cryofixation and FS IEM

We have successfully used HPF and FS to preserve morphology of the embryo sac during fertilization, and immunogold labeling to study cytoskeletal changes in the reproductive cells of *Torenia* and *Nicotiana*. *Torenia fournieri* has a protruded naked embryo sac which is covered by a cuticle and a thick cell wall. It is very difficult to preserve their fine structure by chemical fixation, especially during fertilization. Employing cryofixation and FS, we not only obtained high-quality morphological preservation, but also antigenicity preservation of cytoskeleton elements. The protocol described below can be used for ovules, i.e., *N. tobaccum* and embryo sac, i.e., *T. fournieri*. For details on the HPF protocol, readers may refer to Chap. 7 and references cited therein.

14.5.1 Materials

14.5.1.1 Reagents

1. l-α-lecithin (P-9671XV-E or P-3644IV-S; Sigma)
2. MES (2-[N-morpholino]ethanesulfonic acid) buffer
3. Normal goat serum
4. Cold Fish gelatin (Sigma, USA)
5. Anti-actin monoclonal antibody (N350; Amersham Co. Arlington Heights, IL, USA)
6. 10-nm gold-conjugated goat anti-mouse IgG + Ig M secondary antibody (EY Labs. Inc., San Mateo, CA, USA)
7. Acetone with molecular sieve
8. Chloroform
9. Other reagents, see Reagents section under "Chemical fixation and LR White embedding" and "Cryo-thin sectioning IEM (Tokuyasu method)".

14.5.1.2 Solutions and Preparation

1. L-α-lecithin (100 mg/mL) is dissolved in chloroform.
2. MES buffer: 20 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 2 mM $CaCl_2$, 2 mM KCl and 0.2 M sucrose [23].
3. FS solution: absolute anhydrous acetone with molecular sieve
4. Sato's Lead citrate [24].
5. Blocking solution: 2% BSA and 2% normal goat serum in PBS.

14.5.1.3 Equipment and Freezing Materials

1. Baltec HPM 010 HPF machine (Boeckeler, Tucson, AZ)
2. Freezer at −80 °C
3. Brass freezer hats: Brass type A and type B planchettes (Ted Pella): 2 mm diameter, 0.3 mm or 0.6 mm deep well
4. Copper freeze hats coated with l-α-lecithin and air-dried
5. Styrofoam box
6. 5 mL glass vial
7. See section under "Chemical fixation and LR White embedding" and "Cryo-thin sectioning IEM (Tokuyasu method)"for equipment and supplies.

14.5.2 Method

14.5.2.1 Material and Sample Preparation (see Note 18)

1. Clean Brass freezer hats with acetone in ultrasound cleaner for 5 min, then wash with 95 % ethanol 3–5 times.
2. Brass freezer hats are coated with L-α-lecithin and air dried.
3. For *Nicotiana*, ovules are cut longitudinally into several pieces; for *T. fournieri*, embryo sacs are dissected after pollination. Specimens are incubated in MES buffer for 10–30 min and loaded into the cavity of the freezer hat with a minimum volume of MES buffer. The specimen must be wet using MES buffer before freezing and avoid air bubbles in the cavity.
4. Before HPF, use a piece of filter paper to remove excess MES buffer and put the upper hat on, making a "sandwich" carrier. It is important to ensure that there are no air bubbles trapped within the assembly.

14.5.2.2 Freezing and FS

1. Rapidly load the Brass freezer hats on the machine and perform HPF.
2. After freezing, sample carrier is quickly transferred to the freezing vial containing absolute anhydrous acetone (pre-chilled at −85 °C).
3. Specimens freeze substituted at 80–85 °C for 2 days in freezer with three changes of acetone.
4. Substitute for 1 day at −20 °C.
5. Warm up to 4 °C overnight, then transfer to fresh anhydrous acetone for 4 h (three changes) (substitution solution needs to be pre-chilled at that temperature).
6. Replace with pre-chilled 100 % ethanol and rinse with ethanol three more times *(see Note 19)*.

14.5.2.3 Infiltration and Embedding

1. Infiltrate the specimens with the graduated series of LR White resin (ratio of LR White to ethanol at 1:3. 1:1, 3:1) for 3 days, each step for 12 h to overnight. Place specimen vials on a rotator at low speed.
2. Continue to infiltrate specimens with 100 % LR White for 4 h and change resin three times.
3. Embed in gelatin beam capsule.
4. Polymerize in oven at 50 °C overnight.

14.5.2.4 Ultramicrotomy

See Sect. 14.4.2.3 and *Note 20*.

14.5.2.5 Immunogold Labeling

1. Grids are incubated in a blocking solution consisting of PBS with 2 % BSA and 2 % normal goat serum at pH 7.4 for 30 min.
2. Incubate in anti-actin monoclonal antibody or anti-tubulin monoclonal antibody diluted in the ratio 1:800 with blocking solution for 60 min.
3. Rinse with PBS five times, each for 5 min.
4. Incubate in 10 nm gold-conjugated goat anti-mouse IgG + IgM secondary antibody diluted in the ratio 1:10 with blocking solution for 60 min.
5. Rinse with two drops of PBS, followed by distilled water rinse three times.
6. Stain with 1–2 % UA for 20 min.
7. Rinse with distilled water five times, each for 5 min.
8. Stain with Sato lead citrate for 1 min.
9. Controls: substitution of primary antibody with blocking solution and rabbit anti-mouse IgG for the primary antibody.

14.6 Common Problems and Trouble Shooting (Table 14.1)

14.6.1 Problem I—No Labeling

The most common causes are: too dilute antibody and improper fixation of the antigen (lost or destroyed). Increase the concentration of primary antibodies no matter what protocol is currently being used, or set up a series of dilutions of the antibodies starting from 1:20. Try labeling on ultrathin cryosections with a mild sample fixation (2–4 % PFA only).

Table 14.1 Common technical problems and trouble-shootings

Problems	Possible causes	Solutions
Cell shrinkage	Osmolality in fixative changes; Too severe dehydration, either a large step of concentration change or too prolonged time in high concentration ethanol	Adjust the osmolality, decrease steps of dehydration from 30% ethanol with 10% increase
Ultrastructure is not well preserved or damaged	Not enough fixation; Improper fixation method; Improper resin for embedding; Cutting damage	Extend fixation time or use stronger fixative, e.g., add 0.2% GA. Use cryofix or HPF-FS. Change resin. Trim block smaller and change glass knife or use diamond knife
Section falls apart or holes are present in the sample	Not enough infiltration of embedding medium	Increase time or temperature for infiltration. Use microwave process
Little or no immunogold label	Antigen presence is insufficient. Antigen is damaged, masked, or extracted in process. Antibody is diluted too much	Use a mild fixative or cryofix. Change embedding resin or use Tokuyasu method. Increase antibody concentration
Too much background; Non-specific labeling	Antibody is not purified; Antibody concentration is too high; Antibody expired or degraded; Background blocking is not enough; Sample contains sticky substances	Purify antibody; Dilute antibody and find appropriate concentration; Use fresh antibody; Change blocking solution, e.g., increase serum concentration or add more blocking agents
Sections are contaminated: Precipitates are generated by the reaction of buffer and copper grid; Uranyl acetate precipitate; Methyl cellulose precipitate; Bacteria	PBS reacts with copper grid; Some components of solution react with uranyl acetate or uranyl acetate stores for a long time; Methyl cellulose is not centrifuged enough or stored for too long; Improper or prolonged buffer storage	Replace copper grids with nickel grids. Change with other buffer. Thoroughly clean nickel grid with distilled water before staining with uranyl acetate. Centrifuge methyl cellulose before use. Make fresh and filter all the solution before use

14.6.2 Problem II—High Background

Background labeling can be caused by any of the following reasons: (a) antibodies are too concentrated; (b) blocking is weak; (c) contamination is caused by impurity or bad quality of antibodies; (d) antibody is expired or degraded; (e) support grid charge; (f) the sample contains some sticky substances which cling to antibody physically. For primary antibody incubation, a series of dilutions are always

necessary. Polyclonal antibodies usually cause more background than monoclonal antibodies. Antiserum is very sticky and needs to be diluted much more. Stronger blocking can be achieved by increasing the concentration of BSA or combining BSA with other blocking reagents, including normal goat serum, cold fish gelatin, and nonfat dry milk. Grids sinking into the antibody solution or not enough washing during the labeling process can cause high background staining. Hence, keeping grids floating on the solution is important. Antibodies can degrade in storage and improper handling can cause nonspecific labeling. Primary antibodies have to be stored according to the manufacturer's instructions, and avoid repeat thawing. Gold-conjugated secondary antibodies are usually stored at 4 °C and need to be centrifuged after dilution and before incubation. Metal charge on the grids especially nickel causes gold aggregate to form around the mesh bars. Avoid taking images near the bar areas.

14.6.3 Problem III—Labeling is on Different Locations (Structures)

Pseudo-labeling signals can be obvious on some structures, such as cell wall, mitochondria, and nucleus (nucleolus), for different reasons. Appropriate controls can let you know if those unexpected signals are real or not. Controls may include omitting the primary antibody or using pre-immune serum, normal IgG for the primary incubation. An unrelated antibody can also be used for evaluation of the labeling signal pattern. If possible, a truly negative sample lacking the antigen can be very useful as the control. However, it is not impossible that the same protein localizes to different sites or structures in consideration of their high traffic dynamics and multiple functions.

14.6.4 Problem IV—Some Labeling Signal and Structure Do Not Match or Co-localize Even in a Successful Experiment

This problem is especially common in post-embedding labeling of structures less than 100 nm, such as small vesicles and microtubules. For those samples embedded in resin, cutting randomly through a sample exposes part of the structure, and only exposed antigen on the cutting surface can be labeled by the antibody. Therefore, the same structure can be exposed and labeled at any cutting levels or completely avoided as shown in Fig. 14.6. Consequently, labeling pattern for the same structure can be very different on the same section. Evaluation and interpretation of the labeling pattern are always necessary by looking at multiple locations or serial sections of the target structures.

14.7 Notes

1. LR White is an irritant. Use it in a fume hood and wear appropriate protective equipment.
2. Fixative for immunolabeling: We routinely use 4% PFA with 0.01–0.1% GA in PBS. Materials can be fixed in 4% PFA alone. Formaldehyde-fixed material should be stored and transported in formaldehyde before starting the experiment.
3. Chemical fixation and LR White embedding is the easiest method for plant tissues because of its low viscosity, hydrophilic nature, and minimum equipment requirements for sample processing.
4. Never allow the sample to dry during dissection before fixation. Keeping the sample in buffer or the primary fixative during dissection is highly recommended.
5. Most plant tissues need to be vacuumed, especially for those with air spaces inside the tissues such as developed anthers and leaves. The tissues can be vacuumed together with the fixative until they sink.
6. The dehydration process can start from 30% ethanol with 10% increments for each step. Large changes of ethanol concentration during dehydration or keeping the samples in a high concentration of ethanol for a long period of time might cause severe extraction and generate artifacts.
7. LR White tolerates partial dehydration (as low as 70% ethanol); 2–3 changes of resin are necessary for complete infiltration of the resin into samples.
8. Gelatin capsules are the best and the first choice of capsules for LR White embedding so far, but sample orientation is more difficult. The cap top needs to be flatted or pushed in to avoid air trapping inside the capsule during polymerization. Although the flat embedding mold is easier for sample orientation, incomplete resin polymerization occurs on the block surface, and is not suitable for embedding of LR White samples.
9. The cutting surface is easy to get wet because of the hydrophilic property of the resin. Therefore, water level in the knife boat needs to be lowered as much as possible.
10. LR White thin sections spread well on water surface. Using a Perfect Loop to pick up sections can avoid wrinkles.
11. EM grids are made from different materials. The most common ones are made of copper or nickel. It is best to refrain from using copper grids for immunolabeling because copper grids react with salts in the buffer to produce precipitates. Nickel grids are magnetized, making them difficult to manipulate, and they can cause astigmatism when being examined in the electron microscope. Gold or palladium grids can be used if preferred. Coated grids can be purchased commercially or can be coated in the laboratory.
12. Proper counterstaining can show both the cellular structure and gold particles well, as long as the staining intensity is properly controlled. If the cellular structure is not stained, the 2% aqueous UA can be substituted with 2% UA in 70% (vol/vol) methanol with a shorter time of staining (1–3 min).

13. Small specimens such as pollen, root tips, cells, or cell fragments have to be pre-embedded in gelatin after fixation. Gelatin can easily be removed from specimens or from sections by washing in warm aqueous medium.
14. There are two types of aluminum specimen pins for mounting samples for ultracryo-microtomy: Bullseye and Slotted screw. The slotted screws are used for bigger sample blocks and can hold the block firmly. For those samples smaller than the depth of a slot, the Bullseye pins should be used.
15. A metal wire loop with a diameter of 2 mm is suitable to pick up thin sections. After dipping the loop in a drop of 2.3 M sucrose, allow the sucrose solution on the loop to solidify for a few seconds within the cryo-chamber before approaching and picking up the section from the knife. Ideally, sections with charge may "fly" onto the surface of the sucrose drop. The fragile sections can be broken by a harsh touching of the hard frozen sucrose. Instead of sucrose, sections can be picked up using 1 % methyl cellulose or a 1:1 mixture of sucrose and methyl cellulose. The methyl cellulose has a lower surface tension and can improve the retention of morphology in the section [22]. However, the mixture causes frost which might be a problem for picking up sections.
16. Sections covered with sucrose or sucrose/MC mixture can be stored in a closed box or petri dish at 4 °C before immunolabeling. Drying of the section during storage does not obviously affect the labeling or ultrastructure [25].
17. Specimens that have been fixed with aldehydes can be treated with primary amines which will quench any free aldehyde groups that could crosslink antibodies. Useful compounds include glycine (0.15 %) or ammonium chloride (50 mM) dissolved in PBS.
18. For HPF cryofixation, the critical elements for the success of cryofixation and FS are: (a) the specimen loading step needs to be carried out as quickly as possible; hence, all processing steps should be done near the freezing machine; (b) keep specimen wet with MES buffer in petri dish or freezing carrier; (c) do not load too many samples in the freezing carrier to avoid physical damages; (d) use suitable filler with specimen (e.g., 0.2 M sucrose in MES buffer) and avoid trapping any air bubbles in the cavity of the carrier; (e) keep the sample volume at a minimum with a sample shape that fits the cavity of the carrier; (f) the sample should be kept in the same vial throughout processing; (g) avoid exposing the sample to the air; and (h) all the tools, dehydration agents, and infiltration resin for cryofixation and FS must be pre-chilled before use.
19. Acetone inhibits the polymerization of LR White. After HPF-FS, acetone has to be completely replaced by ethanol or LR White before embedding.
20. Only part of the ovule ultrastructure is well preserved by propane jet or HPF; some may be damaged by ice crystals. Therefore, it would be better to section different parts of the ovules using thick sectioning to evaluate the fixation result.

14.8 Interpretation and Conclusion

There is not a single, ideal method or protocol for immunogold labeling. One has to choose a method that is best for both antigen access and specific ultrastructure preservation according to the sample properties and antigen distribution. For most organelles and general ultrastructure, especially if the label targets are cytoskeletal, such as microtubules or actin filaments, chemical fixation and LR White embedding are an easy choice. Tokuyasu method is a better procedure for labeling proteins associated with the membrane system or membrane-bounded organelles, including ER, mitochondria, Golgi, and their related vesicles. Samples processed with HPF-FS may balance the ultrastructure and label signal but may not work for every type of specimen. For new antibody trials, the Tokuyasu method is the first choice because of its high sensitivity. Resin-embedded sections usually need 10–20 times more concentration of the primary antibodies for achieving the same signal from cryosections.

Acknowledgment We are especially grateful to Prof. Edward Yeung at University of Calgary, for his instruction and critical reading of this chapter. We also thank Tari Parmely and Dorothy Stanley at Stowers Institute for revising the English of the manuscript.

References

1. Griffiths G (1993) Fine structure immunocytochemistry. Springer-Verlag, Berlin
2. Tokuyasu KT (1973) A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol 57:551–565
3. Tokuyasu KT (1986) Application of cryoultramicrotomy to immunocytochemistry. J Microsc 143:139–149
4. Kiss JZ, McDonald K (1993) Electron microscopy immunocytochemistry flowing cryofixation and freeze substitution. Meth Cell Biol 37:311–341
5. Newman GR, Hobot JA (1987) Modern acrylics for post-embedding immunostaining techniques. J Histochem Cytochem 35:971–981
6. Newman GR, Hobot JA (2001) Resin microscopy and on-section immunocytochemistry. Springer–Verlag, New York
7. McDonald K (1999) High-pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. Meth Mol Biol 117:77–97
8. McDonald K (2007) Cryopreparation methods for electron microscopy of selected model systems. Meth Cell Biol 79:23–56
9. McDonald K (2014) Out with the old and in with the new: rapid specimen preparation procedures for electron microscopy of sectioned biological material. Protoplasma 251:429–448
10. Takeuchi M, Takabe K, Momeyuki Y (2010) Immunoelectron microscopy of cryofixed and freeze substituted plant tissues. Meth Mol Biol 657:155–165
11. Karahara I, Kang B-H (2014) High-pressure freezing and low-temperature processing of plant tissue samples for electron microscopy. In: Žárský V, Cvrčková F (eds) Plant cell morphogenesis, vol 1080. Methods in molecular biology. Humana Press, New York, pp 147–157
12. Vaughn KC, Campbell WH (1988) Immunogold localization of nitrate reductase in maize leaves. Plant Physiol 88:1354–1357

13. Kuo J (2007) Processing tissues for ultrastructural study in electron microscopy: methods and protocols. Humana Press, New York
14. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, New York
15. Ripper D, Schwarzt H, Stierhof YD (2008) Cryo-section immunolabeling of difficult to preserve specimens: advantages of cryofixation, freeze-substitution and rehydration. Biol Cell 100:109–123
16. Waguri S, Kohmura M, Gotow T, Watanabe T et al (1999) The induction of autophagic vacuoles and the unique endocytic compartments, C-shaped multivesicular bodies, in GH4C1 cells after treatment with 17beta-estradiol, insulin and EGF. Arch Histo Cytol 62:423–434
17. Dettmer J, Hong-Hersdorf A, Stierholf Y-D, Schumacher K (2006) Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. Plant Cell 18:715–730
18. Slot J, Geuze HJ (2007) Cryosectioning and immunolabeling. Nat Protoc 2:2480–2491
19. Bos E, Sant´Anna C, Gnaegi H, Pinto RF et al (2011) A new approach to improve the quality of ultrathin cryo-sections; its use for immunogold EM and correlative electron cryo-tomography. J Struct Biol 175:62–67
20. McLean W, Nakane PF (1974) Periodate–lysine–paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
21. Wilson SM, Bacic A (2012) Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. Nat Protoc 7:1716–1727
22. Liou W, Geuze HJ, Slot JW (1996) Improving structural integrity of cryosections for immuno-gold labeling. Histochem Cell Biol 106:41–58
23. Ding B, Turgeon R, Parthasarathy MV (1991) Routine cryofixation of plant tissue by propane jet freezing for freeze substitution. J Electron Microsc Tech 19:107–117
24. Hainachi T, Sato T, Hoshimo M, Mizumo N (1986) A stable lead stain by modification of Sato's method. Proc. XIth Int. Cong Electron Microsc Kyoto 2181–2182
25. Griffith JM, Posthuma G (2002) A reliable and convenient method to store ultrathin thawed cryosections prior to immunolabeling. J Histochem Cytochem 50:57–62
26. Han Y, Yu J, Guo FL, Watkins SC (2006) Polysomes are associated with microtubules in fertilized eggs of Chinese pine (*Pinus tabulaeformis*). Protoplasma 227:223–227
27. Guo FL, Yu L, Watkins SC, Han YZ (2007) Orientation of microtubules suggests a role in mRNA transportation in fertilized eggs of Chinese pine (*Pinus tabulaeformis*). Protoplasma 231:239–243
28. Yang D, Guo FL, Liu B, Huang H, Watkins SC (2003) Expression and localization of human lysozyme in the endosperm of transgenic rice. Planta 216:597–603

Chapter 15
Abscisic Acid Immunostaining

Yung-I Lee and Mei-Chu Chung

15.1 Introduction

Phytohormones are produced in an extremely low concentration in one part of an organ or tissue, and then moved to another part, where physiological responses are triggered [1]. Since phytohormones are mobile chemical messengers, the visualization of their dynamic distributions in plant tissues can provide insight to their physiological functions. Visualizing the distribution of phytohormones in plant tissue is challenging, because of their low concentrations, and because they are soluble in solvents used during sample preparations [2]. Zavala and Brandon were the first to show the localization of a cytokinin—dihydrozeatin riboside (DHZR) in maize roots by polyclonal antisera by using a low-temperature preparative technique [2]. Soon after, antisera or antibodies against other phytohormones, that is, abscisic acid (ABA), indole-3-acetic acid (IAA), and cytokinins became available, and the distribution of these phytohormones in plant tissues was successfully observed by immunohistochemical (IHC) staining techniques [3–7].

The phytohormone, ABA is a 15-carbon sesquiterpenoid compound, which regulates a range of processes of plant growth and development, such as seed maturation, dormancy, germination, leaf senescence, and the responses to environmental stress [8]. During plant development, or in response to changing environmental conditions, the biosynthesis and levels of ABA would fluctuate dramatically in specific organs or tissues. In drought stress, ABA was found to accumulate in guard cells in the leaf, causing stomata closure [9, 10]. In addition, the radial transport of external ABA was observed in maize roots under drought stress [11]. During seed

Y.-I. Lee (✉)
Biology Department, National Museum of Natural Science, No. 1, Kuan-Chien Road, 404 Taichung, Taiwan, ROC
e-mail: leeyungi@mail.nmns.edu.tw

M.-C. Chung
Institute of Plant and Microbial Biology, Academia Sinica, No. 128, Section 2, Academia Road, Nankang, Taipei 11529, Taiwan, ROC

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_15

development, the accumulation of ABA usually appears as two peaks in several species with the first peak deriving from maternal sources and the second peak resulting from de novo biosynthesis within maturing seeds [12, 13].

Immunohistochemistry is a useful method to study ABA distribution in plant tissues. Immunostaining procedures of ABA include fixation, dehydration, sectioning, and staining of samples. To immobilize ABA molecules within cells, the samples are treated immediately in a solution containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) after dissection [5, 11, 14]. EDAC helps to cross-link the carboxyl group of ABA molecules with structural proteins of the tissue. After processing and sectioning, the sections are incubated with a primary antibody against ABA. This is followed by visualization using a secondary antibody conjugated to a fluorochrome (e.g., fluorescein isothiocyanate (FITC) or the Alexa Fluor dye series) for light microscope studies [11, 15] or with colloid gold particles that can be visualized using an electron microscope [16, 17].

In early reports, polyclonal antiserum against ABA was used for visualizing ABA in plant cells [5, 16]. Polyclonal antiserum may contain antibodies to other epitopes that give nonspecific or background staining. In recent years, monoclonal antibodies against ABA give high specificity for (+)-*cis*,*trans*-ABA, providing more specific and accurate signals of ABA recognition and detection in plant cells and tissues [11, 14, 15, 18, 19].

Although ABA plays a regulatory role in seed development and dormancy [12, 13], the information of its precise localization in developing seeds is limited [20]. Orchid seeds are minute and have a simple structure, that is, a globular embryo is enveloped by a thin seed coat and an endosperm is absent [21]. In general, terrestrial orchids are considered more difficult to germinate as compared with the epiphytic orchids [22]. The accumulation of ABA in mature seeds is the cause of the low germination reported in a few terrestrial orchids [23, 24]. In this chapter, we provide protocols of ABA immunolocalization through the use of a fluorochrome-conjugated secondary antibody and the immunogold procedure for the seed of a terrestrial orchid, *Cypripedium formosanum*.

15.2 Technical Comments

Before the immunostaining of a specimen, it is imperative to test the specificity of the primary antibody used for an experiment. This is especially important if a primary antiserum is used instead of a monoclonal antibody. Furthermore, one needs to determine the appropriate concentration/dilution of the antibody/antiserum to be used. This can be done using an enzyme-linked immunosorbent assay (ELISA) analysis to probe the diluted ABA standard of different concentrations [25]. A good anti-ABA antibody/antiserum should have high immunoreactivities toward ABA. Where an antiserum or antibody produces no immunoreactivity from an ELISA analysis, it should not be used. The ELISA method is also needed to check that there are detectable levels of endogenous ABA within the tissue extract from the samples

where immunolocalization is intended [24, 25]. The endogenous ABA levels from ELISA analysis can be double-checked for the same samples by gas chromatography–mass spectrometry (GC–MS) [14, 26], whenever possible. The data from ELISA and GC–MS analyses should be compatible.

Additional internal control treatments should be performed whenever possible to ensure that the immunostaining signals are indeed real. The immunostaining results should be compared with endogenous ABA levels. The samples with a higher endogenous level should give stronger immunostaining signals as compared to those samples with lower endogenous ABA levels. Another internal control treatment method is to treat the seeds with the fluridone solution, an ABA biosynthetic inhibitor, to reduce the endogenous ABA levels [27], before tissue fixation and processing. The concentration of ABA by ELISA and the intensity of immunostaining of the fluridone-treated seeds should be much less than the untreated ones. These experiments confirm the specificity and reliability of the immunostaining observations.

15.3 Materials

15.3.1 Plant Materials

The orchid plants *C. formosanum* were maintained in the greenhouse at the highland farm of National Taiwan University, Nantou, Taiwan. The flowers were pollinated manually when they were fully open. The seeds were collected at different developmental stages until maturation (*see Note 1*). The timing of seed maturation may change because of the different average temperature in each area. It is advised to collect the seeds at regular intervals to find the optimum timing.

15.3.2 Equipment and Supplies

1. A rotary retracting microtome (e.g., Leica RM2245; Leica Biosystems Nussloch GbH., Germany), or a similar rotary microtome
2. An ultramicrotome (e.g., Leica Ultracut S, Leica Biosystems Nussloch GbH., Germany or Ultracut E; Reichert-Jung, Vienna, Austria) or a similar ultramicrotome
3. The tungsten Carbide Knife or the diamond knife (e.g., Delaware Diamond Knives, Inc., Delaware, USA)
4. Microscope: epifluorescence microscope, confocal laser-scanning microscope (CLSM), or transmission electron microscope (TEM)
5. General laboratory equipment: ovens, a vacuum pump and a vacuum chamber, a rotary mixer, a refrigerator, a moist chamber, and a high-speed centrifuge

6. Histology supplies: formvar-coated EM grids (nickel slot grids, 1 × 2 mm slot size), embedding capsules (e.g., BEEM), and gelatin capsules (Electron Microscopy Sciences, Hatfield, PA), embedding capsule and gelatin capsule holders (127 mm × 76 mm)
7. General laboratory supplies: vials (13.5 × 48.3 mm, 2.0 mL), droppers, filter paper, Eppendorf tubes, blades, Petri dish, Parafilm®, high-quality glass slides (e.g., Superfrost™, Thermo Fisher Scientific Inc, MA, USA), or the poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA), coverslips, dissecting tools

15.3.3 Embedding Kits, Immunostaining, and Chemical Reagents

1. Technovit 8100 resin (Heraeus Kulzer GbH., Hanau, Germany)
2. London Resin White resin (#14382, hard grade; London Company, Basingstoke, UK)
3. Anti-ABA monoclonal antibody (e.g., PDM 09347; Agdia-Phytodetek, Elkhart, IN, USA)
4. Secondary antibodies: FITC-conjugated anti-mouse IgG (F0257; Sigma-Aldrich) and colloid gold-conjugated anti-mouse IgG (18 nm; RPN422 Auro-Probe EMGAR G18; Amersham, Buckinghamshire, UK)
5. Chemical reagents: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), Tween-20 or Triton X-100, bovine serum albumin (BSA), fluridone (containing 41.7% 1-methyl-3-phenyl-5-[3-trifluoromethyl(phenyl)]−4-(1 H)-pyridinone; Alligare, LLC, Opelika, AL, USA), ethanol, acetone, mounting solution with anti-fadent: 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Burlingame, CA, USA), pre-immune mouse IgG (Vector Laboratories, Burlingame, CA, USA), Toluidine blue O (TBO), uranyl acetate, lead citrate, and liquid nitrogen

15.3.4 Solutions

1. 0.1 M sodium phosphate buffer at pH 7.2
2. 2% Paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2
4. Phosphate-buffered saline and Tween (PBST): 0.15 M NaCl, 0.2 M phosphate buffer at pH 7.2, plus 0.05% Tween-20
5. 3% Normal goat serum and 0.5% BSA in PBST
6. TBO solution: 0.01% TBO in 1% boric acid solution
7. EM staining solution: Reynold's lead citrate solution and aqueous uranyl acetate solution
8. Ethanol series—15, 30, 50, 70, 80, 95, and 100%

15.4 Methods

15.4.1 Tissue Fixation for Immunocytochemistry

1. Remove seeds from capsules and immerse them into a solution with 2% (w/v) EDAC in 0.1 M sodium phosphate buffer (w/v) with 0.1% (v/v) Tween-20 or Triton X-100 at pH 7.2 in the vial (*see Note 1*; refs. [5, 11, 14, 16]).
2. Vacuum the vial containing seeds in the EDAC solution for 30 min or longer if necessary, until the seeds have sunk to the bottom the vial. Keep the vial at 4 °C for at least 24 h (*see Note 2*).
3. Wash the seeds with 0.1 M sodium phosphate buffer for three 15-min rinses, and then fix the seeds in a solution of 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer at 4 °C for at least 24 h.

15.4.2 Sample Preparation for Immunofluorescent Detection

1. Wash the seeds with 0.1 M sodium phosphate buffer for three 15-min rinses, and dehydrate the seeds with a graded ethanol series, 30 min each. Replace 100% ethanol with 100% acetone, three times, each for 60 min.
2. Prepare the infiltration solution according to the procedure *described by* the manufacturer. Mix 100 mL of Technovit 8100 resin and 0.6 g Hardener I (benzoyl peroxide) using a magnetic stirrer until the powder of benzoyl peroxide is complete*ly* dissolved. Store the infiltration solution in a dark bottle at 4 °C for up to 1 month.
3. Infiltrate gradually with an increasing concentration of Technovit infiltration solution, that is, 3:1; 1:1, 1:3; acetone/Technovit resin (v:v). Place the vials at 4 °C in a refrigerator with mild agitation for approximately 24 h per change (*see Note 3*).
4. Replace the infiltration solution with fresh 100% Technovit 8100 resin. Repeat this step at least two more times. Allow for 24 h per change and maintain the vials at 4 °C in the dark.
5. At the time of embedding, select the proper embedding capsule holders. Transfer the seeds with infiltration solution to each embedding capsule. Once all the seeds have been transferred, prepare the embedding solution.
6. Prepare the embedding solution according to the manufacturer's instruction just before embedding. Mix 30 mL infiltrating solution and 1 mL Hardener II for 1 min using a magnetic stirrer. Once mixed, polymerization begins. Quickly remove the infiltration solution from the embedding capsule and replace it with the embedding solution. Rinse the seeds once by replacing the embedding solution quickly with fresh embedding solution. Once completed, carefully cap the capsules (*see Note 4*).

7. Place the entire embedding capsule holder in a refrigerator at 4 °C for 12–24 h allowing for proper polymerization prior to sectioning.
8. Section the resin block using a rotary retracting microtome and place the 3-μm-thick sections, on good quality glass slides or the poly-L-lysine-coated slides (*see Note 5*).

15.4.3 Sample Preparation for Immunogold Labeling

1. After fixation, wash the seeds with 0.1 M sodium phosphate buffer for three rinses of 15 min each, and dehydrate the seeds with a graded ethanol series, 30 min each. Complete the dehydration with two 100 % ethanol washes, 60 min each.
2. Infiltrate gradually with an increasing concentration of LR White resin, that is, 3:1; 1:1, 1:3; ethanol/LR White (v:v). Place the vials at 4 °C in a refrigerator with mild agitation for approximately 24 h per change (*see Note 3*).
3. Replace with 100 % LR White resin. Repeat this step at least two more times. Allow for 24 h per change and maintains the vials at 4 °C in the dark with a mild agitation for 24 h.
4. Transfer the seeds from the vial to gelatin capsules containing the fresh LR White resin. Carefully cap the capsules (*see Note 4*) and polymerize them in an oven at 60 °C for 24 h.
5. Cut the block of LR White resin into ultrathin sections with a diamond knife on an ultramicrotome, and place the ultrathin sections on formvar-coated nickel grids.

15.4.4 Immunostaining of Sections for Epifluorescence Microscope or CLSM Observation

1. Incubate the sections with 3 % normal goat serum and 0.5 % BSA in PBST for 1 h at room temperature to reduce nonspecific binding.
2. Dilute the primary monoclonal antibody against ABA in PBST with 0.5 % BSA at the ratio of 1:200. Add 100 μL of the primary antibody solution to the slide and then put a glass or plastic cover on the slide.
3. Incubate the slides in a humid chamber overnight at 4 °C in darkness (*see Note 6*), then wash the slides thoroughly with PBST.
4. Dilute the fluorochrome-conjugated secondary antibody in PBST at the ratio of 1:20, or pretreat the secondary antibody (*see Note 7*; ref. [14]). Add 100 μL of the secondary antibody to each slide and then put a glass or plastic cover on the slide (*see Note 8*).
5. Incubate the slides in a humid chamber for 1 h at 37 °C and then wash them thoroughly with PBST.

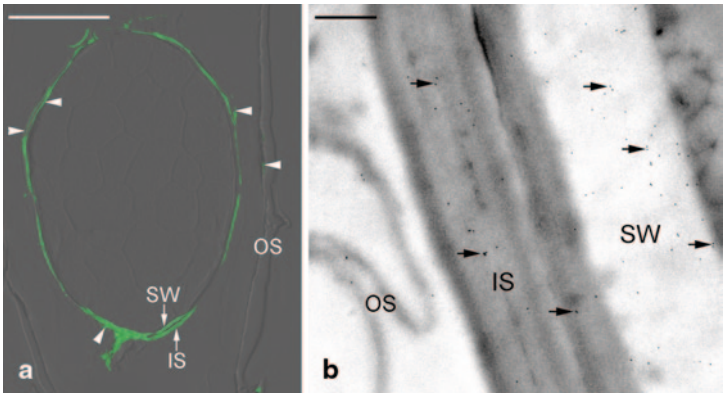

Fig. 15.1 Immunohistochemical localization of ABA in the mature seed of *C. formosanum*. **a** A longitudinal section through a mature seed embedded in Technovit 8100 resin. Sections were incubated with monoclonal ABA antibodies and the secondary antibody with FITC conjugates. The florescence signals of ABA (*arrowheads*) are located on the surface wall (SW) of the embryo proper, as well as the inner (IS) and outer (OS) seed coats. Scale bar = 50 μm. **b** The ultrastructural view showing a portion of a mature embryo embedded in LR White resin. Sections were incubated with monoclonal ABA antibodies and the secondary antibody with colloid gold conjugates (18 nm). Immunogold particle-labeled ABA (*arrows*) mainly accumulates on the surface wall (SW) of the embryo proper, and the shriveled inner (IS) and outer (OS) seed coats. Scale bar = 1 μm

6. After washing with PBST, stain the slides briefly with 0.01 % TBO to quench the tissue autofluorescence [11, 28], and subsequently add an antifadent mounting solution to the slides (*see Note 9*).
7. To confirm the specificity of immunostaining procedures, include the controls as (1) incubation with pre-immune mouse IgG and (2) incubation with the fluorochrome-conjugated anti-mouse IgG, but without the first antibody incubation step.
8. Examine the specific immunofluorescent signals of ABA with an epifluorescence microscope or a CLSM (*see Note 10*). The specific immunofluorescent signals of ABA are shown in Fig. 15.1a.

15.4.5 Immunostaining of Sections for TEM Observation

1. Incubate the sections on grids with 3 % normal goat serum and 0.5 % BSA in PBST for 10 min at room temperature for reducing unspecific binding.
2. Dilute the primary antibody against ABA in PBST with 0.5 % BSA at the ratio of 1:200 and add 5 μL of the antibody solution on clean Parafilm®.
3. Incubate the grid on a drop of the primary antibody solution in a humid Petri dish at room temperature for 1 h and then wash the grid thoroughly with PBST.
4. Dilute the secondary antibody—goat anti-mouse colloid gold conjugates (18 nm; RPN422 Auro-Probe EMGAR G18; Amersham) in PBST at the ratio of 1:20.

5. Incubate the grid on a drop of the secondary antibody solution at room temperature for 20 min and then wash the grid thoroughly with PBST.
6. To confirm the specificity of the immunostaining procedures, controls include as (1) incubation with pre-immune mouse IgG and (2) incubation with colloidal-gold-conjugated anti-mouse IgG, omitting the first antibody incubation step.
7. Counterstain the grid with uranyl acetate, followed by lead citrate. Examine the colloidal gold particles under TEM (*see Note 11*). The colloidal gold particles for ABA localizations are shown in Fig. 15.1b.

15.5 Notes

1. The EDAC pretreatment cross-links ABA to the cellular protein network and preserves antigenicity of ABA. As the seed matures, strong hydrophobic chemicals (e.g., cuticles, lignin, suberin, or wax) accumulate in the seed coat, preventing the diffusion of EDAC into the seed. We usually add 0.1 % (v/v) Tween-20 or Triton X-100 into the EDAC solution, or use ultrasonic treatment to break the seed coat, or dissect the seed to increase permeability.
2. Vacuum will make the penetration of chemical cross-linkers more complete and larger samples should have more vacuum steps during the periods of fixation, dehydration, and infiltration.
3. In general, for the young and fresh materials, the duration of infiltration in Technovit 8100 resin or London Resin White methacrylate resin should be 6–8 h. However, for the mature seed, which is strongly hydrophobic and has a low water content, increase the duration of infiltration to 24 h.
4. Both Technovit 8100 and London Resin White methacrylate resins require an oxygen-free environment during resin polymerization.
5. After cutting the block of Technovit 8100 resin, stretch the sections on distilled water for a few minutes and then transfer the sections carefully onto clean glass slides.
6. To prevent the antibody solution from drying out completely, select a plastic box with a tight closing lid, place a number of layers of clean filter paper at the bottom of the box, and pour distilled water over the filter paper to maintain a high level of moisture within the chamber.
7. To reduce the background in signal detection, the pretreatment of secondary antibody as described in ref [14] is essential. Homogenize about 0.5 g of fresh seeds in a small volume of ice-cold 90 % acetone in liquid nitrogen and refrigerate the mixture overnight at 4 °C. After centrifuging at 13,000 g for 5 min, remove the supernatant and resuspend the pellet in a small volume of 90 % acetone and pass the mixture through a filter paper. Transfer the dried powder to an Eppendorf tube and store it at 4 °C. Add 50 mg of the dried powder to 500 μL PBS containing 1 % BSA and 20 μL of secondary antibody, and incubate the mixture in the dark for 1 day at room temperature. Add 500 μL of PBS to the mixture, centrifugate, and place the pretreated secondary antibody on the slide.

8. When working with the fluorochrome-conjugated secondary antibody, protect the slides from light.
9. It is important to note that the autofluorescence originating from the lignified cell wall will generally give an artifactitious signal [28]. TBO staining will reduce background autofluorescence. To quench the autofluorescence, stain the slides briefly with 0.01 % TBO [11]. It is essential to examine the images from the control samples for unspecific signals and to use them as a reference when observing and judging the specific signals from immunostaining.
10. To minimize tissue autofluorescence in a CLSM, select the proper emission collection filters to reduce some background signals. Using a narrow band-pass filter optimized for the emission of conjugated fluorochrome would reduce emissions coming from tissue autofluorescence.
11. To avoid the formation of precipitates of lead carbonate, keep the staining procedure away from atmospheric carbon dioxide. In TEM, the colloidal gold particles can be recognized as round black dots of regular size that are different from the precipitates of lead carbonate.

15.6 Interpretation of Immunostaining

To evaluate the specific immunofluorescent signals of ABA with an epifluorescence microscope or a CLSM, it is necessary to get rid of the background signals from autofluorescence and/or the unspecific binding of secondary antibody in tissues. Staining the slides briefly with 0.01 % TBO solution is effective in quenching the autofluorescence from lignified cell walls. To reduce background signals from the unspecific binding of secondary antibody in tissues, the pretreatment of the secondary antibody as described in ref [14] is useful. Checking the changes of intensity and locations of fluorescent signals before and after the pretreatments is helpful to discriminate specific immunofluorescent signals of ABA. If the fluorescent signals disappear after the pretreatments, they should be regarded artifacts. To evaluate the specific immunogold labeling of ABA in TEM observation, pretreating the primary antibody with the endogenous ABA extracted from the samples is a reasonable negative control to validate the specific colloidal gold particles of ABA in tissues. After the pretreatment of the primary antibody, the colloidal gold particles of ABA should disappear in tissues. These pretreatments are useful to reduce the unspecific signals and to provide reliable information about the interpretation of immunostaining signals.

Acknowledgments This research was supported by National Science Council, Taiwan, ROC (NSC 97-2313-B-178-001), to Yung-I Lee and by Academia Sinica, Taiwan, ROC, to Mei-Chu Chung. We also thank Dr. Wann-Neng Jane, Miss Mei-Jane Fang, and Miss Yi-Jia Chou (Plant Cell Biology Core lab, IPMB) for the help in the immunogold labeling and the use of a TEM and a CLSM.

References

1. Taiz L, Zeiger E (2010) Plant physiology, 5th edn. Sinauer Associates, Sunderland
2. Zavala ME, Brandon DL (1983) Localization of a phytohormone using immunocytochemistry. J Cell Biol 97:1235–1239
3. Eberle J, Arnscheidt A, Klix D, Weiler EW (1986) Monoclonal antibodies to plant growth regulators. III. Zeatinriboside and dihydrozeatinriboside. Plant Physiol 81:516–521
4. Aloni1 R, Langhans M, Aloni E, Dreieicher E, Ullrich CI (2005) Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream. J Exp Bot 56:1535–1544
5. Sotta B, Sossountzov L, Maldiney R, Sabbagh I et al (1985) Abscisic acid localization by light microscope immunohisto-chemistry in Chenopodium polyspermum L. J Histochem Cytochem 33:201–208
6. Ohmiya A, Hayashi T, Kakiuchi N (1990) Immuno-gold localization of indole-3-acetic acid in peach seedlings. Plant Cell Physiol 31:711–715
7. Ohmiya A, Hayashi T (1992) Immuno-gold localization of IAA in leaf cells of Prunus persica at different stages of development. Physiol Plant 85:439–445
8. Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185
9. Harris MJ, Outlaw WH, Mertens JRR, Weiler EW (1988) Water-stress-induced changes in the abscisic acid content of guard cells and other cells of Vicia faba L. leaves as determined by enzyme-amplified immunoassay. Proc Natl Acad Sci U S A 85:2584–2588
10. Christmann A, Hoffmann T, Teplova I, Grill E, Muller A (2005) Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed *Arabidopsis*. Plant Physiol 137:209–219
11. Schraut D, Ullrich CI, Hartung W (2004) Lateral ABA transport in maize roots (*Zea mays*): visualization by immunolocalization. J Exp Bot 55:1635–1641
12. Bewley JD, Black M (1985) Seeds: physiology of the development and germination. Plenum, NY
13. Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol 90:463–469
14. Peng YB, Zou C, Wang DH, Gong HQ et al (2006) Preferential localization of abscisic acid in primordial and nursing cells of reproductive organs of *Arabidopsis* and cucumber. New Phytol 170:459–466
15. Wachter R, Langhans M, Aloni R, Gotz S et al (2003) Vascularization, high-volume solution flow, and localized roles for enzymes of sucrose metabolism during tumorigenesis by *Agrobacterium tumefaciens* L. Plant Physiol 133:1024–1037
16. Sossountzov L, Sotta B, Maldiney R, Sabbagh I, Miginiac E (1986) Immunoelectron-microscopy localization of abscisic acid with colloidal gold on Lowicryl-embedded tissues of *Chenopodium polyspermum* L. Planta 168:471–481
17. Betrand S, Benhamou N (1992) Immunogold localization of free abscisic acid in tomato root cells. Can J Bot 70:1001–1011
18. Weiler EW (1982) An enzyme-immunoassay for *cis*-(+)-abscisic acid. Physiol Plant 54:510–514
19. Pastor A, Cortadellas N, Alegre L (1995) Immunolocalization of abscisic acid by monoclonal antibodies in *Lavandula stoechas* L. leaves. Plant Growth Regul 16:287–292
20. Welbaum GE, Pavel EW, Hill DR, Gunatilaka MK, Grayson RL (2000) Compartmentation of abscisic acid in developing muskmelon (*Cucumis melo* L.) seeds. In: Black M, Bradford KJ, Vazquez-Ramos J (eds) Seed biology: advances and applications, Proceedings of 6th International Workshop on Seeds. CABI, Merida, Mexico, pp 85–100
21. Arditti J, Ghani AKAR (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytol 145:367–421

22. Lee YI, Lee N, Yeung EC, Chung MC (2005) Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. J Am Soc Hortic Sci 130:747–753
23. Van der Kinderen G (1987) Abscisic acid in terrestrial orchid seeds: a possible impact on their germination. Lindleyana 2:84–87
24. Lee YI, Lu CF, Chung MC, Yeung EC, Lee N (2007) Changes in endogenous abscisic acid levels and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. J Am Soc Hortic Sci 132:246–252
25. Walker-Simmons M (1987) ABA levels and sensitivity in the developing embryos of sprouting and susceptible cultivars. Plant Physiol 84:61–66
26. Li X, Lamotte CE, Stewart CR, Cloud NP et al (1992) Determination of IAA and ABA in the some plant sample by a widely applicable method using GC-MS with selected ion monitoring. J Plant Growth Regul 11:55–65
27. Fong F, Smith JD, Koehler DE (1983) Early events in maize seed development. Plant Physiol 73:899–901
28. Biggs AR (1985) Detection of impervious tissue in tree bark with selective histochemistry and fluorescence microscopy. Stain Technol 60:299–304

Chapter 16
Plant Chromosome Preparations and Staining for Light Microscopic Studies

Subhash C. Hiremath and C. C. Chinnappa

16.1 Introduction

Swiss botanist Wilhelm Von Nägeli in 1842 discovered thread-like structures in plant nuclei and called them in German stäbchen or little sticks in English, which are now known as chromosomes [1]. Waldeyer [2], a German anatomist, coined the word chromosome in 1888 for these structures. In 1886, Mendel [3] formulated the laws of genetics without any knowledge of chromosomes and his work remained unknown. Three botanists Carl Correns (Germany), Hugo de Vries (The Netherlands) and Erich Von Taschermark (Austria) in 1900, independently rediscovered the Mendelian principle of heredity [4]. In 1902, Sutton and Boveri proposed the chromosome theory of inheritance and linked the behaviour of chromosomes with Mendelian principles [5]. Thus, arose the discipline of cytogenetics. Pioneering work of Morgan and his students with fruitfly and McClintock's contribution with maize laid the firm foundation of cytogenetics. It is a hybrid discipline nourished by contributions from cytology, genetics and lately molecular biology. Cytogenetics involves handling of chromosomes, structure, movement, function and behaviour of chromosomes including recombination, transmission and expression of genes [6].

Eukaryotic nuclear genome is constituted of discrete linear chromosomes and apparently, a segregational device for cell division. Each chromosome is made up of a single long linear DNA molecule associated with different protein molecules. It provides a framework structure for linkage groups, allows replication, transcription and transmission of genetic information. Each species is characterized by a precise number of chromosome in the nucleus. Plants show great diversity in chromosome number. In a well-documented work, lowest chromosome number has been reported as $2n=4$ in *Haplopappus gracilis* [7] and in several other species [8]. Highest chro-

S. C. Hiremath (✉)
Department of Applied Genetics, Karnataka University, Dharwad 580003, Karnataka, India
e-mail: subhashhiremath@yahoo.com

C. C. Chinnappa
Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_16

mosome count is known from a fern *Ophioglossum reticulatum* as $n = 720$ [9]. There are a few exceptions to the constancy of chromosome number within a species that shows different chromosome numbers, for example *Eleusine floccifolia* shows diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) races [10]. Several such examples are available in literature. Another source of variation in chromosome number is due to the presence of B chromosomes in addition to the normal chromosomal compliment in some plants [11]. Knowledge of chromosome number is essential in taxonomy, systematics, phylogeny, plant breeding, genetics, physical mapping and DNA-sequencing projects [12].

Light microscopic observation of metaphase chromosomes reveals three distinct features: centromere (primary constriction), nucleolar organizing region (secondary constriction) and telomere at the two free ends of linear chromosomes. Position of centromere determines the shape of the chromosome. Levan et al.'s system of classification and nomenclature of chromosome is widely used by cytogeneticists [13]. Chromosome shape can be metacentric, submetacentric, acrocentric and telocentric depending upon the position of centromere along its length (Fig. 16.1).

Development of staining procedures of root tip squash method and observing condensed chromosomes at metaphase stages have led to the popularity of analysing chromosomes for various cytogenetic studies. At this stage, individual chromosomes are amenable for identification in a genome. In this direction, Levitsky [14] proposed and developed the concept of karyotype, and defined it as phenotypic appearance of somatic metaphase chromosomes complimenting their genic content. Diagrammatic representation of karyotype is often termed as ideogram or karyogram. Karyotype analysis has helped in the identification of individual chromosomes within a genome. Such studies have been undertaken in diverse group of plants over past several decades. These data have been useful in evolutionary and phylogenetic studies at taxonomic level between species and family [12].

With conventional staining methods, it has not been possible to differentiate all the chromosomes in a karyotype of several groups of plants and animals. Various

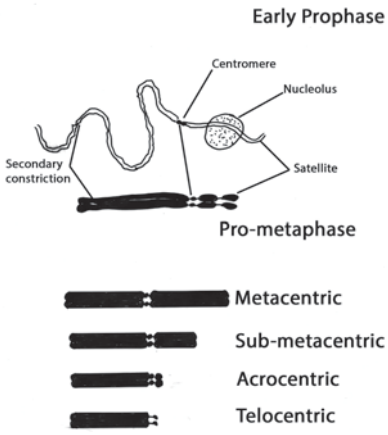

Fig. 16.1 Diagrams showing somatic chromosome morphology (modified [37])

chromosome banding methods were developed after 1960s, which produced transverse dark and light bands along the length of chromosomes. These banding methods are widely used for the identification of individual chromosomes in the karyotype, detection of structural changes in chromosomes, aneuploid identification, chromosome polymorphism and genome analysis of polyploids. Various chromosome banding methods that are reported in usage in plants and animal species are Q-, G-, C-, N-, F-, Hy-, R- and Ag-NOR-banding [15–17]. Among these banding methods, Q- and C-banding are widely used in plants.

Development of in situ hybridization (ISH) technique [18, 19] heralded the dawn of molecular cytogenetics. In this procedure, radioactively labelled nucleic acid sequence or probe was hybridized to chromosomal preparations on the slide. Probe-targeted hybrid sequence along the chromosome was detected by autoradiography. In later techniques, radiolabelling was replaced by fluorochromes or fluorescent stains and DNA: DNA hybridization (probe-target sequence) on chromosomal location was visualized by fluorescent microscopy. This modified ISH is now known as fluorescent in situ hybridization or FISH technique, and various aspects of this procedure have been reviewed [17, 20–22]. FISH technology has enabled cytogeneticists to recognize individual chromosomes for karyotype analysis, and phylogenetic studies to prepare physical maps, to study the role of chromatin at cytological level that regulates gene expression [20, 21]. Modification of FISH is known as genomic in situ hybridization (GISH) in which whole genomic DNA is used as probe instead of specific DNA sequences like 45S or 5S r-RNA genes, etc. GISH allows the identification of whole genome in a polyploidy species or a hybrid. FISH procedures and allied aspects are detailed in Chap. 17 of this book.

Chromosome preparation is a basic indispensable tool in cytogenetics. It is required for recording chromosome numbers and ploidy level, karyopype analysis, chromosome pairing data in species and hybrids, chromosome banding, FISH, GISH and hosts of situations in genetic studies. In modern genomic projects, chromosome preparations provide a framework for physical maps, number of linkage groups and guide in genome sequencing projects [12, 17].

16.2 Materials

16.2.1 General Laboratory Equipment and Supplies

Microscope with digital camera attached, stereomicroscope, slide warmer, water bath, forceps, camel hair brush, slides and coverslips, dissecting needles, Lancet needle, blade, spirit lamp, beakers, vials, cavity block or Syracuse dish, watch glass, and Coplin jars.

16.2.2 Chemicals for Plant Treatment

1. Colchicine (*see Note 1*), 8-hydroxyquinoline (*see Note 2*), p-dichlorobenzene (*see Note 3*), monobromonaphthalene (*see Note 4*)
2. Stains: carmine (*see Note 5*), orcein (*see Note 6*), Basic fuchsin (*see Note 7*), Giemsa (*see Note 8*)
3. Chemicals for fixative preparations: chloroform (*see Note 9*), ethanol (*see Note 10*), propionic acid (*see Note 11*), glacial acetic acid (*see Note 12*)
4. Mounting media: Canada balsam (*see Note 13*), Euparal (*see Note 14*), DPX (*see Note 15*)
5. Other chemicals: hydrochloric acid, barium hydroxide

16.2.3 Preparation of Reagents and Solutions

1. 0.1% Colchicine solution: dissolve 1 g of colchicine in 100 mL dH_2O and use it as stock solution. From this prepare 0.1% solution by dilution. Store in refrigerator.
2. 0.002 M 8-hydroxyquinoline: dissolve 0.3 g of this chemical in 1 L dH_2O. Keep at 60 °C overnight. Store in refrigerator.
3. Paradichlorobenzene-saturated solution: weigh and dissolve 3 g of this chemical in 200 mL dH_2O by keeping the bottle at 60 °C overnight in a stoppered bottle. Store in refrigerator [23].
4. Monobromonaphthalene-saturated solution: prepare saturated solution by adding 1 mL of monobromonaphthalene to 98 mL of water by constant shaking. Later add more of this chemical drop by drop with constant shaking. Let it settle down. Undissolved monobromonaphthalene settles down and can be reused.
5. Preparation of acetocarmine [24]: in a large Erlenmeyer flask, provide heat to boil 100 mL of 45% glacial acetic acid. Add 1 g of carmine powder to the boiling solution and let the solution simmer for 5 min. Allow it to cool and settle. Filter and store in coloured bottle. Keep it in a refrigerator (*see Note 16*).
6. Preparation of acetoorcein: 2% and 1% acetoorcein is prepared in a manner acetocarmine is prepared. Carmine is replaced by orcein. No iron mordanting is needed for acetoorcein staining. Store in refrigerator and use within 2–3 months.
7. Preparation of propino-carmine: propino-carmine is prepared in the same manner as acetocarmine. Instead of 45% acetic acid, 45% propionic acid is used. Addition of ferric salt is recommended.
8. Preparation of Lillie's Feulgen stain [25]: dissolve 1 g of basic fuchsin in 100 mL of 0.15 N HCl (15 mL N HCl to this add 85 mL dH_2O) in a tightly stoppered bottle. To this dye solution, add 2.2 g of sodium metabisulphite. Shake frequently or place on a shaker for 2–4 h. Solution may be clear to pale yellow in colour. Add activated charcoal or carbon and shake for few minutes and filter quickly using coarse filter paper. Good Feulgen stain will be clear with strong sulphur dioxide odour. Store in amber-coloured bottle in refrigerator; it can be stored for a few months.

9. Preparation of de Tomasi's Feulgen stain [26]: pour 200 mL of boiling dH_2O into 500 mL Erlenmeyer flask containing 1 g basic fuchsin powder. Shake well and allow it to cool to 50 °C. Filter into a bottle. Add 30 mL 1 N HCl and 3 g of potassium metabisulphite, stopper the bottle tightly. Shake and leave in dark for 24 h. Solution will be clear. In case it is pale yellow or straw coloured, add 0.5 g carbon and shake for few minutes. Filter quickly using coarse filter paper into a new dry coloured bottle with tight stopper. Store in a refrigerator; it remains in a good condition for 2–3 months. One can get excellent Feulgen reagent using pure pararosaniline instead of basic fuchsin.
10. Preparation of Giemsa stain [27]: dissolve 1 g of Giemsa dye in 66 mL of glycerine at 60 °C with constant stirring for an hour. Add 66 mL methanol and continue stirring at 60 °C for 24 hrs. Filter and keep in refrigerator. The stain solution is useful for up to 2 months. Giemsa stain is available in premade and ready-to-use stock solution of different strength from various venders. From this stock solution, the required per cent of Giemsa stain is prepared by mixing with phosphate buffer (pH 7).
11. Carnoy's Fixative I (*see Note 17*): 3 parts ethanol 95–100 % and 1 part glacial acetic acid.
12. Carnoy's Fixative II (see *Note 18*): ethanol 95–100 %: glacial acetic acid: Chloroform (6: 3: 1; v:v)
13. Preparation of Sorenson phosphate buffer: Part-A, dissolve 9.47 g sodium phosphate dibasic (Na_2HPO_4) in 300 mL dH_2O and make to 1 L. Part-B, dissolve 9.07 g potassium dihydrogen phosphate (KH_2PO_4) in 300 mL dH_2O and make to 1 L. Label and store A and B in separate bottles and leave them at room temperature. Mix 58 mL of part-A and 42 mL of part-B to get 100 mL of buffer at pH 7.0.
14. Preparation of 2x SSC solution: prepare 20X SSC (Saline Citrate Solution) stock solution by dissolving 88.2 gm of trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 175.3 g of sodium chloride (NaCl) per liter dH_2O. This solution can be stored for several months. Dilute 1 part of 20x SSC with 9 part of dH_2O to get 2x SSC.

16.3 Methods of Studying Chromosomes at Mitosis

16.3.1 Specimen Collection

Microscopic examination of any biological specimen for chromosomal analysis involves several steps like sample collection, pretreatment, fixation, staining and mounting. Important aspects of this exercise are to obtain a good chromosomal preparation having large number of cells with well-spread brightly stained chromosomes.

Actively growing meristematic tissues like root tips, shoot tips and leaf tips are favourable material for chromosome preparation. Among these, root tips are chosen as specimen because of the ease of obtaining them at short notice. Root apices can be easily obtained from seeds. They can be easily germinated on moist filter paper at 20–25 °C. Root tips of about 1–3 cm long touching the filter paper are collected in a vial with cold water.

Healthy potted plants at early developmental stage are also good source of actively growing root tips. Take the pot, carefully turn it upside down and remove the pot. The healthy growing roots would be seen on sides of soil mould holding root system. Carefully collect 2–3 cm long root apices with forceps and put them in vial containing cold water. Using a camel hair brush, clean the root tips in running water for any soil particles adhering to them.

16.3.2 Pretreatment

Various pretreating agents have been used to obtain well-condensed chromosomes with clear constriction regions. Further, these agents inhibit spindle formation thereby arrest the chromosomes at metaphase stage with increased number of metaphase cells. All these changes are brought about due to the physical changes in cytoplasm by pretreating agents [28].

Low temperature (2–8 °C) has been widely used as a physical pretreating agent for cereal chromosome analysis. Root apices in cold water are pretreated at low temperature in a fridge for 12–24 h. This causes chromosome condensation with high mitotic index.

Large number of chemicals have been evaluated for the pretreatment of chromosomes. Some chemicals are sparingly soluble in water and these are used as saturated solutions. Only few of the chemicals have been found to be useful as pretreatment chemicals for chromosomes.

16.3.3 Study of Chromosomes at Mitosis

Ability to reproduce is a fundamental property of living organisms. Eukaryotes reproduce by two types of cellular divisions, Mitosis and Meiosis. Mitosis is characterized by perseverance of constant chromosome number. This type of cell division is the property of body cells or somatic tissue. In contrast, meiosis occurs in sporogenous or germinal cells and chromosome number is reduced from diploid to haploid or halved. Both types of cell divisions involve the cell cycle comprising growth (G1), DNA synthesis (S), growth (G2) mitosis or meiosis (M) and cytokinesis (C) phases [29]. Mitotic division involves division of nucleus to form two identical daughter nuclei and this process is referred as karyokinesis or mitosis followed by division of cytoplasm or cytokinesis. Net result of this is the formation of two daughter cells each having one nucleus. In plants, cytokinesis is by cell plate formation.

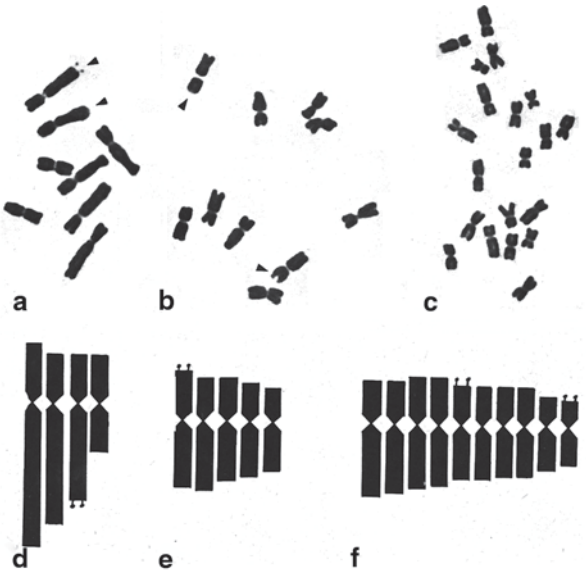

Fig. 16.2 Feulgen stained mitotic chromosomes and karyotype diagrams of three species of *Krigia*. **a** Mitotic metaphase chromosomes of *K. cespitosa* (2*n*=8). *Arrows* show satellite chromosomes. **b** Mitotic metaphase chromosomes of *K. biflora* (2*n*=10). *Arrows* show satellite chromosomes. **c** Mitotic metaphase chromosomes of *K. montana* (2*n*=20). **d**, **e**, **f** Idiograms of haploid sets of karyotype chromosomes of *K. cespitosa, K. biflora* and *K. montana* respectively Modified from Chinnappa, 1981 Cytological studies in *Krigia* (Asteraceae), Can. J. Genet. Cytol. 23. 671–678. [63]

16.3.3.1 Stages in Mitosis

Somatic cell division or mitosis consists of two major phases: karyokinesis or nuclear division or mitosis and cytokinesis. Six stages have been recognized in the mitosis and they are interphase, prophase, metaphase (chromosome morphology for karyotype analysis is normally studied at this stage using various staining procedures), anaphase, telophase and cytokinesis.

16.3.4 Protocols for Root Tip Squash Preparation

Main aim of somatic chromosome preparation is to determine the chromosome number and study chromosome morphology. For this purpose large number of stains have been used to device the staining methods. Among these, most widely used schedules for chromosome preparations are given here. To get good, consistent, high-quality reproducible chromosome preparations, one must use stains certified by the Biological Stains Commission.

16.3.4.1 Acetocarmine Staining Schedule

Material

Collect fresh root tips or leaf tips or any other meristematic tissue. Pretreat them with appropriate pretreating agent.

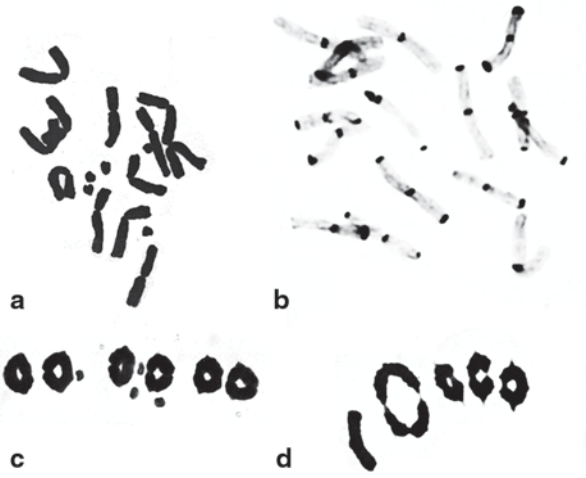

Fig. 16.3 Mitotic and meiotic chromosomes. **a** Mitotic metaphase chromosomes of *Tradescantia hirsuticaulis* $2n=12$ and B chromosomes. **b** Geimsa C-banding chromosomes of *Allium schoenoprassum* $2n=16$. **c** Meiotic metaphase chromosomes of *Tradescantia hirsuticaulis*. Showing 6 ring bivalents and 4 B chromosomes. **d** Meiotic metaphase chromosomes of *Tradescantia hirsuticaulis*, showing 1 rod bivalent, 1 quadivalent and 3 ring bivalents. Fig. 16.3**a**, **c**, **d** Modified from [64] Structural hybridity and supernumerary chromosomes in a diploid *Tradescantia hirsuticaulis* Can. J. Bot. 53: 456–465. Fig. 16.3b. Modified from [45] Giemsa C-banded kazyotypes of seven North American species of *Allium*. Amer. J. Bot. 74: 1087–1092.

Fixation

Wash and fix the material in Carnoy's fixative for 12–24 h and store at −20 °C or transfer it to 70 % alcohol.

Staining Procedures:

1. Take two root tips in a watch glass. Add several drops of 1 N HCl and leave them for few minutes. Root tips are now soft and supple.
2. Transfer one root tip on a slide with forceps and put a drop of 1 % acetocarmine over it. Examine under a dissecting microscope, identify the conical part of a root apex with attached root cap and the zone of elongation at the opposite end. Using a lancet needle, cut and remove root cap and zone of elongation. One must remove the root cap as these cells are tough and prevent uniform spreading of dividing cells. Only 1–2 mm of meristematic tissue is left in carmine solution on the slide.
3. Add a drop of carmine and warm over a gentle spirit lamp flame. Using an iron needle macerate the tissue. Carmine will now have bluish tinge. With a needle evenly spread the tissue in carmine solution (*see Note 19*).

4. Now keep the slide on a flat surface having thick blotters. Pick up a coverslip with forceps and gently place it on the carmine tissue preparation. Gently warm to remove any air bubbles. Do not boil.
5. Place a folded blotting paper over the coverslip on the slide and press hard and firmly on the coverslip with your thumb. This squash process flattens the cells and scatters the condensed chromosomes in the cell. Often the squashing breaks the thin walls of meristematic cells and chromosomes are scattered evenly without any overlap. While squashing, care must be taken not to cause lateral movement of coverslip as this will destroy the well-spread chromosome preparation (Fig. 16.3a).
6. Seal the edges of cover glass with rubber solution or gum mastic.
7. Observe the preparation under microscope. The chromosomes would appear dark translucent red and cytoplasm faintly red.

16.3.4.2 Feulgen Staining Schedule

Feulgen staining has many advantages. In this staining method only chromosomes and nuclei are stained. With this schedule several samples can be processed simultaneously. Stained tissue can be stored. Only disadvantage is that it is long and a little complicated process. Slides can be made permanent using Conger and Fairchild's dry ice method [30] or Celarier's t-butyl alcohol series [31] (*see* Sect. 16.6).

Material and Fixation Protocol

As in subheading "Material" in Sect. 16.3.4.1.

Staining Procedure

1. Transfer root tips from 70% alcohol to water. Give two washes in water for 2–3 min each.
2. Dip the root apices in a vial containing 1 N HCl. Keep the vial in a water bath at 60 °C and hydrolyze the tissue for 8–15 min. The correct time of hydrolysis at which optimum staining occurs must be empirically determined.
3. Remove the vial from the water bath and drain off the hot 1 N HCl and replace with fresh 1N HCl maintained at room temperature. Allow it to stand for 2–3 min.
4. Replace the 1 N HCl in the vial by water. Give two rinses of water for 2–3 min each.
5. Gently transfer the material into vial containing the Feulgen stain. Care should be taken to blot out any carry over water by blotting paper. Keep the vial in dark for 1–1½ h for staining (*see Note 20*).
6. Take a stained root tip on a slide and place a drop of 45% acetic acid. With a lancet needle head, cut and remove the root cap and zone of elongating cells to

expose meristamatic tissue. On the 1–2 mm meristematic tissue, place the cover glass and warm. Squash the cells as in Sect. 16.3.4.1.
7. Seal the edges of coverslip with rubber solution or gum mastic. Observe under microscope (Fig. 16.2a, b, c). Chromosomes and nuclei would appear magenta red and cytoplasm colourless.

16.3.4.3 Acetoorcein Staining Schedule

Acetoorcein schedule is simple and the most widely used method for somatic chromosome preparation. This procedure does not require iron or any mordant. Slides can be made permanent (*see* Sect. 16.6).

Material and fixation protocol

As in subheading "Material" in Sect. 16.3.4.1.

Staining Method According to La Cour [15]

1. Take root tips in vial containing 1N HCl and hydrolyze for few minutes at 60 °C in a water bath. Time of hydrolysis should be empirically determined as it has bearing on separation of cells and staining of chromosomes.
2. Transfer the root tips to a watch glass and put 9–10 drops of 1% acetoorcein solution. Heat over a spirit lamp until fumes start appearing. Heat for 2–3 min. Do not boil. Allow it to cool.
3. Take a clean slide with a drop of 1% acetoorcein and put a stained root tip. Examine under dissecting microscope to locate conical root apex. With a lancet needle remove the root cap and the zone of elongation.
4. Macerate the 1–2 mm meristematic tissue with a needle and apply a clean coverslip. Warm and allow it to cool.
5. Squash the cells and seal the edges of coverslip as in Sect. 16.3.4.1.

16.3.4.4 Giemsa C-banding Schedule

Vosa and Marchi [32] developed this banding procedure. It has undergone several modifications. Improved techniques enable the recognition of individual bands in the chromosomes of plant genome using light microscopy.

Material

As in subheading "Material" in Sect. 16.3.4.1.

Fixation

Fix root tips in 45% acetic acid for few minutes at room temperature or for 20 min at 60 °C [33]. Enzymes like pectinase, cellulase, etc. can be used for softening purpose [6].

Processing and Staining Procedures

1. Squash the root apices on a clean slide in 45% acetic acid.
2. Remove the cover glass by dry ice method [30]. After freezing the coverslip is prised off with a blade.
3. We describe here the standard protocol of Giraldez et al. [34]. Variants from their method are indicated at each step. For dehydration, slides are dipped in 95–100% ethanol overnight. Most workers keep the slides in above alcohol for 1–2 h for good bands in rye, barley, maize and other plants [35].
4. Air dry the slide for few minutes at room temperature. Some investigators air dry the slides overnight [36] to a few weeks [37].
5. Incubate the slides for 2–5 min in 0.2N HCl at 60 °C in water bath. The time and temperature are important for resolution of good bands.
6. For the Barium hydroxide/Saline/Giemsa stain (BSG) procedure, wash the slides briefly in dH_2O.
7. The slides are denatured by incubating the slides in Coplin jar in freshly prepared saturated solution of $Ba(OH)_2$ for 7 min at room temperature or at 50–55 °C in a water bath [34]. Wash carefully in distilled water and ensure all $Ba(OH)_2$ has been removed. Alkali treatment denatures the DNA helix and results into ssDNA. $Ba(OH)_2$ must be prepared fresh for each denaturation reaction.
8. Renaturation of slides is carried out by transferring the slides to a Coplin jar with 2x SSC and incubate it at 60 ºC in water bath for 1 h.
9. Remove the slide from SSC and directly place them in 3% Giemsa stain in Sorenson phosphate buffer pH 7.0 (*see Note 21*).
10. Monitor the staining under microscope. Increase the stain concentration if required. Staining time of 10–45 min would give good results. About 30 min would be optional. In case stain is too blue, increase treatment time of HCl or decrease the time of $Ba(OH)_2$ treatment. In case of deep red staining of cytoplasm, the HCl treatment time is reduced. Giemsa stain should be from good source with stain commission certification for chromosomal staining purpose.
11. Dip the slides in distilled water and air-dry them.
12. Keep the slides in xylene overnight and mount in Euparal or Canada balsam (Fig. 16.3b). Slides can be kept for years at room temperature without any loss of contrast.

16.4 Methods for Karyotype Analysis

The number of chromosomes found in the somatic cells is termed as somatic chromosome number and is referred as 2*n*. Whereas, the number of chromosomes present in gametic cells is half of the somatic number and is termed as gametic chromosome number or n. Every eukaryotic species is normally characterized by a definite number of chromosomes in their cells and it is a species-specific trait.

Levitsky [14] proposed the concept of karyotype. He defined the karyotype as phenotypic appearance of somatic metaphase chromosome in contrast to their genic content. In a simpler term, karyotype is the number and appearance of chromosomes at somatic metaphase stage. Diagrammatic representation of a karyotype is referred to as ideogram or karyogram (Fig. 16.2d, e, f).

Six features of karyotype are recognized and compared [37]. They are differences in (i) absolute size of chromosomes, (ii) position of centromere, (iii) relative size of chromosome, (iv) basic chromosome number, (v) number and position of satellite, and (vi) degree and distribution of heterochromatin. Analysis of karyotype helps us in identifying each chromosome pair in the chromosome complement. This has several theoretical and practical applications. Karyotype and its significance in genetics, evolution and systematics has been discussed by eminent geneticists [37–42].

16.4.1 Chromosome Morphology and Classification

Position of centromere along the length of chromosome is the most important criterion for identification and classification of chromosome in the karyotype. Several systems of classification and nomenclature have been proposed [13, 41, 43, 44]. Levan et al.'s [13] system of nomenclature and classification of chromosomes is widely used in karyotype analysis. This system of classification is given in Table 16.1. Position of centromere is correlated to the resulting type of chromosome. Arm ratio (R) that is long arm/short arm is an important parameter in determining the position of centromere on the chromosome. In a karyotype, chromosomes can be identified as metacentric (M or m), submetacentric (Sm), subtelocentric (St), acrocentric (T) and telocentric depending upon the arm ratio (Table 16.1, Fig. 16.1).

Satellite is a spherical body separated from the main body of chromosome by a secondary constriction. It is connected to the main body of chromosome by thin

Table 16.1 Classification of chromosomes. (Based on data from [13])

Chromosome type	R	Symbol	Position of centromere
Metacentric	1.0	M	Median point
Metacentric	1.0–1.7	M	Median
Submetacentric	1.7–3.0	Sm	Submedian
Subtelocentric	3.0–7.0	St	Subterminal
Acrocentric	7.0	T	Terminal
Telocentric	∞	T	Terminal point

chromatin fibre. Nucleolus is formed at secondary constriction [41]. Each chromosome complement or karyotype contains two or more satellites. Secondary constriction is constant in position on a chromosome. This feature is used in identifying particular chromosome. A chromosome possessing satellite is termed as satellite chromosome or SAT-chromosome.

Battaglia [41] classified the satellite based on position and size. Based on position, terminal and intercalary satellites are recognized. In the former, satellite is on the extreme end of secondary constriction and in the latter, the satellite is between two nuclear constrictions. Satellites have been further classified based on size: (a) microsatellite—small spherical satellite, that is having diameter equal or less than one half of the chromosomal diameter; (b) macrosatellite—large spheroidal satellite, that is having diameter greater than one half of the chromosome diameter; and (c) linear satellite—a satellite having the shape of a long chromosome segment.

16.4.2 Construction of Karyotype and Idiogram

About 15–20 complete metaphase plates with well-spread clear chromosomes from three to eight individuals from each population are analyzed. Obtain photo prints of 10 metaphase plates and select 5 plates for measurements of chromosomes. Classification of chromosome types is based on Levan et al.'s [13] system. Karyogram is prepared by arranging chromosomes in decreasing order.

16.5 Study of Chromosomes at Meiosis

Meiosis is a characteristic feature of sexually reproducing organisms. In animals, meiosis normally takes place in germinal cell leading to formation of haploid gametes. In contrast, in plants, meiosis occurs in sporogenous tissue of anther and ovule to form haploid microspores or megaspores. These divide mitotically to form male and female gametophytes. Two haploid gametes fuse to form a diploid zygote that develops into a diploid sporophyte.

16.5.1 Outline of Meiotic Process

It consists of two cell divisions, meiosis-I and meiosis-II. DNA synthesis or chromosome duplication occurs prior to meiosis-I. In meiosis-I, the chromosome number is halved and sometimes this division is referred to as reduction division. Second meiotic division is typically a mitotic division. The net result of meiosis is from one diploid sporogenous cell four haploid cells are produced. From these, later by mitotic divisions, gametes are formed. Thus meiosis plays a crucial role in reproduction of eukaryotic organisms.

16.5.1.1 Meiotic Division I

The first meiotic division is basically a reduction division. The sporogenous cells contain diploid or 2*n* chromosome number. These chromosomes pair during prophase of this division. The paired chromosomes are known as homologous chromosomes and they are normally identical in size, shape and gene sequence. This pairing of homologous chromosomes is the basis for orderly process that leads to reduction of chromosome number to half or haploid condition. The reduction of chromosome number takes place in such a way that two haploid cells of Division I receive one chromosome from each chromosome pair.

Stages in Meiotic division I: prophase I—leptotene, zygotene, pachytene, diplotene, diakinesis; metaphase-I; anaphase-I and telophase-I. Exchange of chromosome segments by a process called crossing over is visualized at diplotene, diakinesis and metaphase stages. This results in segregation of genes.

16.5.1.2 Meiotic Division II

Mechanistically, meiotic division II is similar to somatic mitotic process. It follows prophase-II, metaphase-II, anaphase-II and telophase-II as in typical somatic mitosis (prophase, metaphase, anaphase and telophase). Anther pollen mother cells (PMCs) and megaspore mother cells (MMCs) in ovule are both diploids. They undergo meiosis and produce four haploid uninucleate microspores and megaspores. These haploid cells develop into male and female gametes. The result of meiotic division of a single diploid cell leads to formation of four haploid cells. Unlike mitotic products, these four cells are not genetically identical.

16.5.2 Protocol for Meiotic Chromosome Preparation and Staining

Meiotic investigations provide cytogenetic data useful in genetic analysis, plant breeding and evolutionary studies. Generally, all protocols for meiotic chromosome preparations utilize classic anther squash technique using variety of stains. Commonly used stains are carmine, Feulgen and Giemsa. These protocols are described here.

16.5.2.1 Acetocarmine Staining Schedule

This staining schedule is widely used in meiotic chromosome preparation in plants. Its popularity is due to simple staining schedule and intense staining of chromosomes with bright contrast between chromosomes and cytoplasm.

Material and Fixation

Collect inflorescence or young flower buds and fix them in freshly prepared Carnoy's fluid for 12–24 h. Store them at −20 °C or transfer them to 70 % alcohol at 0–4 °C. Volume of fixative should be 10–20 times more than the volume of the material.

Staining Procedure

1. Take out the inflorescence or bud from storage vial and place it on a watch glass containing few drops of 70 % alcohol. Work from young bud to older ones in a graded manner.
2. Place the bud on the slide and dissect out the anthers and discard the remaining floral parts. Keep all the anthers in a watch glass with few drops of 70 % alcohol.
3. Put an anther on the slide in a drop of 1 % acetocarmine. Crush it with a lancet needle head. Warm over a spirit lamp. Check the presence of PMC with meiotic stages under a microscope. In case PMC do not show the meiotic stage, discard the slide. Repeat the above process with next older bud until you find a bud having anther with meiotic stages.
4. Now take the anther with desired meiotic stage on a clean slide and put a drop of 1 % acetocarmine over it. With a needle, tease and macerate the anther so as to free the PMC from the anther wall. Remove all the debris with a needle and put a cover glass, warm over the spirit lamp (*see Note 22*).
5. Put a folded blotting paper over this slide and press down over the cover slip with your thumb. This pressure will flatten the meiocytes and chromosomes will appear in one plane. Generally, all the meiocytes in a given bud tend to be in the same stage of meiosis. They follow synchronous division. Anthers from different flower buds may show different stages.
6. Examine the slide under compound microscope first under low power and then in high power. Instead of acetocarmine one can use propinocarmine or acetoorcein and follow the above schedule.
7. Slides can be made permanent using Celeriar's method [31] or McClintock's method [42] or Conger and Fairchild's dry ice method [30].

16.5.2.2 Feulgen Staining Schedule

Material and Fixation

Collect inflorescence or flower buds. Fix them in freshly prepared Carnoy's fluid (3:1) for 12–24 h and store them at −20 °C or transfer to 70 % alcohol.

Staining Procedures

1. Take out the inflorescence or flower bud from the storage vial and keep in a watch glass with few drops of 70% alcohol. Work from the young to mature buds.
2. Steps 2 and 3 as in acetocarmine meiotic staining schedule, *see* subheading "Staining Procedure" in Sect. 16.5.2.1.
3. Collect large number of anthers at different meiotic stages. Give them two washes of dH_2O for 2–3 min.
4. Transfer all the anthers to a vial containing 1 N HCl. Keep the vial in a water bath at 60 °C and hydrolyze them for 8–15 min. Correct time of hydrolysis at which optimum staining occurs must be empirically determined.
5. Take out the vial from water bath and drain off the hot 1 N HCl. Pour 1 N HCl maintained at room temperature and allow it to stand for 2–3 min.
6. Replace the 1 N HCl in vial by dH_2O. Give two changes of dH_2O for 2–3 min each.
7. Transfer all the anthers into vial containing Schiff's reagent or Feulgen stain and put a stopper (*see Note 23*). Keep the vial in dark for 1–1½ h. Stained anthers can be stored. Give a wash in dH_2O and transfer the stained anthers to 70% alcohol at −20 °C. They can be stored up to 3 months.
8. Take a right anther on a clean slide and put a drop of 45% acetic acid over it. With a needle, tease and macerate the anther so as to free PMC's from anther wall. Remove all the debris with a needle and put a cover glass. Warm over spirit lamp.
9. Squash the preparation as in subheading "Staining Procedure" in Sect. 16.3.4.1. Seal the edges of cover glass with rubber solution or gum mastic. Normally all the PMC in a given bud tend to be in same stage of meiosis. They follow synchronous division. Anther from different flower buds may show different stages.
10. Slides can be made permanent as in Sect. 16.6 (Fig. 16.3c, d).

16.5.2.3 Giemsa C-banding Schedule

Material and Fixation

As in acetocarmine schedule for meiotic chromosome preparation.

Staining Procedures

1. Make an anther squash preparation as in acetocarmine meiotic staining schedule (Steps 1–5). Instead of acetocarmine, use 45% acetic acid for squash preparation.
2. Remove the cover glass by dry ice method. After freezing the slide prise off the cover glass with a blade.

3. Dip the slides in ethanol for 1–2 h and air-dry them. The procedure followed here is from Cai and Chinnappa [45].
4. Place the air-dried slides in a Coplin jar with 0.2N HCl for 2 min at room temperature or at 60 °C [34].
5. BSG Procedure ($Ba(OH)_2$/saline/Giemsa stain)—denaturation**:** Transfer the slides to freshly prepared saturated solution of $Ba(OH)_2$ for 7 min at room temperature or at 50–55 °C in water bath [34]. Wash all the $Ba(OH)_2$ with dH_2O or deionized water.
6. Renaturation: Keep the slides in a Coplin jar with 2x SSC and incubate at 60 °C in a water bath for 1 h.
7. Remove the slides from SSC solution and place them in 3 % Giemsa solution with pH 7.0. Some investigators wash the slides from SSC in distilled water three times and air dry them. Then place them in 3 % Giemsa stain [34]. Staining time of about 10–45 min would give good results. Dip the slides in distilled water and air dry them.
8. Keep the slides in xylene overnight and mount them in Euparal or Canada balsam.

16.6 Permanent Slide Preparation

Permanent preparations of cytological slides are much desired. These are required for later observation and record keeping. We describe here the widely used methods for making the slides permanent.

16.6.1 McClintock's Method [42]

1. Scrape the sealing and dip the slide in a Coplin jar containing 10 % acetic acid. Wait for 10–15 min for cover glass to separate from the slide. In case it does not separate flick off the cover glass with a blade.
2. Transfer the slide and cover glass to a next Coplin jar having a mixture of 1:1 glacial acetic acid and absolute alcohol. Leave them in this fluid for 15 min.
3. Pass through acetic acid—absolute alcohol 3:1 and 1:9 mixture for 5 min at each step.
4. Keep the slides and cover glass in absolute alcohol for 5 min. Give one more change of absolute alcohol.
5. Next give two changes in absolute alcohol for 5 min each.
6. Mount the cover glass on a new slide and use new cover glass to mount on old slide using Euparal (*see Note 24*).

16.6.2 Dry Ice Method (Conger and Fairchild [30])

1. Keep the squashed slides on the flat bed of dry ice for about 2–3 min. Freezing for longer duration is harmless.
2. Pry off the cover glass with a blade while the slide is still on the dry ice bed. Most of the material will be sticking to slide only.
3. Quickly dip the frozen slide into a Coplin jar containing absolute or 95% alcohol for 5 min.
4. Transfer the slide to a next Coplin jar with absolute alcohol or 95% alcohol. Allow it to remain in this fluid for 10 min (*see Note 25*).
5. Mount the preparation in Euparal or Canada balsam.

16.6.3 Celarier's Method [31]

1. Keep the squashed or smeared slides in a rectangular horizontal staining jar containing mixture of 1:1 glacial acetic acid and t-butanol. Cover glass will loosen after 5–10 min and with a needle flick off the cover glass. Allow them in this fluid for another 10–20 min.
2. Pick up the slide and cover glass with forceps and blot the fluid on a blotting paper. Place them in the next staining jar having mixture of 1 part glacial acetic acid and 3 parts butanol. Retain them in this dehydration grade for 15–30 min.
3. Transfer the slide and cover glass after blotting away the excess dehydration fluid to a next staining jar containing pure butanol. Leave them for 10 min.
4. Remove the slide from butanol and mount in a drop of Euparal. Mount the cover glass in a drop of Euparal on a new slide.

16.7 Notes

1. Colchicine ($C_{22}H_{25}NO_6$, MW-399.4) is an alkaloid obtained from autumn crocus *Colchicum autumnale* used in the treatment of gout. Colchicine is a pale yellow crystal or powder soluble in water. Low concentration from 0.05 to 0.5% in water for 1–3 h at room temperature brings about the shortening of chromosome arms and constriction regions become highly conspicuous. Higher concentrations of this chemical induces polyploidy. Roots should be washed properly after pretreatment and fixed in a fixative. This pretreatment appears to allow the easy penetration of fixative at later stages. Handling and disposal of colchicine should be done with great care as it is a toxic compound. Washing of root tips after pretreatment should not be prolonged as the dividing cells may enter into interphase.
2. 8-hydroxyquinoline (C_9H_7NO, MW-145.6) is a light-yellow crystalline solid organic compound and sparingly soluble in water with melting point of 72–74 ºC.

It is a most popular pretreating agent for medium to long chromosomes. Excised root tips are pretreated with 0.002 M 8-hydroxyquinoline at 12–16 ºC for 3–5 h results into same shortening of chromosomes as after colchicine [46]. Pretreatment is followed by washing and fixation. Pretreatment above 18 ºC causes clumping of chromosomes. Unlike colchicine, hydroxyquinoline allows the metaphase chromosomes to maintain their relative arrangement at equatorial plane.

3. p-dichlorobenzene ($C_6H_4Cl_2$, MW-147) is a chlorinated aromatic hydrocarbon compound used as a fumigant, insecticide and repellent. The compound is a white solid and sublimes into gas at room temperature with poor solubility in water. Plants with small chromosomes are pretreated with this chemical agent. A saturated aqueous solution is used to pretreat the somatic tissue for 3–5 h at 12–16 ºC or at room temperature [47].
4. Monobromonaphthalene ($C_{10}H_7Br$, MW-207.07) is a clear-yellow liquid with high density (1.4 g/mL at 20 ºC) and it is sparingly soluble in water but miscible in alcohol, ether and benzene. Somatic tissue is pretreated with saturated solution of this chemical for 2–4 h at room temperature [38]. This pretreatment has been found to be effective for *Lolium* spp., tomato, egg-plant, pepper, wheat and barley chromosomes [48].
5. Caramine is a most frequently used dye to stain chromosomes in plants, animal and human tissue. Carmine is obtained from female cochineal insects *Coccus cacti* growing on cactus plants *Opuntia coccinellifera* in South America. Large quantity of carmine is produced in South America for food, cosmetic, drug, art and textile industries. Only a fraction of it has usage in science. Cochineal is a crude dried material containing dead dry female insects and plant remains. Carmine of commerce is obtained from this by precipitation with aluminium or other metal ions. Dye available in market is inconsistent in quality. It is recommended to use carmine certified by stain commission. Recently, Dapson [49] has reviewed various aspects of this dye. Dye is a mixture of carminic acid and carmine. Carminic acid has anthroquinone nucleus having methyl, carboxylic acid and hydroxyl groups with attached sugar moiety [50]. It could be in free acid form or salt of sodium and potassium. In contrast, carmine appears to be made up of two molecules of carminic acid co-ordinately bound to single atom of aluminium at carboxyl-hydroxyl pairs [51]. Some dye samples probably contain amino-carmine. As occasional extraction of dye from cochineal by ammonium hydroxide produce aminated carmine, it is used in food industry. Dapson [49] proposed that in acetocarmine staining, aluminium ions of carmine are replaced by ferric ions and carmine forms a tightly bound coordination complex with DNA. Carmine has no potential for covalent bonding to tissue or DNA. Chromosome appears red with bluish tinge. Low pH of acetocarmine solution prevents the staining of cytoplasm. Carmine is soluble in water and alcohol. It is the most popular dye for staining meiotic chromosomes and its use in staining somatic chromosomes is less prevalent in plants.
6. Orcein is a deep purple dye obtained from two species of lichens *Rocella tinctoria* and *Lacanora parella*. Lichen extract, which contains colourless parent substance orcinol, is treated with ammonia in the presence of air resulting in the

formation of orcein dye. Orcein is a variable mixture of 14 different compounds and are phenoxazone derivates. Among these eight compounds α, β, γ amino orcein, α, β, γ hydroxy orcein and β amino orceimine, γ amino-orceimine are the major components constituting 98–99 % of orcein dye. Remaining 1–2 % are secondary components [52]. Orcein is soluble in acetic acid, ethanol, acetone and aqueous alkali and not soluble in water, benzene, chloroform and ether. One or two per cent of orcein in 45 % acetic acid is used for staining chromosomes of plants and animals. Orcein is available in synthetic form but natural orcein appears to give better results. Mechanism of orcein staining is not clearly known, as the orcein itself is a mixture of several related compounds. The stain may interact at acid pH with negative-charged group or possibly interact hydrophobically with chromatin [53]. This is a widely used stain for somatic chromosomes and infrequently used these days for staining meiotic chromosomes in plants.

7. Feulgen and Rossenbeck [54] for the first time demonstrated that DNA can be localized in the cell using Schiff's reaction for aldehyde. With Schiff's reagent, only the chromosomes are stained magenta red and rest of the part of cell appears colourless. Basic fuchsin used in cytology is a variable mixture of triphenyl methane analogoue. Pararosaniline (= magenta = 0), rosaniline (Magenta I), Magenta-II (Basic fuchsin) and new Magenta-III (new fuchsin). Rosaniline, magenta-II and magenta-III have 1,2,3 methyl groups. Pararosaniline is unmethylated [55]. It is easily soluble in water and alcohol. Preparation of Feulgen reagent involves dissolution of basic fuchsin dye in water. Addition of HCl and sodium metabisulphite to above solution releases sulphur dioxide in the reaction medium and it reacts with water to produce sulphurous acid. This reacts with basic fuchsin to yield colourless fuchsin sulphurous acid or Leuco-basic fuchsin or Schiff's reagent. When the tissue is hydrolyzed with 1 N HCl, the purine containing fraction of DNA in chromosome is separated from sugar and aldehyde group is left free on sugar moiety. Fuchsin sulphurous acid of Feulgen reagent reacts with free aldehyde group of DNA to give a magenta-red colour [28]. Using this staining method, one can quantify the DNA amount in a nucleus using microdensitometer.
8. Giemsa dye is a mixture of methylene blue and its oxidation products Azures especially Azure B and eosin Y. It is generally prepared by dissolving the Giemsa powder in glycerine and methanol. Dye is also available as premade stock solution from various companies. Required percentage of stain solution is prepared from this by dilution with buffer. This dye stains the chromatin red and cytoplasm blue. Giemsa is not a general purpose stain for chromosomes. It is used in producing unique C-banding pattern in plants and animal including human chromosomes. This stain is also highly acclaimed in revealing characteristic G-bands in animal chromosomes. In plants G-bands appear to be of doubtful origin and are not popular [56, 57].
9. Chloroform is a sweet smelling colourless liquid and is sparingly soluble in water. It is miscible in all proportions with alcohol and acetic acid and acetone. Chloroform is a good solvent for lipids and this characteristic is useful in formulating fixatives. It makes tissue highly brittle and is seldom used in smear preparations.

10. Ethanol is a colourless fluid and soluble in water in all proportions. Ethanol is a component of most fixatives. This alcohol has great penetration power and dehydrating property. This causes tissue to shrink, harden and makes it brittle. Alcohol replaces the water molecules in the tissue. Alcohol denatures proteins and precipitates nucleic acids [58]. Ethanol as a single fluid is not used as a fixative. Ethanol is available as 95% or absolute alcohol (100%). Denatured spirit refers ethanol to which methanol is added to prevent its use for drinking.
11. Propionic acid is a colourless liquid with acrid odour. Propionic acid is miscible in all proportions with water, alcohol and many other organic solvents. It is used sometimes as a substitute for acetic acid. This causes less swelling of cells than the acetic acid and also a good solvent for aniline dyes.
12. Glacial acetic acid is a colourless liquid having pungent odour and miscible with water and alcohol in all proportions. It does not denature protein and has greater penetrating power than alcohol [59]. The most striking feature of acetic acid is its swelling effect on cellular structure and tissues fixed in it are soft unlike fixed in alcohol. When combined with alcohol it offsets the shrinkage caused by alcohol [58]. This is a good solvent for aniline dyes and one can easily prepare aceto-carmine or acetoorcein.
13. Canada balsam is collected as bark exudates from balsam fir tree *Abies balsamea* which naturally grows in North America. Canada balsam is a thick lightly yellow, transparent liquid and composed of resins solubilised in the essential oil forming oleoresin. On evaporation of essential oil hard resin is left which is soluble in xylene, but not in alcohol. Canada balsam is an ideal mountant and its refractive index is same as that of a glass. Main drawback of this mountant is it dries slowly and basic dyes fade due to its acidic nature during long storage [40]. It is desirable to keep a marble piece in the Canada balsam bottle [59]. It is useful to mount in Canada balsam from after xylene not after dehydration from alcohol series. Neutral balsams are also available [40, 58, 59].
14. Euparal is a mounting medium widely used in cytological studies. It is a synthetic resin with refractive index of 1.483 and is soluble in xylene, butanol and alcohol. Euparal is a mixture of eucalyptol, sandarac (a resin from the tree *Tetraclinin articulata* grown in North-West Africa), paraldehyde and camsal (camphor and phenyl salicytate). Slides can be directly mounted in euparal from 95% alcohol [60].
15. DPX is a neutral, colourless mounting medium with refractive index of 1.522 and most stains are well preserved. It is a mixture of plastic, polysterene dissolved in xylene [61, 62]. The disadvantage of this mountant is it sets quickly and retracts from the edge of coverslip. This has been overcome by adding a plasticizer [58].
16. Belling [24] originally recommended the addition of trace amount of ferric hydrate dissolved in 45% of acetic acid until the acetocarmine solution turns bluish red and avoids precipitation. Recent modification is adding few drops of ferric chloride or ferric acetate in 45% acetic acid.

17. Carnoy's Fixative I is prepared fresh whenever required. Root apices, flower bud, leaf tips and animal tissue are fixed in this fixative from 30 min to 24 h at room temperature. Finally they are stored in the same fluid at −20 °C until use. In case deep freezer is not available, transfer the material to 70 % alcohol and leave it to 0–4 °C or room temperature for 1–2 months. Leaving the material in 70 % alcohol longer than 2 months would lead to over staining of cytoplasm and contrast would be lost.
18. Carnoy's Fixative II is popular with animal cytologists and fixing of flower buds. Duration of fixation and other conditions are similar to Carnoy's I fixative. This fixative makes the plant tissue more brittle [28, 58].
19. The iron in the needle will react with carmine solution and iron acts as a mordant. This helps in deep staining of chromosomes. In case iron is already added in the carmine there is no need to use iron needle.
20. If long term storage is required, wash the Feulgen stained root apices in water and transfer them to 70 % alcohol. The material can be stored up to 3 months.
21. Some investigators wash the slides form SSC in distilled water three times, air dry them, and finally place them in Giemsa stain solution [6].
22. In case the anther is large, then cut one end of it with a sharp blade. Squeeze the anther from the other end to facilitate the release of PMC into a pool of acetocarmine stain. Discard the anther wall.
23. In case anthers are very small, then whole bud can be hydrolyzed and stained in Feulgen reagent. Dissect out the stained anthers from flower bud and check for meiotic stages. Squash anthers having meiotic stages.
24. One must keep track of which side of cover glass and slide contains the tissue. One disadvantage of this method is lot of tissue is lost during transfer through dehydration grades.
25. At final stage it is possible to dehydrate the tissue with two changes of xylene for 5 min each instead of alcohol and mount in Canada balsam. This method is widely used due to its simple process.

References

1. Geitler L (1938) Chromosomenbau. Verlag Gebrüder Borntraeger, Berlin
2. Waldeyer W (1888) Über Karyokinese und ihre Beziehung zu den Befruchtung svorgängen. Arch Mikr Anat 32:1–222
3. Mendel G (1886) Versuche uber Pflanzen-Hybriden. Verhandlungen des Natur-forschenden Vereines in Brunn 4:3–47 (First English translation in 1901, J Royal Horticult Soc 26:1–32)
4. Snustad DP, Simmons MJ (2012) Genetics, 6th edn. Wiley, New York
5. Sutton WS (1902) On the morphology of the chromosome group in *Brachystola magna*. Biol Bull 4:24–39
6. Singh RJ (2003) Plant cytogenetics, 2nd edn. CRC Press, Boca Raton
7. Jackson RC (1957) New low chromosome number in plants. Science 126:1115–1116
8. Roberto C (2005) Low chromosome number in angiosperms. Caryologia 58:403–409
9. Khandelwal S (1990) Chromosome evolution in the genus *Ophioglossum* L. Bot J Linn Soc 102:205–217

10. Hiremath SC, Chennaveeraiah MS (1982) Cytogenetical studies in wild and cultivated species of *Eleusine* (Gramineae). Caryologia 35:57–69
11. Jones RN, Viegas W, Houben A (2008) A century of B-chromosomes in plants: so what? Ann Bot 101:767–775
12. Heslop-Harrison JS, Schwarzacher T (2011) Organization of the plant genome in chromosomes. Plant J 66:18–33
13. Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52:201–220
14. Levitsky GA (1931) The morphology of chromosomes. Bull. Appl Bot Pl Breed 27:19–174
15. La Cour L (1941) Aceto-orcein: a new stain-fixative for chromosomes. Stain Tech 16: 169–174
16. Fedak G, Kim NS (2008) Tools and methodologies for cytogenetic studies of plant chromosomes. Tsitol Genet 42:64–80
17. Figueroa DM, Bass HW (2010) A historical and modern prospective on plant cytogenetics. Brief Funct Genomics 9:95–102
18. Gall JG, Pardue ML (1969) Formation and detection of RNA-DNA hybrid molecules in cytogenetical preparations. Proc Nat Acad Sci USA 63:378–383
19. John HA, Birnstiel ML, Jones KW (1969) RNA-DNA hybrids at cytological level. Nature 223:582–587
20. Lavania UC (1998) Fluorescence in situ hybridization in genome, chromosome and gene identification in plants. Curr Sci 74:126–133
21. Schwarzacher T (2003) DNA, chromosomes and in situ hybridization. Genome 46:953–962
22. Jiang J, Gill BS (2006) Current status and future of fluorescence in situ hybridization (FISH) in plant genome research. Genome 49:1057–1068
23. Palmer RG, Heer H (1973) A root tip squash technique for soybean chromosomes. Crop Sci 13:389–391
24. Belling J (1921) On counting chromosomes in pollen mother cells. Amer Nat 55:573–574
25. Lillie RD (1951) Simplification of the manufacture Schiff's reagent for use in histochemical procedures. Stain Tech 26:163–165
26. De Tomasi JA (1936) Improving the technique of Feulgen stain. Stain Tech 11:137–144
27. Kimber G, Gill BS, Rubenstein JM, Barnhill GL (1975) The technique of Giemsa staining of cereal chromosomes. Missouri Agri Exp Stn Res Bull 1012
28. Sharma AK, Sharma A (1983) Chromosome techniques: theory and practice, 3rd edn. Butterworth & Co., London
29. Smith MU, Kindfield ACH (1999) Teaching cell division: basics and recommendations. Amer Biol Teach 61:366–371
30. Conger AD, Fairchild M (1953) A quick freeze method for making smear slides permanent. Stain Tech 28:281–283
31. Celarier RP (1956) Tertiary butyl alcohol dehydration of chromosome smears. Stain Tech 31:155–157
32. Vosa CG, Marchi P (1972) Quinacrine fluorescence and Giemsa staining in plants. Nature New Biol 237:191–192
33. Marks GE (1975) The Giemsa-staining centromeres of *Nigella damascena*. J Cell Sci 18: 19–25
34. Giráldez R, Cermeno MC, Orellana J (1979) Comparison of C-banding pattern in the chromosomes of inbred lines and open pollinated varieties of rye, Z. Pflanzenzücht 83:40–48
35. Singh RJ, Robellen G (1975) Comparison of somatic Giemsa banding pattern in several species of rye. Z. Pflanzenzücht 75:270–285
36. Linde-Laursen IB (1975) Giemsa C-banding of the chromosomes of 'Emir' barley. Hereditas 81:285–289
37. Stebbins GL (1971) Chromosomal evolution in higher plants. Edward Arnold, London
38. Darlington CD, La Cour LF (1976) Handling of chromosomes, 6th edn. George Allen & Unwin, London

39. White MJD (1973) Animal cytology and evolution, 3rd edn. Cambridge Univ Press, Cambridge
40. Darlington CD (1958) Evolution of genetic systems, 2nd edn. Oliver & Boyd, Edinburgh
41. Battaglia E (1955) Chromosome morphology and terminology. Caryologia 8:179–187
42. McClintock B (1929) A method for making acetocarmin smear permanent. Stain Tech 4: 53–56
43. Naranjo CA, Poggio L, Brandham PE (1983) A practical method of chromosome classification on the basis of centromere position. Genetica 62:51–53
44. Matern B, Simak M (1968) Statistical problems in karyotype analysis. Hereditas 59:280–288
45. Cai Q, Chinnappa CC (1987) Giemsa C-banded karyotypes of seven North American species of *Allium*. Amer J Bot 74:1087–1092
46. Tjio JH, Levan A (1950) The use of oxyquinoline in chromosome analysis. An Estac Exp. Aula Dei 2:21–64
47. Meyer JR (1945) Prefixing with paradichlorobenzene to facilitate chromosome study. Stain Tech 20:121–125
48. Jahier J, Chevre AM, Delourme R, Eber F, Tanguy AM (1996) Technique of plant cytogenetics. Science Pub Inc., New Hampshire
49. Dapson RW (2007) The history, chemistry and mode of action of carmine and related dyes. Biotechnic Histochem 82:173–187
50. Bhatia SB, Venkataraman K (1965) The position of carboxyl group in carminic acid. Indian J Chem 3:92–93
51. Meloan SN, Valentine LS, Puchtler H (1971) On the structure of carminic acid and carmine. Histochemie 27:87–95
52. Beecken H, Musso H (1961) Phenoxazines IV. The condensation of hydroquinones with O-phenylenediamine and o-aminophenols. Chem Ber 94:601–613
53. Tonzetich J (1994) Orcein staining and the identification of polytene chromosomes. Methods Mol Biol 247:249–256
54. Feulgen R, Rossenbeck H (1924) Mikorskopisch-Chemischer Nachweis einer Nuclein saure vom Typus der thymonucleisaure und die darauf beruhende elektive Farbung vom Zellkernen in mikroskopischen Praparaten. Z Physiol Chem 135:203–248
55. Schulte E, Wittekind D (1989) Standardization of the Feulgen—Schiff technique. Histochemistry 91:321–331
56. Greilhuber J (1977) Why plant chromosomes do not show G-bands. Theor Appl Genet 50:121–124
57. Schubert I, Reiger R, Dobel P (1984) G and/or C-bands in plant chromosomes? J Cell Sci 71:111–120
58. Baker JR (1966) Cytological techniques, 5th edn. Chapman & Hall, London
59. Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York
60. Lillie RD, Zirkle C, Dempsey EW, Greco P (1953) Final report of the committee on histological mounting media. Stain Technol 28:57–80
61. Kirkpatrick J, Lendrum AC (1939) Mounting medium for microscopical preparations giving good preservation of colour. J Path Bact 49:592–594
62. Kirkpatrick J, Lendrum AC (1941) Further observations on the use of synthetic resin as substitute for Canada balsam, precipitation of paraffin wax in medium and on improved plasticizer. J Path Bact 53:441–443
63. Chinnappa CC (1981) Cytological studies in the genus *Krigia* (Asteraceae). Can J Genet Cytol 23:671–678
64. Chinnappa CC (1975) Structural hybridity and supernumerary chromosomes in a diploid *Tradescantia hirsuticaulis*. Can J Bot 53:456–465

Chapter 17
Chromosome Techniques and FISH

Mei-Chu Chung

17.1 Introduction

Fluorescence in situ hybridization (FISH) allows us to visualize the physical position of a specific DNA sequence in chromosomes or a nucleus spread on microscope slides. FISH results show cytological features of the distribution and organization of DNA sequences in chromosomes, providing an opportunity to study chromosomes at molecular level. This method involves hybridization of a specific DNA sequence (probe) onto the complementary DNA (target) spread on slide and visualization of the hybridization sites as fluorescent signals via immunological detection. Since FISH was first introduced by Gall and Pardue [1] and John et al. [2], continuous refinement in protocol and improvement in imaging facilities have made it as one of the most appropriate methods for cytogenetic and genomic research. The preparation of chromosome spreads is crucial for satisfactory FISH/GISH results with plant materials. Here we share our experience in preparing plant chromosome spreads for FISH analysis. A protocol routinely used for FISH to plant chromosomes in our laboratory is also provided.

17.1.1 Development of FISH Technique

In situ hybridization was first described as a method to detect the locations of DNA:RNA hybrids in cytological preparations with the use of a radioactive probe and autoradiography [1, 2]. This technique, with good sensitivity, had been extensively used for gene mapping, mainly repetitive sequences, in humans [3] and plants [4, 5], even for the detection of single-copy genes [6]. Because of the poor resolution and the hazardous, complicated, and slow procedure, radiolabeled probes were soon replaced by nonradioactive probes. Nonradioactive probes, could be made

M.-C. Chung (✉)
Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, ROC
e-mail: bomchung@gate.sinica.edu.tw

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_17

by conjugating biotin to nucleotides [7] or by incorporating biotin into DNA/RNA molecules following enzymatic or chemical methods [7–9].

The hybridization sites of biotin-labeled probes can be visualized as colored precipitates produced by an enzyme conjugated to streptavidin or avidin that has high affinity to biotin [10, 11]. The hybridization sites can also be directly detected as fluorescent signals generated by fluorescein isothiocyanate (FITC) conjugated to streptavidin or avidin [12]. However, biotin occurs endogenously in almost every tissue and cell, and may cause nonspecific binding of streptavidin or avidin to result in unexpected signals. Indirect detection with an immunological method to use an anti-biotin antibody conjugated to a fluorescent molecule is considered more specific than direct detection [12–14]. Anti-biotin antibody has more specificity than streptavidin or avidin to biotin-labeled probes. Digoxigenin (DIG) is a highly antigenic plant steroid molecule found exclusively among foxglove plants (*Digitalis* spp.). Antibodies directed against DIG will not cross-react with antigens from other organisms, making DIG-labeled probes superior in nonradioactive probes for FISH. A DIG-labeled probe is usually preferred in FISH mapping a single probe. For bicolor FISH, the sequence with low copy is suggested to be labeled with DIG, whereas sequences that may generate strong and conspicuous signals, such as rDNA, telomeres and centromeres, are labeled with biotin.

Fluorescence as well as biotin and DIG are commonly used for probe labeling, and highly specific antibodies conjugated to various fluorophores are commercially available for use. With these advantages, it is possible to combine probes with different labels in one hybridization mixture and detect each probe with separate antibodies in one experiment (multicolor FISH). Multicolor FISH simultaneously shows the distribution patterns of those sequences on chromosomes and the correlation among those sequences participating in the genomic organization. The demand for mapping more and more genes in one FISH experiment provokes continuously developing new methods for probe labeling and progressing great advances in microscope and detector hardware. FISH has become one of the major methods for gene mapping, clinical studies, and molecular cytogenetic studies. This technique and its modifications, such as multicolor FISH (M-FISH, SKY, CCK), primed in situ labeling (PRINS), and comparative genomic hybridization, are now important complementary tools in human genetic diagnostics. These techniques are reviewed elsewhere [15, 16].

17.1.2 Application and Achievements of FISH in Plant Studies

The use of in situ hybridization to plant chromosomes was first reported in the early 1980s, including the mapping of rDNA or repetitive DNA sequences on wheat chromosomes by use of radioactive probes [17–19], by colorimetric detection [10], or in many cases, by fluorescence [11]. A modification of the FISH method, genome in situ hybridization (GISH), with total genomic DNA labeled

as a probe, was done later [14]. GISH allows the user to differentiate the chromosome set of one parent (probe) from the other parent(s) in an interspecific hybrid. Since then, FISH and GISH have opened new perspectives for studying molecular cytogenetics in plants (*see*, e.g., refs. [20, 21]). Some of the achievements are considered significant milestones in plant molecular cytogenetic studies, including the model plant *Arabidopsis thaliana* [22] and many crops such as rice, wheat, maize, tomato, *Brassica*, and *Festulolium* (*see*, e.g., refs. [23–30]). Today, FISH is a unique and reliable technique to physically map DNA sequences in chromosomes, revealing the association of the molecular information for DNA sequences and cytological features along chromosomes and within genomes (*see*, e.g., refs. [23, 31]).

Higher plant genomes are known to contain various and abundant repetitive DNA sequences [32]. Some of these repetitive sequences are tandem repeated and with biological functions, for example, the sequences of 45S/5S rRNA genes (rDNAs) (Fig. 17.1a–c), telomere, and centromere. FISH with these tandem repeats may show specific distribution patterns on chromosomes to facilitate chromosome identification [33]. Results of rDNA-FISH in many plants have revealed variations in the number and chromosomal position of rDNA loci among related species, which are valuable evidence of genome evolution at chromosomal levels (*see*, e.g., refs. [34, 35]). The number of 5S rDNA or 45S rDNA loci may reflect the ploidy levels. For example, FISH results showed two 5S rDNA sites and six 45S rDNA sites in a chromosome complement of *Iris giganticaerulea* Small (Fig. 17.1a). The number of 5S rDNA sites indicates it is a diploid ($2n=2x=44$). Also, the number and feature of 45S rDNA sites presented in *Kalanchoe garambiensis* Kudo (Fig. 17.1b) also indicate it is a diploid ($2n=2x=68$). Five pairs of chromosomes of *A. thaliana* can be identified by the distribution patterns of 5S rDNA and 45S rDNA sites [36]. On the basis of rDNA-FISH, a mutant of *A. thaliana* of low fertility was identified as a trisomy with triplex chromosome 2 (Fig. 17.1c) [37]. FISH with genome- or chromosome-specific repeats, together with 45S/5S rDNA, allowed for precise karyotyping in maize [25]) and *Arabidopsis* [22].

The whole-genome sequencing of several crops has been completed and provides a huge amount of DNA sequencing data and numerous bacterial artificial chromosome (BAC) libraries. These publicly available resources provide a new scenario for studying the genome and chromosomes by FISH. FISH with BAC-based probes (BAC-FISH) has played a role in these genome sequencing projects to visualize the distribution of supercontigs (continuous set of BAC clones) and the gaps between contigs on chromosomes and to estimate the distances of those gaps [36, 38–40]. The BAC containing a single gene with agronomical importance can be identified with the help of whole-genome sequencing data, which allows us to easily map a single gene on a chromosome by FISH with this BAC as probe (*see,* e.g., ref. [23]). An integrated map displaying the genetic linkage map based on restriction fragment-length polymorphism (RFLP) markers and the physical maps based on DNA sequence database (pseudomolecule) on a particular chromosome with cytological features can be established by FISH with a set of BACs anchored

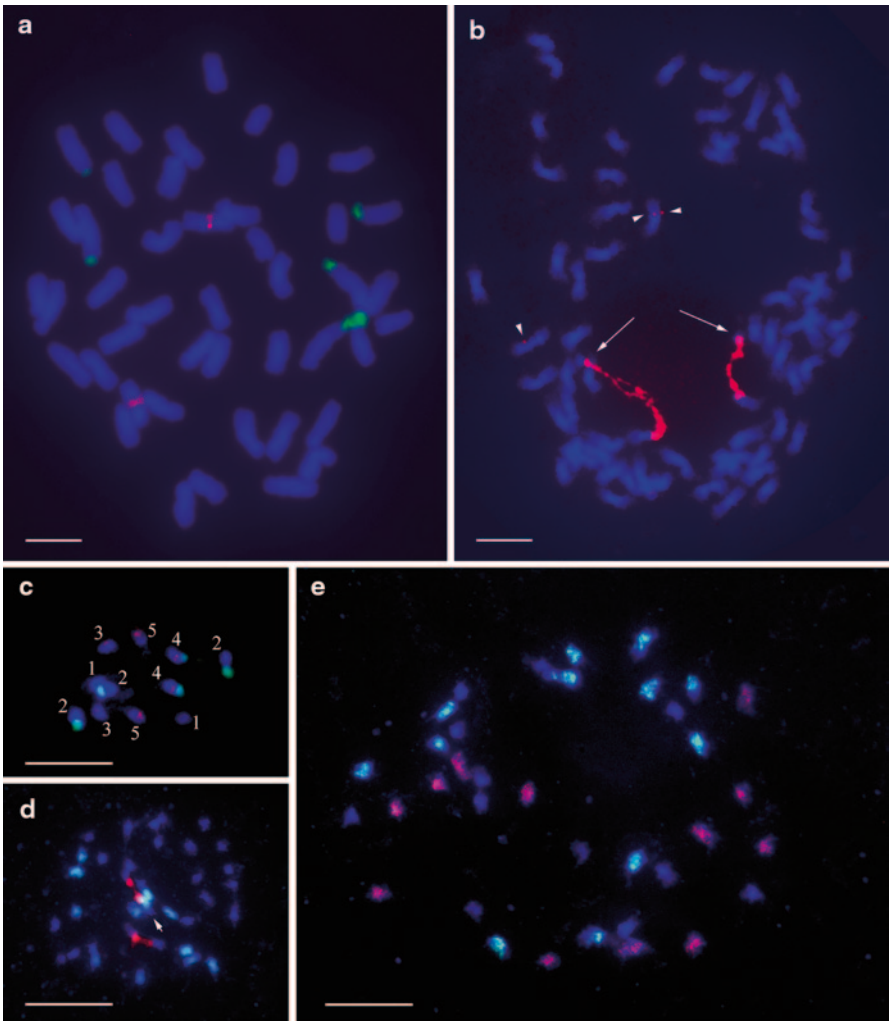

Fig. 17.1 Examples of FISH and GISH images of plant chromosomes. **a** FISH mapping 5S (*red*) and 45S (*green*) ribosomal DNA sites on somatic metaphase chromosomes of *Iris giganticaerulea* Small (giant blue iris or giant blue flag; $2n=2x=44$). **b** FISH mapping 45S rDNA sites on somatic chromosomes of *Kalanchoe garambiensis* Kudo ($2n=2x=68$). The stretched rDNA arrays on a pair of chromosome with satellite (*arrows*) were labeled with bright fluorescent signals of rDNA-FISH whereas the other rDNA arrays (*arrowheads*) at proximal regions are shorter than that at the former. **c** FISH with 5S rDNA sites (*red*) and 45S rDNA sites (*green*) on the somatic metaphase chromosomes of *Arabidopsis thaliana* revealing a trisomy ($2n=11$) with triplex chromosome 2. **d**, **e** Combined FISH and GISH on a meiotic metaphase I spread from an interspecific hybrid (BCE, $2n=36$), *Oryza minuta* (BBCC, $2n=48$) × *O. australiensis* (EE, $2n=24$). **d** FISH and GISH indicating three 45S rDNA (*red*) sites derived from BC genome and one from E genome (*arrow*). Chromosomes labeled in *green* are derived from E genome. **e** GISH with total genomic DNA of CC genome (*red*) and of EE genome (*green*) distinguishing different chromosome sets of this interspecific hybrid (BCE, $2n=36$). Chromosomes of B genomes are unlabeled. The chromosomes are counterstained with DAPI (*blue*). *Scale bar* = 10 μm

with those RFLP markers [41]. Successful examples have been reported in rice [31, 42, 43] and sorghum [44]. Chromosome-specific BACs, together with repetitive repeats, are useful landmarks for chromosome identification [25, 42]. A comparative map between related species can be constructed by using FISH with a set of chromosome-specific BACs. The different distribution of these BACs indicates the rearrangements and different linearity of chromosomal organization between species, thus revealing the evolutionary stories of their karyotypes (*see,* e.g., refs. [21, 45, 46]). A pool of BACs belonging to a certain chromosome has been used for painting that individual chromosome of *Arabidopsis* [47, 48]. However, painting chromosomes with BAC-FISH is relatively difficult in plants with abundant repetitive sequences, which cause high background noise. In this case, unlabeled genomic DNA is required as a blocking agent to suppress the repetitive sequences in the BACs to hybridize on the chromosomes.

17.1.3 Characterization of Genome Organization of Hybrids and Polyploids by GISH

GISH, which is a modified version of FISH, uses total genomic DNA being labeled as a probe [14, 49]. It allows us to visualize the constitution and variation in allopolyploid, discriminate the parental genomes in an interspecific hybrid (Fig. 21.1d, e), and detect intergeneric recombination [30, 50, 51]. It allows studies of homoeologous chromosome pairing at meiosis of interspecific hybrids in detail, thus revealing the affinity and phylogenetic distance of parental genomes [50]. The power of GISH relies on the differences in components and organization of repetitive sequences between genomes to be discriminated [52]. For better discrimination, unlabeled genomic DNA of another parent is used as a blocking agent in the hybridization mixture [53]. The ratio of probe to unlabeled blocking DNA is critical for specific probes, which is determined by the genome affinities between two species [50].

17.1.4 Sensitivity and Resolution for Visualizing DNA Sequences by FISH

Any source of DNA or RNA can be labeled as a probe for FISH. Preliminary information for the probe, including copy number (single or repetitive), genomic organization (spread or cluster), and species specificity, will help for predicting and explaining FISH results. For example, it is considered difficult to map a sequence in a single copy or dispersed repeats by FISH. The specificity of the probe will determine the amount of blocking DNA applied in GISH. The probe size determines the performance of FISH analysis. The length of DNA fragment influences thermal stability and the rate of the renaturation of DNA in solution. A long probe gives maximal hybridization rate and structural stability. However, long fragments may

give a high level of fluorescent background due to a large networking formation. Also, a long probe may have difficulty in penetrating the dense matrix of cells or chromosomes [54]. The rate of renaturation is directly proportional to the concentration of complementary sequences. Therefore, both the concentration of probe in hybridization mixture and the copy number of targeted sequences in genomes affect the rate of renaturation.

The efficiency of FISH analysis is determined by the detectable size of the target (sensitivity) and the distinguished distance apart two close targets (resolution). FISH may be used to detect a sequence existing as a single copy or repetitive units on mitotic chromosomes, meiotic chromosomes, interphase nuclei, or extended DNA fibers. Many factors affect the resolution of FISH analysis, including chromatin compactness, genome size, nuclear division stage, cell type, and the distribution of heterochromatin/euchromatin. Repetitive sequences, which are short but arranged in tandem arrays such as telomeres, centromeres, and 45S/5S rDNAs, can be readily detected by FISH [22]. A cluster of tandem repetitive 45S rDNA repeats provides a large target for hybridization with a single probe to present strong and conspicuous FISH signals [34, 55]. Therefore, using 45S rDNAs as a probe is a good start to practice FISH. The structure and coding sequences of 45S rDNA are highly conserved among higher plants, which allows for detecting rDNA sites by using a probe derived from other plants. For example, plasmid p*TA71* containing the rDNA unit of wheat [56] has been used as a probe in rDNA-FISH analysis in many plants, including *Oryza* species [34], *Lycoris* species [35], *Sorghum* species [57], and *A. thaliana* [58]. Otherwise, a segment of the coding region of 45S rDNA or the segment between 18S and 28S rDNAs containing 5.8S rDNA and two internal transcribed spacers (ITSs) can be easily amplified and labeled simultaneously by PCR [55]. In this case, primers can be designed with the publicly available published sequences. Also, 45S rDNA can be used in bicolor FISH analysis as a reference for chromosome identification and for checking the efficiency of the whole FISH procedure.

However, mapping a single gene on a chromosome is difficult. A single gene as short as 2–2.5 kb has been detected on maize chromosomes at somatic metaphase [59, 60]. Two close probes < 1 Mbp apart are difficult to resolve on somatic metaphase chromosomes because chromatin is highly compacted at this stage. The resolution can be improved with meiotic pachytene chromosomes or interphase nuclei. FISH with extended DNA fiber can resolve probes 10–500 kbp apart. Fiber FISH is useful to examine the ordering, overlaps, and gaps in BAC contigs [38, 61] as well as the organization of genes [55].

17.1.5 FISH to Plant Materials

The basic procedure of FISH involves four parts: (1) preparing the specimen spreads on slides (target), (2) preparing and labeling the probe, (3) hybridizing the probe to the target, and (4) visualizing fluorescent signals (Fig. 17.2). The preparation of chromosome spreads is considered the most crucial part for performing FISH to plant materials. A slide qualified for FISH analysis should have numerous dividing

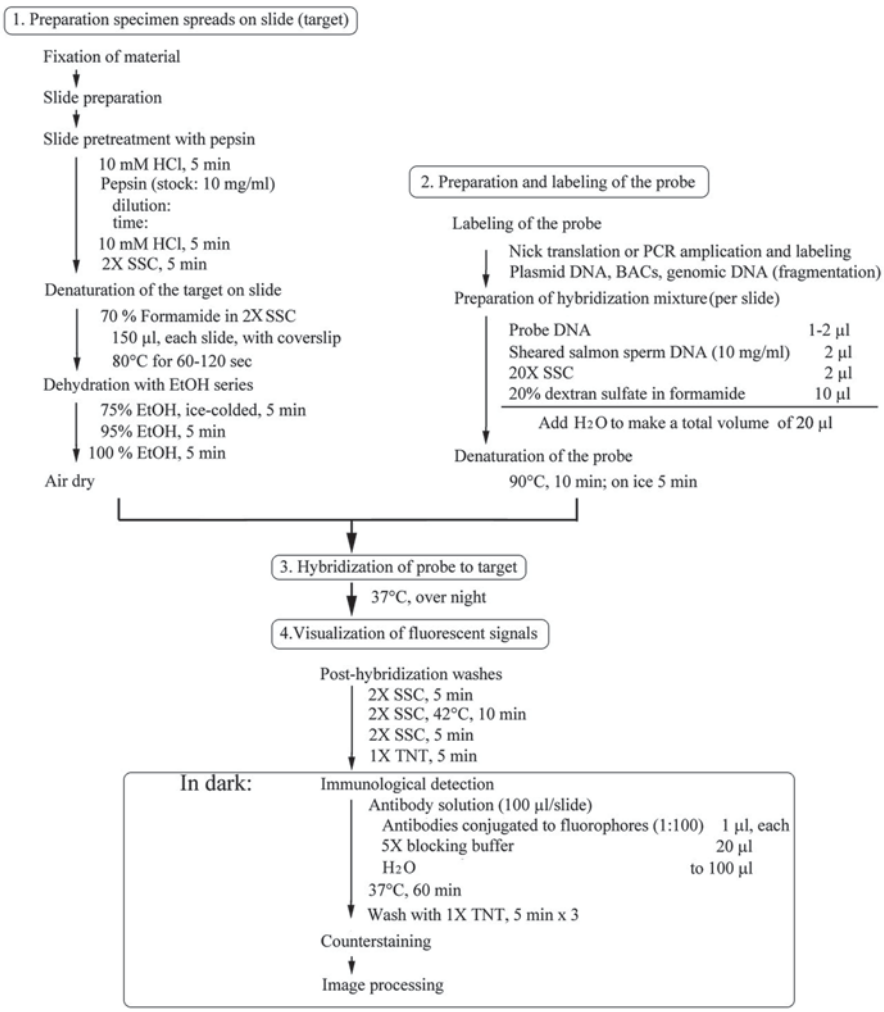

Fig. 17.2 Diagram for fluorescence in situ hybridization

nuclei at the proper stage, with well-spread chromosomes, and little cellular debris on the chromosome spreads. FISH to plant chromosomes is thought to be more difficult than to animal species mainly because of the cell wall and viscous cytoplasm. Cellular debris includes the cytoplasm and cell wall, which may prevent the penetration of probes and antibodies to reduce FISH signals and may generate high background noise. In some cases, low metaphase index and similar chromosome morphology are also problems. Young and healthy plant tissues are generally active in mitosis. Root tips collected from newly germinated seedling are an ideal tissue for preparing mitotic chromosomes. Water control is important for obtaining roots in active growth. Usually watering after a period of dryness may induce formation

of new lateral roots. The developing pollen mother cells (PMCs) are absolutely the best material for preparing meiotic chromosomes. In most cases, collecting materials at the right stage is critical. The developing stage of PMCs can be roughly identified by the size of flora buds. However, for inflorescences located within the stem before maturation, some other indexes are needed such as the position of the flag leaf (the last leaf) node in rice, plant height (~40 cm) in barley [62], and number of fully developed leaf sheaths in maize [63].

Many comprehensive FISH protocols for plants have been detailed elsewhere. The book, *Practical In Situ Hybridization* [64], which introduced the theory, concepts, protocol, and troubleshooting of this technique, is a valuable reference for FISH. Here we describe a protocol routinely used for FISH to plant chromosomes in our laboratory and share our experience in preparing plant chromosome spreads for FISH analysis. Some successful results based on this protocol have been reported [31, 33–35, 37, 50, 51, 55].

17.2 Materials and Equipment

Prepare all buffers and solutions with distilled water or double-distilled water (ddH_2O). Sterilize stock solutions by autoclaving or filtration. Store buffers at room temperature unless otherwise specified. Stock solutions dispensed in a small volume are stored at −20 °C. Frozen stocks are thawed at room temperature and thereafter kept on ice.

17.2.1 Fixation and Chromosome Preparation

1. Farmer's fluid (fixative): 95% ethanol/glacial acetic acid = 3:1, freshly prepared before use.
2. 8-Hydroxyquinoline (2 mM): Dissolve 29 mg of 8-hydroxyquinoline (MW = 145.15) in 100 mL distilled water. Let it sit at 37 °C overnight or add a small amount of ethanol to speed the 8-hydroxyquinoline crystal going into solution. Store at 4 °C.
3. Citrate buffer (10 mM, pH 4.5): Make a 10 mL solution with 445 μL of 0.1 M trisodium citrate, 555 μL of 0.1 M citric acid, and 9 mL ddH_2O. Keep the 0.1 M sodium citrate and 0.1 M citric acid solutions at 4 °C and make the citrate buffer when required.
4. Maceration enzyme mixture: Dissolve 200 mg cellulose (Onozuka R-10; Yakult Honsha, Tokyo, Japan) in 9.8 mL of 0.01 M citrate buffer and let it sit at 55 °C for 10 min to enhance enzyme solubility and inactivate proteases. Cool to room temperature, and then add 0.2 mL pectinase (Sigma Chemical Co., St. Louis, MO). Dispense aliquots of 100 μL and store at −20 °C. Alternatively, the enzyme mixture can be prepared with a hypotonic solution (75 mM potassium chloride); in this case, adjust the final solution to pH 4.0 with a few drops of 1 M HCl.

17.2.2 Probe Preparation

1. Sources of DNA probes: Different types of DNA molecules, including plasmid DNA, genomic DNA, or PCR products, can be labeled as probes.
2. Nick translation mixture: Reaction mixture (5×) contains reaction buffer (250 mM Tris–HCl, pH 7.5, 25 mM $MgCl_2$), labeled-dNTPs mixture (0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP, and 0.08 mM digoxigenin-11-dUTP/biotin-16-dUTP), DNA polymerase I, and DNase I. Commercial Nick translation kits, such as DIG/Biotin Nick Translation Mix from Roche (Roche #1-745-824; #1-745-816), are convenient.
3. PCR labeling mixture: Concentrated reaction mixture (10×) is a composite of reaction buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), labeled-dNTPs (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.9 mM dTTP, and 0.1 mM digoxigenin-11-dUTP/biotin-16-dUTP), Taq DNA polymerase, and primers. Concentrated reaction buffer (10×) is available from many companies (e.g., HotStarTaq Master Mix Kit from Qiagen, Venlo, The Netherlands). Labeled-dNTPs mixture (10×) is also available from Roche Applied Science (Basel, Switzerland). Primers can be specific to the gene of interest, designed according to the poly-linker region (e.g., nucleotide sequencing primers) or a random hexanucleotide primers mixture.
4. 0.5 M EDTA (pH 8.0): Dissolve 148 g ethylenediaminetetraacetic acid (EDTA) into 800 mL distilled water by stirring vigorously and adding solid NaOH (~30–40 g) slowly; the solution becomes clear as the pH nears 8.0. Add distilled water for a final volume of 1000 mL, autoclave, and then store at room temperature.
5. 10× TBE buffer: Dissolve 121.1 g Tris base (1 M), 61.8 g boric acid (1 M) in 800 mL distilled water, and then add 40 mL 0.5 M EDTA (0.02 M, pH 8.0) and bring to a final volume of 1000 mL with distilled water.
6. 1% Agarose gel: Melt 1 g agarose in 100 mL of 0.5× TBE buffer by microwave, pour into a casting tray after cooling to ~60 °C (just too hot to keep holding in bare hands). Leave it to set for at least 30 min, preferably 1 h.

17.2.3 Hybridization

1. Store ethanol series (75, 95, 100%) in Coplin jars at room temperature. Keep a jar of 75% ethanol at −20°C.
2. 20× Saline-sodium citrate buffer (20× SSC): Dissolve 175.3 g NaCl (3 M) and 88.2 g trisodium citrate (300 mM) in 800 mL distilled water, adjust to pH 7.0 with a few drops of concentrated HCl; then add ddH_2O to a final volume of 1 L. Sterilize by autoclaving, then store at room temperature.
3. Formamide (deionized): Add 1% (w/v) ion-exchange resin (20–50 mesh, AG 501-X8 Resin; Bio-Rad Berkeley, California) into formamide, stir for 30 min at room temperature, filter through filter paper (No. 1), and then store at −20 °C.
4. 70% Formamide in 2 × SSC (10 mL): Prepare a 10 mL solution with deionized formamide (7 mL), 20 × SSC (1 mL), and ddH_2O (2 mL). Store at −20 °C.

5. 20% Dextran sulfate (average MW >500,000; D8906; Sigma) in formamide: Dissolve 2 g dextran sulfate in 10 mL formamide (deionized) by stirring at room temperature overnight. Dispense aliquots of 1 mL and store at −20 °C.
6. Pepsin stock (10 mg/mL): Add 25 mL ddH_2O to a 250-mg bottle available through Sigma (P7012) and let it sit at 37 °C for 10 min. Dispense aliquots of 100 μL and store at −20 °C.
7. Sheared salmon sperm DNA (10 mg/mL): Dissolve salmon sperm DNA in ddH_2O and autoclave to break DNA molecules into 100- to 300-bp fragments. Dispense aliquots of 1 mL and store at −20 °C.
8. Rubber cement.

17.2.4 Washing and Detection

1. 2 M Tris–HCl (pH 7.5): Dissolve 242.28 g Tris (hydroxymethyl)aminomethane in 800 mL ddH_2O, adjust to pH 7.5 with 60–70 mL concentrated HCl, and then add ddH_2O to a final volume of 1 L. Sterilize by autoclaving, and store at room temperature.
2. 5 M NaCl: Dissolve 292.2 g NaCl in 700 mL distilled water, bring the volume to 1 L with distilled water, and stir vigorously. Slight heating will help it go into solution. Sterilize by autoclaving and store at room temperature.
3. 10× TNT: Mix 250 mL 2 M Tris–HCl (pH 7.5), 150 mL 5 M NaCl, and 2.5 mL Tween-20, then add distilled water to a final volume of 500 mL.
4. 5× Blocking buffer: Dissolve 0.25 g blocking reagent (Roche) in a beaker with 5 mL 10× TNT and 5 mL ddH_2O by stirring and heating slightly. Sterilize by filtration (0.2 μm filter) and store in aliquots at −20 °C.
5. Fluorochrome-conjugated antibodies: Anti-DIG or anti-biotin antibodies conjugated to fluorochrome, for example, anti-DIG-Rhodamine (Roche Applied Science) and anti-biotin-FITC (SP-3040; Vector Lab, Burlingame, CA).
6. Counterstaining solution: 4′,6-Diamidino-2-phenylindole (DAPI, 2 μg/mL) in antifade mounting medium, e.g., VectaShield Mounting Medium with DAPI (H-1200; Vector Lab). The concentration of DAPI can be diluted with DAPI-free VectaShield (H-1000).
7. Plastic coverslips: Cut 24 × 50 mm pieces from heatproof plastic film such as plastic autoclavable waste disposal bags, microwave cooking bags, overhead projector sheets, or any other available plastic.

17.2.5 Equipment

1. Imaging system for capturing FISH signals: Epi-fluorescence microscope equipped with optical filter sets (for DAPI, FITC, and Rhodamine), a digital charge-coupled device (CCD) camera, an image acquisition software, and an image editing software.

2. Microscope with phase contrast optics for checking the chromosome preparations.
3. Thermal cycler or water bath set to 15 °C for probe labeling reaction and an agarose gel electrophoresis system for checking the size of probes.
4. Incubator at 37 °C, water bath at 42 °C, heating block at 85 °C, oven or hot plate with exact temperature control at 80 °C, vacuum desiccator or SpeedVac, and a shaker.
5. Mini microcentrifuge (6000 rpm) for quick spin.
6. Vortex for mixing components in a solution.
7. Six Coplin jars: three for ethanol series (75, 95, and 100 %) at room temperature, one for 75 % ethanol at −20 °C, one for washing at room temperature, and one for pre-warming at 42 °C for washing.
8. Humidified box: An airtight and lightproof box for hybridization and immune-detection steps. The bottom is lined with a piece of dish sponge moistened with water to provide a humid environment.
9. Space: It is convenient to have all needed equipment in one room. A totally dark room is not necessary; however, since overexposure may cause fluorochrome bleaching, a room with a switch for light control but without daylight or sun coming through a window is needed.

17.3 Methods

17.3.1 Preparing Chromosome Spreads

17.3.1.1 Meiotic Chromosome Preparations

1. Collect developing inflorescences or floral buds and immediately immerse specimens into freshly prepared Farmer's fluid (*see Note 1*). Vacuum to speed the infiltration of fixative into the tissue. Leave samples in fixative at room temperature for 24 h, and replace with fresh prepared fixative. Store at −20 °C. Prepare the chromosome spreads within 2–3 months (*see Note 2*).
2. The developing stage of PMCs can be roughly identified by the size of floral bud/anther. Place an inflorescence in a Petri dish with fresh fixative, display individual buds on a branch, or order buds according to their sizes. Discard the buds with yellow anthers because they may contain mature pollens. If you are not familiar with this plant material, choose the medium-sized bud first for checking its developing stage.
3. Dissect the anther(s) from the bud and put in a drop of 45 % acetic acid (*see Note 3*) on a slide (*see Note 4*). Squash PMCs from the anther by using a dissection needle (*see Note 5*), remove larger fragments (if any), and then add a coverslip over the PMC suspension. Examine the developing stage of PMCs under a phase contrast microscope. Repeat this step until the anthers at the correct stage are located. Collect buds in a similar size for a specific stage in a tube with fresh fixative. Samples can be stored at −20 °C.

4. Squash the PMCs from the anthers and check the developing stage of PMCs as described in step 3. For the slide with the PMCs at the proper meiotic stage, warm the slide gently and evenly over the flame of an alcohol lamp to make the PMCs swell. Heat thoroughly but do not boil. Check if the chromosomes are spread well in swollen PMCs (*see Note 6*), then press the slide with the coverslip down firmly on a filter paper. Check the slide under a phase contrast microscope if the chromosome spreads are qualified for FISH analysis.
5. Soak the slide in liquid nitrogen for 5 min or let it sit in a deep freezer (−80 °C) for at least 2 h, flick the coverslip off by using a razor blade, and dry the slide on a hot plate at 40 °C for 1 h. The slide is ready for FISH or can be stored in a desiccator at room temperature for 2 weeks before use. Slides, with coverslip, also can be stored in a deep freezer (−80 °C) for several years. Before using for FISH, take the frozen slides from the freezer, flick the coverslip off by using a razor blade, and immediately dip them in ice-cold fixative for 5 min, dehydrate through an ethanol series (75, 95, 100 %, each for 5 min, at room temperature), and then dry them on a hot plate at 40 °C for 1 h.

17.3.1.2 Mitotic Chromosome Preparation

1. Any plant tissue active in cell division (e.g., apical meristems of young root tip and shoot, or a developing flora bud) is a suitable source of mitotic chromosomes. In most cases, root tips must be pretreated with 8-hydroxyquinoline for chromosome contraction and to improve spreading of chromosomes.
2. Collect the specimen active in cell division from healthy plants usually at noon on a sunny day. Wash excised tissues briefly with ddH_2O, blot on tissue paper to remove excess water, and pretreat with 2 mM 8-hydroxyquinoline at 18 °C for 2–3 h (the time varies by the species) to accumulate metaphase chromosomes. Wash tissues three times with ddH_2O, blot on tissue paper to remove excess water, and transfer to freshly prepared fixative overnight at room temperature. Replace the fixative with newly prepared solution. Samples can be kept in fixative and stored at −20 °C for months.
3. For FISH analysis, the fixed tissues are usually softened with maceration enzyme mixture; never soften the fixed tissue by warming or by boiling in HCl. Wash the plant tissue two times with ddH_2O for 5 min each. Place the tissue in a drop of water and dissect the meristem tissue and remove away the rest of the parts. Blot out water with tissue paper and transfer the meristem tissues to a drop of maceration enzyme mixture on a slide. Place the slide in a Petri dish lined with moist filter paper and incubate at 37 °C for 1–2 h (the time varies by the species and tissue).
4. Remove the enzyme solution with a piece of filter paper and wash the tissues with a drop of water added from the edge of the tissues without disturbing the softened tissues. Let it sit for 5 min. Repeat the wash step twice, then leave sample in a drop of ddH_2O or 45 % acetic acid.

5. Spread chromosomes in the way you are familiar with. The flame-dry method is preferred in our laboratory to prepare mitotic chromosomes because it is easy and no coverslip is used, so steps for removing the coverslip and sequential dehydration with ethanol series can be omitted. Put a little clump of softened tissues for one preparation (depending on the size of plant tissue) on a slide. Blot out solution from tissues with a piece of filter paper and add 2–3 drops of freshly prepared fixative on tissue. Squash and spread the tissue evenly and quickly by using a forceps before the fixative dry out. Add 3–4 more drops of fixative evenly on the slide and pass the slide over a flame of an alcohol burner to make the fixative on the slide ignite. Place the slide on a tube tracker until the fixative burns up. Blot excess solution from the edges of slides on a paper towel, then air-dry at room temperature.
6. Screen the spreads under a phase contrast microscope to choose suitable slides for FISH (*see Note 7*). Slides are ready for FISH. Slides also can be stored in a desiccator at room temperature for 2–3 months or they can be stored at −80 °C for years. Before being used for FISH, frozen slides are treated as described in Sect. 3.1.1 (Fig. 17.3).

17.3.2 Probe Labeling

1. DNA templates: DNA fragments cloned in plasmid, BAC, or yeast artificial clone (YAC) and DNA isolated from total genome are usually labeled by nick translation. DNAs in small quantities or short inserts (< 1 kb) cloned in a plasmid are amplified and labeled by one run of PCR (*see Note 8*).
2. DNA fragmentation: For efficient labeling by nick translation, large DNA molecules (e.g., genomic DNA or inserts in BAC or YAC) are fragmented into short pieces of 2–10 kb before being labeled. Several methods recommended for DNA fragmentation include autoclaving, sonication, DNase digestion, and shearing through a fine needle [64, p. 23]. We usually cook the genomic DNA in a boiling water bath for 30–60 min (depending on the plant species). Check the size and concentration of DNA sample by gel electrophoresis before using as a template for labeling.
3. Nick translation: Reaction mixtures for probe labeling can be purchased, e.g., DIG-nick translation mix (catalog no. 11-745-816-910) and Biotin–nick translation mix (catalog no. 11-745-824-910) from Roche Applied Science. High-yield and good-quality probes can be harvested using the manufacturer's instructions. Check the probe length and concentration by gel electrophoresis (*see Note 9*). In our experience, labeled probes of about 200–600 bp give satisfactory FISH results. Labeling probes are stored at −20 °C (*see Note 10*).
4. PCR labeling: The probe can be amplified and labeled simultaneously by PCR as for amplification of the specific DNA fragment but including DIG- or biotin-dTTP in the dNTPs mixture. Check the length and concentration of probe by gel electrophoresis.

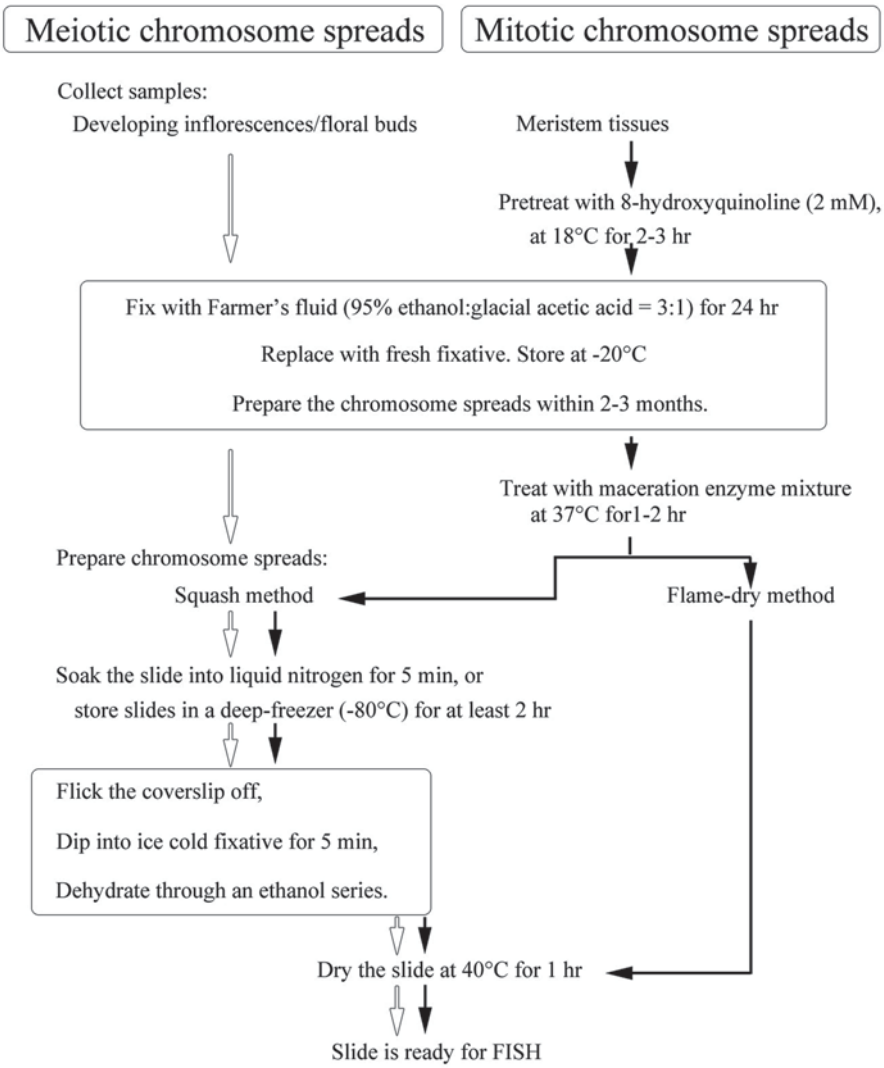

Fig. 17.3 Diagram for chromosome preparation

17.3.3 Hybridization of the Probe to Chromosomes

Handle the slides with chromosome preparations carefully. Remove coverslip from slide when dipped in solution because chromosomes may be lost by constrained detachment of the coverslip from the slide. Avoid touching the slide surface with the micropipette tip when applying solution to the slide because chromosomes on slide may be scratched by the tip.

17.3.3.1 Pretreatment with Pepsin (see Note 11)

1. Dip slides for FISH in a Coplin jar with 50 mL of 10 mM HCl; gently shake for 5 min.
2. Drain (never dry) the slides on paper towels.
3. Add 100 µL pepsin (1–10 µg/mL, dilute the 10-mg/mL stock solution with 10 mM HCl), add a plastic coverslip, place in a humidified box, and incubate at 37 °C for few minutes to hours. Adjust the working concentration and duration of pepsin digestion for getting more penetration of probes and less loss of cells.
4. Wash with 10 mM HCl for 5 min.
5. Wash with 2× SSC for 5 min.
6. Pour out the solution. Blot slides on paper towels. Slide is ready for denaturing.

17.3.3.2 Denaturing Chromosome Preparations

1. Pre-warm the hot plate or oven to exactly 80 °C. Putting a metal plate in the oven may keep the temperature stable.
2. Add 150 µL of 70 % formamide in 2× SSC solution on each slide. Apply a plastic coverslip over the denatured solution carefully without forming air bubbles.
3. Heat the slides at 80 °C for 60–120 s. The temperature and duration of denature must be adjusted to obtain good FISH signals and maintain chromosome features.
4. Dip the slides immediately into ice-cold 75 % EtOH and shake gently to detach the coverslips from slides. Transfer slides to 95 and 100 % EtOH each for 5 min, and then air-dry.

17.3.3.3 Preparing the Hybridization Mixture

1. Add the following components to a microcentrifuge tube (per slide): Add H_2O to make a final volume of 20 µL (*see Note 15*)

Probe DNA	1–2 µL (*see Note 12*)
Sheared salmon sperm DNA (10 mg/mL)	2 µL (*see Note 13*)
20× SSC	2 µL
20 % dextran sulfate in deionized formamide	10 µL (*see Note 14*)

2. Vortex to mix the solution well and briefly spin down the solution.
3. Denature the probe DNA in this mixture by heating at 90 °C (use a water bath or heating block) for 10 min and immediately chill the mixture on ice. Spin down the solution before it is applied to a denatured slide (*see Note 16*).

17.3.3.4 Hybridization

1. Apply 20-μL hybridization mixture to denatured chromosome spreads on a slide and add a 24 × 40-mm coverslip.
2. Seal the coverslip with rubber cement and place the slides in a humidified box. Incubate at 37 °C overnight. For mapping highly repetitive sequences, the hybridization time may be reduced to 6 h. For mapping a single-copy sequence, the hybridization time can be extended.

17.3.4 Washing

All the wash steps in the following procedure are performed in a Coplin jar with gentle shaking on a shaker at room temperature (25–30 °C) unless otherwise specified (*see Note 17*). Blot excess solution from the bottom and edges of slides on paper towels, but never let slides dry out between changes of solution. Uneven drying out from the surface of a slide may damage the chromosome features and raise the background noise due to unspecific binding of probes and antibodies on the slide surface.

1. Set the water bath at 42 °C and pre-warm 2× SSC (50 mL) in a Coplin jar.
2. Peel the rubber cement off by using a forceps. Dip the slides into 2× SSC in a Coplin jar. Gently shake for 5 min to detach the coverslips from the slides. Remove the coverslips from the solution in the jar.
3. Transfer slides to the jar with pre-warmed 2× SSC (42 °C) and gently shake at 42 °C for 10 min. If you do not have a shaker at 42 °C, keep the jar in a water bath and shake gently by hand occasionally.
4. Transfer slides to fresh 2× SSC for 5 min.
5. Replace the 2× SSC with 1× TNT and shake for 5 min.
6. Pour out the solution. Blot slides on paper towels.

17.3.5 Detection (Switch Lights off During the Following Procedures)

1. Prepare antibody solution (100 μL per slide) with 5× blocking buffer (20 μL), ddH_2O (80 μL), and antibody conjugated to fluorophores (1 μL). Antibodies to different labeled DNA probes can be mixed in one solution.
2. Add 100 μL antibody solution on each slide and cover with a plastic coverslip. Place slides in a humidified box (lightproof) and incubate at 37 °C for 60 min.
3. Dip the slides into a Coplin jar with 1× TNT. Remove the coverslips. Wash the slides in 1× TNT three times for 5 min each.
4. Blot excess solution from bottom and edges of slides (never dry) on paper towels. Add 20 μL VectaShield with DAPI (H1200; Vector Lab) on each slide and

cover with a 24 × 40-mm coverslip (no. 0, thin). Slides can remain flat in a dark box and stored at 4 °C for several days before observation (*see Note 18*).

17.3.6 Image Processing

1. Check FISH results by using a fluorescence microscope equipped with appropriate filter sets for individual channels.
2. Find ideal chromosome spreads on a slide first, and then check if there are hybridization signals (bright bands or dots) on chromosomes.
3. Acquire the images of the frame of interest at the individual channel—chromosomes (blue), FITC signals (green), and rhodamine signals (red)—by using a cooled CCD camera. A set of digital micrographs will be obtained; these are gray-scale images recording the intensity, features, and positions of fluorescent signals at individual channels under the same field of view. Save the raw data without any improvement (*see Note 19*).
4. Merge a set of gray-scale images (source) into an RGB figure (color composite), which will show FISH signals in red or green on specific locations on chromosomes in blue (*see Note 20*). Save this figure as a new TIFF file.
5. Adjust the signal strengths of the individual colors with an image-processing program until the images are well aligned and the relative brightness and contrast of each are acceptable; then the final image can be saved, printed, and so on. Two popular programs for image processing are Adobe Photoshop and NIH ImageJ.

17.3.7 Reprobing

Chromosome spreads can be reprobed two or more times; however, chromosome spreads may be lost or chromosomal features may be destroyed by the repetitive denaturation process.

1. Remove the coverslip with a razor blade and wash the slide with 1× TNT three times for 5 min each (*see Note 21*).
2. Wash with 2× SSC for 5 min.
3. Denature as usual.

17.4 Notes

1. The development stage of PMCs within an anther is usually synchronized; therefore, pretreatments to interfere in cell division are not necessary. Moreover, some agents for arresting mitosis may interfere with the process of meiosis and pairing [64].

2. Keep the materials in fixative and stored at −20 °C, and prepare chromosome spreads as soon as possible. Long-term storage in fixative, even at low temperature, will make cytoplasm viscous, which results in low FISH signals and high background noise. Slides with chromosome spreads can be stored at −20 °C to −80 °C for years.
3. Increasing the concentration of acetic acid from 45 to 60% could make cytoplasm clear and help cells adhere to the slides [65].
4. Chromosomes may detach from greasy slides during FISH processing. Use of clear slides is important to have materials adhere tightly. A reused slide is not recommended because remnants of the last sample and unavoidable scratches may interfere with subsequent experiments. New slides can be cleaned simply by dipping in 95% ethanol and wiping the slides clean with Kimwipes before use. However, never clean a coverslip with ethanol. If both the coverslip and slide are cleaned with ethanol, cells or chromosomes will attach to the coverslip rather than the slide. Slides coated with poly-lysine permit electrostatic coupling of the sample to the slide for adhering specimens and are recommended for preparing large chromosomes. Commercially supplied ready-coated slides (e.g., Sigma) work well but are expensive.
5. Maceration is usually not necessary for spreading chromosomes from PMCs because the cell wall surrounding the PMC is thin and fragile. Enzyme maceration is needed for those PMCs with thick callose deposition [39, 66].
6. The volume of solution (45% acetic acid) under a coverslip is important. A small volume of solution gives less room for cells or nuclei to spread and swell, but a large volume may slop over the edge of the coverslip and carry cells or nuclei away.
7. It is not necessary to completely digest the cell wall as for protoplast isolation, but sufficient maceration allows tissue to be easily spread into a single layer and most of the nuclei/chromosomes be released from cytoplasm. Ideal chromosome spreads keep all the chromosomes in a complement and individual chromosomes are kept apart with less overlap.
8. In addition to nick translation and the PCR methods mentioned here, several other methods for probe-labeling different template DNA are available [64].
9. DNase I, which may decay after storage, determines the size of end products of nick translation. The size of labeled probes should be checked by gel electrophoresis. Labeling probes with a ready-made stock mixture from commercial suppliers is highly recommended for beginners.
10. For mapping single and small-DNA sequences by FISH, high-purity and efficiently labeled probes are required. Labeled probes can be purified by precipitation with ethanol or by passing the reaction products through a spin column of a PCR clean-up kit to remove most of the unincorporated nucleotides. The efficiency of labeling is examined by a dot-blot test [64, p. 49].
11. In addition to pepsin digestion, several pretreatments of chromosome spreads for FISH, including RNase treatment and paraformaldehyde fixation, have been recommended in some protocols [64, p. 97]. Adopt any of these treatments if necessary.

12. Accurately estimating the concentration of the probe harvested from the labeling reaction is thought to be difficult. The amount of labeled probe can be approximately assessed by the condition given for nick translation, that is, if 1 μg template DNA in a 20-μL reaction volume is labeled by nick translation, the final concentration of probe in that reaction mixture is 50 ng/μL (1 μg/20 μL). Usually 0.5 μL of such labeled probe (25 ng) is enough for each slide. In most of the protocol, excess probe is often used in the hybridization mixture; however, use of too much probe may cause unspecific signals and is costly.
13. The hybridization mixture usually contains excess unlabeled blocking DNA (e.g., sheared salmon sperm DNA) to outcompete the hybridization of probe to undesired target sequences.
14. Dextran sulfate, which favors the formation of probe networks, can accelerate the rate of hybridization. A stock of 50 % (w/v) solution in water is often suggested in other protocols; here, we make a less viscous 20 % (w/v) solution in formamide, which can be prepared easily and used conveniently. In some protocols, the hybridization mixture includes sodium dodecyl sulfate (SDS, 0.1–0.2 %) to help with penetration of the probe. Be careful to avoid generating bubbles when pipetting the solution with SDS.
15. Add water to make a total volume of 20 μL for each slide or decrease the volume of probe by a vacuum desiccator or SpeedVac if necessary.
16. Labeled probe DNA and target DNA on the slide can be denatured together after applying the hybridization mixture on the slide. In this case, the coverslip on the slide must be sealed with rubber cement before denaturing to prevent the solution from drying out. We prefer to denature probe DNA and target DNA separately because probe DNA in the hybridization mixture must be fully denatured before applying to the chromosome spreads, whereas extreme conditions (high temperature and long duration) applied to chromosome preparations may cause DNA loss and damage chromosome features.
17. The stringency used in this protocol is about 75–80 %. For removing nonspecific binding, the stringency for washing is usually slightly higher than that for hybridization. Because of the hazards and costs associated with formamide, we do not include formamide in washing buffer as suggested in other protocols. The washing series described here provides satisfactory differentiation for most of the probes we have performed.
18. In some GISH experiments, probe derived from total genomic DNA can stain all chromosomes without counterstaining. Overstaining chromosomes may obscure weak hybridization signals. Dilute DAPI/PI-containing VectaShield with DAPI-free VectaShield (H-1000) if necessary.
19. Keep one copy of the original image data before any processing. The contrast and brightness of FISH images can be adjusted, but are never overprocessed. Images must always be kept at high resolution. High-resolution images can be compressed to a JPEG format for transferring the image data across the Internet to submit data for publication, for example. However, compressing an image file, which will irreversibly lose information, should not be performed more than once.

20. The FISH image often displays signals in red or green and chromosomes in blue on a black background. Such an image appears in high contrast and informative on a computer screen but not in hard copy. In this case, selecting the bright (black/white) instead of blue for the channel of chromosomes may improve the contrast of the printed figures.
21. If more pepsin digestion is needed, follow the steps in Sect. 3.3.1.

17.5 Interpretation and Conclusions

In the past decades, FISH and GISH have become fast, sensitive, and important complementary tools in cytogenetic and genomic studies. The use of FISH/GISH for fundamental research at the chromosomal level will continue.

Several important points should be considered before carrying out FISH experiences in your laboratory. The imaging system is the most expensive instrument in a FISH laboratory. Several manufacturers make high-quality fluorescence microscopes, cooled CCD cameras, or both. Software associated with the CCD camera allows for image acquisition, image processing, and chromosome analysis. The knowledge and after-sales service of local representatives are as important as price for selecting the manufacturer. The imaging system that works well in our system has been described previously [34, 51]. It is important to determine the target (metaphase chromosome, pachytene chromosome, interphase nucleus, or DNA fiber) based on the question to be addressed. As well, the combination of probes in a FISH reaction is important to displaying the most information in a single figure.

FISH signals that may present as bright dots or bands should be located at the same focal plane of a chromosome. Theoretically, FISH signals should appear at two sister chromatids of a chromosome or at corresponding positions of homologous chromosomes (Fig. 17.1a–c). However, signals may be missed from corresponding positions because of low hybridization efficiency (Fig. 17.1b), cytoplasm masking the target, or other unknown reasons. Generally, at least five chromosome complements with consistent results are required to give reliable FISH results. Here we provide a brief procedure for FISH (Fig. 17.2) and chromosome preparation (Fig. 17.3) that is convenient for a beginner who wants to start FISH analysis. A routine FISH/GISH protocol will be established by continuous practice and improvement of processes based on the principles and notes described here, and then high-quality FISH images for publication can be readily obtained.

References

1. Gall JG, Pardue ML (1969) Formation and detection of RNA–DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci U S A 69:378–383
2. John H, Birnstiel ML, Jones KW (1969) RNA–DNA hybrids at the cytological level. Nature 223:578–582

3. Ferguson-Smith MA (1991) Putting the genetics back into cytogenetics. Am J Hum Genet 48:179–182
4. Hutchinson J, Lonsdale LM (1982) The chromosomal distribution of cloned highly repetitive sequences from hexaploid wheat. Heredity 48:371–376
5. Wu HK, Chung MC, Wu T, Ning CN, Wu R (1991) Localization of specific repetitive DNA sequences in individual rice chromosomes. Chromosoma 100:330–338
6. Harper ME, Saunders GF (1981) Localization of single copy DNA sequences on G-banded human chromosomes by in situ hybridization. Chromosoma 83:431–439
7. Langer PR, Waldrop AA, Ward DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc Natl Acad Sci U S A 78:6633–6637
8. Brigati DJ, Myerson D, Leary JJ, Spalholz B et al (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissues using biotin-labelled hybridization probes. Virology 126:32–50
9. Trask BJ (1991) Fluorescent in situ hybridization. Trends Genet 7:149–154
10. Rayburn AL, Gill BS (1985) Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. J Hered 76:78–81
11. Jiang J, Gill BS (1994) Nonisotopic in situ hybridization and plant genome mapping: the first 10 years. Genome 37:717–725
12. Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A 83:2934–2938
13. Langer-Safer PR, Levine M, Ward DC (1982) Immunocytological method for mapping genes on *Drosophila polytene* chromosomes. Proc Natl Acad Sci U S A 79:4381–4385
14. Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) In situ localization of parental genomes in a wide hybrid. Ann Bot 64:315–324
15. Tönnies H (2002) Modern molecular cytogenetic techniques in genetic diagnostics. Trends Mol Med 8:246–250
16. Tönnies H (2010) Molecular cytogenetics in molecular diagnostics. In: Patrinos G, Ansorge W (eds) Molecular diagnostics, 2nd edn. Elsevier, Burlington, pp 133–153
17. Gerlach WL, Miller TE, Flavell RB (1980) The nucleolus organizers of diploid wheats revealed by *in situ* hybridization. Theor Appl Genet 58:97–100
18. Gerlach WL, Peacock WI (1980) Chromosomal locations of highly repeated DNA sequences in wheat. Heredity 44:269–276
19. Miller TE, Hutchinson J, Reader SM (1983) The identification of the nucleolus organiser chromosomes of diploid wheat. Theor Appl Genet 65:145–147
20. de Jong H (2003) Visualizing DNA domains and sequences by microscopy: a fifty-year history of molecular cytogenetics. Genome 46:943–946
21. Jiang J, Gill BS (2006) Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. Genome 49:1057–1068
22. Siroky J (2008) Chromosome landmarks as tools to study the genome of *Arabidopsis thaliana*. Cytogenet Genome Res 120:202–209
23. Ohmido N, Fukui K, Kinoshita T (2010) Recent advances in rice genome and chromosome structure research by fluorescence in situ hybridization (FISH). Proc Jpn Acad Ser B 86:103–116
24. Mukai Y (2005) Perspectives in molecular cytogenetics of wheat. Wheat Information Service 100:17–31
25. Kato A, Lamb JC, Birchler JA (2004) Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc Nat Acad Sci U S A 101:13554–13559
26. Gupta PK, Kulwal PL, Rustgi S (2005) Wheat cytogenetics in the genomics era and its relevance to breeding. Cytogenet Genome Res 109:315–327
27. Stack SM, Royer SM, Shearer LA, Chang SB et al (2009) Role of fluorescence in situ hybridization in sequencing the tomato genome. Cytogenet Genome Res 124:339–350
28. Szinay D, Bai Y, Visser R, de Jong H (2010) FISH applications for genomics and plant breeding strategies in tomato and other *Solanaceous* crops. Cytogenet Genome Res 129:199–210

29. Snowdon RJ (2007) Cytogenetics and genome analysis in *Brassica* crops. Chrom Res 15:85–95
30. Kopecký D, Lukaszewski AJ, Doležel J (2008) Cytogenetics of *Festulolium* (*Festuca* x *Lolium* hybrids). Cytogenet Genome Res 120:370–383
31. Kao FI, Cheng YY, Chow TY, Chen HH et al (2006) An integrated map of *Oryza sativa* L. chromosome 5. Theor Appl Genet 112:891–902
32. Kubis S, Schmidt T, Heslop-Harrison JS (1998) Repetitive DNA elements as major components of plant genomes. Ann Bot 82 (Suppl. A):45–55
33. Fang SA, Eu TI, Chung MC (2011) Isolation and characterization of genome-specific markers in *Oryza* species with the BB genome. Plant Sci 181:300–308
34. Chung MC, Lee YI, Cheng YY, Chou YJ, Lu CF (2008) Chromosomal polymorphism of ribosomal genes in the genus *Oryza*. Theor Appl Genet 116:745–753
35. Chang YC, Shii CT, Chung MC (2009) Variations in ribosomal RNA gene loci in spider lily (*Lycoris* spp.). J Am Soc Hortic Sci 134:567–573
36. Fransz P, Armstrong S, Alonso-Blanco C, Fischer TC et al (1998) Cytogenetics for the model system *Arabidopsis thaliana*. Plant J 13:867–876
37. Lo KL, Wang LC, Chen IJ, Liu YC, Chung MC, Lo WS (2014) Transcriptional consequence and impaired gametogenesis with high-grade aneuploidy in *Arabidopsis thaliana*. PLoS ONE 9(12):e114617. doi:10.1371/journal.pone.0114617
38. Cheng ZK, Buell CR, Wing RA, Jiang J (2002) Resolution of fluorescence in-situ hybridization mapping on rice mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers. Chrom Res 10:379–387
39. Kulikova O, Gualtieri G, Geurts R, Kim DJ et al (2001) Integration of the FISH pachytene and genetic maps of *Medicago truncatula*. Plant J 27:49–58
40. Cheng CH, Chung MC, Liu SM, Chen SK et al (2005) A fine physical map of the rice chromosome 5. Mol Gen Genom 274:337–345
41. Harper LC, Cande WZ (2000) Mapping a new frontier; development of integrated cytogenetic maps. Plants Funct Integr Genomics 1:89–98
42. Cheng ZK, Buell CR, Wing RA, Gu M, Jiang J (2001) Toward a cytological characterization of the rice genome. Genome Res 11:2133–2141
43. Cheng ZK, Presting GG, Buell CR, Wing RA, Jiang J (2001) High resolution pachytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination along chromosome 10 of rice. Genetics 157:1749–1757
44. Islam-Faridi MN, Childs KL, Klein PE, Hodnett G et al (2002) A molecular cytogenetic map of sorghum chromosome 1: fluorescence in situ hybridization analysis with mapped bacterial artificial chromosomes. Genetics 161:345–353
45. Kato A, Vega JM, Han F, Lamb JC, Birchler JA (2005) Advances in plant chromosome identification and cytogenetic techniques. Cur Op Pl Biol 8:148–154
46. Lysak MA, Lexer C (2006) Towards the era of comparative evolutionary genomics in Brassicaceae. Pl Syst Evol 259:175–198
47. Lysak MA, Fransz PF, Ali HBM, Schubert I (2001) Chromosome painting in *Arabidopsis thaliana*. Plant J 28:689–697
48. Lysak MA, Pecinka A, Schubert I (2003) Recent progress in chromosome painting of *Arabidopsis* and related species. Chrom Res 11:195–204
49. Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1989) *In situ* localization of parental genomes in a wide hybrid. Ann Bot 64:315–324
50. Lee YI, Chang FC, Chung MC (2011) Chromosome pairing affinities in interspecific hybrids reflect phylogenetic distances among lady's slipper orchids (*Paphiopedilum*). Ann Bot 108:113–121
51. Chang YC, Shii CT, Lee YC, Chung MC (2013) Diverse chromosome complements in the functional gametes of interspecific hybrids of MT- and A-karyotype *Lycoris* spp. Plant Sys Evol 299:1141–1155

52. Anamthawat-Jonsson K, Reader SM (1995) Preannealing of total genomic DNA probes for simultaneous genomic in situ hybridization. Genome 38:814–816
53. Anamthawat-Jonsson K, Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS (1990) Discrimination between closely related *Triticeae* species using genomic DNA as a probe. Theor Appl Genet 79:721–728
54. Salvo-Garrido H, Travella S, Schwarzacher T, Harwood WA, Snape JW (2001) An efficient method for the physical mapping of transgenes in barley using in situ hybridization. Genome 44:104–110
55. Chang KD, Fang SA, Chang FC, Chung MC (2010) Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant *Oryza* species. Genomics 96:181–190
56. Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucl Acids Res 7:1869–1885
57. Sang Y, Liang GH (2000) Comparative physical mapping of the 18S-5.8S-26S rDNA in three *Sorghum* species. Genome 43:918–922
58. Murata M, Shibata F, Yokota E (2006) The origin, meiotic behavior, and transmission of a novel mini-chromosome in *Arabidopsis thaliana*. Chromosoma 115:311–319
59. Lamb JC, Danilova T, Bauer MJ, Meyer J et al (2007) Single gene detection and karyotyping using small target FISH on maize somatic chromosomes. Genetics 175:1047–1058
60. Kato A, Albert PS, Vega JM, Birchler JA (2006) Sensitive FISH signal detection using directly labeled probes produced by high concentration DNA polymerase nick translation in maize. Biotech Histochem 81:71–78
61. de Jong HJ, Fransz P, Zabel P (1999) High-Resolution FISH in plants: techniques and applications. Trends Plant Sci 4:258–263
62. Higgins JD (2013) Analyzing meiosis in barley. Meth Mol Biol 990:135–144
63. Li X, Topp CN, Dawe RK (2012) Maize antibody procedures: immunolocalization and chromatin immunoprecipitation. In: Bass HW, Birchler JA (eds) Plant cytogenetics: genome structure and function. Springer, New York, pp 273–274
64. Schwarzacher T, Heslop-Harrison P (2000) Practical *in situ* hybridization. Bios Scientific Publishers, Oxford
65. Lysak MA, Fransz P, Schubert I (2006) Cytogenetic analyses of *Arabidopsis*. In: Salinas J, Sanchez-Serrano JJ (eds) Arabidopsis protocols, 2nd edn. Humana Press, Totowa, NJ, pp 173–186
66. Geurts RI, de Jong H (2013) Fluorescent in situ hybridization (FISH) on pachytene chromosomes as a tool for genome characterization. Meth Mol Biol 1069:15–24

Chapter 18
Detection of S-Phase of Cell Division Cycle in Plant Cells and Tissues by Using 5-Ethynyl-2′-Deoxyuridine (EdU)

Soujanya Kuntam and Ferhan Ayaydin

18.1 Introduction

Assaying cell proliferation is crucial in assessing cell health, characterizing cellular responses to various treatments or genetic modifications. It is also essential to determine the degree of synchronicity in cell division cycle synchronization experiments. Most cell proliferation assays estimate the number of cells by either incorporating a modified nucleotide during cell proliferation or by measuring the total nucleic acid content of lysed or isolated cells. The most accurate method is direct measurement of new DNA synthesis, which traditionally was achieved by the incorporation of tritium-labeled thymidine and detection by autoradiography [1, 2]. This radioactive method has been replaced by others, such as the incorporation of the thymidine analog, 5-bromo-2-deoxyuridine (BrdU), into DNA followed by immunodetection with a specific antibody raised against BrdU [3, 4]. Although effective and sensitive, this method requires DNA denaturation or digestion (using hydrochloric acid, heat, or DNase) to expose BrdU to the antibody [5, 6]. The use of acid or heat often destroys cell morphology and damages the epitope of many proteins. This hinders their immunocytochemical detection with fluorescence-labeled antibodies. Using DNase, however, poses difficulty in obtaining reproducible levels of DNA digestion while analyzing different cell types or samples [7].

The antibody-based detection method of BrdU assay also necessitates cell wall digestion in experiments carried out on plant cells. Therefore, protoplasts or partially cell wall-digested cells/organs/tissue sections are often used for BrdU-based detection of proliferative activity in plants [8]. However, a significant wounding and osmotic stress is imposed on live plant cells due to treatment with cell wall-digesting enzymes. Moreover, specific optimization of the digestion medium, of the type and concentration of the enzymes, and their osmolarity is required for

F. Ayaydin (✉) · S. Kuntam
Cellular Imaging Laboratory, Biological Research Centre, Hungarian Academy of Sciences, Temesvári körút 62, 6726 Szeged, Hungary
e-mail: ayaydin.ferhan@brc.mta.hu

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_18

each plant species, organ, and cell type under investigation [9]. Partial cell wall digestion or release of protoplasts not only prolongs the experimental duration but also causes substantial reorganization of cytoskeleton and activation of stress- and defense-related genes. It is also possible to first chemically fix the cells and then partially digest their cell wall to diminish stress-related artifacts. However, this approach requires highly pure and expensive cell wall digestion enzymes, as crude enzyme preparations contain impurities such as proteases and nucleases that can significantly compromise cellular integrity [10].

Recently, a new agent 5-ethynyl-2′-deoxyuridine (EdU) based assay has emerged as a remarkable alternative to the abovementioned methods. EdU is a terminal alkyne containing nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis [11]. The EdU detection method is based on click chemistry. "Click" chemistry (Huisgen 1, 3-dipolar cycloaddition) is a type of chemical reaction that occurs at room temperature and is catalyzed by copper Cu(I), resulting in the formation of a strong covalent bond between an azide (present in the structure of the fluorochrome) and an alkyne group (present in EdU) [12, 13] (Fig. 18.1).

The alkyne group is quite unreactive in biological systems and thus allows use in living cells [14, 15]. The small molecular size of the detection fluorochrome, compared to that of antibodies required for BrdU-based immunodetection, enables efficient penetration into plant cells, without the need for cell wall digestion or harsh DNA denaturation treatments [16]. This considerably simplifies the procedure and reduces the duration of the assay. In addition, the mild non-caustic EdU proliferation assay components keep the proteins intact, allowing their parallel immunocytochemical detection with fluorescence-labeled antibodies. Although initially developed for application in cultured mammalian cells, the EdU assay has been successfully applied in a wide variety of species including bacteria [17], yeast [18, 19], and a broad spectrum of animals [20–23] and plants [16].

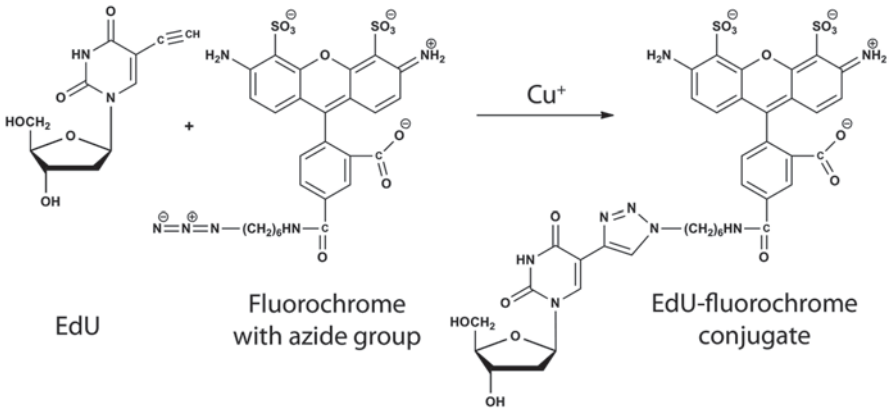

Fig. 18.1 Copper-catalyzed azide-alkyne cycloaddition reaction. *EdU* incorporated into DNA during the S-phase of the cell cycle is detected by copper(I)-catalyzed click coupling to an azide-derivatized fluorophore. The reaction of *EdU* with Alexa Fluor 488 azide is shown as an example

The EdU-based assay has already been applied in several plant tissues and organs such as *Arabidopsis* root tips [24–29], leaf/petiole junction [30], and inflorescence [31]. The assay was also used to differentiate cells in early and late S-phase in root tips of *Arabidopsis* seedlings [32]. The assay was successfully applied in alfalfa root tips [33] and suspension-cultured cells [34], tomato root tips [35, 36], field bean root tips [37], asparagus cladodes [38], tobacco suspension-cultured cells [39], rice suspension-cultured cells [40], and maize root tips [41]. Further, this assay was used to determine the proliferation activity of different cell types of the anther locule of maize [42]. Here, we describe detailed cell synchronization and EdU-detection protocols for both monocot (rice)- and dicot (*Arabidopsis*)-cultured cells and roots.

18.2 EdU Labeling of Suspension-Cultured Cells

18.2.1 Materials

1. *Arabidopsis thaliana* ecotype Landsberg erecta (cell line MM1) culture medium: Murashige and Skoog (MS) medium with 0.5 mg/L naphthalene acetic acid (NAA) and 0.05 mg/L kinetin.
2. *Oryza sativa* L. ssp japonica cv. "Unggi-9" cell culture medium: G1 medium with 1 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) [16].
3. 5-ethynyl-2′-deoxyuridine (EdU) stock solution: 10 mM EdU is prepared in dimethyl sulfoxide (DMSO) as a 1000× concentrated stock solution (*see Note 1*).
4. Hydroxyurea (HU): HU prepared as a 1 M solution in water and sterilized with a filter such as 0.22 μm pore-sized Millipore mixed cellulose ester membrane. HU should be freshly prepared for each experiment as prolonged storage in aqueous medium may cause decomposition (*see Note 2*).
5. Formaldehyde stock solution (8 % w/v): Dissolve 8 g paraformaldehyde powder in 100 mL water by heating to 60–70 °C in a fume hood. Add drops of 5 M KOH to the warm milky solution until it becomes completely clear. Heating at alkaline pH depolymerizes paraformaldehyde. After cooling to room temperature, adjust pH to between 6.5 and 7.5 with 5 % (v/v) H_2SO_4. Aliquots of this stock solution can be stored frozen for 6 months (*see Note 3*).
6. Fixation solution (4 % formaldehyde in PBS with Triton X-100): Mix 8 % formaldehyde stock solution with equal volume of 2× PBS (2× PBS: 5.4 mM KCl, 2.94 mM KH_2PO_4, 274 mM NaCl, and 16 mM Na_2HPO_4, pH 7.4) and add Triton X-100 to a final concentration of 0.05 % (*see Note 5*).
7. EdU detection cocktail: 1× PBS, 40 mM sodium ascorbate, 0.5 % Triton X-100, 4 mM $CuSO_4$, 2.5–20 μM Alexa Fluor 488 azide (Invitrogen). To prevent oxidation of the formed Cu (I) to non-catalytic Cu (II) species, prepare the detection cocktail freshly and use within 15 min. The sequence of adding the components is important. Follow the sequence given above.

8. DNA staining (DAPI) solution: Prepare 1 mg/mL DAPI (4,6-diamidino-2-phenylindole) solution in DMSO (10,000× stock) and dilute to 100 ng/mL in 1× PBS (*see Note 4*).
9. Mounting solution: For short-term mounting of samples, use 1× PBS which prevents cell shrinkage. For long-term preservation of samples, use an anti-fade mounting solution such as Fluoromount-G (Southern Biotech; *see Note 7*).
10. Consumables: 1.5 mL microfuge tubes, 4 mL cylindrical polypropylene tubes with cap, microscope slides, and circular coverslips.
11. Equipment: Laminar flow hood, fume hood, roller/rocker plate, desktop centrifuge with swing-out rotor, fluorescence microscope, or laser scanning fluorescence confocal microscope with appropriate filter sets.

18.2.2 Method

18.2.2.1 Synchronization of Rice Suspension-Cultured Cells

1. Seven-day-old suspension culture of rice is kept in the same medium for two more days to induce partial nutrient starvation and subcultured in a 1:3 ratio of conditioned medium to fresh medium ratio on the 9th day following previous subculturing.
2. Freshly prepared, filter sterilized HU at a final concentration of 20 mM is immediately added to the cells and incubated for a period of 36 h to block them in the S-phase of cell cycle.
3. HU block is removed by washing the cells 3 × 10 min with the sterile supernatant of a 1-day-old rice suspension culture (*see Note 8*).
4. Samples are taken before wash, after wash and at 2 h intervals following the wash.

18.2.2.2 Synchronization of *Arabidopsis* Suspension-Cultured Cells

1. Seven-day-old suspension culture of *Arabidopsis* is kept in the same culture medium for one more day (8th day) to induce partial nutrient starvation and subcultured by diluting 20× with fresh medium.
2. Freshly prepared, filter sterilized HU at a final concentration of 5 mM is immediately added to the cells and incubated for a period of 36 h to block them in the S-phase of cell cycle.
3. HU block is removed by washing the cells 3 × 10 min with the sterile supernatant of a 36-h-old *Arabidopsis* suspension culture (*see Note 8*).
4. Samples are taken before wash, after wash and at 2 h intervals following the wash.

18.2.2.3 EdU Labeling

1. Incubate 1 mL of cells in a 1.5 mL microfuge tube on a roller with 10 μM final concentration of EdU for 30 min in its own culture medium at culture growth temperature.
2. Settle cells by centrifuging for 5 min at 400*g*. Wash the pellet in 1.5 mL 1× PBS for 5 min (in a roller) and transfer to 4 mL tube, recentrifuge and discard the supernatant.
3. Fix the cell pellet for 45 min (rice) or 30 min (*Arabidopsis*) in 4 mL fixation solution on a roller. Centrifuge and replace the fixative with the same volume of 1× PBS. At this stage, the samples can be kept in the refrigerator for several weeks until further processing.
4. Wash cells 3 × 5 min with 1× PBS. Collect 200 μL (1/20th of total fixed cells) of cells containing the smallest clusters (*see Note 9*). Collect cells by centrifugation, discard the supernatant and incubate the cells in 150 μL EdU detection cocktail by rotating for 30 min at room temperature in 0.2 mL microfuge tube (*see Note 6*).
5. After 2 × 5 min washes with 1× PBS (0.2 mL each), label the nucleus with 1× PBS containing DAPI (100 ng/mL final concentration) for 5 min. Mount 20 μL aliquot of the cells onto a microscope slide with a coverslip and gently press with a tissue paper to flatten the clusters. The rest of the labeled cells can be kept in the fridge, in a dark container for several days.
6. Using a fluorescence microscope, count the number of EdU-positive cells among 500–1000 DAPI-labeled nuclei and calculate the percentage of S-phase nuclei. Note that some cells which are at the very beginning or at the very end of DNA synthesis may display spotty or patchy EdU signal. As an additional indicator of cell cycle status, mitotic index of the samples can also be calculated in parallel using the same sample (Fig. 18.2a, b).

18.3 EdU Labeling of Root Tissue

18.3.1 Materials

1. Sterile half-strength MS agar plates containing 0.7% agar with no hormones.
2. *Oryza sativa* L. ssp japonica cv. “Nipponbare” and *Arabidopsis thaliana* ecotype Columbia-0 seeds.
3. Sterilization solutions: 70% ethanol and 4% (v/v) commercial bleach (e.g., domestos containing 4.8 g sodium hypochlorite per 100 g) in water.
4. 5-ethynyl-2′-deoxyuridine (EdU) stock solution: see Sect. 18.2.1
5. Formaldehyde stock solution (8% w/v): see Sect. 18.2.1
6. Fixation solution (4% formaldehyde in PBS with TritonX-100): see Sect. 18.2.1

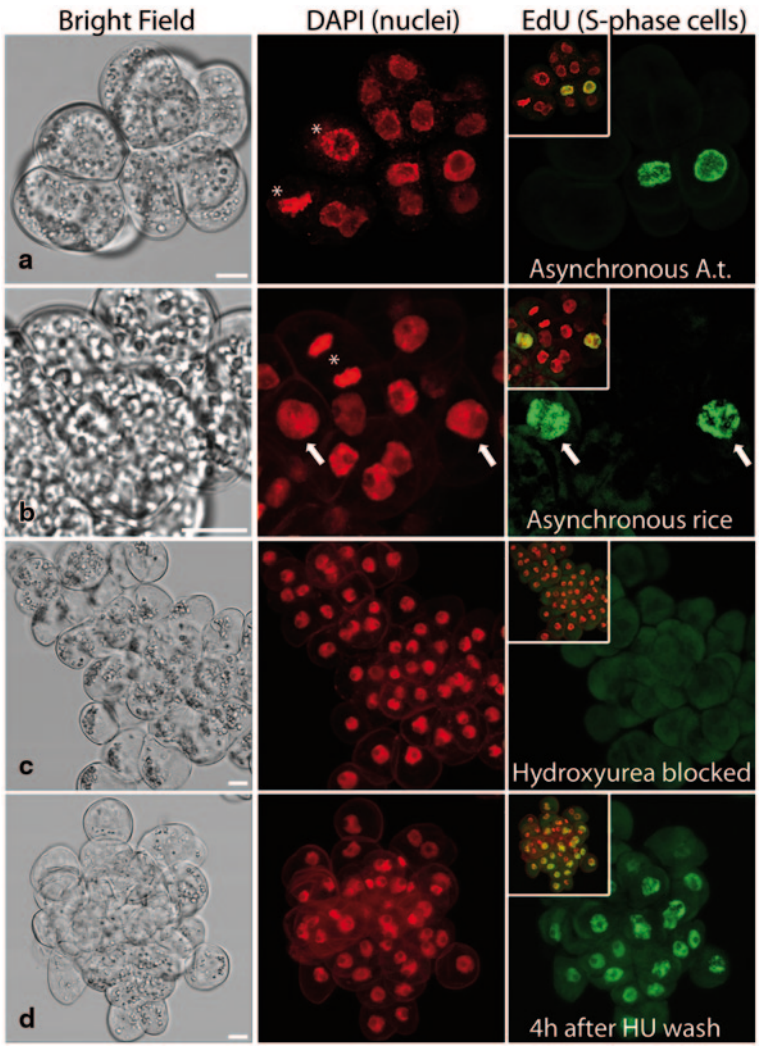

Fig. 18.2 *EdU* labeling of S-phase nuclei on suspension-cultured cells of *Arabidopsis* (cell line "MM1," Panel **a**) and rice (cv. "Unggi-9," Panels **b**–**d**). Asynchronously dividing (**a**, **b**), hydroxyurea (HU)-blocked (**c**) and HU-released (*4 h after HU wash*) cells were treated with 10 mM *EdU* for 30 min. Nuclei were labeled with DAPI (*middle column*) and mitotic cells of asynchronously dividing cultures were highlighted with asterisks. *Arrows* indicate *EdU*-incorporated nuclei. Scale bars = 10 μm

7. EdU detection cocktail: see Sect. 18.2.1
8. DNA staining (DAPI) solution: see Sect. 18.2.1
9. Mounting solution: see Sect. 18.2.1
10. Consumables: see Sect. 18.2.1
11. Equipment: see Sect. 18.2.1

18.3.2 Method

1. Dehusk rice seeds before sterilization. Place rice or Arabidopsis seeds in a 1.5 mL microfuge tube containing 70% ethanol and roll for 1 min.
2. Replace ethanol with 4% (v/v) commercial bleach and roll for 15 min.
3. Wash seeds 5 × 5 min in sterile distilled water and place them on sterile half strength MS agar plates. Seeds grown at 22 °C in long day conditions (16 h light/8 h dark cycle).
4. Seven-day-old seedlings were incubated in half strength liquid MS medium with 10 μM EdU for a period of 30 min to 2 h at room temperature.
5. Roots of rice seedlings were cut directly under fixation solution in a petri dish. Arabidopsis seedlings were fixed as whole. Both rice roots and Arabidopsis seedlings were fixed for 30 min at room temperature (*see Note 10*).
6. Replace the fixer with 1× PBS. At this stage, the samples can be stored in the refrigerator for several days.
7. Wash the root tips/seedlings 3 × 5 min in 1× PBS. Cut Arabidopsis root tips under 1× PBS after washes. Incubate root tips in 200 μL detection cocktail by rotating for 30 min at room temperature in 0.2 mL microfuge tube (*see Note 6*).
8. After 3 × 5 min washes with 1× PBS, counterstain the nucleus with 1× PBS containing DAPI (500 ng/mL) at room temperature for 20 min.
9. Mount root tips onto a microscope slide using a coverslip and mounting solution (e.g., Fluoromount-G) and gently press with a tissue paper to flatten the root tips and to blot the excess mounting solution (*see Note 7*).
10. For quantification of labeling, individual labeled nuclei in a given root region (root tip, stele, etc., Fig. 18.3) can be counted (see Sect. 18.2.2.3). Alternatively, total fluorescence intensity of the EdU-linked fluorochrome can be measured in a predetermined region of interest [16].

18.4 Notes

1. Caution: EdU is toxic, use appropriate precautions. EdU can interfere with cell cycle progression in long-term experiments. For long-term cell cycle studies (i.e., EdU incubation during complete cell division cycle), a less toxic derivative, F-ara-EdU can be used [43–45].
2. Caution: HU is toxic and carcinogenic; use appropriate precautions. More efficient but significantly more expensive inhibitor, aphidicolin can also be used instead of HU.
3. Paraformaldehyde is very hazardous in case of skin contact, eye contact, or inhalation (irritant/corrosive). Work in a fume hood and wear protective equipment. Avoid repeated freeze-thaw cycles of frozen aliquots. Thawed aliquots may require reheating to 60–70 °C for complete dissolution. Discard the aliquot if reheating does not clear up the precipitate. A fixation solution prepared from powdered formaldehyde is a better fixative than commercially available liquid formaldehyde solution [10].

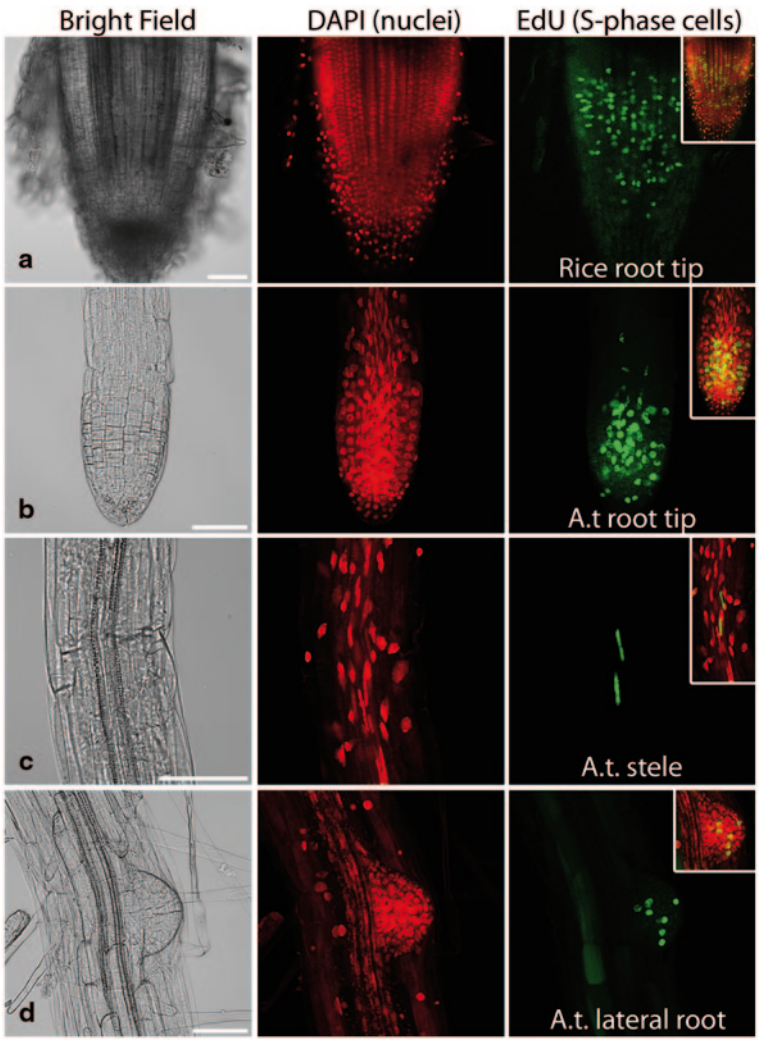

Fig. 18.3 EdU labeling of S-phase cells on various root tissues of *Arabidopsis* (Ecotype "Columbia-0") and rice (cv. "Nipponbare"). Roots of seedlings were immersed in 10 μM EdU for 30 min (rice) or 2 h (A.t: *Arabidopsis*). EdU-incorporated S-phase cells at root tips (**a**, **b**), stele (**c**) and lateral root meristem (**d**) are shown. Nuclei were labeled with DAPI (*middle column*). Scale bars = 50 μm

4. Caution: DAPI which is used as a nuclear counterstain is a known mutagen; use appropriate precautions. Other nucleic acid dyes such as Hoechst (DNA specific) or Propidium iodide (DNA/RNA stain) can also be used to locate the nuclei and chromosomes. All nucleic acid intercalating dyes should be handled with extreme care due to health hazards.
5. The addition of Triton X-100 to the fixation solution provides uniform fixation with reduced cell shrinkage.

6. Fluorochrome-containing solutions should not be exposed to strong light; therefore, incubations should be performed at dark or under dim light. The simplest solution is to wrap the samples in aluminum foil during incubations.
7. Glycerol-based (or high osmolarity) mounting media may cause cell shrinkage but they better suit imaging with high numerical aperture oil immersion objectives. Mounting the samples in PBS or water-based anti-fade solutions prevents cell shrinkage; however, care must be taken not to dry out the sample. Sealing the coverslip or occasional PBS loading may be necessary for prolonged observations to prevent sample drying.
8. One-day-old (rice) or 36-h-old (*Arabidopsis*) conditioned culture mediums were prepared by subculturing separate cultures 1 day (or 36 h in case of *Arabidopsis*) before the time of HU wash. We have found that using such conditioned mediums for HU washes is far more efficient than using fresh culture mediums for both rice and *Arabidopsis*.
9. A brief settling on bench settles the largest and heaviest clusters leaving the smaller clusters in suspension. Nylon mesh can also be used to collect a population of finer clusters as long as the smaller size of the cluster is not due to genetic variation. Selecting smaller clusters makes S-phase and mitotic index counting easier.
10. Since the fixer contains Triton X-100, plants with thin and fragile seedlings (like *Arabidopsis*) can also be fixed as a whole without cutting the roots (as shown in Fig. 18.3b–d). However, we have found that roots have to be cut before incubation with the EdU detection cocktail for fast and efficient penetration of the azide containing fluorochrome.

18.5 Interpretation and Conclusions (Troubleshooting)

Faint/no EdU labeling can be caused by:

1. The sequence of adding the components of the EdU detection cocktail is important. If the detection cocktail turns milky or develops a precipitate after the addition of all the components, it will not work efficiently.
2. Sodium ascorbate may have degraded. Stock solutions must be stored at $\leq -20\,^{\circ}C$. Thus stored, the solution is stable for several months. If the solution has turned brown, it has degraded. Discard and prepare new stock solution.
3. EdU may have degraded due to improper storage. Aliquots of EdU stock solution must be stored at $\leq -20\,^{\circ}C$. At this storage condition, the solution is stable for at least a year.
4. At the time of EdU addition, the cell culture or the tissue under consideration may not contain DNA synthesizing cells due to suboptimal growth conditions, stress, inhibitory chemicals, or altered genetic makeup. For asynchronously dividing cultures and young tissues, DAPI labeling of fixed samples and detection of mitosis can help to quickly assess the proliferative status of the culture.

References

1. Taylor JH, Woods PS, Hughes WL (1957) The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium labeled thymidine. Proc Natl Acad Sci U S A 43:122–128
2. Mendelsohn ML (1962) Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. The growth fraction. J Natl Cancer Inst 28:1015–1029
3. Gratzner HG (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218:474–475
4. Leif RC, Stein JH, Zucker RM (2004) A short history of the initial application of anti-5-BrdU to the detection and measurement of S phase. Cytometry A 58:45–52
5. Dolbeare F, Gratzner H, Pallavicini MG, Gray JW (1983) Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acad SciU SA 80:5573–5577
6. Moran R, Darzynkiewicz Z, Staiano-Coico L, Melamed MR (1985) Detection of BrdUrd incorporation by monoclonal antibodies: role of the DNA denaturation step. J Histochem Cytochem 33:821–827
7. Holm M, Thomsen M, Hoyer M, Hokland P (1998) Optimization of a flow cytometric method for the simultaneous measurement of cell surface antigen, DNA content and in vivo BrdUrd incorporation into normal and malignant hematopoietic cells. Cytometry A 32:28–36
8. Stroobants C, Sossountzov L, Miginiac E (1990) DNA Synthesis in excised tobacco leaves after Bromodeoxyuridine incorporation: immunohistochemical detection in semi-thin spurr sections. J Histochem Cytochem 38:641–647
9. Fowke LC, Cutler AJ (1994) Plant protoplast techniques. In: Harris N, Oparka KJ (eds) Plant cell biology: a practical approach. IRL, Oxford, pp 177–196
10. Goodbody KC, Lloyd CW (1994) Immunofluorescence techniques for the analysis of the cytoskeleton. In: Harris N, Oparka KJ (eds) Plant cell biology: a practical approach. IRL, Oxford, pp 221–243
11. Salic A, Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis *in vivo*. Proc Natl Acad Sci U S A 105:2415–2420
12. Kolb HC, Sharpless KB (2003) The growing impact of click chemistry on drug discovery. Drug Discov Today 8:1128–1137
13. Darzynkiewicz Z, Traganos F, Zhao H, Halicka HD, Li J (2011) Cytometry of DNA replication and RNA synthesis: historical perspective and recent advances based on "click chemistry". Cytometry A 79:328–337
14. Best MD (2009) Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules. BioChemistry 48:6571–6584
15. Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR (2006) A comparative study of bioorthogonal reactions with azides. ACS Chem Biol 1:644–648
16. Kotogány E, Dudits D, Horváth GV, Ayaydin F (2010) A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. Plant Meth 6:5
17. Ferullo DJ, Cooper DL, Moore HR, Lovett S (2010) Cell cycle synchronization of E. coli using the stringent response, with fluorescence labeling assays for DNA content and replication. Methods 48:8–13
18. Hua H, Kearsey SE (2011) Monitoring DNA replication in fission yeast by incorporation of 5-ethynyl-2′-deoxyuridine. Nucl Acids Res 39:e60
19. Lopez-Serra L, Lengronne A, Borges V, Kelly G, Uhlmann F (2013) Budding yeast Wapl controls sister chromatid cohesion maintenance and chromosome condensation. Curr Biol 23:64–69
20. Kaiser CL, Kamien AJ, Shah PA, Chapman BJ, Cotanche DA (2009) 5-ethynyl-2′-deoxyuridine labeling detects proliferating cells in the regenerating avian cochlea. Laryngoscope 119:1770–1775

21. Warren M, Puskarczyk K, Chapman SC (2009) Chick embryo proliferation studies using EdU labeling. Dev Dyn 238:944–949
22. Diermeier-Daucher S, Clarke ST, Hill D, Vollmann-Zwerenz A, Bradford JA, Brockhoff G (2009) Cell type specific applicability of 5-ethynyl-2′-deoxyuridine (EdU) for dynamic proliferation assessment in flow cytometry. Cytometry A 75:535–546
23. Limsirichaikul S, Niimi A, Fawcett H, Lehmann A, Yamashita S, Ogi T (2009) A rapid non-radioactive technique for measurement of repair synthesis in primary human fibroblasts by incorporation of ethynyl deoxyuridine (EdU). Nucl Acids Res 37:e31
24. Notaguchi M, Wolf S, Lucas WJ (2012) Phloem-mobile Aux/IAA transcripts target to the root tip and modify root architecture. J Integr Plant Biol 54:760–772
25. Kakar K, Zhang H, Scheres B, Dhonukshe P (2013) CLASP-mediated cortical microtubule organization guides PIN polarization axis. Nature 495:529–533
26. Perilli S, Perez-Perez JM, Di Mambro R, Peris CL, Diaz-Trivino S, Del Bianco M, Pierdonati E, Moubayidin L, Cruz-Ramirez A, Constantino P, Scheres B, Sabatini S (2013) RETINOBLASTOMA-RELATED protein stimulates cell differentiation in the *Arabidopsis* root meristem by interacting with cytokinin signaling. Plant Cell 25:4469–4478
27. Zhu Y, Weng M, Yang Y, Zhang C, Li Z, Shen WH, Dong A (2011) *Arabidopsis* homologues of the histone chaperone ASF1 are crucial for chromatin replication and cell proliferation in plant development. Plant J 66:443–455
28. Xu D, Huang W, Li Y, Wang H, Hunag H, Cui X (2012) Elongator complex is critical for cell cycle progression and leaf patterning in *Arabidopsis*. Plant J 69:792–808
29. Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J (2013) Glucose–TOR signalling reprograms the transcriptome and activates meristems. Nature 496:181–186
30. Ichihashi Y, Kawade K, Usami T, Horiguchi G, Takahashi T, Tsukaya H (2011) Key proliferative activity in the junction between the leaf blade and leaf petiole of *Arabidopsis*. Plant Physiol 157:1151–1162
31. She W, Grimanelli D, Rutowicz K, Whitehead MW, Puzio M, Kotlinski M, Jerzmanowski A, Baroux C (2013) Chromatin reprogramming during the somatic-to-reproductive cell fate transition in plants. Development 140:4008–4019
32. Hayashi K, Hasegawa J, Matasunaga S (2013) The boundary of the meristematic and elongation zones in roots: endoreduplication precedes rapid cell expansion. Sci Rep 3:2723
33. Bazin J, Khan GA, Combier JP, Bustos-Sanmamed P, Debernardi JM, Rodriguez R, Sorin C, Palatnik J, Hartmann C, Crespi M, Lelandais-Briere C (2013) miR396 affects mycorrhization and root meristem activity in the legume *Medicago truncatula*. Plant J 74:920–934
34. Ayaydin F, Kotogány E, Abraham E, Horváth GV (2011) Synchronization of *Medicago sativa* cell suspension culture. Meth Mol Biol 761:227–238
35. Ron M, Dorrity MW, de Lucas M, Toal T, Hernandez RJ, Little SA, Maloof JN, Kliebenstein DJ, Brady SM (2013) Identification of novel loci regulating interspecific variation in root morphology and cellular development in tomato. Plant Physiol 162:755–768
36. Kuznetsova MA, Sheval EV (2013) Detection of replication sites in plant cell nuclei by using semithin sections. Cell Tissue Biol 7:375–378
37. Schubert I, Schubert V, Fuchs J (2011) No evidence for "break-induced replication" in a higher plant—but break-induced conversion may occur. Front Plant Sci 2:8
38. Nakayama H, Yamaguchi T, Tsukaya H (2012) Acquisition and diversification of cladodes: leaf-like organs in the genus Asparagus. Plant Cell 24:929–940
39. Tresch S, Schmotz J, Grossmann K (2011) Probing mode of action in plant cell cycle by the herbicide endothall, a protein phosphatase inhibitor. Pestic Biochem Physiol 99:86–95
40. Dudits D, Abraham E, Miskolczi P, Ayaydin F, Bilgin M, Horváth GV (2011) Cell-cycle control as a target for calcium, hormonal and developmental signals: the role of phosphorylation in the retinoblastoma-centred pathway. Ann Bot 107:1193–1202
41. Bass HW, Wear EE, Lee TJ, Hoffman GG, Gumber HK, Allen GC, Thompson WF, Hanley-Bowdoin L (2014) A maize root tip system to study DNA replication programmes in somatic and endocycling nuclei during plant development. J Exp Bot 65:2747–2756

42. Kelliher T, Walbot V (2011) Emergence and patterning of the five cell types of the *Zea mays* anther locule. Dev Biol 350:32–49
43. Neef AB, Luedtkel NW (2011) Dynamic metabolic labeling of DNA *in vivo* with arabinosyl nucleosides. Proc Natl Acad Sci U S A 108:20404–20409
44. Cruz-Ramirez A, Diaz-Trivino S, Wachsman G, Du Y, Arteaga-Vazquez M, Zhang H, Benjamins R, Blilou I, Neef AB, Chandler V, Scheres B (2013) A SCARECROW-RETINOBLASTOMA protein network controls protective quiescence in the *Arabidopsis* root stem cell organizer. PLOS Biol 11:e1001724
45. Qu D, Wang G, Wang Z, Zhou L, Chi W, Cong S, Ren X, Liang P, Zhang B (2011) 5-ethynyl-2′-deoxycytidine as a new agent for DNA labeling: detection of proliferating cells. Anal Biochem 417:112–121

Chapter 19
Staining Methods for Programmed Cell Death

Owen S. D. Wally and Claudio Stasolla

19.1 Introduction

Together with cell division and differentiation, programmed cell death (PCD) is a naturally occurring event regulating growth and development. Manifestation of PCD in plants is apparent during stress conditions, including plant pathogen interactions and tolerance to adverse environmental conditions. By triggering the suicide mode the plant sacrifices cells, tissue, and/or organs to increase chances of survival. During pathogen attack, for example, PCD is triggered as a component of the hypersensitive response to dismantle specific cells around the site of infection to physically isolate the pathogen, thus delaying or preventing its spread.

Execution of the death program is not only linked to suboptimal growing conditions but also observed in normal developmental processes where it modulates cell and tissue homeostasis through the selective elimination of damaged and/or undesired cells. Well-studied examples of PCD occur in both embryonic and post-embryonic phases of development. During embryo and seed formation PCD is responsible for the removal of the suspensor once the embryo has attained a defined stage, and the degradation of nucellus tissue and aleurone layers within the endosperm. Developmentally regulated examples of PCD are also observed during post-embryonic growth in both vegetative and reproductive phases. Tracheary elements differentiation, senescence, and leaf sculpturing are examples of PCD during vegetative development, while sculpturing of the male and female organs, as well as pollen–stigma interaction and petal senescence are processes mediated by PCD during reproductive growth.

Some of the ultrastructural alterations mediated by PCD and contributing to the dismantling of cellular components in plant cells are similar to those occurring during mammalian apoptosis and autophagy. These include the condensation of chromatin and nuclear envelope, as well as the fragmentation of the DNA. Systemic

C. Stasolla (✉) · O. S. D. Wally
Department of Plant Science, University of Manitoba, 222 Agriculture Bld,
Winnipeg R3T 2N2, Manitoba, Canada
e-mail: Claudio.Stasolla@umanitoba.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_19

fragmentation of DNA occurs in two distinct steps and is regulated by nuclease activity [1]. The first step produces fragments of 50–300 kbp through the cleavage of the DNA in the proximity of interloop sites of the chromatin at the inter-rosette [2]. Oligonucleosomal DNA fragments are subsequently produced by cleavage at the internucleosomal sites which is mediated by the endonuclease enzyme DNase1 [2].

While the DNA laddering obtained with conventional gel electrophoretic procedures is effective in identifying internucleosomal cleavage in whole tissues and/or organs undergoing PCD, it cannot be used in the identification and localization of dying cells. Three in situ detection methods: in situ end labeling (ISEL), in situ nick translation (ISNT), and terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) can be utilized for the in situ detection of PCD. Both ISEL and basic ISNT use DNA polymerase I large fragment Klenow to incorporate fluorescent-labeled nucleotides into the DNA strands that were nicked during PCD. Labeled nucleotides are then incorporated into the fragmented DNA at the 3′-hydroxy ends of overhanging DNA in a template-dependent manner, with no labeling possible on blunt-ended DNA fragments. More detailed information about these two methods is available in the literature [3, 4].

Developed more than two decades ago [5], TUNEL is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3′-hydroxy ends of overhanging DNA. Compared to ISEL and ISNT, one major benefit of TUNEL technique is that it labels all types of DNA in a template-independent manner (through use of TdT), leading to an overall stronger signal intensity, which also occurs at a much faster rate than Klenow [6, 7]. Additionally, TUNEL preferentially labels cells that are undergoing apoptosis rather than necrosis, due to the nature of chromosomal breakage, thus differentiating between the two death types [7, 8]. Finally, visualization of TUNEL-positive cells can be conducted colorimetrically, or fluorometrically, in whole-mount or sectioned tissues. This flexibility is important as it allows the utilization of the technique in a large variety of experimental systems.

While TUNEL has been widely adopted as the standard for apoptosis detection in mammalian cells there have been some concerns about false-positive detection within sectioned plant tissues, due to chromosomal shearing during sample preparation [9]. These false TUNEL results can be mitigated through appropriate use of control tissues and using longer and more consistent fixing protocols [10]. Another consideration to keep in mind is the nonspecificity of TUNEL, which recognizes and labels free 3′-hydroxyl termini which might have also been generated by other processes not related to PCD. Examples of nonspecific TUNEL reactivity include cells undergoing DNA repair or active transcription [11, 12]. Simple tissue section procedures have also been shown to increase the incidence of the TUNEL-positive signal in non-apoptotic cells [11]. Despite these drawbacks, however, TUNEL is far more sensitive and specific than existing chromosomal labeling and classical cell death labeling techniques and should be considered the “gold standard” for PCD detection in plant materials. As a general rule, it is always preferable to confirm the TUNEL signal using other PCD-specific techniques/assays.

Given the cost and the time to perform TUNEL, it is advisable to conduct some preliminary studies to assess the presence of cell death in the desired tissue. A simple

staining procedure involves the use of trypan blue, a relatively specific stain that is only taken up by dead cells or by the hyphal cell walls of fungi and oomycetes [13]. This stain is based on the principle of dye exclusion. Living cells, characterized by a functional and intact plasma membrane, exclude the dye, while cells with a damaged plasma membrane, that is, dying cells, are permeable to trypan blue. Based on this principle, trypan blue is often used to visualize cell damage or death at a whole tissue and/or organ level, as well as microlesions (occurring after exposure to an incompatible pathogen interaction) at a cellular level. Examples of trypan blue staining include whole *Arabidopsis* leaves [14], small leaf discs taken from larger leaves [15], and cultured cells [16]. The procedure involves boiling the samples within alcoholic trypan blue staining solution for a short period of time to allow the stain to permeate into the necrotic plant cells, followed by a longer incubation to allow the completion of stain penetration. The dye is then removed along with residual chlorophyll in the tissue using chloral hydrate. The samples can then be visualized using light microscopy. While unable to differentiate between PCD and necrosis, trypan blue can be employed as an initial screening procedure.

The purpose of this chapter is to provide a detailed methodology aimed at visualizing cell death on excised leaves using trypan blue staining, and examining sectioned tissues using both fluorescent and colorimetric TUNEL assays.

19.2 Materials

19.2.1 Trypan Blue Assay

Lactophenol solution: glycerol, phenol, ultrapure H_2O (premade lactophenol solution is also available: EMD Millipore # R03266-74), trypan blue (Sigma # T6146), magnetic stirrer, dry bath or water bath, chloral hydrate, 70% glycerol, and 95% ethanol.

19.2.2 TUNEL

19.2.2.1 Sample Fixation

Paraformaldehyde (Sigma #P6148), PBS buffer (NaCl, KCl, Na_2HPO_4, and KH_2PO_4), glass sample vials (for larger samples: 24 mL, 30 mm diameter, 52 mm height (PELCO® #12706), for minute samples: 4 mL, 15 mm diameter, 48 mm height (PELCO® #411)), vacuum oven chamber, razor blades, forceps, scalpel, tissue rotator (PELCO® R2 Rotary Mixer #1050), and rotator head (PELCO® #1051 for 24 mL vials and PELCO® #1054 for 4 mL vials).

19.2.2.2 Dehydration

Xylene, 95% ethanol**,** 2-thiobarbituric acid (TBA), tissue rotator, and pipettes.

19.2.2.3 Embedding, Block Preparation, and Sectioning

Paraplast® Plus Tissue Embedding Medium (Fisher Scientific #23-021-400), oven, Peel-A-Way® Disposable Histology Molds (for larger samples: Peel-A-Way® 22 mm × 30 mm × 20 mm deep, rectangular (TED PELLA #27112), for minute samples: Peel-A-Way®, Truncated, 22 mm square top, tapered to 8 mm bottom (TED PELLA #27116)), forceps, small spatula, water tray, bucket filled with ice, embedding rings (Fisher Scientific #22-038-197), slide warmer, Superfrost® Plus microscope slides (Fisher Scientific #12-550-15), and coverslips.

19.2.2.4 Dewaxing

Nine Coplin staining jars (Fisher Scientific # S17495), xylene, and PBS buffer (NaCl, KCl, Na_2HPO_4, and KH_2PO_4).

19.2.2.5 TUNEL Analysis

PBS buffer (NaCl, KCl, Na_2HPO_4, and KH_2PO_4), proteinase K solution (Roche # 03115887001), and recombinant RNase-free DNAse I (Roche # 04716728001).

Fluorescent TUNEL Assay

Calf thymus recombinant Terminal Transferase (TdT) (Roche #03333566001) (400 u/μL in 60 mM KH_2PO_4 pH 7.2, 150 mM KCl, 1 mM 2-mercaptoethanol, 0.5% v/v Triton X-100, 50% v/v glycerol), KCl, KH_2PO_4, glycerol, Triton X-100, TdT label mix (Roche # 11767291910) (containing final concentration of 0.5 mM of each unlabeled dATP, dTTP, dCTP, and dGTP, 0.05 mM of fluorescein labeled dUTP, 200 mM $KC_2H_6AsO_2$ (potassium cacodylate), 25 mM Tris-HCl pH 6.6, 1 mM $CoCl_2$ and 0.25 mg/mL of ProLong® Gold Antifade Reagent (Life Technologies #P36930), staining dish (Wheaton® stain dish (Ted Pella # 21054)), slide glass rack with handle (Ted Pella #21057), rocking platform, and slide humidity incubation box (Lab Scientific #HIC).

Colorimetric TUNEL Alkaline Phosphatase Assay

Additional reagents required:

1. TUNEL AP converter reagent (Roche # 11772457001), which contains ready-to-use anti-fluorescein polyclonal antibody, Fab fragment raised in sheep conjugated with alkaline phosphatase (AP) in triethanolamine buffer.
2. Substrate solution: 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma #B6149), nitroblue tetrazolium (NBT; Sigma #N6876), dimethyl formamide, Tris, HCl, NaCl, and $MgCl_2$. Premade substrate solutions are also commercially available, for example, Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega #S3841).
3. Mounting medium: Aqua-Poly/Mount (Polysciences Inc #18606-20).

19.3 Methods

19.3.1 Trypan Blue Assay

1. If not using a premade lactophenol solution combine a 1:1:1:1 ratio of concentrated lactic acid, glycerol, phenol, and ultrapure H_2O. The solution must be mixed constantly on a magnetic stirrer in the fume hood for at least 1 h.
2. Prepare the trypan blue staining solution by dissolving trypan blue (25 mg) in 10 mL of lactophenol solution. Once dissolved, dilute the solution with 20 mL of 95% ethanol.
3. Place the samples to be examined (small leaf disks or cultured cells), into suitable sized centrifuge tubes *(see Note 1)*.
4. Completely cover the samples with the trypan blue solution and place the samples in a 100℃ dry bath for 1 min.
5. Allow the samples to cool slowly at room temperature. Incubate the samples for 24 h at room temperature *(see Note 2)*.
6. During the last phases of incubation, prepare the chloral hydrate solution. In a fume hood dissolve 50 g of chloral hydrate in 20 mL of H_2O with constant agitation *(see Note 3)*.
7. With a pipettor gently remove the trypan blue staining solution from the samples and dispose it in the appropriate waste container. In a fume hood cover the tissues completely with chloral hydrate solution. Agitate the samples gently on a bench rocker or orbital shaker at low speed for 3 h at room temperature. If the samples are not fully cleared, change with fresh chloral hydrate solution and incubate overnight. The clearing of the tissues is complete when the tissue background becomes transparent.
8. With a pipettor discard the chloral hydrate solution in the appropriate waste container and cover the tissues with 70% glycerol.
9. With forceps, place the samples on a glass microscope slide with cover slip and examine the samples by light microscopy under 40–100× magnification. Dead cells accumulating trypan blue appear dark while living cells exclude the dye and remain colorless.

19.3.2 TUNEL Assay

19.3.2.1 Sample Fixation

1. Prepare 1× PBS buffer by combining 4 g of NaCl, 0.1 g of KCl, 0.72 g of Na_2HPO_4, and 0.12 g of KH_2PO_4 in 400 mL of water. Adjust the pH to 7.4 with HCl and bring the volume to 500 mL with water.
2. Prepare 250 mL of 4% paraformaldehyde in PBS pH 7.4. Warm 200 mL of the 1× PBS to 60 ℃ on a hot plate and add 10 g of paraformaldehyde with continuous stirring *(see Note 4)*. Once the solution is clear bring the volume to 250 mL with 1× PBS. Cool at room temperature and store in the fridge.
3. Aliquot about 15–20 mL of the fixative into capped glass vials and store in ice.
4. With the help of a scalpel/razor blade and forceps, dissect the tissue to be examined and place it rapidly into the vials containing the fixative *(see Note 5)*. Scarification and small incisions might be required for hard tissue to favor the penetration of the fixative.
5. Transfer the vial to a small tray full of ice and place the tray in a vacuum chamber/oven. Loosen the caps and apply a vacuum of about 24 mm Hg for 20 min *(see Note 6)*. As the air is removed, air bubbles start forming from the tissue which will eventually sink to the bottom of the vial. An additional 20 min of vacuuming might be required. Alternative infiltration protocols are available for those tissues characterized by thick, hydrophobic cuticles *(see Note 7)*.
6. Once the infiltration is complete, gently release the vacuum. The vials, with their caps tightened, can be transferred to a rotary mixer and incubated overnight at 4 ℃.

19.3.2.2 Dehydration

1. The following dehydration solutions can be prepared in advance. D_1 (80 mL 95% ethanol, 20 mL TBA, and 100 mL H_2O), D_2 (100 mL 95% ethanol, 40 mL TBA, and 60 mL H_2O), D_3 (100 mL 95% ethanol, 70 mL TBA, and 30 mL H_2O), D_4 (90 mL 95% ethanol and 110 mL TBA), D_5 (50 mL 95% ethanol and 150 mL TBA), D_6 (100 mL TBA), and D_7 (100 mL TBA).
2. After the overnight incubation at 4 ℃ the fixative can be gently decanted from the vials with the help of a glass pipette. Quickly, add 15 mL of 1× PBS and incubate on the rotator for 2 h.
3. Replace PBS with 15 mL of solution D_1 *(see Note 8)*. Incubate on the rotator overnight at 4 ℃.
4. Repeat the procedure with the remaining solutions $D_{2–7}$ with an overnight incubation for each solution *(see Note 9)*. During this procedure it is imperative to add excess volumes of solutions to fully cover the samples.

19.3.2.3 Embedding

1. While the samples are dehydrating, in a fume hood set an oven at a temperature of 56–60 ℃. Fill a plastic beaker with pellets of Paraplast® Plus Tissue Embedding Medium and melt the wax in the oven *(see Note 10)*. Complete melting might require several hours.
2. Add a few pellets of solid Paraplast® Plus Tissue Embedding Medium in the vials (about ¼ of the total volume) and transfer the vials to the rotator for 2 h at room temperature.
3. After the pellets are completely melted add a few more pellets and continue the incubation on the rotator for 2 additional hours.
4. The capped vials can now be placed in the 56–60 ℃ oven for about 1–2 h.
5. Decant about ½ of the TBA/wax solution from each vial with the help of a glass pipette and add an equal volume of melted wax. Leave the vials uncapped to allow the complete evaporation of TBA.
6. Remove by decantation all the wax in the vials and replace it with melted wax *(see Note 11)*. Leave the uncapped vials in the oven overnight.
7. Repeat the changes with melted wax (step 6) twice.

19.3.2.4 Block Preparation

1. During the initial steps of embedding, place the Peel-A-Way® Disposable Histology Molds and a spatula in the 56–60 ℃ oven *(see Note 12)*.
2. By swirling the glass vials quickly pour the wax and the samples into the molds. Arrange the samples in the desired orientation at the bottom of the molds making sure they are evenly separated and away from the edges of the mold. The wax will tend to harden rapidly, so this procedure needs to be executed rapidly *(see Note 13)*. Alternative preparative methods are available for samples which are difficult to orient and require more time *(see Note 14)*.
3. Transfer the molds from the oven in a box (or the lid of a petri dish) and place the box into a tray containing ice and water. The low temperature will favor a uniform solidification of the wax. After 30 min, or when the blocks are fully solidified, they can be transferred into a plastic bag and stored at 4 ℃ for up to 6 months.
4. The embedding rings can be prepared days in advanced by sealing one end of the ring with adhesive tape and pouring melted wax into the other end so as to fill their cavity. Once the wax is fully solidified, the rings can be stored at room temperature for an indefinite period of time.
5. The plastic of the molds can be pealed to release the sample-containing block of wax. In a fume hood, and with the aid of an alcohol burner, warm up a spatula and reduce the height of the block and flatten its surface opposite to the samples.
6. Sandwich the hot spatula between the wax support and the embedding ring. As soon as the wax on either side starts melting, rapidly retract the spatula and allow

the two surfaces to melt together by applying gentle pressure with a finger on the block of wax. As the wax starts solidifying, release the pressure.
7. Once fully solidified, the blocks can be stored in the fridge and used immediately for sectioning or left for long-term storage.

19.3.2.5 Sectioning

1. Trim the block to the appropriate size with a single-edged razor blade, ensuring the right and left sides are parallel, while the top is slightly shorter than the bottom. The surface of the trimmed block should have the shape of a trapezoid.
2. The embedding ring can be inserted in the holder of a rotary microtome with the long side of the block down. The cutting angle should be set at 7–9° while the section thickness should be 10 μm.
3. Label the slides with a pencil and place them on a slide warmer at 37 ℃. Using a glass pipette gently add water onto each slide.
4. Start sectioning and discard the first sections as they might not contain the sample. The ribbon, which can be obtained when using a rotary microtome, is placed on a piece of cardboard with the help of forceps and a small brush. Using a sharp razor blade cut the ribbon into segments and place one segment on each slide.
5. As they come in contact with water the ribbon segments start expanding; additional water can be added to facilitate expansion.
6. Once the ribbon segments are fully expanded (it will take a few minutes) drain the water from each slide on a paper towel with the help of a small brush. Make sure the sections remain flat.
7. Place the slides back on the slide warmer and let them dry overnight at 37 ℃. Once fully dry, they can be stored in a slide box and used later.

19.3.2.6 Dewaxing

1. Prepare the following solutions (*c1,* xylene; *c2,* xylene; *c3,* 100 % ethanol; *c4,* 100 % ethanol; *c5,* 95 % ethanol; *c6,* 70 % ethanol; *c7,* 50 % ethanol; *c8,* 1 × PBS; and *c9,* 1 × PBS) and fill 9 Coplin jars with each solution *(see Note 15)*.
2. Insert the slides into the grooves of the first jar *(c1)* using a pair of forceps. It is important that slides do not stick to one another as this might compromise the full removal of the wax *(see Note 16)*. Leave the slides for 15–20 min.
3. With the forceps, remove one slide from the jar, tap it gently on a paper towel to get rid of excessive xylene and transfer it into the second jar *(c2)*. After repeating the procedure for the remaining slides incubate for 15–20 min.
4. Transfer the slides into the third jar *(c3)* after removing the excess xylene. Let them rest for 2 min.
5. Continue the transfer into the other jars *(c4–9)* with a 2 min wash.

19.3.2.7 Fluorometric TUNEL Assay

Ten slides will be used for this procedure: one negative control slide, one positive control slide, and eight reaction slides.

1. Drain and blot excess PBS from the slides using a paper towel. Place the slides horizontally onto a slide holder and apply 200 µL of protein kinase solution (20 µg/mL) to each slide. Gently place a cover slip onto each slide and incubate at room temperature for 15 min.
2. Carefully remove the cover slip by tilting the slides, and place each slide in a staining dish using the appropriate rack with handle. Fill the dish with 1 × PBS and wash with gentle agitation on a rocking platform for 5 min at room temperature. Repeat this step 2 times.

Steps 3–9 must be carried out under minimum light conditions to retain the fluorescence of the labeled probes.

3. While the slides are washing in 1 × PBS, defrost the TdT enzyme solution and the TUNEL labeling solution on ice *(see Note 17)*.
4. In a microcentrifuge tube, aliquot 100 µL of the TUNEL labeling solution to be used as the negative control. Store on ice.
5. In another microcentrifuge tube, add 100 µL of the TdT enzyme solution and 900 µL of TdT labeling solution, yielding a total of 1000 µL TdT reaction mixture. Mix by pipetting and store on ice.
6. Remove a slide (positive control) from the staining dish, drain and blot excess PBS, and place it horizontally onto a slide holder. Pipette 100 µL of recombinant DNase I solution and incubate at room temperature for 10 min.
7. Prepare the slide humidity incubation box by wetting paper towels with water and placing them at the bottom of the box. Transfer the 10 slides onto the tray (after the DNase I solution has been carefully drained and blotted from the positive control slide) and place the tray in the box *(see Note 18)*. Quickly add 100 µL of the TdT labeling solution on the negative control slide and a similar volume of the TdT reaction mixture on each of the remaining 9 slides. Cover with glass coverslips and seal the box with Parafilm. Place the box in a 37 ℃ oven for 1 h.
8. Carefully remove the slides from the humidity incubation box, tilt each slide gently to remove the coverslip and wash in PBS (see step 2) for 10 min at room temperature. Repeat this step twice.
9. Drain and blot PBS from each slide, add two drops of the ProLong® Gold Antifade Reagent and store in the dark *(see Note 19)*. After 24 h the slides can be examined using fluorescence microscopy.

19.3.2.8 Colorimetric TUNEL AP Assay

The fluorescent signal of UDP-fluorescein labeled sections can be converted into a colorimetric signal using anti-fluorescein antibodies conjugated with AP. This

conversion is particularly useful for tissues characterized by high auto-fluorescence, or when facilities for high quality fluorescent imaging are not available.

1. Proceed to step 8 of the fluorometric TUNEL assay protocol.
2. Remove excess of PBS by draining and blotting, and place the slides horizontally onto a slide holder. Apply 50 μL of TUNEL-AP solution onto each slide, gently cover with a glass coverslip, and transfer the tray into a humidity incubation box. Incubate 37 ℃ for 30 min.
3. Remove the slides from the humidity incubation box, tilt each slide gently to remove the coverslip and wash in PBS (see step 2 of the Fluorometric TUNEL assay) for 10 min at room temperature. Repeat this step twice.
4. The detection solutions (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$), NBT solution (30 mg NBT in 1 mL of 70 % dimethyl formamide) and the BCIP solution (15 mg BCIP in 1 mL of dimethyl formamide) can be prepared in advanced *(see Note 20)*.
5. Add 1 mL of NBT and BCIP solutions each to 98 mL of detection solution. Mix and transfer the contents into a Coplin jar. Remove the slides from the PBS wash and place them into the jar. Cover the jar with aluminum foil and incubate at room temperature for 6–8 h.
6. If using Western Blue® stabilized substrate, drain the excess PBS solution from each slide and place the slides horizontally into a slide holder. Apply 200 μL of Western Blue®. Add coverslips and incubate at room temperature for 4 h *(see Note 21)*.
7. When the desired color is achieved (either with the NBT/BCIP method or with Western Blue®; *see Note 22*), rinse each slide briefly in deionized water to remove some background stain, remove excess water by draining and apply two drops of Aquamount per slide. Apply a glass coverslip and let the slides dry overnight in the dark.
8. Slides can now be visualized under light microscopy.

19.4 Notes

1. Many researchers prefer to use 12-well microtiter plates for processing samples with trypan blue. However, microtiter plates are difficult to seal and this can result in leaking of the stain (in alcoholic lactophenol) which can remove labels written with felt pens. Wax pencils are recommended for labeling plates. Additionally, only very small leaves or other tissue samples can be used within the plates. Finally, we find that microtiter plates are difficult to heat to boiling temperatures and require a specialized setup.
2. Depending on the degree of tissue necrosis, incubation time needs to be empirically adjusted. The samples should be checked every hour and processed further once the desired staining intensity is reached.

3. Trypan blue staining works better in plant tissues when chloral hydrate is used to destain. The use of chloral hydrate is inconvenient since it is a controlled substance requiring special ordering protocols from manufacturers and special disposal procedures. The handling of chloral hydrate should be performed in a fume hood with proper face and hand protection. The use of alcoholic lactophenol (1:2 lactophenol: 95% ethanol) is often used as a chloral hydrate alternative, albeit with higher background signals.
4. Paraformaldehyde is a toxic substance and gloves should be worn. The whole procedure described in step 2 can be carried out in a fume hood.
5. In order to preserve the structural integrity of the tissue to be examined, it is highly recommended to proceed rapidly. Ideally, the tissue should be trimmed to produce a flat surface which will facilitate the orientation of the samples in the mold during the embedding steps.
6. A less effective infiltration procedure can also be achieved by placing the samples in a desiccator.
7. Samples which do not infiltrate properly in the vacuum/chamber oven can be placed in a small syringe with 4 mL of fixative after the piston has been removed. The piston is reinserted and pressed gently to remove excess air while the syringe outlet is facing up. Once all the air has escaped, the outlet is then plugged with a gloved finger and the piston is pulled in order to create a vacuum. Shaking the syringe several times will facilitate the removal of residual air from the tissue. Vacuum can be applied for about 5–10 min depending on the type of tissue.
8. A simple strategy to prevent the loss of minute tissue is to cover the tip of the pipette with a nylon mesh while removing the solution from the vial.
9. The length of each dehydration step can be adjusted depending on the size and characteristics of the samples. For small and "more permeable" samples the length of time can be reduced to a few hours.
10. Temperature higher than 62 ℃ can alter the properties of the wax and compromise further steps.
11. Wax tends to solidify quickly and this step has to be carried out very quickly. If dealing with several vials it is recommended to stagger the changes of solution in order to minimize the time the oven remains open.
12. Depending on the size of the samples different Peel-A-Way® Disposable Histology Molds can be used (*see* the Materials section). For example, molds with smaller bottom areas are highly recommended for minute samples as they can be oriented and grouped together.
13. The use of weighing boats is a suitable alternative to the Peel-A-Way® Disposable Histology Molds, especially if many samples are available, as in the case of suspension cultures. The large wax blocks obtained with weighing boats will need to be cut into smaller blocks to fit the embedding rings.
14. A hot plate set up at 60 ℃ can be used to prepare the blocks outside the oven and slow down the solidification of the wax. After filling one third of the mold with the samples and wax, the mold can be transferred onto the hot plate. The samples can then be oriented slowly with the help of a magnifying lens and using a pre-warmed spatula. Once the desired orientation is reached, the mold

can be transferred onto a flat surface for a few seconds, just to let the thin layer of wax solidify. The mold can then be filled with more melted wax and rapidly placed on a tray containing water and ice for about 30 min to allow the remaining wax to solidify completely.

15. It is recommended to use fresh solutions after each jar has been used three to four times. This is especially important for the xylene and 100% ethanol washes.
16. A commonly used procedure is to "sandwich" the slides downside (non-sample) together in order to double the number of slides in each jar. From our experience, we discourage this procedure, especially for the initial washes *(c1–4)* as some xylene and ethanol might remain trapped between the slides and be carried into the remaining jars.
17. The TUNEL label mix reaction buffer contains $KC_2H_6AsO_2$ (potassium cacodylate) which is extremely toxic on inhalation and absorption by the skin, in addition to $CoCl_2$ which is a known carcinogen. The Roche in situ Cell Death Detection Kit, Fluorescein (Roche #11684795) including all the reagents required for TUNEL assay, does not contain cacodylate in the enzyme mix. However, many other commercially available kits, including older Roche kits, do contain it. Special care should be taken to avoid potential contamination and accidental inhalation.
18. While ensuring proper drainage of the solutions from the slides, it is important to proceed quickly in order to prevent the slides from drying as this will increase the background signal.
19. The ProLong® Gold Antifade Reagent enhances the signal and prevents photobleaching, especially if the slides need to be examined at a later date and/or stored. The reagent is not required if the slides are examined immediately and in this case glycerol can be used as the mounting medium.
20. While the detection solution can be stored in the fridge, the NBT and BCIP solutions should be freshly prepared under low light conditions.
21. The use of Western Blue® substrate solution is highly recommended as being as sensitive as other NBT/BCIP formulations but producing a more uniform signal among slides and experiments. Alternative premade substrates for AP such as SIGMAFAST™ BCIP®/NBT (Sigma #B5655-5TAB) are also commercially available.
22. The incubation time can vary from a few hours to overnight. It is highly recommended to check the slides every hour and to stop the reaction when the desired signal is reached. During the NBT/BCIP staining procedure if some precipitate forms it is suggested to change the solution.

19.5 Interpretation and Conclusions

Cell death is an important process which together with cell division and differentiation shapes the body plan. The ability to localize and identify dying and/or dead cells is therefore crucial in many biological studies and staining techniques have

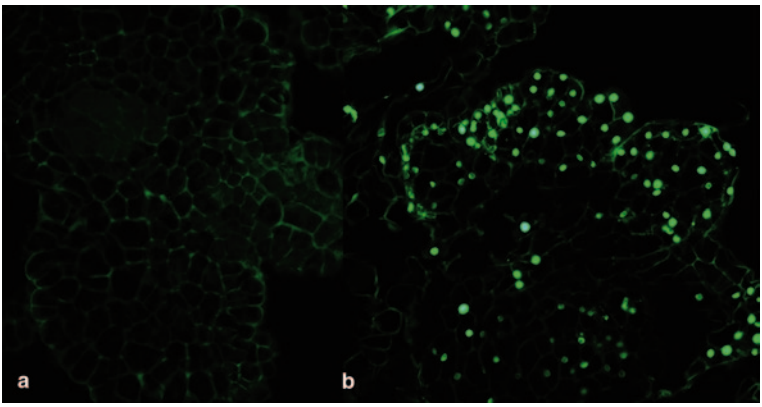

Fig. 19.1 Maize suspension control cells devoid of the TdT enzyme solution with no TUNEL signal in their nuclei (**a**) and cells showing many TUNEL-positive nuclei (**b**). Scale bar = 30 μm

emerged over the years. The most relevant factor when interpreting the results is establishing whether the observed death event is genetically programmed, or un-programmed. This protocol suggests the combined use of trypan blue staining prior to the TUNEL analysis (which is much more expensive and time-consuming) to determine the presence of dead cells in the tissue. Once death is observed using the staining method, the nature of the death (programmed or non-programmed) must be established using TUNEL, which is specific to PCD. In addition, before embarking in the TUNEL experiments, it might be worthwhile to perform an electrophoretic analysis of the DNA to verify the presence of the DNA fragmentation which is a characteristic of death in cells. It must be noted, however, that DNA fragmentation is visible on the gel only if there are a significant number of dying/dead cells within the tissue.

As with any protocol, the TUNEL assay must be optimized for any tissue, as well as for different developmental stages of the same tissue. Incubation periods, length of tissue permeabilization, and the choice between fluorometric and colorimetric detection must be carefully selected to prevent high background signals which can compromise the interpretation and validity of the results. Compared to nuclei of cells undergoing PCD, nuclei of control tissue should be devoid of any signal (Fig. 19.1). Clear results should be obtained for several sections before claiming the presence (or absence) of PCD in the tissue analyzed. Caution in the interpretation must also be applied when working with cells or tissues characterized by a high autofluorescence. In these cases the colorimetric detection or the choice of the right fluorophore is highly recommended.

References

1. Schwartzman RA, Cidlowski JA (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocr Rev 14:133–151

2. Peitsch MC, Polzar B, Stephan H, Crompton T, MacDonald HR, Mannherz HG, Tschop J (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J 12:371–377
3. Wijsman JH, Jonker RR, Keijzer R, van de Velde CJ, Cornelisse CJ, van Dierendonck JH (1993) A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. J Histochem Cytochem 41:7–12
4. Hashimoto S, Koji T, Niu JZ, Kanematsu T, Nakane PK (1995) Differential staining of DNA strand breaks in dying cells by nonradioactive in situ nick translation. Arch Histol Cytol 58:161–170
5. Gavrieli Y, Sherman Y, Bensasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
6. Gorczyca W, Gong JP, Darzynkiewicz Z (1993) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 53:1945–1951
7. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H (1994) Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest 71:219–225
8. Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong JP, Melamed MR, Darzynkiewicz Z (1993) Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. Leukemia 7:659–670
9. Wang H, Li J, Bostock RM, Gilchrist DG (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8:375–391
10. Ning SB, Wang L, Song YC (2002) Identification of programmed cell death in situ in individual plant cells *in vivo* using a chromosome preparation technique. J Exp Bot 53:651–658
11. Sloop GD, Roa JC, Delgado AG, Balart JT, Hines MO, Hill JM (1999) Histologic sectioning produces TUNEL reactivity—a potential cause of false-positive staining. Arch Pathol Lab Med 123:529–532
12. Kanoh M, Takemura G, Misao J, Hayakawa Y, Aoyama T, Nishigaki K, Noda T et al (1999) Significance of myocytes with positive DNA in situ nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy—not apoptosis but DNA repair. Circulation 99:2757–2764
13. Keogh RC, Deverall BJ, Mcleod S (1980) Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. Trans Br Mycol Soc 74:329–333
14. Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT (1999) The gain-of-function *Arabidopsis acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell 11:1695–1708
15. Hamberg M, Sanz A, Rodriguez MJ, Calvo AP, Castresana C (2003) Activation of the fatty acid alpha-dioxygenase pathway during bacterial infection of tobacco leaves—formation of oxylipins protecting against cell death. J Biol Chem 278:51796–51805
16. Gain P, Thuret G, Chiquet C, Dumollard JM, Mosnier JF, Burillon C, Delbosc B, Herve P, Campos L (2002) Value of two mortality assessment techniques for organ cultured corneal endothelium: trypan blue versus TUNEL technique. Br J Ophthalmol 86:306–310

Chapter 20
Laser Microdissection of Plant Tissues

Jenna L. Millar, Michael G. Becker and Mark F. Belmonte

20.1 Introduction

Access to uniform populations of plant cells and tissues represents a major challenge for researchers interested in understanding cell- and tissue-specific programing of biological processes. Biological processes are rarely carried out homogeneously within multicellular organisms and are often separated between cells, tissues, and organs in both space and time [1, 2]. Further, these biological processes can be controlled via biotic and abiotic cues and stresses [3]. Traditional methods of isolating cells and tissues of the plant require fine forceps. However, these methods often result in contamination from adjacent tissues thus biasing downstream interpretation of the data [4]. Laser microdissection (LMD) is one of the only tools providing researchers the accessibility of individual cells from heterogeneous tissues. This technique uses classic histological methods combined with an LMD system; it can be performed on most organisms and requires no a priori knowledge of the system.

To date, there are two commonly used histological methods for processing and sectioning plant material for LMD: tissue freezing for cryostat sectioning [5–7] and paraffin embedding [8–10]. Cryosectioning may be performed on fresh-frozen tissue, or tissue fixed and infused with a cryoprotectant, such as 10–15 % sucrose. Although excellent for preserving the molecular profile of cells, perturbations to cell morphology may make target cell and tissue identification difficult [10, 11]. Conversely, paraffin embedding of plant tissues allows for excellent retention of cellular morphology and high-quality nucleic acid integrity for directed quantitative PCR [12], microarray [7, 8], and RNA-sequencing strategies [9, 13].

When preparing plant tissues using the paraffin-embedding method, there are a number of steps that require attention during the preparation, fixation, and processing of each sample. Further, when designing a histological protocol to

M. F. Belmonte (✉) · J. L. Millar · M. G. Becker
Department of Biological Sciences, University of Manitoba,
50 Sifton Road, Winnipeg, Manitoba R3T 2N2, Canada
e-mail: mark.belmonte@umanitoba.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_20

prepare tissues for LMD, the downstream application must be considered as molecules of interest may be affected by many steps of the tissue processing protocol. The balance between cellular morphology and RNA integrity is generally achieved through modifications to solvents or their concentrations and/or through alterations to incubation times and temperature [14]. Below are a series of steps that can be used to prepare most plant tissues for LMD:

1. Sample collection: The organ of interest is collected from plants at a desired stage. Organs should be collected from a number of different plants to ensure there is sufficient biological diversity within the sample. Depending on the size of the organ of interest, tissues are hand dissected with a fresh RNase-free double-sided razor blade to make processing more manageable; this is described in more detail below.
2. Fixation: Fixatives are used to preserve the anatomical structure of the sample. This is imperative if specific tissues or cell types are to be obtained, since the anatomy must be distinguishable using general light microscopy (*see Note 1*). Commonly, fixatives for plant material include acetone, ethanol, ethanol–acetic acid, and methacarn as they preserve anatomical integrity and have much less interference with the extraction of target molecules [11]. Conversely, cross-linking fixatives, such as formaldehyde–acetic acid–ethanol, demonstrate improved anatomical preservation but hinder the subsequent recovery of RNA, DNA, or proteins [11, 15].
3. Dehydration: The fixed sample is dehydrated with a graded ethanol series to remove water from the sample. Since water is a major source of nucleic acid degradation [14], it is important that the material is thoroughly dehydrated.
4. Transitional infiltration: Xylenes are gradually infiltrated into the sample. This acts as a transitional solvent to enable infiltration of paraffin wax into the cells.
5. Paraffin infiltration: The sample is gradually infiltrated with heated paraffin. Since heated paraffin is a source of RNA degradation [16], the minimum time necessary for the cells to be sufficiently infiltrated should be used. The timing of this step is critical; if there is inadequate infiltration, the embedding and cutting stage will be difficult and in some cases impossible.
6. Embedding: The sample in molten paraffin is allowed to cool and solidify in a weigh boat. Careful and tedious work is necessary for alignment of tissues along the same plane. If this step is performed correctly during the sectioning stage when the target area is reached in one seed/leaf, it should be present in all other seeds/leaves maximizing the area of target tissue on each polyethylene naphthalate (PEN) membrane slide.
7. Blocking and sectioning: The sample is prepared for microtome sectioning by cutting excess paraffin from the processed material and mounting it on a sample holder (embedding ring). The sample is trimmed into a trapezoid shape on the embedding ring and sectioned at 5–10 μm with a rotary microtome. Cutting the sample into a trapezoid makes it easier to form ribbons during sectioning. Ribbons are consecutive sections that attach to each other during sectioning and allow the researcher to accurately target their cell or tissue of interest. It is much

easier to transfer, mount, and arrange ribbons rather than individual sections on the membrane slide.
8. De-waxing: Finally, paraffin is removed from the membrane slide using xylene baths, leaving only the sections of processed tissue on the slide. This allows for easier cutting during LMD, easier extraction of molecules, and increased clarity of the sections.

Most plant organs can be processed for LMD following the eight general steps listed above. However, depending on the cellular characteristics of the tissue, the protocol must be modified to accommodate different anatomical features. For example, soft, porous tissues of leaves are more easily infiltrated than thicker, more ridged cell walls of the seed coat. Thus, protocols must be modified for different organs and sometimes tissues within the same organ to optimize cellular integrity and improve RNA, DNA, or protein quality and yield. With downstream applications in mind, it cannot be stressed enough that clean, sterile, and nuclease-free techniques must be used throughout the entire process in order to obtain acceptable results. For example, all glassware should be baked at 180 °C for 24 h, all water should be DEPC treated, all workspace should be nuclease free, and all solution changes should be performed in a fume hood. In addition to the detailed procedures for seed and leaf tissue processing using the paraffin-embedding method for LMD, we also provide useful troubleshooting tips to help those interested in the processing of tissues for LMD.

20.2 Materials

All procedures in preparation for LMD, as well as all those after LMD, require an RNase-free workspace, equipment, and chemical reagents; this is accomplished by wiping counters and necessary equipment with a nuclease degrading solution (e.g., RNase AWAY spray), using nuclease-free or DEPC-treated water for all solutions, and baking all glassware at 180 °C for 24 h. In addition, while preparing for and/or performing LMD gloves should be worn at all times and should be periodically sprayed with RNase AWAY spray.

1. *Brassica napus* (canola) seeds and leaves (collected at specific developmental stages).
2. Fixative solutions: 100 % glacial acetic acid and 85 % ethanol.
3. Dehydration solutions: 70 %, 85 %, 95 % and 100 % ethanol.
4. Transitional infiltration solutions: 3:1 ethanol/xylenes, 1:1 ethanol/xylenes, 1:3 ethanol/xylenes, and 100 % xylenes.
5. Infiltration materials: 100 % xylenes, paraffin chips [Paraplast® Plus™ (McCormick Scientific)], and molten paraffin wax (60 °C).
6. Cutting and LMD solutions: Nuclease-free water and RNA lysis solution [RNAqueous ®—Micro kit (Ambion®; Life Technologies)].
7. Glassware: Beakers (250 mL), glass Pasteur pipettes, and glass vials (10 mL).

8. Consumable supplies: Disposable gloves, PEN membrane slides (Leica Microsystems), filter pipette tips (100 1000 μL), microcentrifuge tubes (0.5 mL), soft-bristle paintbrush, double-edged razor blades, RNase AWAY spray (Molecular BioProducts), plastic weigh boats, and embedding rings (available from Fisher Scientific or Thomas Scientific).
9. Equipment: Pipettes (100 and 1000 μL); refrigerator (4 °C); freezer (−80 °C); incubator or, more preferably, vacuum oven (42 and 60 °C); fume hood; ethanol burner; vacuum or vacuum oven (if possessed); Leica 2520 rotary microtome (or similar); and a Leica LMD 7000 system.

20.3 Methods

LMD tissue processing protocols have been developed and optimized for both seed and leaf tissues. The same general steps are used for both organs. However, time spent on each step varies and should be modified to accommodate your tissue of interest. Regardless of the tissue, the sensitivity of experiments involving RNA must not be overlooked; anything coming in contact with the sample must be RNase free to prevent RNA degradation from contaminating nucleases. If cleanliness and sterile techniques are neglected, days of tedious work, along with hundreds of dollars, can be completely wasted. To avoid this, remember that both prior to and during tissue processing, all equipment and workspace should be either baked at 180 °C for 24 h or wiped with RNase AWAY spray. Gloves should be worn at all times, replaced often, and periodically sprayed with RNase AWAY spray.

20.3.1 Five-Day Processing Protocol of Paraffin-Embedded Seed Tissues for Laser Microdissection

1. Day 1. Siliques (or ovaries) containing seeds are collected at the desired stage and placed immediately on ice. Depending on the developmental stage of the seeds, the seeds are either processed still attached to their siliques or dissected from their siliques so only the seeds are processed. Young seeds (ovule to 10 days after fertilization) are soft and fragile and therefore are processed attached to their siliques for support. The siliques are cut into 1 cm fragments with a fresh double-edged razor blade. Cuts can also be made to the silique wall (lengthwise and perpendicular to the replum) to prevent air bubbles from interfering with downstream processing steps (*see Note 2*). Older, more mature seeds, such as the mature green seeds, are removed from their siliques to make dehydration and infiltration easier. These mature seeds have thick seed coats and therefore are difficult to infiltrate. Thus, before the seeds are removed from the silique, a cut is made along the length of the silique, parallel to the replum, that removes some silique wall as well as some of the seed coat from the seeds inside. This

gives the molten paraffin easier access to seed tissues and helps with infiltration. After cuts to the seeds are made, using forceps, the silique wall is removed and seeds are removed from the replum by excising the funiculus from the replum (*see Note 2*).

2. Seeds are transferred to a glass vial containing 4 mL ice cold fixative solution (1:3 glacial acetic acid:85 % ethanol). The sample is fixed overnight at 4 °C on a rotary mixer if possible (*see Note 3*).
3. Day 2. The sample is gradually dehydrated with ethanol. All solution changes should be performed in a fume hood. The fixed sample is rinsed 3 × 5 min with 70 % ethanol. The solution is gradually replaced with a higher concentration of ethanol, starting with 85 %, then 95 %, and lastly, two changes of 100 % ethanol, with 30 min between each change. Samples should be kept at 4 °C at all times to minimize RNA degradation.
4. Xylene is used as a transition solvent and must be gradually infiltrated into the sample. The ethanol is first replaced with ice cold 3:1 ethanol/xylene, then ice cold 1:1 ethanol/xylene, ice cold 1:3 ethanol/xylene, and finally ice cold 100 % xylene, with an hour between each change. If necessary, these steps can be performed in a vacuum with the samples on ice to speed infiltration. The sample in 100 % xylene is then stored at 4 °C overnight.
5. Day 3. A second ice cold 100 % xylene change is performed and should only fill the vial halfway to avoid overspill. After 2 h, add six paraffin chips to the sample vial and continue to infiltrate at 4 °C for 2 h. Add additional 10 paraffin chips and store vial at 4 °C overnight.
6. Day 4. The sample is incubated at 42 °C for 1.5 h, followed by incubation at 60 °C for 30 min. Performing the incubation in a vacuum oven will speed up infiltration (*see Note 4*) but if not available, a non-vacuum oven will suffice. During the incubation, fumes can build up in the vial and result in an explosion. To prevent the buildup of pressure in the vial, unscrew the lid and loosely place it on the top of the vial. Inadequate infiltration of paraffin can cause problems in the subsequent microtome steps and should be performed thoughtfully and thoroughly.
7. After the 30 min incubation, the xylene/paraffin solution is replaced with 100 % molten paraffin for 1 h. An additional paraffin change is completed and the sample is incubated overnight at 60 °C. Paraffin changes must be performed quickly to avoid solidification of the wax. Repeated solidification and melting of the paraffin can compromise the cellular integrity of the sample making identification of target cells or tissues difficult.
8. Day 5. At least three changes of molten paraffin are made at 1 h intervals between each change before the sample is embedded.

This 5-day tissue processing protocol for seeds can be modified for younger seed stages (ovule to heart) that do not require as much time for paraffin infiltration. Reducing the days and hours in heated paraffin can enhance RNA quality and thus should be reduced whenever possible (Fig. 20.1). The only difference between the 5-day protocol and the 4-day protocol is that the last day is removed and the three

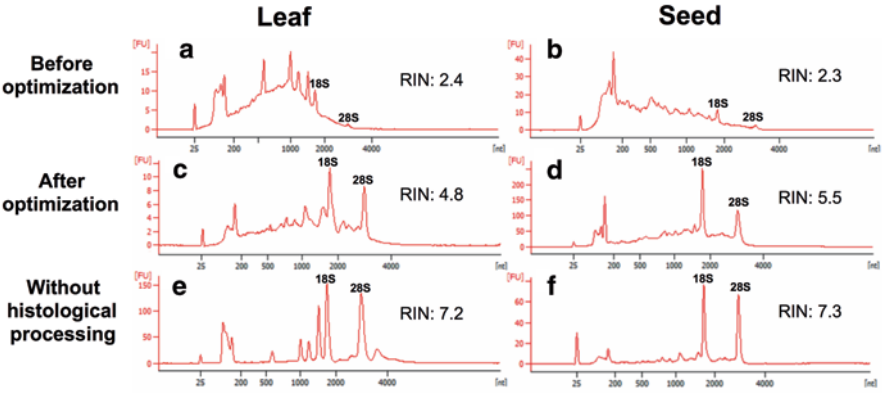

Fig. 20.1 Electropherograms produced using an Agilent 2100 Bioanalyzer, showing RNA quality of paraffin-embedded *Brassica napus* seed and *leaf tissues before and after optimization* of tissue processing. **a, b** RNA isolated from *leaf* and *seed* tissue subjected to a 5-day tissue processing protocol shows high degradation of RNA, represented by the very low numbers of 18 S and 28 S ribosomal transcripts. **c, d** RNA isolated from *leaf* and *seed* tissue subjected to a shorter, 4-day tissue processing protocol demonstrates superior RNA quality, represented by the noticeable peaks of the 18 S and 28 S ribosomal transcripts. **e, f** High-quality RNA isolated from whole, fresh-frozen leaf and seed tissue *without histological processing*

100 % paraffin changes and sample embedding take place on the fourth day of tissue processing.

Refer to section "Paraffin embedding, blocking, and sectioning tissues for laser microdissection" for embedding and sectioning for laser microdissection.

20.3.2 Four-Day Processing Protocol of Paraffin-Embedded Leaf Cross-Sections for Laser Microdissection

Reminder: As with the seed processing protocol, all workspace and equipment must be RNase free (refer to the protocol section for necessary cleaning and treatment of equipment and workspace). *Notes 1, 3–6* are applicable to this protocol and should be reviewed before the start of tissue processing.

1. Day 1. Collect leaves at desired stage of development. Using a razor blade cut 1 cm strips about 3 mm in width and immediately fix in 4 mL ice cold fixative solution containing 1:3 glacial acetic acid:85 % ethanol. Fix samples at 4 °C overnight.
2. Day 2. Dehydrate samples in a graded ethanol series. First, wash samples 3 × 5 min in 70 % ethanol. Dehydrate samples 20 min each in 85 %, 95 %, and 2 × 100 % ethanol. Leaf tissues are much more porous than seed tissues and less time is required between changes. Additional washes of 100 % ethanol are suggested until the sample no longer contains chlorophyll.

3. Immediately following ethanol dehydration, the sample is gradually infiltrated with xylene for 1 h each starting with a 3:1 ethanol/xylene mix, followed by 1:1 ethanol/xylene, 1:3 ethanol/xylene, and finally 100 % xylene. The sample is stored at 4 °C overnight.
4. Day 3. The sample is washed with 100 % xylene for 2 h at 4 °C. A second xylene change is performed but should only fill the vial half way. Six paraffin chips are added to the sample and allowed to gradually dissolve for 3 h. Ten paraffin chips are added to the vial and allowed to infiltrate overnight at 4 °C.
5. Day 4. Incubate the sample at 42 °C for 1.5 h and then at 60 °C for 30 min. Gradually replace the solution with 100 % molten paraffin and keep the sample at 60 °C. At least three changes of 100 % molten paraffin should be made with an hour between each change before embedding. Paraffin changes should be performed quickly to avoid solidification and melting of the sample.

20.3.3 Paraffin Embedding, Blocking, and Sectioning Tissues for Laser Microdissection

1. Molten paraffin is poured into a plastic weigh boat. Swirl the sample around in the vial to mobilize tissues and pour into the weigh boat containing molten paraffin. Tissue organization and arrangement must be performed quickly since molten paraffin can solidify within minutes.
    a. Seeds in siliques are arranged with heated forceps into rectangles about 1.5 cm × 1 cm at the bottom of the boat (Fig. 20.2a). Siliques should be oriented with the replum perpendicular to the bottom of the weigh boat. This orientation will maximize the area of target tissue on the membrane slide (*see Note 5*).
    b. Seeds without siliques are arranged with the cut side face down on the bottom of the weigh boat in rectangles about 1.5 cm × 1 cm. Positioning the seeds face down will ensure all the seeds are aligned on the same plane.
    c. If interested in cross-sections, leaf strips should be oriented so the blade of the leaf is perpendicular to the bottom of the weigh boat. Leaf samples will sometimes float in the paraffin making aligning difficult. To overcome this, wait until the paraffin is just starting to solidify and has a gel-like consistency at the bottom of the boat. At this time, leaf samples can be placed into the solidifying paraffin and will stay in position. With this orientation, sectioning yields cross-sections of leaves, which is often the plane of section that is most easily interpreted.

Once the tissue is arranged, the paraffin is allowed to solidify and is stored in the refrigerator at 4 °C until the blocking stage (Fig. 20.2b).

2. Embedding rings are filled with molten paraffin and allowed to completely solidify for at least 1 h (Fig. 20.2b).
3. Excess paraffin is cut from the area containing the sample using an RNase-free razor blade or a heated metal spatula. A small piece of paraffin is cut and melted

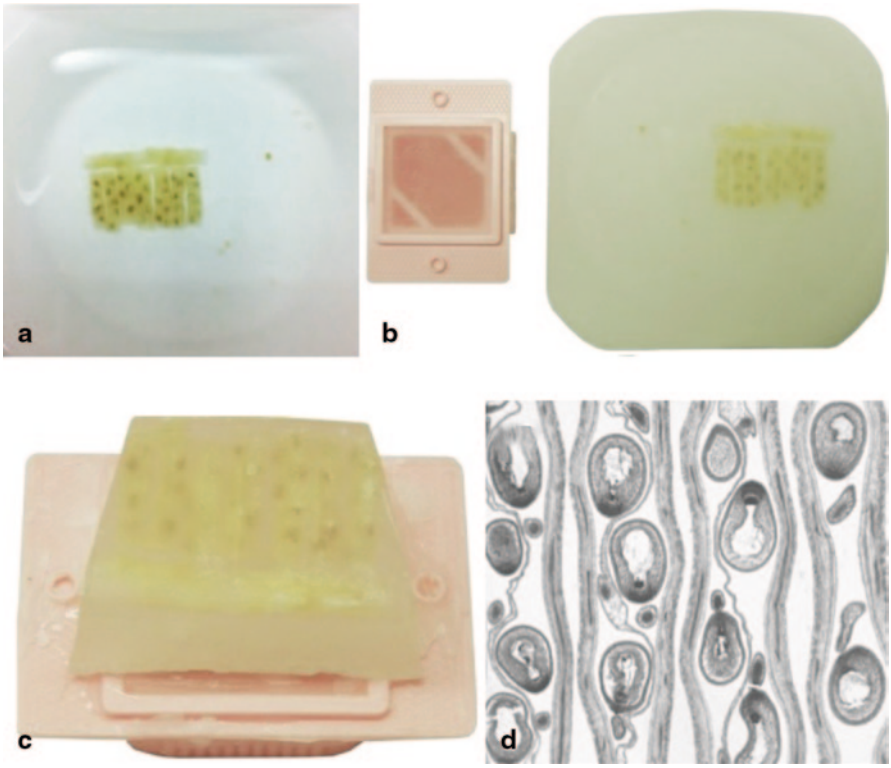

Fig. 20.2 Embedding and aligning seeds in paraffin wax for laser microdissection. **a** Siliques infiltrated with paraffin wax are poured into a weigh boat and aligned into a rectangle. **b** Wax is *left* to solidify and mounted on an embedding ring. **c** The block is shaped into a trapezoid in preparation for sectioning. **d** Unstained paraffin-embedded histological section of globular stage canola seeds

over an ethanol burner with a metal spatula. The molten paraffin is poured on the flat face of the embedding ring and the rectangle of paraffin containing the sample is placed on the flat face of the embedding ring and allowed to solidify (Fig. 20.2c). The metal spatula is then heated with the flame and is used to melt the edge of the block to the paraffin in the embedding ring.

4. The sample and embedding ring are then allowed to solidify at 4 °C for at least 1 h before trimming and sectioning.
5. Using a single-edged razor blade, the face of the rectangular sample is trimmed into a trapezoid (Fig. 20.2c). The top and bottom edges of the trapezoid should be parallel for successful ribbons and to maximize the surface area of the sample on the PEN membrane slide.
6. The sample is now ready to be cut into ribbons using the rotary microtome (Fig. 20.3a). Cutting the sample into ribbons makes the sections easier to transfer onto the PEN membrane slide. Sections should be cut between 5–10 µm thick (*see Note 6*). Using disposable glass microscope slides and a light microscope, during sectioning, sections are mounted and analyzed under the microscope until the desired area in the seed/leaf is reached.

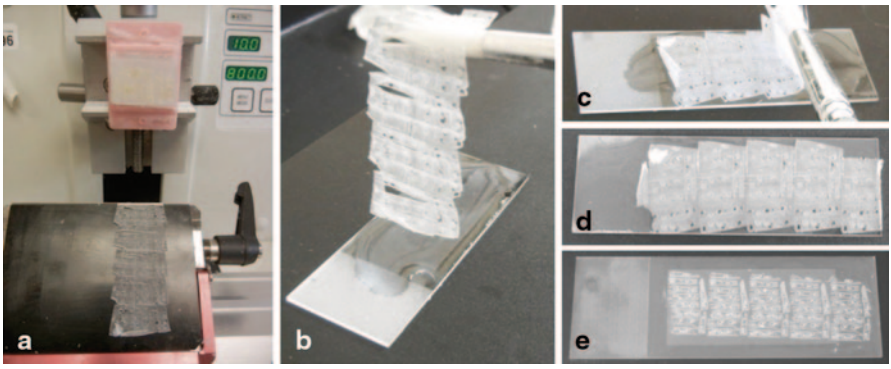

Fig. 20.3 Sectioning paraffin-embedded plant material for laser microdissection. **a** Paraffin-embedded sections cut 5–10 µm thick using a rotary microtome. **b, c** Ribbons transferred to a *PEN* membrane slide using a soft-bristle paintbrush. **d** Paraffin-embedded sections mounted on a *PEN* membrane slide. **e** Paraffin-embedded sections on a *PEN* membrane slide after being de-paraffinized using xylene baths

7. Place a PEN membrane slide on a clean surface at room temperature. Flood the slide with nuclease-free water. The ribbon with the target area of interest is then transferred from the microtome to the membrane slide using a soft-bristle paintbrush (Fig. 20.3b–d). The PEN membrane slide is delicate and should only be handled at the edges with care. Never touch the membrane (*see Note 7*).
8. Excess water can be removed from the slide using a delicate task wipe. This may cause shifting of the sections and should be performed carefully without touching the membrane. Dry the slide on a clean surface at room temperature for 1–2 h.
9. De-paraffinization of sectioned tissues in xylene baths. Fill two rectangular glass containers (about 10 cm long, 8 cm wide, and 7 cm deep) with 100% xylenes. Place the slides in a slide holder and submerge in xylene bath #1 for 30 s. Transfer the slide holder to xylene bath #2 for 30 s.
10. Allow the xylene to completely evaporate from the slides in a fume hood for at least 30 min. De-paraffinized tissues will appear white (Fig. 20.3e). The membrane slides are kept in an RNase-free slide box until LMD is performed.

20.3.4 Laser Microdissection of Paraffin-Embedded Plant Tissues

The tissue sample is now ready for LMD. For optimal RNA results, LMD should be performed within 2 weeks from the date of fixation. All microscope work should also be RNase free and thus all microscope parts and workspace should be sprayed with RNase AWAY spray.

1. Prior to LMD, a thorough examination of the anatomy of the sample is required to confidently isolate pure populations of target cells. Plastic embedded sections are an excellent starting point (e.g., *see* Chap. 4). After becoming familiar with the tissue of interest using plastic sections, it is easier to identify cellular features in paraffin-embedded sections with decreased resolution. Time should be spent establishing the criteria and cutting boundaries of your target area in paraffin before performing LMD. Figure 20.4 shows examples of plastic embedded tissue compared with paraffin-embedded tissue in seeds (Fig. 20.4a–c) and leaves (Fig. 20.4d–f).
2. Next, a laser strength that frees the dissected area yet limits the amount of burning on the slide is determined. If the laser strength is too high, the dissected section can burn to the slide, making it very difficult to free the section (Fig. 20.5). For paraffin-embedded seeds cut at 10 µm, optimal laser settings on the Leica LMD 7000 system are power = 45, aperture = 5, speed = 5, specimen balance = 24, head current = 70 %, and pulse frequency = 3773. For paraffin-embedded leaves cut at 7 µm, optimal laser settings on the Leica LMD 7000 system are power = 45,

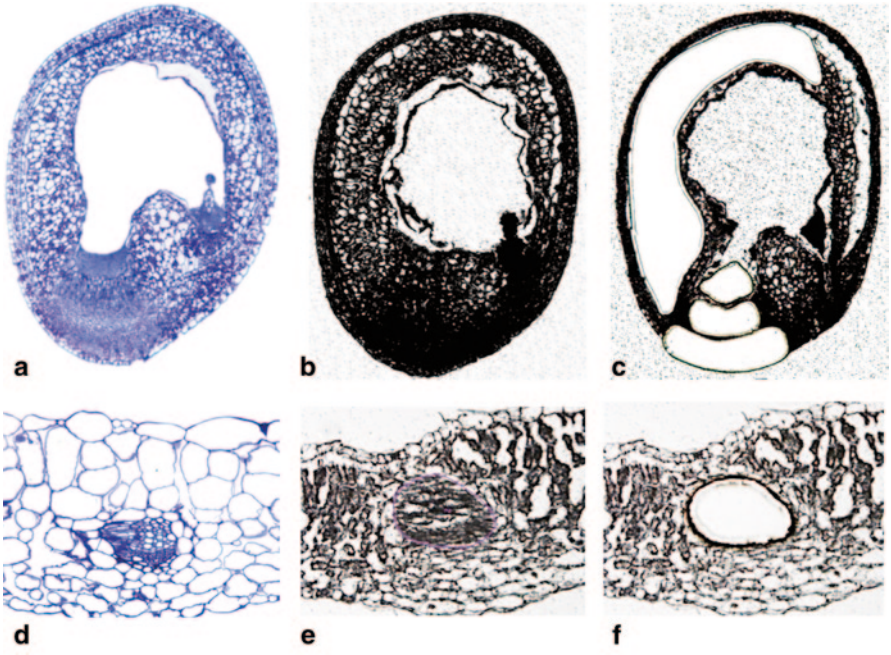

Fig. 20.4 Micrographs of plastic embedded seed (**a**) and leaf (**d**) tissues, and paraffin-embedded tissues before (**b** seed; **e** leaf) and after laser microdissection (**c** seed; **f** leaf). Plastic embedded tissues were fixed with a glutaraldehyde:paraformaldehyde solution in 1× phosphate-buffered saline. Plastic sections were cut 3 µm thick and stained with toluidine blue O and periodic acid Schiff's reagent; these sections show much higher clarity than the paraffin-embedded sections. The paraffin-embedded seeds were fixed with acetic acid:ethanol solution and were sectioned 10 µm thick

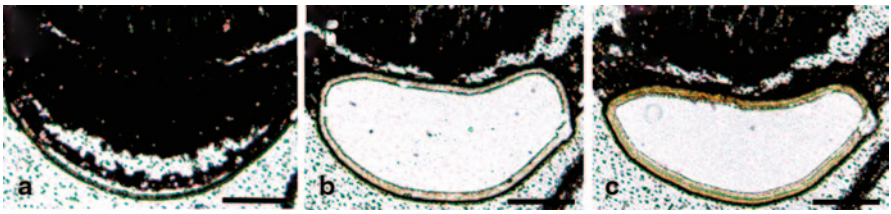

Fig. 20.5 Micrographs of paraffin-embedded seeds showing optimal laser intensity. **a** Paraffin-embedded seed sectioned at 10 µm cut with a low laser intensity (*head current* = 50; *laser power* = 45), resulting in a failed cut of the LMD element. **b** Paraffin-embedded seed sectioned at 10 µm cut with an optimal laser intensity (*head current* = 70; *laser power* = 45), releasing the LMD element. **c** Paraffin-embedded seed sectioned at 10 µm cut with a high laser intensity (head current = 85; laser power = 60), resulting in burning of the slide. Scale bars = 125 µm

aperture = 5, speed = 5, specimen balance = 24, head current = 60 %, and pulse frequency = 3773. On occasion, these parameters are modified for cutting different tissues and even for cutting different areas of the slide (if the membrane is touched, often a higher power is required); thus, although these settings are a good starting point, they may have to be adjusted depending on what is being dissected.

3. The membrane slide is positioned on the slide holder and inserted into the LMD system. For the Leica LMD 7000 system, the slide must be placed on the slide holder with the membrane facing down, with the label on the right-hand side.
4. Next, a collection tube (0.5 mL) is placed in the tube holder of the LMD system. A total of 30 µL of lysis buffer is pipetted into the cap of the collection tube. The tube holder is inserted into the LMD system. Lysis buffer generally evaporates after 1 h. If crystallization occurs, add 10 µL lysis buffer to resuspend the sample.
5. Tissues are dissected from the membrane (*see Note 8*). Folded areas should not be collected because of the risk of contamination from underlying (folded) cells. In addition, cells of interest should be undercut to prevent contamination from adjacent cells.
6. Following tissue collection, the cut LMD elements in RNA lysis solution are stored at −80 °C until the RNA is isolated.

20.3.5 RNA Isolation of Laser Microdissected Samples

RNAs from the laser microdissected samples are isolated using an RNAqueous®—Micro kit (Ambion® by Life Technologies) as per the manufacturer's instructions. An aliquot (4 µL) for each RNA sample should be set aside for analysis on the Agilent Bioanalyzer to test for RNA quality. RNA samples and aliquots are stored at −80 °C until further use.

20.4 Notes

1. Staining paraffin-embedded samples and autofluorescence. If LMD targets are difficult to identify, different histological stains may be used (with caution) to help distinguish the target area from adjacent tissues [17]. However, always test the effect of the stain on the quality of the RNA. Another alternative to histological stains is autofluorescence [18]. Autofluorescence is a quick and easy option that can be used to distinguish some cell types as well as cellular compounds. This requires a fluorescent filter, which can be attached as a module to the LMD system.
2. Preparing collected siliques and seeds for fixation. Young siliques that are processed whole can be cut along their length to prevent air bubbles from interfering with downstream processing steps. Because the canola silique is composed of two fused ovaries, two cuts should be made (avoiding cutting the replum) along the length of each ovary wall. Air bubbles cause siliques to float in molten paraffin and makes embedding and aligning of the tissue nearly impossible. For mature seeds, pick the seed off by the funiculus to avoid pinching the seed. Picking up the seed using fine forceps can damage cell and tissue layers thus resulting in histological artifacts.
3. Incubating at 4 °C. During all of tissue processing, when the sample is stored at 4 °C, it is sufficient to let it stand upright in the vial. If accessible, rotating the sample on a rotary mixer improves infiltration.
4. Using a vacuum oven. During fixation, dehydration, and transitional infiltration, the sample can be kept in a vacuum on ice to hasten infiltration. Once the sample is subjected to the later heating steps, it needs to remain at 60 °C to avoid solidification. If a vacuum oven is available, we advise that the remaining paraffin infiltration stages can be performed in the vacuum (heated to 60 °C) and will likely speed up infiltration thus shortening the time in wax.
5. Aligning tissues. Aligning tissues during embedding will reduce cost and save time. Having more tissue of interest on a membrane slide will reduce the number of membrane slides required and thus reduce costs. Organ fragments should be arranged in rectangles large enough to span the membrane on the PEN membrane slide (about 1.5 cm × 1 cm). If sections are cut at this size, only one ribbon will have to be mounted, saving time and effort during the transferring step, as well as maximizing the total surface area covered for each slide. In addition, if the individual leaves/seeds are embedded in the same plane in the block, each section should reveal the same area in each of the samples (Fig. 20.2d). Although packing in the maximum amount of tissue into the block has benefits, it can also make sectioning with the microtome difficult given the lack of paraffin to support the sample. Thus, if there is an issue with the sample falling out of the block or if sections are compressed and wrinkled, during embedding, evenly distribute the tissue and leave empty wax between the tissues.
6. Section thickness. If the target area is difficult to identify with thick section (10 μm), reducing the section thickness to 5 μm can help with the resolution of the section. Further, section thickness ranging from 3 to 10 μm has little effect

on the recovery of RNA from harvested cells [15]. However, it is important to note that thinner sections require the dissection of more target sample.

7. Damaged membrane slides. If the PEN membrane on the slide is damaged, water or xylenes can infiltrate between the slide and the membrane during the mounting and de-waxing of the sections. When laser microdissection is performed, areas with a damaged membrane are difficult to cut and often do not release from the slide. Always handle slides from their edges avoiding contact with the membrane.
8. Laser microdissection element shape and area. Whenever possible the largest area should be cut using the LMD system. Preliminary results have shown that LMD elements with larger areas (80,000 μm^2), and less cells subjected to the laser in proportion to the area, have higher RNA quality (RIN = 4.3) than RNA isolated from LMD elements of a smaller area (5000 μm^2) (RIN = 2.8). Further, shapes that have the smallest perimeter with respect to the area of the shape (e.g., circle), will have the lowest number of cells ablated by the laser and should have higher RNA quality.

20.5 Interpretation and Conclusion

The protocols provided here are an effective means for the efficient generation of anatomically conserved and molecular-rich sections for LMD in canola leaves and seeds. Qualitative analysis with the Agilent 2100 Bioanalyzer confirmed an increase in RNA quality (Fig. 20.1) with optimized protocols. Sample quality achieved through these protocols is sufficient for downstream applications such as quantitative real-time PCR and next generation sequencing. Although these protocols are optimized specifically for *B. napus* seeds and leaves they provide a template for the establishment of processing protocols for similar organisms and tissues. Collectively, LMD serves as a valuable tool for plant biologists interested in understanding the cellular and molecular mechanisms underlying biological processes separated in both space and time.

Acknowledgments This work was supported through the National Science and Engineering Research Council Discovery Grants program to M.F.B. and through NSERC postgraduate fellowships to J.L.M. and M.G.B.

References

1. Brady SM, Orlando DA, Lee JY et al (2007) A high-resolution root spatiotemporal map reveals dominant expression patterns. Science 318:801–806
2. Du TG, Schmid M, Jansen RP (2007) Why cells move messages: the biological functions of mRNA localization. Semin Cell Dev Biol 18:171–177
3. Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63:3523–3542

4. Hsieh T-FF, Shin J, Uzawa R et al (2011) Regulation of imprinted gene expression in *Arabidopsis* endosperm. PNAS 108:1755–1762
5. Abbott E, Hall D, Hamberger B, Bohlmann J (2010) Laser microdissection of conifer stem tissues: isolation and analysis of high quality RNA, terpene synthase enzyme activity and terpenoid metabolites from resin ducts and cambial zone tissue of white spruce (*Picea glauca*). BMC Plant Biol 10:106. doi:10.1186/1471-2229-10-106
6. Burgemeister R, Gangnus R, Haar B et al (2003) High quality RNA retrieved from samples obtained by using LMPC (laser microdissection and pressure catapulting) technology. Path Res Pract 199:431–436
7. Spencer MWB, Casson SA, Lindsey K (2007) Transcriptional profiling of the *Arabidopsis* embryo. Plant Physiol 143:924–940
8. Belmonte MF, Kirkbride RC, Stone SL et al (2013) Comprehensive developmental profiles of gene activity in regions and subregions of the *Arabidopsis* seed. PNAS 110:E435–E444
9. Osaka M, Matsuda T, Sakazono S et al (2013) Cell type-specific transcriptome of Brassicaceae stigmatic papilla cells from a combination of laser microdissection and RNA sequencing. Plant Cell Physiol 54:1894–1906
10. Takahashi H, Kamakura H, Sato Y et al (2010) A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J Plant Res 123:807–813
11. Nelson T, Tausta SL, Gandotra N et al (2006) Laser microdissection of plant tissue: what you see is what you get. Ann Rev Plant Biol 57:181–201
12. Chandran D, Inada N, Hather G et al (2010) Laser microdissection of *Arabidopsis* cells at the powdery mildew infection site reveals site-specific processes and regulators. PNAS 107:460–465
13. Schmid MW, Schmidt A, Klostermeier UC et al (2012) A powerful method for transcriptional profiling of specific cell types in eukaryotes: laser-assisted microdissection and RNA sequencing. PloS ONE 7:e29685
14. Ludwig Y, Hochholdinger F (2013) Laser microdissection of plant cells. In: Žárský V, Cvrčková F (eds) Plant cell morphogenesis, vol 1080. Humana Press, Totowa NJ, pp 249–258
15. Kerk NM, Ceserani T, Tausta SL et al (2003) Laser capture microdissection of cells from plant tissues. Plant Physiol 132:27–35
16. Evers DL, He J, Kim YH et al (2011) Paraffin embedding contributes to RNA aggregation, reduced RNA yield, and low RNA quality. J Mol Diagn 13:687–694
17. Fang J, Schneider B (2013) Laser microdissection: a sample preparation technique for plant micrometabolic profiling. Phytochem Anal 25:307–313
18. Liang Z, Sham T, Yang G et al (2013) Profiling of secondary metabolites in tissues from *Rheum palmatum L*. using laser microdissection and liquid chromatography mass spectrometry. Anal Bioanal Chem 405:4199–4212

Chapter 21
RNA In situ Hybridization

Shalini Mukherjee, Claudio Stasolla and Belay T. Ayele

21.1 Introduction

Information about the spatiotemporal distribution of transcripts is an essential requirement for the molecular characterization of a single gene, or a set of genes participating in specific pathways. Over the past few years, several methods have been developed and optimized to quantify the level of gene transcripts. While some of these techniques are routinely used for single genes, such as reverse transcription-polymerase chain reaction (RT-PCR), nuclease protection assays (NPA), Northern blot analysis, and more recently quantitative (q) RT-PCRs, other methods, such as cDNA microarray analyses and whole transcriptome shotgun sequencing, are employed for obtaining information on global changes in transcript levels. As the starting material for all these techniques are tissue extracts, the cellular relationships and resolution are lost and the results obtained refer to the average of the whole tissue, which in many instances is composed of a highly heterogeneous population of cells. Initially described by Pardue and Gall [1] for the localization of DNA:RNA hybrids in several cytological preparations, the RNA hybridization technique is based on the assumption that, if adequately preserved, nucleic acids can be detected directly in histologic specimens through the hybridization of a complementary strand of labeled nucleic acid. Different from the quantitative techniques described above, RNA in situ hybridization maintains cellular relationships and allows for the sensitive detection of transcripts in specific cells within tissues and organs. The advantage of localizing mRNAs in a cellular environment makes this a handy tool for studying gene expression in a specific time-dependent study, such as during a particular developmental period, or after specific treatments [2].

Soon after its inception, the RNA in situ hybridization technique was applied mainly in animal tissues for the detection of transcripts encoding globin [3], viruses

C. Stasolla (✉) · S. Mukherjee · B. T. Ayele
Department of Plant Science, University of Manitoba,
222 Agriculture Bld., Winnipeg, Manitoba R3T 2N2, Canada
e-mail: Claudio.Stasolla@umanitoba.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_21

[4], hormones [5], and prolactin [6]. All these studies employed radioisotopes as labels because of the elevated sensitivity, which allowed for the detection of low-abundance transcripts with a detection limit of five molecules/cell [7]. Because of safety concerns, and other limitations including long exposure times and low resolution [8, 9], radioisotopes were soon abandoned and replaced with nonradioactive methods, where some of them employed biotin which could be easily incorporated into nucleotides and detected with an avidin system [10]. However, compared to the previously used radioactive method, the biotin–avidin system is less sensitive and can only be used for the detection of highly abundant transcripts. A significant improvement to the technique was brought about by the introduction and utilization of digoxigenin (DIG) in 1987, a plant steroid which can be linked to nucleic acids and easily detected by anti-DIG antibodies conjugated with a variety of enzymes, including alkaline phosphatases and peroxidases [11]. Compared to biotin, DIG increased the detection sensitivity and has now become the label of choice. DIG ribo-probes are effective for moderately to strongly expressed genes [12]. Table 21.1 summarizes the different kinds of labeled probes used for in situ hybridization in the past.

The RNA in situ hybridization technique was developed before the refinement of molecular cloning techniques and, therefore, the initial probes were double-stranded DNA molecules obtained by simple digestions [20]. The DNA probes were first denatured with high temperatures or in basic solutions, and subsequently applied to the tissue. While relatively easy to perform, this protocol is not very sensitive, as the chances for the two strands of the probes to reanneal prior to hybridizing to the endogenous transcripts are high. This problem can be overcome with the use of single-stranded oligonucleotide probes obtained either from templates generated by PCR reaction or from plasmids (Fig. 21.1). Templates generated by PCR reactions are amplified using primers containing adaptors for RNA polymerase binding sites, which are then utilized as starting docks for in vitro transcription reactions containing labeled (DIG-labeled in the case of Fig. 21.1a) nucleotides and generating sense and anti-

Table 21.1 Examples of different probes used for in situ hybridization in animals and plants

Kind of probe	Label on probe	Reference	Tissue material
DNA	Radioactive labeled with thymidine-^{3}H	[1]	Oocytes of the toad, *Xenopus laevis*
DNA	Biotinyl-dUTP derivative	[13]	Chicken muscle tissue
Viral DNA	Biotin-labeled analogs of TTP (biotin-labeled dUTP)	[14]	Guinea pig embryo cell cultures
cDNA	^{32}P-labeled cDNA	[15]	Rat neuron cells
cDNA	^{3}H-labeled deoxycytidine triphosphate and thymidine triphosphate	[16]	Rat pituitary tumor
RNA (ribo-probe)	Digoxigenin (DIG)	[17]	Rat pituitary cells
RNA	DIG	[8]	Liver of adult male Sprague-Dawley rats
cDNA	^{33}P-labeled dCTP	[18]	Barley developing seeds
RNA	DIG	[19]	Cell aggregates and single cells of Norway spruce

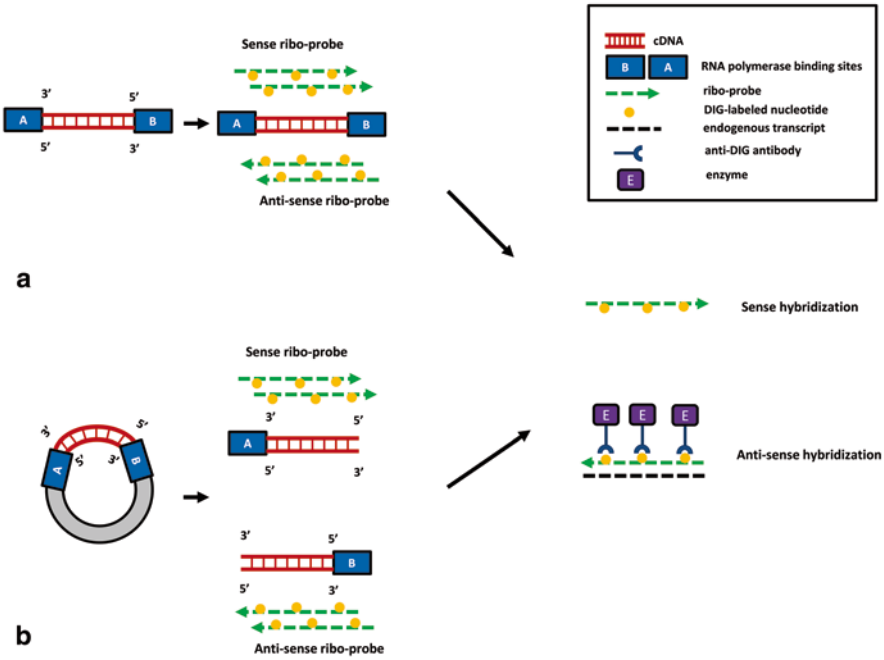

Fig. 21.1 Generation of probes using a PCR-produced template (**a**), and from plasmid (**b**)

sense ribo-probes (Fig. 21.1a). Alternatively, the cDNA of interest can be ligated into a plasmid already containing the RNA polymerase binding sites. Restriction enzymes cut and linearize the cDNA fragments linked to the respective RNA polymerase binding sites, which are then used for RNA transcription reactions (Fig. 21.1b). Labeled single-stranded ribo-probes obtained from either method are ready to be applied onto the tissue and only the antisense probe should hybridize to the endogenous transcripts (Fig. 21.1). The tissue is incubated first with enzyme-linked antibodies binding to the labeled nucleotides and then with the suitable substrate which, after reacting with the enzyme, generates a "visible" signal. No hybridization should occur in the sense hybridization, which is often used as a negative control.

21.2 Basic Steps of RNA In situ Hybridization

The RNA in situ hybridization procedure can be quite tedious and time-consuming. To simplify for the reader, this section analyzes three basic steps: tissue processing, hybridization, and detection. This preliminary information will be accompanied by a more in-depth description in the Methods section.

The selected protocol presented in this chapter uses a DIG-labeling system which we have been using successfully over the past 10 years on a variety of probes, some of which detect a very specific and low-abundant target genes. As reminded

previously, caution must be exercised when reproducing this protocol as optimizations of one or more steps are often needed to enhance the positive signal and reduce the background noise. Critical steps of the protocols are accompanied by Notes, which might facilitate the execution of the step or propose alternative methods.

21.2.1 Tissue Processing

The main purpose of tissue processing is the retention of structural integrity while exposing the endogenous transcript pool to the probes. This fine balance can be achieved with the judicious choice of chemicals and tissue manipulations which must be empirically determined. The choice of the fixative is paramount. Unlike early studies using frozen tissue and producing many artifacts [7], the use of the right fixative on fresh tissue can minimize cellular degradation. While alcohol-based fixatives cause precipitation of cellular components and poor tissue preservation, aldehydes seem to best preserve the tissue (*see* Chap. 2). Because of their ability to cross-link proteins, aldehydes are effective in immobilizing and "fixing" nucleic acids, which in a cellular environment are encased and surrounded by proteins. A varying degree of fixation is found among aldehydes. Although used in some studies, glutaraldehyde is a very strong fixative, effective in preserving the structural integrity of the specimen, but less suitable for target availability. This problem can be overcome with subsequent digestions of the tissue using proteolytic enzymes, as discussed below. Optimal combinations between tissue preservation and exposure of endogenous transcripts are achieved using paraformaldehyde, the fixative of choice in the protocol outlined in this chapter. An alternative fixative is formalin, which at a suitable concentration (usually 10%) can produce similar results as paraformaldehyde [7]. Despite the choice of the fixative, a crucial step is the quick fixation of the tissue, as the RNA pool is labile and subject to rapid degradation. This is especially important for low-abundance transcripts.

Exposure of the target transcripts can be facilitated by conducting a rapid proteolytic digestion of the tissue prior to hybridization. This step, highly recommended when aldehydes are used as fixatives, can make use of different proteolytic enzymes, including pronase and proteinase K. In our hands both enzymes are highly effective, and their concentration and duration of incubation need to be carefully optimized. While for abundant transcripts longer digestions with a high concentration of enzyme are often acceptable, over-digestion for less abundant targets can result in complete target loss. It is therefore imperative to carefully evaluate the digestion parameters depending upon the type and nature of transcript to be localized.

21.2.2 Hybridization

This step can be considered as the "black box" of the whole RNA in situ hybridization procedure, as the chemistry of probe–target hybridization is unknown.

According to Brahic and Haase [4], hybridization on a tissue has a melting temperature of 5 °C lower and a rate of hybridization of 10 % slower than a hybridization occurring in solution. Significant variations in these values further occur depending on the nature of the tissue where the hybridization takes place.

For proper hybridization to occur, the antisense ribo-probe must "find" the complementary endogenous transcript and bind to it through formation of hydrogen bonds: three bonds between guanine and cytosine, and two between adenine and thymidine. The stability of the duplex must remain unaltered after all the washes to which the tissue is subjected during the procedure aimed at removing unpaired probes. According to Brown [7], several factors affect the stability of the duplex and must therefore be considered to optimize the procedure. These include the hybridization temperature, the availability of cations in the hybridization solution, and the length of the probe. The choice of the right temperature is important for ensuring only complementary strands hybridize successfully. Lower-than-optimal temperatures can result in duplex formation between non-fully complementary strands, leading to increased nonspecific signal, while higher-than-optimal temperatures might preclude formation of duplexes between complementary strands causing a loss of signal. As a general rule, hybridization should be carried out at a temperature 25 °C lower than the T_m, where T_m is the melting temperature required to achieve 50 % hybridization between two single strands.

Stability of double-stranded nucleic acid molecules is highly affected by the presence of monovalent cations, which, due to their positive charges, decrease the negative repulsion exercised by the phosphate groups between the two strands. Absence or low concentrations of cations in the hybridization buffer reduces the formation of duplexes.

The length of the probe also plays an important role and should be optimized for enhancing the signal. Although longer probes tend to produce a stronger signal because of the presence of a large number of labeled nucleotides, they might not penetrate the tissue completely. This is in contrast to short probes, containing few labeled nucleotides but able to penetrate the tissue effectively. As a general rule, probes between 15 and 500 bases in length should be used, although longer probes have provided us with a satisfactory signal. The length of the probe is often dictated by the type and nature of the gene, especially in the events where the target transcripts are encoded by a member of a gene family, or by genes containing domains conserved among other genes. The probe should be designed to hybridize only with the target of interest.

21.2.3 Detection

The detection method is totally dependent upon the choice of the label. As shown in Fig. 21.1, detection of DIG-labeled nucleotides is achieved by incubating the tissue with enzyme-linked anti-DIG antibodies. The enzymes utilized can be alkaline phosphatases or peroxidases, although the former has higher sensitivity and

produces a stronger signal. The signal is produced by a colorimetric system which employs 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT). The precipitate formed by the reaction of the substrate with the enzyme is dark and easily identifiable. Like other detection procedures, the strength of the signal depends on the abundance of the target nucleic acid and the nature (length) and strength (level of labeling) of the probe. Incubation time with the substrates should be optimized.

21.3 Materials

21.3.1 Synthesis of RNA Probes

1. DNA fragment of interest (template)—generated as shown in Fig. 21.1.
2. pGEM-T-Easy vector (Promega #A1360).
3. 10X Transcription buffer (Roche #11465384001).
4. 10X DIG RNA labeling mix (Roche #11277073910).
5. RNA polymerase—SP6 (Roche #10810274001) or T7 (Roche #10881767001) as required for sense and antisense strands.
6. RNaseOut ribonuclease inhibitor (Life Technologies #10777-019).
7. Diethylpyrocarbonate (DEPC)-treated water.
8. TMS buffer (50 mM Tris-HCl, pH 7.8; 100 mM NaCl; 10 mM $MgCl_2$).
9. tRNA (100 mg/mL) (Roche #10109495001).
10. DNase I, RNase-free (Roche #04716728001).
11. Carbonate buffer (0.2 M sodium bicarbonate, pH 10.2).
12. Sodium acetate (3 M, pH 5.5).
13. Absolute (100 %) ethanol.
14. Agarose.
15. 37 °C incubator.
16. Dry bath at 60 °C.
17. Blotting membrane (Zeta-Probe, Bio-Rad #162-0165).
18. Buffer B1 (100 mM Tris-HCl, pH 7.5; 150 mM NaCl).
19. Buffer B1 with Triton X-100 (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.3 % Triton X-100).
20. Buffer B2 (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 10 mM $MgCl_2$).
21. Anti-DIG-AP Fab fragments (Roche #11093274910).
22. Blocking reagent (Roche #11096176001).
23. Western Blue® stabilized substrate for alkaline phosphatases (Promega #S3841).
24. Rocking platform.
25. Tabletop centrifuge.
26. Electrophoresis unit.
27. UV cross-linker.

21.3.2 Tissue Fixation in Paraformaldehyde

1. Plant tissue.
2. Small glass vials.
3. Glass beaker.
4. Magnetic stirrer with heater.
5. 4% paraformaldehyde.
6. Phosphate-buffered saline (PBS) (0.13 M NaCl; 0.27 mM KCl; 7 mM Na_2HPO_4; 3 mM KH_2PO_4; pH 7.2).
7. Vacuum oven chamber.
8. Tissue rotator (PELCO® R2 Rotator #1050).
9. Rotator head (PELCO® #1054 for 4 mL vials).
10. Pipette.
11. RNaseZap® (Life Technologies #AM9780) (optional).

21.3.3 Tissue Dehydration, Embedding, and Block Preparation

1. PBS (0.13 M NaCl; 0.27 mM KCl; 7 mM Na_2HPO_4; 3 mM KH_2PO_4; pH 7.2).
2. Ethanol (95 and 100%).
3. DEPC-treated water.
4. Xylene.
5. Tissue rotator (PELCO® R2 Rotator #1050).
6. Rotator head (PELCO® #1054 for 4 mL vials).
7. Paraplast® Plus Tissue Embedding Medium (Fisher Scientific #23-021-400).
8. Forceps.
9. Small spatula.
10. Peel-A-Way® Disposable Histology Molds (Fisher Scientific #18-41).
11. Embedding rings (Fisher Scientific #22-038-197).

21.3.4 Tissue Sectioning

1. Paraplast® Plus Tissue Embedding Medium (Fisher Scientific #23-021-400).
2. Slide warmer.
3. Microscope slides.
   a) Uncoated slides for general histology: Plain Glass Microscope Slides (Fisherbrand® #12-550-A3).
   b) Slides with adherent surface: Superfrost® Plus Microscope Slides (Fisherbrand® 12-555-15).

21.3.5 Tissue De-waxing

1. Ethanol (95 and 100 %).
2. Coplin jars (Fisher Scientific #S17495).
3. Xylene.
4. PBS.
5. Hydrochloric acid (HCl).
6. DEPC-treated water.

21.3.6 Pre-hybridization

1. Pronase (20 mg/mL) kept at −20 °C (Roche #10165921001).
2. Pronase buffer (100 mM Tris-HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid (EDTA)).
3. Glycine (also known as pronase blocking solution) 2 mg/mL prepared in 1X PBS.
4. Acetic anhydride.
5. Triethanolamine.
6. Paraformaldehyde.

21.3.7 Hybridization

1. 10X salt stock (3 M NaCl; 0.1 M Tris-HCl, pH 7.0; 50 mM EDTA; 0.1 M Na-phosphate buffer, pH 6.8; DEPC water).
2. Deionized formamide.
3. tRNA (100 mg/mL) (Roche #10109495001).
4. Dextran sulfate (0.5 g/mL) (Phytotechnology Lab #D308).
5. Denhart's solution (Amresco #E 717).
6. Hybridization solution (600 µL 10X salt stock; 2400 µL deionized formamide; 1200 µL dextran sulfate; 420 µL DEPC water; 60 µL tRNA; 120 µL Denhart's solution). Warm the dextran sulfate to 65 °C prior to pipetting.
7. NTE buffer (500 mM NaCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA).
8. 20X SSC (3 M NaCl; 0.3 M Na-citrate; volume made up to 1 L with DEPC water and adjust pH to 7.0. Prepare 2X and 0.1X SSC by dilution from 20X stock fresh on the day of use).
9. Buffer B1 (100 mM Tris-HCl, pH 7.5; 150 mM NaCl).
10. Buffer B1 with Triton X-100 (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.3 % Triton X-100).
11. Buffer B2 (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 10 mM $MgCl_2$).
12. Blocking solution: 0.5 g blocking reagent (Roche #11096176001) in 100 mL Buffer B1.

13. Blocking–washing buffer: 1 g bovine serum albumin (BSA) in 100 mL Buffer B1 with Triton X-100. To be chilled on ice until BSA dissolves. Do not shake or agitate.
14. Antibody solution: 2 μL anti-DIG-AP Fab fragments in 2 mL blocking–washing buffer.

21.4 Methods

21.4.1 Creating an RNase-free Environment

Before initiating the procedure it is imperative to work in an RNase-free environment. This can be ensured by dedicating a separate bench area for RNA in situ hybridization work and keeping it clean. Glassware and metals should be baked at 180 °C overnight (at least 16 h) to remove RNases. Plasticware may be cleaned with RNaseZap® or by soaking in 1 M NaOH for at least 30 min and then washed thoroughly with DEPC-treated water *(see Note 1)* to remove the NaOH. All reagents should be prepared by treating them with 0.1 % DEPC. Tris buffer reacts with DEPC and cannot be treated with DEPC; dissolve the desired amount of Tris in 0.1 % DEPC-treated water.

The solutions/reagents that need to be stored at −20 °C should be aliquoted in small volumes and discarded after each use. All purchased plasticware, that is Eppendorf tubes, PCR tubes, and pipette tips, should be nuclease-free. The pipettors should be cleaned well with RNaseZap and care must be taken while pipetting to avoid cross-contamination and the undesirable introduction of RNase. If possible, dedicate a set of pipettors for the procedure. Gloves should be worn at all times and changed frequently. Precautions should be taken in avoiding touching any surface that may be contaminated with RNases (e.g., face, hair, door handles, switches, and refrigerator handles).

21.4.2 Synthesis of the Unhydrolyzed Probe

1. Prepare the cDNA template for in vivo transcription *(see Note 2)*. This can be done by direct PCR (Fig. 21.1a) or by cloning the desired fragment into a plasmid, followed by restriction analysis (Fig. 21.1b). Our plasmid of choice is pGEM-T-Easy vector.
2. Verify the integrity of the cDNA fragment by agarose gel (0.8−2 % depending on the size of the expected band) and purify the band using any commercially available gel purifying kit (e.g., Qiagen #28704).
3. After gel purification, run about 2 μL of the purified product on a 1 % agarose gel to ensure the presence of the fragment.

4. The following day before starting the transcription reaction, turn on the incubator and set the temperature to 37 °C.
5. Prepare two different reactions in two Eppendorf tubes (placed on ice). Add 1 μg of template cDNA, 2.5 μL of 10X transcription buffer, 2.5 μL of DIG RNA labeling mix, 1 μL of RNaseOUT ribonuclease inhibitor, and 1 μL of the appropriate RNA polymerase (T7 in one tube and SP6 in the other and label them as the sense or antisense probe depending on the orientation of the transcript). Add nuclease-free water to bring the final volume to 25 μL.
6. Incubate the tubes at 37 °C for at least 2 h.
7. Stop the reaction by adding 75 μL of TMS buffer, 2 μL of tRNA, 2 μL of DNase I RNase-free. Mix well by gently tapping the tube and spin down the reaction mixture for a few seconds. Incubate at 37 °C for 15 min.
8. Remove 1 μL of each probe and store in a new tube. This unhydrolyzed probe will be run on a gel to compare with the hydrolyzed probe.
9. Add 12 μL 3 M sodium acetate and 200 μL absolute alcohol to precipitate the RNA.
10. Incubate the tubes at −20 °C for at least 1 h *(see Note 3)*.
11. Centrifuge the tubes at 13,000 rpm at 4 °C for 15 min and wash the pellets with 70 % ethanol. Leave the tubes open on a clean workbench to air-dry the pellets. Once the pellet in each tube is fully dried, resuspend it in 52 μL of DEPC-treated water.
12. If your unhydrolyzed probe is of the desired length, then check its integrity on an agarose gel *(see Note 4)*, and assess the incorporation of DIG (*see* Sect. 21.4.4). If the probe needs to be hydrolyzed continue with Sect. 21.4.3.

21.4.3 Carbonate Probe Hydrolysis

1. Ensure a sample of unhydrolyzed probe has been set aside in a new tube. This will be run on a gel with the hydrolyzed probe (*see* Step 5).
2. To the 51 μL remaining in each tube, add an equal volume of carbonate buffer and incubate at 60 °C for the time calculated by the following equation:

$$\text{Time} = [(L_{\text{initial}} - L_{\text{final}})/K \times (L_{\text{initial}} \times L_{\text{final}})]$$

where L_{initial} is the initial length (in kb) of the RNA probe, L_{final} is the final desired probe length (in kb), and K is a constant = 0.11 kb/min).

3. After hydrolysis at 60 °C, place the tubes on ice and precipitate the RNA with 12 μL of 3 M sodium acetate and 200 μL absolute ethanol. Incubate the tubes at −20 °C for 1 h or overnight.
4. Remove the tubes from the −20 °C freezer, centrifuge them at 13,000 rpm at 4 °C for 15 min, and wash the pellets with 70 % ethanol. Leave the tubes open on a clean workbench and allow the pellets to air-dry. Once fully dried, resuspend the pellets in 52 μL of nuclease-free or DEPC-treated water. Remove 1 μL of hydrolyzed RNA from each tube and store in a new tube.

5. Run unhydrolyzed and hydrolyzed (Step 4) RNA probes on an agarose gel. The unhydrolyzed RNA should produce a clean band, while the hydrolyzed RNA a smear *(see Note 4)*.

21.4.4 Checking DIG Incorporation

The DIG labeling of the probes (hydrolyzed or unhydrolyzed) must be checked by dot blot using anti-DIG antibodies.

1. Spot 1 μL of the sense and antisense probe solutions onto a small piece of blotting membrane (Zeta-Probe, Bio-Rad) and label the location with pencil. Fix the DNA by UV cross-linking. Briefly wet the membrane in Buffer B1.
2. Incubate for 30 min in Buffer B1 containing 0.5 % blocking reagent on a rocking platform.
3. After a brief wash with Buffer B1, incubate the membrane with Buffer B1 containing anti-DIG antibody (1.5 μL in 150 μL) for 15 min at room temperature in the dark. Pipet the antibody solution onto the membrane.
4. Wash for 5 min in Buffer B1. Repeat the wash step.
5. Wash for 2 min in Buffer B2.
6. Add Western Blue® substrate solution to cover the membrane (0.8 mL) and incubate in the dark. Allow color to develop for 45 min, wash the membrane with water to stop color development, and observe the intensity of the spots. For precise quantifications, RNA controls of known concentrations can also be used.
7. Store the DIG-labeled RNA probes at −80 °C.

21.4.5 Tissue Fixation

1. Label clean RNase-free glass vials and cover the label with transparent tape as the label might wash away by the organic solvents used in subsequent steps.
2. Prepare 500 mL of 4 % paraformaldehyde in PBS buffer pH 7.2. Warm 400 mL of the buffer at 60 °C on a hot plate and add 16 g of paraformaldehyde *(see Notes 5 and 6)*. Using a magnetic stirrer, stir it for about 15 min to completely dissolve the paraformaldehyde powder in the buffer. Bring the volume to 500 mL with PBS buffer and let the solution cool down. The solution should be chilled in the fridge before proceeding to Step 3.
3. Cut the tissue using a sharp razor and place it in a cold fixative that was kept on ice *(see Note 7)*. For hard tissues such as seeds, or tissues with waxy cuticles such as leaves, it might be necessary to scarify the surface in order to allow the solution to penetrate. Close the caps of the vials lightly, and then place the vials in a vacuum oven chamber and apply vacuum for 15–20 min at 24” Hg. If samples are difficult to vacuum, the time can be extended to 45–50 min.
4. Remove the vials from the vacuum chamber, close the caps tightly, and place them in a rotator at 4 °C overnight *(see Note 8)*.

21.4.6 Tissue Dehydration, Embedding, and Block Preparation

1. Drain off the fixative from the vials and wash the tissues twice in 1X PBS for 5 min to remove all traces of paraformaldehyde. During the washing steps, keep the vials at 4 °C.
2. Using DEPC-treated water, ethanol, and xylene prepare the following dehydration solutions: 50, 70, 95, and 100 % ethanol, and 100 % ethanol:xylene (1:1).
3. Using a pipette, remove the PBS solution from the vials and add 50 % ethanol so as to submerge all the tissue. Place the capped vials back on the rotator and incubate at 4 °C for 1 h. If the tissue contains chlorophyll, all the dehydration steps can be carried out at room temperature *(see Note 9)*.
4. Repeat Step 3 with all the dehydration solutions prepared in Step 2.
5. Remove the 100 % ethanol:xylene (1:1) solution and add fresh xylene. Incubate for 1 h at room temperature.
6. Before the last dehydration step, fill a beaker (500 mL) with pellets of Paraplast® Plus tissue embedding medium. Melt the wax in an oven at 56–60 °C *(see Note 10)*.
7. Add a few Paraplast® Plus pellets (roughly the same volume as xylene) into the vials and leave them uncapped in an oven at 54–55 °C overnight which will allow xylene to evaporate. The oven should preferably be placed in a fume hood as xylene and Paraplast® Plus can produce vapors which are harmful if inhaled *(see Note 11)*.
8. The next day replace the xylene-wax solution in the vials with the melted wax prepared in Step 6. Place the vials back into the oven without their caps and incubate for 5–6 h. The tissues will settle to the bottom of the vials.
9. Remove the vials from the oven, and quickly decant the wax into a waste container in a fume hood. Refill the vials with fresh molten wax and place them back in the oven, without cap.
10. The next day repeat the changes with melted wax (Step 8) twice *(see Note 12)*.
11. During the last change of wax, pre-warm a spatula and the Peel-A-Way® Disposable Histology Molds by placing them in the oven.
12. Quickly pour the content of the vials (melted wax and samples) into the molds. If the volume of the melted wax exceeds that of the molds, discard some of the wax into a waste container. Before the wax hardens, use the spatula to make sure the specimens settle at the bottom of the molds and place them in the right orientation *(see Note 13)*. The specimens should not be in contact with the walls of the mold. Be sure to label the molds with sample identification.
13. Place the molds in a tray containing some water and ice to accelerate the hardening of the wax. Once fully solidified, the molds can be placed in a plastic bag and stored in the fridge for a maximum of 6 months.
14. Prepare the embedding rings by pouring molten wax into their cavity inside a fume hood. One opening of the rings must be sealed with adhesive tape. Once the wax is solidified completely, it will provide a flat support for the block

(*see* Chap. 3). To simplify the clean-up, it is good to carry out this step and Steps 9, 10, and 12 on a sheet of aluminum foil.

15. Wear plastic gloves and peal the plastic of the molds to release the block containing the tissue. Warm a spatula, and place one of its warm sides on the wax support of the embedding ring. Immediately place the tissue block on top of the spatula. As the wax on the block starts melting, slide the spatula out and allow the two surfaces to fuse/solidify together. Gently apply pressure on the top of the wax block. This step should be performed in a fume hood.
16. The blocks can be stored at 4 °C to ensure the wax solidifies completely.

21.4.7 Tissue Sectioning

1. Using a razor blade, trim the blocks with the tissue embedded into a trapezoid with parallel and smooth sides. The edges have to be smooth and clean to ensure formation of a proper ribbon during sectioning.
2. Insert the block into the embedding ring of the rotary microtome, setting the cutting angle at 7–9° and the thickness at 10 μm.
3. Set the slide warmer at 37 °C. Label Superfrost® Plus Microscope slides with pencil and place them on the warmer. Add about 0.5–1 mL of DEPC water onto each slide.
4. Start sectioning the block. The first few sections usually do not contain any tissue and can be discarded. The subsequent sections will form a ribbon which should be placed on a cardboard with the dull side facing up. Using a razor blade, the ribbon should be cut in segments of about 4–5 cm (about 2/3 of the length of the slides).
5. Using a wet paint brush and forceps, apply the segments of the ribbons onto the slides. To maximize the use of the slides, two segments can be placed on one slide. The shiny side of the ribbon should be placed down onto the slide as it will adhere to the slide surface better when dry. When placing the ribbon onto a slide, start by placing one end of the segment on the edge of the slide and then with a rapid movement, drop the whole ribbon segment which will float on the water on top of the slide *(see Note 14)*. The ribbon will expand immediately.
6. Let the slides dry on the slide warmer at 37 °C overnight.

21.4.8 Hybridization

21.4.8.1 Pre-hybridization of Sections—Day 1: Part 1

1. Prior to starting the hybridization procedure, set an incubator at 37 °C, a Boekel hybridization oven at 50 °C and a dry bath at 65 °C. Prepare 25 mL of 4% paraformaldehyde in the fume hood and cool it on ice (*see* Step 2 in Sect. 21.4.5).

2. Prepare the hybridization solution that will be required in subsequent steps (*see* Sect. 21.4.8.2). Mix well by pipetting up and down carefully without generating bubbles as dextran sulfate is very viscous. Do not vortex. Keep the tube in an oven at 37 °C until further use. The volume of hybridization buffer to be prepared needs to be calculated on the basis of the number of slides that is being used. Typically, about 4.8 mL of solution is prepared for ten slides *(see Note 15)*.
3. In a fume hood, arrange 10 Coplin jars as follows: C1 and C2 with xylene, C3 with absolute (100 %) ethanol, C4 with 95 % ethanol, C5 with 70 % ethanol, C6 with 50 % ethanol, C7 and C8 with DEPC water, C9 with 1.6 % hydrochloric acid, and C10 with 1X PBS.
4. Using forceps insert the slides in the first Coplin jar C1 and keep it there for 15 min. Ensure the slides do not stick to each other.
5. Remove the slides one by one and place them into the second jar C2 and keep them in the jar for another 15 min.
6. Transfer the slides after draining off the excess xylene from the slides to the third jar C3 containing absolute alcohol and keep them in the jar for 1–2 min. Repeat the process as you transfer the slides through jars C4–C6.
7. Once removed from jar C6, transfer the slides to jar C7 and keep them in the jar for 5 min. At this step you can use a rocking platform.
8. Transfer the slides to jar C8 and wash for 5 min.
9. Transfer the slides to jar C9 and keep the slides inside for 10 min. As this jar contains HCl, caution is required while handling *(see Note 16)*.
10. Wash the slides again in jar C8 containing fresh DEPC water for 5 min. Then transfer the slides to jar C10 containing 1X PBS for 2 min.
11. Blot the excess buffer from the slides by tilting them on a paper towel while holding with forceps. Place them onto the horizontal slide holder.
12. Incubate the sections in the pronase incubation solution (pronase buffer 10 mL + 60 μL pronase incubated at 37 °C for 1 h) by adding 1 mL to each slide, carefully placing a coverslip, and then keeping the slide holder at 37 °C for 15 min *(see Note 17)*.
13. Tilt the slides to remove the coverslips and decant pronase solution. Rinse the slides in 1X PBS in jar C10 at room temperature for 2 min. Remove excess PBS, and transfer the slides with forceps back onto the slide holder. Apply 2 mL glycine solution to each slide and incubate them at room temperature for 2 min. This step stops the action of pronase.
14. Tilt the slides to remove glycine and rinse the slides in fresh 1X PBS twice, each for 1 min while keeping the jar on a rocking platform.
15. Post-fix the tissues by removing the excess PBS from the slides and putting them back on the slide holder. Apply 2 mL of 4 % paraformaldehyde on each slide and incubate the slides for 15 min.
16. Rinse the slides in 1X PBS twice for 5 min each. In between the washes, prepare the acetic anhydride solution that is required for the next step.
17. Transfer the slides into a new jar for acetic anhydride treatment. This step reduces nonspecific binding of the probes. In the jar add 3 mL of triethanolamine to 200 mL of DEPC water and mix it well for 3–4 min with the help of a small magnetic stir bar. Just prior to transferring the slides, add 1 mL of acetic

anhydride and mix for another minute. Incubate the slides for 10 min while stirring the solution *(see Note 18)*.
18. Rinse the slides well in 1X PBS twice for 2 min each time.
19. Dehydrate the slides in a new set of Coplin jars containing DEPC water (C1), 50 % ethanol (C2), 70 % ethanol (C3), 95 % ethanol (C4), and absolute alcohol (C5) by keeping them in each jar for 1–2 min. After removing the slides from C5, air-dry the slides by placing them on the slide holder.

21.4.8.2 Hybridization of Sections—Day 1: Part 2

The protocol is optimized for ten slides. Normally the concentration needs to be optimized for each probe; however, the method outlined here will give an idea about the starting point which can be increased or decreased as required. The concentration of the probes needs to be measured at the end of the probe synthesis, and if it is around 2 μg/μL, we typically use 4 μg of probe per slide, but the amount of probe needs to be determined empirically depending upon DIG labeling.

1. Remove the hybridization buffer (prepared earlier) from the 37 °C oven. Add 8 μL of probe to an Eppendorf tube containing 12 μL of DEPC water and 20 μL of deionized formamide. Care should be taken to label the sense and antisense tubes properly at this step in order to avoid any error at the later steps. Incubate the tubes containing the probe at 60 °C for 5 min and chill on ice for a minute. Add hybridization buffer (300 μL per slide) and mix well by pipetting up and down (take care not to introduce any bubbles).
2. Place about 8–10 paper towels to the metal slide holder and wet them with 2X SSC and 25 mL formamide. Formamide is added at this step in order to minimize any evaporation of liquid from the slide/slide holder. Place the air-dried slides on the slide holder. Arrange the slides designated for sense and antisense probes carefully so as to avoid any error while adding the respective probes.
3. Add 270–300 μL of the probe mix onto the slides and place the coverslips carefully to make sure the hybridization mix containing the probe is distributed throughout the length of the tissue with no apparent air bubbles underneath the coverslip *(see Note 19)*.
4. Replace the cover of the metal slide holder and wrap the edge of the holder with parafilm and the entire slide holder with plastic cling wrap. Place the slide holder inside the hybridization oven set at 50 °C for overnight *(see Note 20)*.

21.4.8.3 Washing of Sections—Day 2

1. Before proceeding with washing, prepare 20X SSC, Buffers B1, B1 with Triton X-100, B2, and NTE. Pre-warm NTE at 37 °C overnight.
2. After removing the slide holder from the hybridization oven, gently remove the coverslips from each slide by tilting the slide. If a coverslip adheres to a slide, then soak the whole slide in DEPC water *(see Note 21)*.

3. Wash the slides four times in 2X SSC, each time for 15 min on rocking platform.
4. Wash with pre-warmed NTE buffer twice for 10 min each *(see Note 22)*.
5. Wash once in 50 % formamide in 2X SSC for 20 min at 50 °C.
6. Wash once in 0.1X SSC for 5 min on rocking platform.
7. Incubate the slides in a Coplin jar containing the Blocking solution for 60 min on rocking platform with gentle agitation.
8. Wash the slides for 45 min in the blocking–washing buffer for 45 min on rocking platform.
9. Prepare the antibody solution at 1:200 dilution (about 250 µL per slide; add 15 µL anti-DIG-AP Fab fragments to 3 mL blocking–washing buffer).
10. Reassemble the slide holder by placing some paper towel moistened with water at the bottom of the holder. Gently remove the slides from the blocking–washing buffer, blot them dry, and place them on the slide holder. Pipette 250 µL of the antibody solution and place the coverslips on the slides. Incubate at room temperature in the dark for 2 h.
11. After incubation, remove the coverslips as described in Step 2 and wash the slides as follows on a rocking platform:

    a. Wash four times with Buffer B1 with Triton X-100 each time for 15 min.
    b. Wash with Buffer B1 for 5 min.
    c. Wash with Buffer B2 for 5 min.

12. Blot the slides dry and place them on the slide holder. Add 400 µL of Western Blue® substrate solution onto each slide, place the coverslips, and incubate at room temperature in the dark overnight (or 24–48 h, *see Note 23*).

21.4.8.4 Preparation for Microscopy—Day 3

1. Wash the slides gently by dipping them carefully in a sterile jar containing DEPC water.
2. Drain the slides, blot-dry, add a few drops of water, and examine the slides immediately.
3. The slides can also be mounted for later examination by first rinsing them in DEPC water, and then dehydrating them quickly in 10, 30, 50, 70, 80, 95, and 100 % ethanol for 5 s each and two washes of xylene for 2 min each. Dehydrated slides can be mounted on a toluene-based mounting medium.

21.5 Practical Considerations and Interpretation of Results

When compared with other molecular biology techniques used for analyzing the expression of specific genes, RNA in situ hybridization has the advantage of identifying specific cells where the transcripts are localized. This “spatial” component

is very crucial for defining and characterizing the function of a specific gene. In the opinion of the authors, proper interpretation of the results requires an advanced knowledge of histology of the samples analyzed, and proper controls. Before embarking in the RNA in situ hybridization procedure it is advisable to conduct preliminary studies to become familiar with the structure and anatomy of the sample. Free-hand sections as well as paraffin and/or Technovit sections can assist in this effort (*see* Chaps. 1–4). It would also be useful to have some information on the expected staining patterns, such as, is the gene of interest expected to be expressed in specific cells/domains of the tissue? Results from other expression studies, such as microarray analyses, can be used to obtain this preliminary information.

Finally, the utilization of proper controls can minimize possible artifacts leading to inaccurate interpretations. Staining in the negative control should be minimized by optimizing the concentration of probes, the stringency of the tissue digestion and washes. Sense hybridizations are often used as negative controls. It must be noted, however, that the background signal in a sense hybridization might be different from that of an antisense hybridization. Sense and antisense probes, in fact, might have a different affinity for the tissue; consequently, a comparison between the two probes is not necessarily valid. A more suitable negative control would include a sample known to be devoid of the target RNA, or plants where the expression of the target gene is experimentally knocked out. Selection of the appropriate tissue would however require some knowledge on the behavior of the gene. Alternative negative controls can include tissues pretreated with RNAses, and/or omission of the antibody.

Although seldom utilized, positive controls might also assist in the proper interpretation of the results. They might consist of samples known to express the gene of interest (or even better, plants overexpressing the desired gene), or the utilization of probes generated from genes with a similar expression pattern to the gene of interest.

Therefore, while providing valuable information on the localization of transcripts, the results of the RNA in situ hybridization must always be interpreted with caution. While the inclusions of appropriate controls are advisable, the integration of additional analyses, possibly including quantitative expression data, can facilitate the interpretation of the results.

21.6 Notes

1. DEPC is carcinogenic, so proper safety procedures must be followed. DEPC-treated water can be prepared several days in advance and stored. In a fume hood, mix water and DEPC (0.1 %) in a flask. Incubate overnight on a stirrer and autoclave the following day to degrade DEPC. We usually prepare 5 L of DEPC-treated water per RNA in situ hybridization.
2. For this procedure we use T7 and SP6 RNA polymerases. The use of a specific polymerase must be decided in advance in order to design the cDNA fragments with the specific RNA-polymerase binding sites.

3. Longer incubation times at −20 °C are acceptable, but an overnight incubation is not recommended.
4. When running RNA on agarose gel, make sure that the electrophoretic unit is RNase-free. Carefully wipe the unit with RNaseZap® and prepare RNase-free running and loading buffers. In some instances, we have been successful in retaining the RNA integrity by using a non-RNase-free unit and running the gel for only 10 min.
5. Paraformaldehyde is highly toxic and should be handled with care. Work in a fume hood and avoid inhaling the powder and the vapors of the solution.
6. Ethanol-based fixatives such as formalin–acetic–alcohol (FAA) or formalin–propionic acid–alcohol (FPA) are sometimes used for moderate to highly impervious tissues. They have a good penetrating capacity and hence are suitable for tissues such as woody stems, tough herbaceous stems, and old roots.
7. It is extremely important to fix the tissue immediately in ice-cold fixative to preserve its structural integrity and most importantly the integrity of the target RNA.
8. The time of fixation is critical and it should be optimized accordingly. Often the overnight incubation at 4 °C can be replaced by incubation at room temperature for a period of 3–4 h. Under-fixation causes loss of tissue integrity and possible target RNA degradation, while over-fixation can make the target RNA inaccessible to the probe. The suggested incubation times should be taken as the initial reference and possibly modified depending on the type and nature of the specimen.
9. As chlorophyll interferes with the signal, it has to be completely removed from the tissue. Often, dehydration at 4 °C might not be sufficient to clear the tissue and therefore incubation at room temperature might be required, even if this might result in the partial degradation of the target RNA. If chlorophyll is not fully removed at the end of the dehydration period, it is recommended to repeat the 100 % ethanol wash overnight at 4 °C.
10. The temperature should not exceed 62 °C as elevated temperatures alter the properties of the wax. Also, it is recommended to melt only the amount of wax needed for the experiment, as melted wax should be fresh every time.
11. Xylene vapors are extremely harmful to the mucous lining of the human body, and can also depress the central nervous system hence care should be taken. Always use a fume hood.
12. To completely remove residual xylene and air from the tissue, a vacuum step can be performed at this stage. Make sure that the temperature of the vacuum oven is set at about 4 °C above the wax melting temperature. If there is no visible sign of air bubbles, the vials can be placed back in the oven.
13. The proper orientation of the samples is critical for obtaining adequate sections. While longitudinal sections are easier to obtain as the sample can be placed flat at the bottom of the mold, cross-sections might require some additional handling. For example, partially solidified wax can be used to orient the samples vertically. In some instances, a hot plate might be used to extend the time of the

embedding procedure and facilitate the handling of the tissue, and a magnifying glass to see to orient small samples.

14. The tissue sections from the wax block should form a flat ribbon for best results. Care should be taken while placing these tissue ribbons on the slide which should already contain water. If there is not enough water on the slide, the ribbon containing the sections will not extend fully, and hence will form wrinkles and will not lie in one plane. This can result in the formation of air bubbles which can compromise the subsequent hybridization steps. Excess water on the slide should be carefully removed with a Kimwipe, otherwise the section will float away from the slide surface.
15. In our procedure we hybridize ten slides per RNA in situ hybridization experiment. This is the maximum number of slides that our Coplin jars can hold and which, in our opinion, can be handled successfully. Therefore if a different number of slides is used, the volumes of pre-hybridization and hybridization buffers should be adjusted accordingly.
16. Hydrochloric acid helps dissolving calcium pectate from the wall of adjacent cells and hence makes the tissue more permeable to the probes.
17. Pronase pretreatment is used to degrade proteins that may surround the target RNA and hence facilitate probe binding. The concentration may be further optimized if the recommended amount does not work well. However, care should be taken not to overexpose the cells to pronase as this will destroy cellular integrity. The action of pronase is stopped with glycine. Proteinase K can also be used instead of pronase.
18. Treatment with acetic anhydride and triethanolamine decreases the background and is also thought to inactivate RNases, thereby increasing the signal strength.
19. The presence of any air bubbles in the slide is undesirable. Care should be taken while placing the coverslip on top of the tissue sections as described in the Methods section. Should any tiny air bubble be seen trapped at the hybridization step, it should not be a big concern as the higher temperatures during hybridization will get rid of the air bubble. But bigger bubbles should be avoided as they will hinder the process.
20. Optimal hybridization temperature is dependent on the GC content of the designed probe. A probe with an average GC content (50–65 %) usually works well at hybridization temperature of 55–60 °C. A higher GC content will require a higher temperature. Optimization of the hybridization temperature can often give a better result.
21. A coverslip adhering to the slide is an indication that the section might have dried during hybridization. This might result in high background.
22. The NTE buffer should be kept at 37 °C. This temperature favors the detachment of nonspecific probes attracted to the tissue by van der Waal's forces.
23. Incubation time varies from probe to probe. For weaker signals the incubation in Western Blue® can be extended up to 48 h, while for stronger signals it should be reduced to a few hours. As a general rule, the reaction should be stopped as soon as the background signal appears in the negative control.

References

1. Pardue ML, Gall JG (1969) Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc Natl Acad Sci U S A 64:600–604
2. Darby IA, Hewitson TD (eds) (2006) *In situ* hybridization protocols, 3 edn. Humana Press Inc, Totowa
3. Harrison PR, Conkie D, Paul J, Jones K (1973) Localisation of cellular globin messenger RNA by *in situ* hybridization to complementary DNA. FEBS Lett 32:109–112
4. Brahic A, Haase T (1978) Detection of viral sequences of low reiteration frequency by hybridization. Proc Natl Acad Sci U S A 75:6125–6129
5. Hudson P, Penschow J, Shine J, Ryan J, Niall H, Coghlan J (1981) Hybridization histochemistry: use of recombinant DNA as a "homing probe" for tissue localization of specific mRNA populations. Endocrinology 108:353–356
6. Pochet R, Brocas H, Vassart G, Toubeau G, Seo H, Refetoff S, Dumont JE, Pasteels JL (1981) Radioautographic localization of prolactin messenger RNA on histological sections by *in situ* hybridization. Brain Res 211:433–438
7. Brown C (1998) *In situ* hybridization with riboprobes: an overview for veterinary pathologists. Vet Pathol 35:159–167
8. Braissant O, Wahl W (1998) A simplified in situ hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica 1:10–16
9. Lee D, Xiong S, Xiong W-C (2013) General introduction to in situ hybridization protocol using non-radioactively labeled probes to detect mRNAs on tissue sections. In: Zhou R, Mei L (eds) Neural development: methods and protocols. Meth Mol Biol 1018:165–174
10. Green NM (1975) Avidin. Adv Protein Chem 29:85–133
11. Holtke H, Kessler C (1990) Non-radioactive labeling of RNA transcripts in vitro with the hapten digoxigenin (DIG). Nucleic Acid Res 18:5843–5851
12. Brewer PB, Heisler MG, Hejátko J, Friml J, Benková E (2006) In situ hybridization for mRNA detection in *Arabidopsis* tissue sections. Nat Protoc 1:1462–1467
13. Singer RH, Ward C (1982) Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. Proc Natl Acad Sci U S A 79:7331–7335
14. Brigati A (1983) Detection of viral genomes in cultured with embedded tissue sections using biotin-labeled hybridization probes. Virology 126:32–50
15. Bloch B, Popovici T, Levin MJ, Tuil D, Kahn A (1985) Transferrin gene expression visualized in oligodendrocytes of a rat brain by using in situ hybridization and immunohistochemistry. Proc Natl Acad Sci U S A 82:6706–6710
16. Lloyd RV, Landefeld TD (1985) Detection of prolactin messenger RNA in rat anterior pituitary by in situ hybridization. Am J Pathol 125:35–44
17. Farquharson J, Cockburn F, Patrick WA, Jamieson EC, Logan RW (1992) Infant cerebral cortex phospholipid fatty-acid composition and diet. Lancet 340:810–813
18. Radchuk V, Borisjuk L, Radchuk R, Steinbiss HH, Rolletschek H, Broeders S, Wobus U (2009) Jekyll encodes a novel protein involved in the sexual reproduction of barley. Plant Physiol 150:190–204
19. Palovaara J, Hallberg H, Stasolla C, Luit B, Hakman J (2010) Expression of a gymnosperm PIN homologous gene correlates with auxin immunolocalization pattern at cotyledon formation and in demarcation of the procambium during *Picea abies* somatic embryo development and in seedling tissues. Tree Physiol 30:479–489
20. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Part III
Preparative Methods for the Study of Wood Anatomy

Chapter 22
Microscopic Examination of Wood: Sample Preparation and Techniques for Light Microscopy

Jacques C. Tardif and France Conciatori

22.1 Introduction

22.1.1 Brief Historical Information

The study of wood anatomy has had a long history. In the *Historia Plantarium*, Theophrastes (371–286 BC) described fibers, vessels, and the pith as components of wood [1]. Centuries later, the development of both the microscope and the microtome constituted major technical innovations that increased the ability to study plant anatomy. The oldest description of a compound microscope dates back to 1625 [2] with the first published record on the use of a microscope dating from 1685 [3]. The books on timber by Hill in 1770 included references to the first (automatic) microtome, advanced techniques such as staining methods, the use of spirit of wine to preserve wood material, and accurate information on plant anatomy [4, 5]. Half a century later, the term "microtome" was coined by Chevalier in 1839 and the term "microtomy" became in use in 1884 [4–6]. The mechanical microtome, invented by Rivet in 1868 devised for cutting plant tissue, is considered as the origin of today's microtome [5–7]. Other technical advances of the nineteenth century include botanists boiling samples to soften them and the introduction of various stains including safranin by Ehrlich in 1877 [4, 5]. While the history of microtechnique is beyond the scope of this chapter, it is worth mentioning that in recent years, new microtome models and sectioning techniques adapted to the need of dendrochronologists and stem anatomists have been developed [8–11].

J. C. Tardif (✉) · F. Conciatori
Centre for Forest Interdisciplinary Research (C-FIR), Departments of Biology/Environmental Studies and Sciences, University of Winnipeg, Winnipeg, Manitoba R3B 2E9, Canada
e-mail: j.tardif@uwinnipeg.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_22

22.1.2 Range of Applications

"What kind of wood is that?" [12]. This question not restricted to the amateur wood anatomist is commonly asked in many scientific disciplines. Wood identification is crucial in fields such as anthropology, archaeology, conservation of historic woody material, forensic sciences, paleobotany, and in industrial processes to name a few [13–18]. Wood anatomy also constitutes a major component of wood sciences as well as of tree physiology, pathology, and ecology [1]. In tropical wood, the concept of "chemical growth rings" was developed following wood anatomical studies [19]. In recent decades, the impact of environmental changes on the anatomical structure of annual rings or growth rings from trees and shrubs has also been of particular interest to dendrochronologists [8–11, 20]. The importance of wood anatomy can also be stressed by the numerous resources that exist on wood terminology, wood structure, and/or wood identification (e.g., [12, 17, 21–31]) and specifically referring to sectioning techniques applied to biological, botanical, and to wood materials (e.g., [3, 7, 9, 19, 32–55]).

22.1.3 Skills Needed

Making thin sections of wood is not an easy task and there is a wide array of details to which the practitioner must pay attention. The quality of thin sections may be influenced by a range of variables including the specimen itself, its preparation, the softening technique, fiber orientation, the blade angle, the cutting angle (slicing), and, the staining, dehydrating, clearing, and mounting procedures. There are few shortcuts in microtomy [45]. Three needed skills (patience, organization, and intolerance to dirt and grease) have been emphasized to become a good microtomist [45]. Cleanliness, accuracy, adaptability, and patience [55] as well as enthusiasm as an attitude needed to overcome the initial lack of experience [46] have also been stressed. Excellent note keeping may be added as there is no one-fits-all technique or recipe. The ability to stay focused is likewise important and the use of cell phone and music headphones is not permitted in many laboratories. These apparatus constitute a major source of distractions, diverting attention away and increasing the risk for errors and accident. Sectioning wood makes use of many senses, hearing the sound of the blade slicing through a specimen is informative and it may reveal a lack of softening or blade maladjustment.

Fifty-five years after the publication of "Suggestions to beginners on cutting and mounting wood sections for microscopic examination" in 1926 [41], it was still admitted that there was a lack of a plainspoken sufficiently detailed "cook book" on wood microtechniques [45]. A handbook that would guide the beginner who has no access to an experienced technician and speed up the learning process was

missing. Since this call, *Identifying Wood: Accurate Results with Simple Tools* [12] published in 1990 provided a detailed introduction to wood microtechniques with a review of the basic equipment and technique used when mounting non-permanent slides. In 1999, *Plant Microtechnique and Microscopy* [3] was published updating past instruction manuals of plant microtechniques. In 2001, *A Guide to Wood Microtomy* [46] was published providing another detailed guide to help beginners avoid common pitfalls and mistakes. Also intended to fill this gap, the first international course on wood anatomy of tree rings was offered in 2002 [56]. A decade of teaching to an international audience has resulted in the publication of *Microscopic Preparation Techniques for Plant Stem Analysis* [9]. This largely illustrated booklet mainly focuses on providing practical instructions for the efficient production of high-quality thin sections of xylem and phloem of plant stems, branches, and roots using simple techniques. Given the terminological gaps that exist between the technical and botanical meaning of "wood," it was also recently proposed to use the botanically neutral term "stem anatomy" in lieu of "wood anatomy" for better integration of xylem and bark of trees, shrubs, and herbs in all taxonomic units among conifers, monocotyledons, and dicotyledons [57].

22.1.4 Objectives

This chapter aims to be practical and informative. It describes simple procedures for routine preparation of sections of stem/wood specimens that has proved to be satisfactory for microscopic examination (Fig. 22.1). In this chapter (1) we provide a list of materials and chemicals that are useful when preparing stem material for light microscopy. (2) We cover sample preparation and provide an overview of the main methods used to soften wood. The techniques presented do not require fixation (killing and fixing of the protoplasm) or embedding. (3) We cover microtoming and common problems associated with stem sectioning. The emphasis will be on obtaining thin sections from a sliding microtome. (4) We cover basic staining techniques also including dehydration and clearing. Hundreds of stain and staining recipes exist and the objective is not to provide an exhaustive list and description of methods associated to the theory and reactivity of staining. (5) We cover permanent mounting of thin sections on slides for light microscopy and image analysis. The topics of microscopy and image analysis are, however, outside the scope of this chapter. Throughout the chapter, we will address general difficulties or pitfalls encountered and provide clues on how to resolve them. Our aim is to provide an up-to-date synthesis of simple and practical procedures to assist the practitioner in obtaining high-quality thin sections of stem material. The readers will also be able to extend their knowledge by consulting the extensive list of references provided.

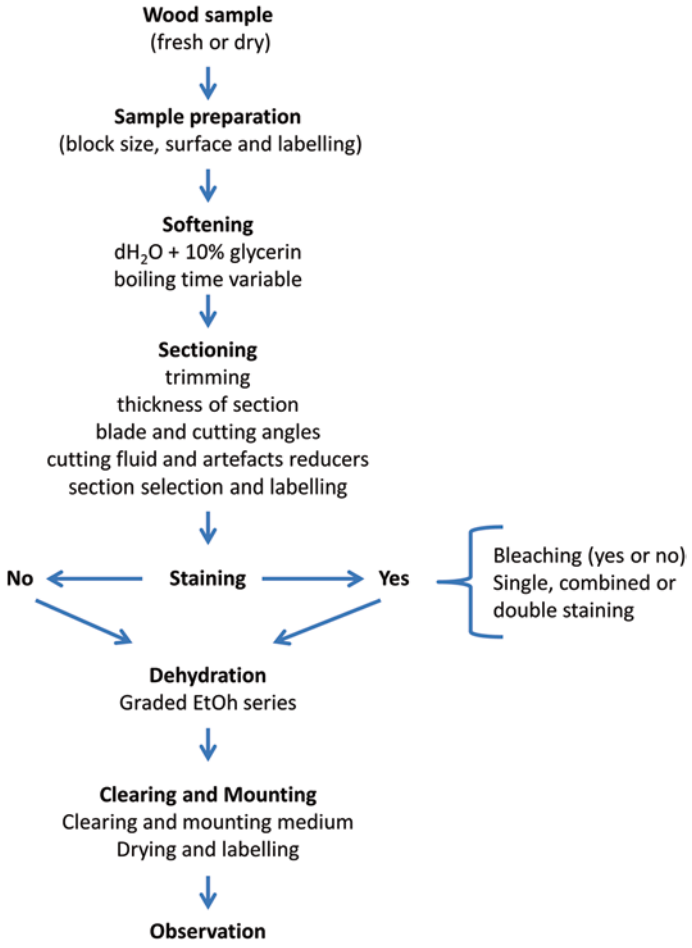

Fig. 22.1 Overview of the general procedures and decision-making involved in making thin sections of stem/wood material

22.2 Materials

Table 22.1 provides an itemized listing of materials and chemicals that are handy when preparing stem specimens for anatomical studies. It is by no means exhaustive or a "must all have" list. Various stains, clearing solvents, and mounting media exist. Each practitioner develops their own preferences (e.g., bottle sizes, staining solutions, etc.) so this list is highly adaptable. The quantity needed will also vary according to the wood anatomy projects pursued. It is recommended to prepare an orderly work layout in which all chemicals, reagents, and solutions are properly labeled and arranged so once prepared they can be easily accessed and replenished (Fig. 22.2). An orderly layout also helps to organize and manage the work flow.

Table 22.1 Suggested laboratory equipment and chemicals for wood/stem anatomical studies

Equipment	Chemicals
Well-lighted and ventilated laboratory with a fume hood	Distilled water (dH_2O, use to prepare various solutions)
Microtome (e.g., GLS1, sliding, or others) with conventional knife, or special holder for disposable blades (S35 type, Feather Safety Razor Co.; NT-Cutter blades (A-type, 0.38)	Ethyl alcohol (95% and absolute). 2,2-dimethoxypropane
Stereoscopic and compound microscopes to quickly assess quality of thin sections	Glycerin (for several uses) Corn starch (to prepare non-Newtonian fluid)
Hot plate for boiling samples Slide warmer plate or dry-off stove with numerous light lead weights or weak magnets	Common stains (safranin O, astra blue, aniline blue, and Fast Green FCF). Acetic acid/tartaric acid (to prepare stain solution)
Electronic precision scale (0.001 g) Weight dishes	Sodium hypochloride (household bleach) to bleach and remove cell content
Beakers and Erlenmeyer (various size 500 mL to 1L) Graduate cylinders (various sizes: 10, 25, 50, 100 mL)	Clearing solvents (e.g., d-limonene, Histoclear, xylene). Some are more toxic than others...
Wash bottles (250 mL) for dH_2O and various alcohol solutions. Amber glass bottles with glass eye dropper (30 mL) for stain solution. Glass bottle for clearing solvent	Mounting media (e.g., Canada balsam, Euparal, Permount)
Vials to store fresh and softened materials. Petri dishes for storing of thin sections during microtoming Several watch glasses for staining and dehydrating thin sections	Epoxy glue, skin crack care, spray bandage. These products may be used with fragile samples
Containers to properly dispose of chemicals waste	
Laboratory log book to note trials, errors, and success	
Ballpoint pen (resist better to alcohol than waterproof fine-point pen), markers/pencils	
Fine camel hair, paint, or aquarelle brushes Numerous standard pipettes	
Fine-tip tweezers (Dumoxel 11231–30 by Dumont) Angled dissecting needle Razor blades (single edged) or scalpel	
Applicator C-Tips to clean the excess of the mounting media (once dry) on the microscope slide	
Plain or write-on microscope slides (one end forsted), stadard size: 26 mm × 76 mm (1" × 3") Nalgen polypaper label size: 2.22 cm × 2.22 cm (7/8" × 7/8")	
Slides and coverslips (various sizes, a popular one being 22 mm × 40 mm	
Nitrile gloves Stopwatch for staining procedure Absorbent filter paper to remove excess stain, clearing solvent, and mounting medium	
Wood working tools (handsaw, large knife, or chisel and a hammer) to prepare and split samples radially and tangentially to the size needed for sectioning	

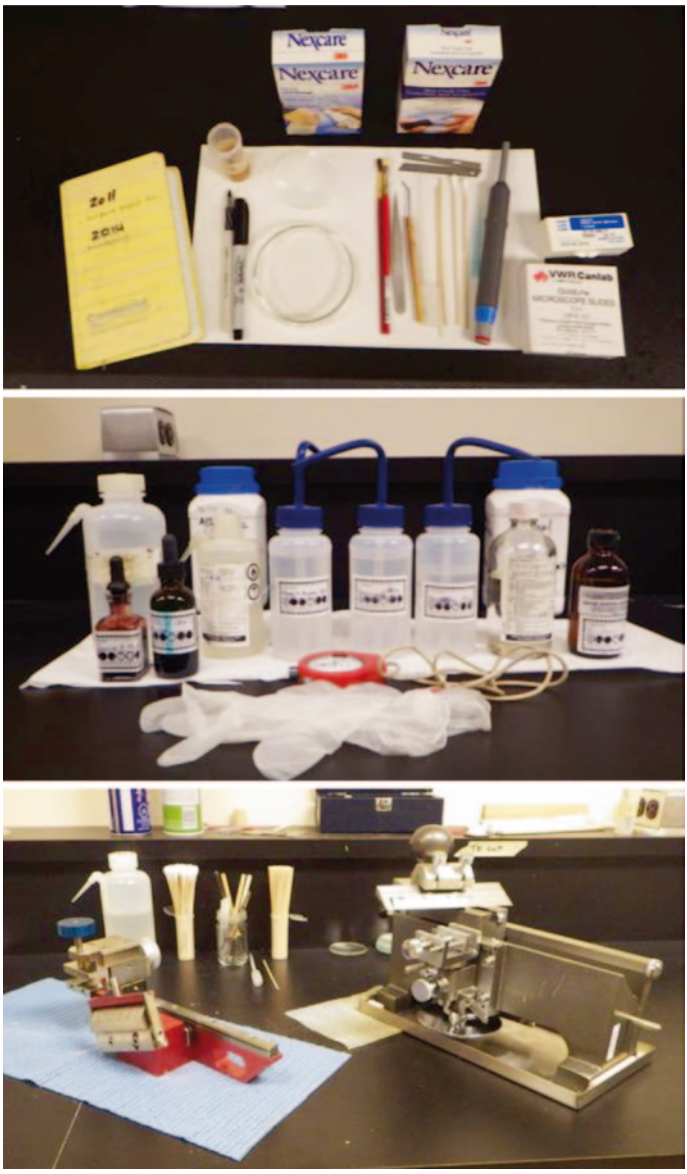

Fig. 22.2 Overview and organization of equipment that can be used to make thin sections of stem/wood material

The practitioner also needs to be aware of the proper usage, storage, and disposal of materials and chemicals as well as the safety procedures to be used when working with hazardous substances. Wearing proper protective clothing (gloves, goggles, etc.) and following proper safety procedures (labeling, fume hood, etc.) should not be ignored.

22.3 Sample Preparation and Softening Methods

22.3.1 Sampling Stem Material

Samples from fresh plant material can take various forms (cylinders, blocks, flakes, sawdust, etc.) and may be obtained using either destructive (excavation of roots, sawing a stem, etc.) or less destructive (chisel, increment borers, microborers, etc.) procedures. Fresh stem material, once collected, will usually be cleaned, washed, and stored in 40 % ethanol prior to sectioning [9, 31,53]. Dry stem material will also come in various forms and sizes that often will be specific to the discipline (e.g., archeology, ecology, forensic science, paleobotany). Dry material may also present seasoning checks originating from the drying process and that depending on their extent (surficial or deep) may affect the final results [42–44, 54].

Both beginners and experienced practitioners may anticipate making a large number of unsatisfactory sections when beginning a new project and, if feasible, either extra and/or larger specimens should be collected for practicing purposes. All material should also be properly labeled as soon as collected with minimally providing a specimen number or species name, location and site conditions, type of specimen (e.g., root, stem, or branch), date of collection, and photograph number. This information will often be specific to the research project. If wood identification is pursued, sample should include, if possible, at least one annual ring or preferably several ones so that complete transversal, radial, and transverse sections can be made. Unless it constitutes the object of the study, samples with growth anomalies (e.g., growth suppression or release, compression wood, traumatic resins ducts, false ring, or frost rings) or from the roots, branches, or heartwood may be avoided. It should be stressed that sapwood will cut more readily than heartwood [45], the latter being usually more difficult to section due to extractive deposits (e.g., gums, crystals, tannins). Uniformed softening may also be more difficult to achieve in a specimen including both sapwood and heartwood [37]. The research question (and the sampling design) will however often determine what a representative sample is and how it will be collected and prepared.

In recent years, specific tools like the Trephor microborer have been developed to extract microcores (2 mm in diameter and 15 mm in length) from trees and shrubs [60]. This microborer often associated with xylogenesis studies (e.g., [60], *see also* Chap. 23) can also be used like standard increment borers (4–5 mm in diameter and variable length) for microscopic studies [9, 61]. No matter the instrument obtaining quality thin sections will invariably relate to the sharpness of the cutting tube. A dull borer will induce undue mechanical stresses on the extracted cores (e.g., compressed cores, partly squeeze, broken, or twisted cores) making their use difficult for microscopic preparation [9]. This is especially true if good-quality continuous anatomical sequences of annual rings or growth rings are needed. If cores are to be mounted, cell orientation is crucial and the use of a waterproof adhesive is indicated if samples are to be exposed to water (i.e., cutting fluid).

Once collected, fresh cores or microcores can be stored in labeled vials (e.g., Eppendorf microtubes, screw top vials) in an ethanol (usually 40–50 %) solution [60–62]. When xylogenesis is not the object of the study nor is paraffin embedding an option, the cores can also simply be glued on wood mounts. Waterproof adhesives (epoxy, gel, etc.) have successfully been used to mount cores [62–64] and are indicated if mounted samples are to be exposed to water during sectioning (i.e., cutting fluid). In the case of fresh microcores, they can first be left to dry out for about 30 s on a piece of tissue to increase the glue adherence [62]. A layer of adhesive is then deposited on the wood holder and the microcore is pressed with forceps and covered completely with glue. Proper orientation of the cells (e.g., tracheids in cross-sectional view) is critical at this stage if unbiased measurement of cell dimensions is to be obtained and it cannot be overstressed. The sample is then let to sit at room temperature for 24 h for the glue to dry. The wood holder can then be clamped into the microtome and sectioning made directing the blade toward the pith to prevent the bark from breaking-off during cutting [62].

22.3.2 Preparing Stem Sample (Size and Surface Planes)

If microborers and/or standard increment borers are used for sampling stem material, the size of the specimen will be given. Small cylindrical stems will usually need support so that they can be held by microtome clamps and the use of cork and other materials (e.g., carrots, elder pith, and polystyrene) has been largely described [9, 17, 49, 53]. If large stem samples are obtained, the first step will involve their reduction into blocks of a convenient size (Fig. 22.3) depending on the need to obtain transversal and/or longitudinal sections (radial or tangential). It should be remembered that while smaller samples (especially transverse plane) are easiest to cut (less wear on the microtome blade) they are more difficult to handle and offer less room for errors. Cubes of approximately 1 cm^3 are often used (e.g., [30–31, 37, 40, 41, 45, 65]) but other dimensions have also been recommended [30, 42, 54, 66]. Taking advantage of larger blocks when working with soft woods and reducing the transverse plane when working with very hard woods can also be indicated [43]. In addition, larger blocks may be desirable to allow for trimming of the oversoftened and often damaged exterior block portions following the softening procedure [45]. The block size as well as the slide coverslip dimension should also be in line with the sample size that the microtome holder can accommodate. Recently developed microtomes for wood anatomy allow handling samples up to a size of 2 cm × 6 cm [11]. In addition, progress is being made toward developing a technique that will allow to easily cut microsections from entire increment cores (0.5 cm × 40 cm; H. Gärtner, personal communication, 2014).

If reference anatomical slides are to be made, wood blocks should be carefully prepared so that true transversal, radial, and tangential planes are identified (Fig. 22.3) and presented to the microtome blade [9, 18, 30, 41, 45, 67]. To obtain properly oriented sections in all three planes, the wood sample should always first be split longitudinally along the tracheids/fibers. Depending on the size of the

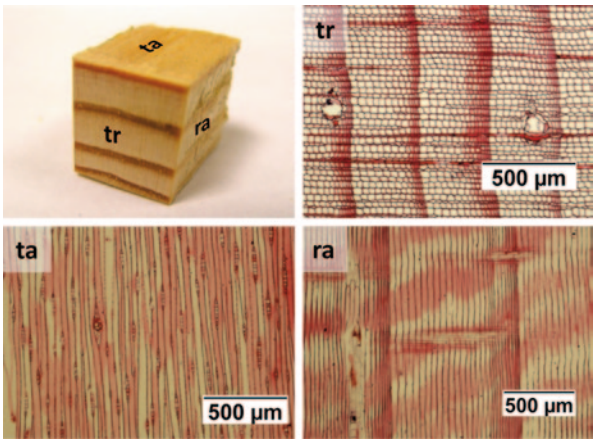

Fig. 22.3 Wood blocks showing transverse *(tr)*, radial *(ra)*, and tangential *(ta)* surfaces and example of thin section showing the same surfaces. If transverse cell measurements (e.g., tracheid lumen, cell wall thickness) are to be obtained, splitting of the wood sample along the radial and tangential surfaces is essential so that the angle of the fibers/tracheids in transversal view be properly determined

sample, position a stable knife (chisel, scalpel, or razor blade and hammer) on the transverse surface and carefully split the block to produce true radial and tangential surfaces [9]. Having the sidewalls of the blocks split off more or less parallel to each other will also offer optimum stability when clamped to the microtome holder. Given that the sample will always split longitudinally along the fibers/tracheids, the proper orientation of the transverse section can also be assessed and corrected if needed [9]. Small adjustments may also be made during sectioning. A quick look under a stereoscopic microscope prior to and after sectioning can also help assure the cells are properly oriented. The importance of sample preparation and proper cell alignment cannot be overstressed as the practitioner needs to avoid later disappointment as there may be very little leeway during sectioning if excessive trimming or alignment is required after the softening treatment [54]. Again, obtaining unbiased measurements of cell dimension in the transverse plane requires proper preparation of the sample [9]. In our laboratory, we often will prepare three blocks (one for each plane) because microtome clamps (according to model) will often damage the other planes when sectioning one. As previously mentioned, the research questions and study objectives will often direct sample preparation and cell alignment prior to sectioning.

22.3.3 Proper Sample Identification

When preparing multiple wood blocks for sectioning, it is important that they always be properly labeled. In a project involving blocks from numerous trees and sites, proper identification becomes crucial especially if samples are to undergo a

softening treatment all at once. It has been proposed to carve roman numbers on the longitudinal (radial) surface not to be sectioned [19, 42]. Grooves and notches can be made after removing one or more of the block's corners and a record of these markings kept [37, 38, 41, 45–47]. A system identifying up to 45 wood samples was proposed [40]. It was also suggested to use a soft pencil or to wrap the samples in labeled heat resistant textiles [9]. Labeling with a soft pencil could be done on the radial surface not to be sectioned. It was also suggested to use a single digit code number and to apply it to all surfaces not be sectioned [54]. If soft pencils or permanent ink pens are to be used, trials should be made to assure labeling will not dissolve or be removed during the softening treatments. Furthermore, using pencil or ink is not indicated if samples are to be analyzed for their isotopic or chemical contents.

22.3.4 Softening Methods

According to the project, wood samples will either be air-dried or be fresh (or stored in a solution often containing alcohol, dH_2O, and/or glycerin). Good sections are difficult to obtain from material that is too soft (too softened) or that has been inadequately softened. In some cases, the material may be too fragile to be sectioned (i.e., decayed wood, fossil wood, charcoals) before being stabilized (see Sect. 22.4.1). If embedding cannot be avoided, the reader is referred to previously cited works and Chap. 23. As a general rule, fresh (green) material is much easier to cut than dry (seasoned) material and it can often be sectioned directly without any softening treatments [27–41–67]. Fresh material or soft wood may simply need to be soaked a few minutes in dH_2O prior to sectioning [12]. Tap water may be used if no chemical/isotopic analyses are to be conducted.

In comparison, air-dried (seasoned) wood provides more difficulties and will more than often need to be softened. Obtaining longitudinal sections (radial and tangential) will normally require less softening than transverse sections. Hard wood and especially tropical wood may also need "vigorous" softening. Numerous publications have addressed in detail the topic of softening wood material prior to sectioning [17, 29, 30, 37, 45, 46, 54, 65, 68–73]. A wide range of methods have been evaluated from the use of water to that of more corrosive chemicals like hydrofluoric acid (HF). No matter the softening procedures to be used, it may be helpful to group wood samples according to their specific gravity to limit the range of variability in their permeability making the sectioning process simpler [54]. One point to also remember is that softening of wood may alter its chemistry [70]. Softening may thus be counterindicated in wood microchemistry studies whereas it is usually of little consequences for wood identification and morphological characterization. For example, it was reported that slight loss of lignin may occur from prolonged boiling in dH_2O [74] or from exposure to steam [75].

22.3.4.1 Soft Boiling and Glycerin Methods

In our laboratory, the main softening method used and that has proven effective with North American shrubs and tree species is the soft boiling method. This widely used method serves to expel the air and to dissolve water-soluble infiltrates [9, 12, 13, 16–18, 27, 30, 31, 36–49, 67]. Properly identified samples are softly boiled on a hot plate in a 500-mL beaker (or Erlenmeyer flask) filled with tap or dH_2O. The samples are left boiling until they sink. The boiling duration will depend on factors affecting wood density and regrouping samples by specific gravity (e.g., species, sapwood/hardwood, etc.) will help reduce the variability in boiling time. It should be noted however that the heaviest/densest wood may sink without being softened [46].

As a starting point, samples may be immersed in dH_2O and softly boiled for 1–2 h. Boiling time may range from a few minutes to many hours. We usually boil samples for a few hours. A few porcelain chips may be added to reduce "bumping" when producing the boiling water [29]. Boiling times of 12–20 h were reported for New Zealand species [42]. If boiling is prolonged water level will need to be monitored. If available, a reflux condenser may be used but an aluminum foil cover may also suffice. To speed up the process, the samples may be periodically removed from the boiling water, immersed in cold water for a few minutes, and replaced in boiling water if not waterlogged and sinking. The process continues until the samples sink. Alternatively, they can be brought to boil, transferred to a container with cold water, and boil again until they become waterlogged and sink. If a vacuum pump is available, the process may be further accelerated by vacuuming dH_2O into the sample [19, 30, 37, 41]. A vapor pressure cooker may also be used to soften extremely dense wood like ebony wood [9]. It was also suggested to boil dense wood in a 10% solution of glycerin in water with a boiling time of about 8–9 h [46]. If a glycerin solution is used, only hot dH_2O should be added to the boiling liquid to prevent potential explosion [47]. Boiling samples directly in glycerin has also been mentioned [44] and recommended [76]. No noticeable extra softening was however reported after boiling samples in glycerin at 200 °C for 4 h after an initial softening in boiling water [70]. In our laboratory we commonly boil samples in dH_2O with 10% glycerin [46, 47]. After recording the time it took for samples to sink, we extend boiling for about the same time with periodic transfer from boiling to cold dH_2O.

Once softened (waterlogged and sunk), wood samples should be sectioned while still warm and wet. If not, they may be stored in a mixture of alcohol, dH_2O, and glycerin. The glycerin helps to prevent the sections from drying and also contributes to the softening. Various mixtures have been proposed [30, 41] and care should be taken to avoid extra softening when using glycerin [37]. If waterlogged samples are stored for a long period, it was recommended that a slow softening treatment be maintained by reducing the glycerin to no more than 10% to avoid troubles during sectioning [45]. For long-term storage of softened samples, a 40% alcohol solution may also be used and storing vials should be air tight to prevent alcohol from evaporating.

As an alternative to boiling, many authors have reported obtaining sufficient softening by simply immersing wood samples in various mixtures containing glycerin, alcohol, and water for one to several weeks [9, 16, 27, 31, 44, 67]. It was also suggested to add thymol to glycerin to prevent the growth of fungi in the solution [32]. No matter the recipe, care should be taken so that alcohol and water do not evaporate. Trials and good data recording are required when initially setting up the methodology to avoid excessive softening. In our laboratory we will often start a new project by submitting extra samples to various methods and/or glycerin mixture to determine the optimum mixture. Our experience with softening dried (seasoned) wood blocks by simple immersion in a glycerin:alcohol:dH_2O solution for long periods has however not been satisfying and we do not use it.

22.3.4.2 Direct Jet of Steam

A softening method that has been reported to allow immediate sectioning of wood samples consists in applying a constant jet of superheated steam directly to the surface of the sample being sectioned [17, 29, 30, 35, 37, 39, 68, 69, 75, 77]. Steaming affects all the strength properties of wood [78]. The hot steam produced by boiling water softens the wood and condenses keeping the sample wet. Sections as thin as 10 μm free from air and curling could be produced using this method [68]. The steam method was claimed to be easier/faster than the boiling method [69]. Sectioning artifacts (torn cambial cells, split, and cracked sections) were also reported to be reduced. Issues raised with the steam method concern the coolness/warmness of the steam jet and the extra care needed to properly clean the microtome to avoid rust problems [45]. Simple steam generating apparatus have been described [17, 29, 68, 69] and experimenting with boiling kettles or other devices may be indicated.

22.3.4.3 Other Softening Methods

In some cases, very hard woods (e.g., tropical species) may require more drastic treatments and the degree of softening will often need to be determined by trial and error [45]. Expulsing air from the wood blocks through soft boiling in water will often remain the first step as it will allow for optimal penetration of various reagents. It was found that many of these alternate methods had a pronounced impact on wood chemistry compared to boiling in water [70] and thus may not be always indicated. Many of these more drastic treatments are also not without risks [28] and proper care and manipulation of chemicals need to be stressed. Safe laboratory practices are indicated. For many of these methods, a thin line also exists between softening and maceration, and the practitioner will need to exert caution and experiment with various concentration and boiling/immersion time to avoid sample degradation (e.g., [15, 16, 46, 67]).

Ethylene Diamine

The use of ethylene diamine (ETD) to soften wood has been described in detail [45, 46, 54, 67, 79]. The method was also used to soften wood prior to embedding in paraffin [72] or in polyethylene glycol [50]. ETD is a swelling agent that will slightly increase cell wall volume thus reducing density and making sectioning of dense wood easier. The induced swelling is reported to be practically lost during the dehydration procedure. The method described uses ETD reduced to a 4% solution using dH_2O. Specimens are submerged and a vacuum-type desiccator is used. The ETD treatment may or may not be followed by a HF treatment [54]. Results obtained with ETD were reported to be superior to those obtained with the HF method [79]. The ETD method was adapted so it could also be used with an autoclave or a thermos bottle [79] and numerous modifications to the original method were proposed [46, 50]. This method was recommended for softening wood having a specific gravity between 0.5 and 0.9 [46]. It was however not recommended for samples comprising both xylem and bark due to the differential swelling degree of the two tissues [79].

Hydrofluoric Acid

Softening wood with HF has been widely utilized in the early- to mid- twentieth century [17, 19, 36, 38–46, 54, 67, 70, 73, 79–85] and especially with tropical and very hard woods containing silica and crystalline material (mineral deposits). HF will dissolve silica particles, affect lignin, and partially degrade cellulose resulting in a loss of its tensile strength [55]. Various HF concentration and time exposure have been proposed according to the softness/hardness of the material. Previously waterlogged wood blocks are transferred into a 30–60% HF solution and regularly examined to determine optimal softening time. The use of 10% HF has also been proposed to remove silica [17, 80]. Soft pines and poplars may take 1–2 weeks of soaking, oak 3 weeks, and certain extremely hard tropical woods may be softened after 5 months [41, 42]. Shortening the HF treatment may be attained by pretreating the samples in alcohol using a dental vulcanizer heated at about 160 °C for 1–4 h [70, 82]. The HF treatment could also be reduced by half the time by using a pressure cylinder [83]. Neutralizing the HF after softening is a crucial step and the samples are often boiled in at least six changes of water and the residual acidity tested using litmus paper [73]. After rinsing, the samples needing further softening may be left in a mixture of alcohol and glycerin for several days or weeks [40, 43]. While papers published in the first part of the twentieth century recommended using HF as a routine softening technique for almost all species, it only had limited use in recent decades due to the method being expensive, difficult, and time consuming [86]. HF is also extremely corrosive and cannot be used with glassware. As previously written, practitioners sooner or later tend to become careless with this very active acid and HF burns are far from being pleasant [41].

Glacial Acetic Acid and Hydrogen Peroxide

Softening was obtained by boiling wood samples for 1–3 h in a mixture of one part by volume of glacial acetic acid and two parts by volume of hydrogen peroxide in a reflux condenser under a fume hood [65]. Boiling should cease when samples get a whitish color indicating over-softening of the outer portion and sectioning should occur after proper rinsing in running water [36, 48, 49, 65]. A variation of the method in which samples are immersed in a 50/50 volume to volume solution and placed in loosely caped bottles in an oven at 50 °C was also proposed [67]. Complete maceration and delignification may take place after 2 or 3 days with shorter period causing the over-softening of the outer portion of the blocks. Reducing the glacial acetic acid proportion and using the solution at room temperature may also provide a more gradual softening [67]. This method will partially delignify the wood and should not be used if microchemical investigation of the cell wall is required [45]. While this method was said to yield successful results with many types of wood [37, 48, 49], it was also not recommended based on unsatisfactory results [40, 46, 70].

Miscellaneous Softening Methods

Various other approaches have been described to soften wood. Successful results were reported from boiling samples in a 2–4 % solution of potassium hydroxide [44] or in a 5 % sodium hydroxide solution [70]. Another method requires placing waterlogged wood samples in pure acetone for 1–2 h, followed by immersion in a solution of cellulose acetate (12 % cellulose acetate in acetone) for 2–14 days [87]. In addition to softening, this method was said to infiltrate tissues with a matrix facilitating sectioning [44]. Indirect evidence of satisfactory results with this method was reported [41] but others did not recommend it [29, 70]. Another method includes heating previously waterlogged samples in 50–100 mL of triethylene glycol [67, 71]. The Foster and Gifford's fluid has also been advocated to soften refractory material [15, 16, 49, 67]. Various recipes for this fluid exist and it was suggested to increase the proportion of lactic acid for very hard material. Other methods referred to as the Sam Buck's #1 and #2 softening methods were also described [67, 73]. These methods use various chemicals such as acetic acid, commercial ammonia, or commercial antifreeze (ethylene glycol). Another softening method referred to as the Versteegh method includes the storage of fresh wood in a 5/5/90 mixture of formalin, acetic acid, and ethyl alcohol. It was mentioned that samples can be stored for years without the need for any other softening treatments [67]. Softening was also achieved by storing blocks of hard wood in commercial formalin for a week or longer [44, 88]. Softening wood with hot 95 % alcohol was also reported [55, 88]. A reagent called "diaphanol" (a chitin softening agent) has also been reported to be useful in softening wood [32]. The use of Contrad 70 (a liquid detergent) to soften wood was also reported [3].

22.4 Stem Sectioning

22.4.1 Reducing Cutting Artifacts, Non-Newtonian Fluid, and Other Techniques

Making thin sections of woody stem samples like accurately dating tree rings is not as easy as it sounds. It requires considerable skill and experience and also the best microtomes and blades. Even in the best condition, making thin sections of woody stem material will more than often produce cutting artifacts (Fig. 22.4). These refer to minute dislocations in the structure of the cell wall that follows mechanical

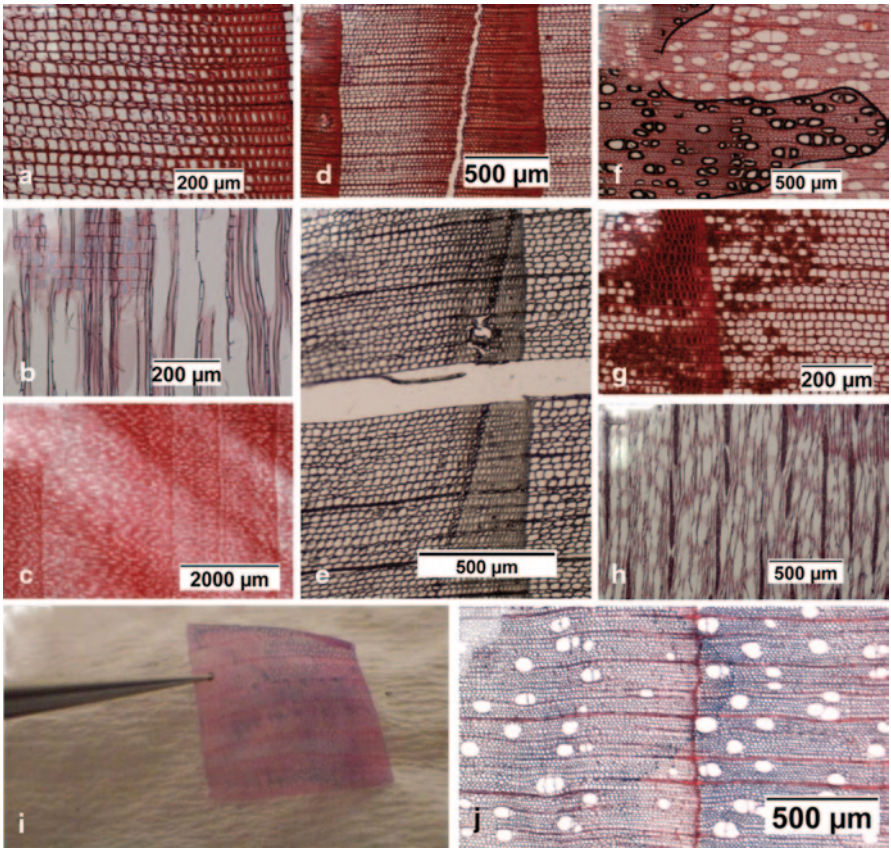

Fig. 22.4 Various sectioning and preparation artifacts. **a** Detached secondary walls in earlywood tracheids, **b** broken fibers in radial view, **c** variations in section thickness, **d** split section, **e** blade trace, **f** air bubbles having been drawn under the coverslip, **g** inadequate rinsing after using the non-Newtonian (Cornstartch-Water-Glycerin) CWG fluid, **h** broken fibers in a tangential view, and **i and j** staining problem associated with Fast Green being washed out by the clearing solvent resulting in uneven staining of the section

failure of wood under stress [89]. Many artifacts may occur during the mechanical sectioning of wood [6, 9, 90, 91]. When sectioning coniferous wood, it is frequently observed that the secondary wall, especially of earlywood cells, breaks or separates from the primary wall and bends into the cell lumen (Fig. 22.4a). Soft non-lignified cells (i.e., cambial cells, differencing xylem cells, parenchyma cells) may also be squeezed, torn, and distorted [10, 72]. Sectioning artifacts constitute a problem when cell measurements are to be obtained and time-consuming corrections often need to be applied manually to digital images to avoid generating erroneous data [66, 92].

Potential sectioning artifacts are associated to a certain degree with the specimen itself, cell wall density, wood block size, blade type and sharpness, blade angles, and human dexterity/experience. Using a sharp blade will help to reduce splitting of the primary walls in conifers and using the full length of the blade (slicing effect) will reduce breakage of the secondary walls [9]. To further minimize sectioning artifacts in un-embedded samples, it may be indicated to apply a non-Newtonian fluid to stabilize the cells during sectioning [10]. The basic principle is to fill the open wood cells with a solution (fluid phase) that hardens instantly (solid phase) as pressure is exerted by the cutting blade. The non-Newtonian CWG fluid (10 g of cornstarch, 8 mL of water, and 7 g of glycerin) is applied to the wood block with a fine brush prior to sectioning. The consistency of the preparation is adequate when the paste "crumbles" when stirred and "flows" when at rest [9]. The stabilizing effect of the CWG fluid enables cutting the wooden material as if it would have no pores making it comparable to embedded specimens [10]. It must however be noted that the CWG fluid has no cohesive properties and will not be beneficial for friable or fragmented samples. We successfully adopted this simple procedure (CWG fluid) when sectioning transverse sections. After sectioning, thin sections need to be thoroughly washed in dH_2O to remove starch grains thus avoiding contamination of the final slide (Fig. 22.4g).

At times, it may be necessary to use fluids with cohesive properties to allow proper sectioning of friable un-embedded material. It was suggested to apply a 2% solution of celloidin (in equal part of anhydrous ether and methyl alcohol) to the wood surface when sectioning to better support the tissues [30, 38, 39]. The celloidin may be removed by immersing the thin sections in acetone. A 0.5% solution of celloidin in alcohol, ether, or acetone was also recommended [49]. The use of polyvinyl lactophenol for embedding brittle specimens was also proposed [93]. The brittle wood samples acquire a soft pliable rubber-like nature after having been immersed and warmed gently for about 30 min, drained and allowed to cool for 24 h prior to sectioning. The application of a two-component adhesive (e.g., epoxy resins) on brittle samples and charcoals has also been used [9, 14]. The adhesive fills the cavities and polymerizes within 30 min. Combining the two resins in various quantities also allows controlling the final hardness.

A small piece of transparent self-adhesive tape (e.g., scotch tape) may also be attached to the sectioned surface prior to sectioning to maintain the section cohesion [49, 50, 94]. Cellulose tape was also utilized [95]. With severely decayed samples, removal of the tape without further sample deterioration may be difficult and both acetone [58] and xylene [95] were used for tape removal. The use of a

liquid polymer plastic substance like Nobecutan to coat the longitudinal sections prior to cutting was also proposed [94]. In our laboratory, we often will use 3M spray bandage or Nexcare 3M skin crack care (Fig. 22.2 top) with fragile and brittle stem samples. The spray bandage provides a protectant shield and may be used with lightly "damaged" surface. Skin crack care may be used for more severely "damaged" samples (i.e., severe frost ring, traumatic resin ducts) as the product contains silicone and acts as a sealant. The product is applied directly on the wood surface before sectioning and will take about 2–3 min to dry. The excess alcohol or dH_2O is first slightly drained from the wood surface with an absorbent paper to allow the product to penetrate the sample. The application of the product will be repeated after each sectioning. It was also recently proposed to use a polystyrene foam solution as an anti-tearing agent to be applied on the sample prior to cutting with a microtome [50].

22.4.2 Microtome Types

The two main techniques used today to obtain thin sections of stem material are either freehand sectioning or sectioning with a microtome. Freehand sectioning with a razor blade or with a hand-microtome device remains among the simplest ways of producing thin sections ([12, 45, 46], also *see* Chap. 1). These authors provide abundant information on the use of razor blades for freehand sectioning of wood material. One of the problems however concerns the inability to control the thickness and quality from one section to the next [44, 51, 67, 91]. This problem of constant thickness is partially resolved by the use of mechanical microtomes, which allows thin sections of a predetermined thickness (usually 5–30 µm) to be repeatedly made.

Microtomes are precision-mechanical instruments. Depending on their type, the sample holder and/or the knife holder can be either fixed or adjustable and an automatic feeding mechanism may be coupled with the cutting action. Generally, the microtomes used for wood anatomy are of two types: rotary or sliding. Freezing or cryostat sectioning has been advantageously used with small, fragile, or severely decayed material (e.g., [13, 58, 86, 96]) but will not be covered here. A rotary microtome is usually recommended for material that is not too hard [46]. A disadvantage of the rotary microtome is that the blade holder is fixed at 90° (perpendicular) to the direction of cut. In the absence of embedding, cutting artifacts associated with structure compression may occur because the blade is not slicing but planning the sample [11].

The sliding microtome (Fig. 22.2 bottom) has been a standard instrument for sectioning wood samples. The ability to set the blade obliquely to the axis of movement allows for a greater portion of the knife to be used when sectioning hard material. In recent years, new microtome models were developed for wood anatomical studies. The GSL1-microtome (Fig. 22.2 bottom left) was precisely designed for the production of thin sections for tree-ring analysis and general wood anatomy [11]. It has a special sample holder for increment cores, standard samples, and micro-cores. The fixed position of the sample holder may constitute a disadvantage, however as fine

tuning of the sample orientation is not possible. In contrast to standard sliding microtome, the GSL1 blade angle is fixed but the cutting angle (slicing) remains adjustable. The WSL Core-microtome was developed to ease the preparation of the surface of increment cores for automatic image analysis of tree-ring anatomical features [8]. The WSL Lab-microtome is a full sliding microtome designed to be an upgraded version of the discontinued Reichert OmE sliding microtome [9]. In contrast to most sliding microtomes, it has like the GLS1 a fixed-sledge guidance system with no internal play allowing great stability when cutting wood sections. In our laboratory we routinely use a GLS1 microtome as well as a Euromex small sliding microtome (Fig. 22.2 bottom right) and a Leitz Wetzlar Minot 1212 rotary microtome.

22.4.3 Blade Types and Microtome Maintenance

The current trend in wood microtomy is to move away from conventional microtome knives to the benefit of disposable blades. If a conventional knife is used, it is recommended that a type-C knife be used for wood sectioning [31, 46, 49, 94]. The main disadvantage with conventional knife lies in the sharpening process and ample information on sharpening can be found in botanical microtechnique publications (e.g., [6, 37, 41, 46, 94]). In contrast, disposable blades do not require sharpening and can constitute a cheap alternative. These blades (S35 Feather type), are essentially refined razor blades held in a modified microtome knife holder. It was also proposed to use NT-Cutter knife blades (A-types, 0.38) for microtome sectioning of wood [97]. The GLS1 microtome [11] and the WSL microtome [9] were developed with a special blade holder allowing the use of NT-cutter knife blades. The NT-cutter knife blade however is not compatible with the modified microtome knife holder (S35 Feather type) as the NT-cutter blade is wider than the S35 feather blade.

When working with wood, no matter if a conventional microtome knife, disposable blades, or razor blades are used, it is essential that they be well sharpened. Woody tissues will quickly blunt whatever blade is used [51]. Obtaining quality thin sections depends on keeping the blade absolutely free from nicks, scratches and always extremely sharp. The microtome used to section wood should always be properly maintained. It is essential when using a sliding microtome that the track of the knife carriage be cleaned and properly oiled so the blade mechanism slides smoothly. A microtome should be thoroughly cleaned, dried, and properly lubricated after each usage. When not in use it should also be stored in a dust-free environment [45]. Microtomes are not all rust proof and the practitioner will not save time by not planning for proper cleaning, drying, and lubricating after each work session.

22.4.4 Sectioning Procedures

Making thin sections with a sliding microtome is not easy and a certain degree of dexterity and experimentation is required. The procedures described below may need to be altered depending on the specific microtome or material being sectioned

as each wood specimen, each microtome, and each blade are unique. For example, the GSL1 microtome offers no possibility to adjust the blade angle and sample holder (Fig. 22.5), a rotary microtome will not allow adjusting the blade holder (cutting angle) for a slicing effect, and a standard sliding microtome may offer maximum flexibility. The practitioner needs to become familiar with the microtome and the material to be cut and he/she should not be afraid to sacrifice a few days, extra samples, and a few disposable blades to purposely make sectioning errors (Fig. 22.4) and correct them. Practical exercises should be systematic with good record keeping. For example, what are the consequences of a cutting motion that is too slow or too fast? What are the consequences of having a blade angle that is too obtuse or acute or a cutting angle that is too narrow or wide (slicing effect)? How is sectioning affected by varying the thickness of the sections and how do thin sections be compared under a microscope? Each time we start a new project, we take some time to initially explore and re-assess various techniques given the specificity of the research questions and the material to be sectioned.

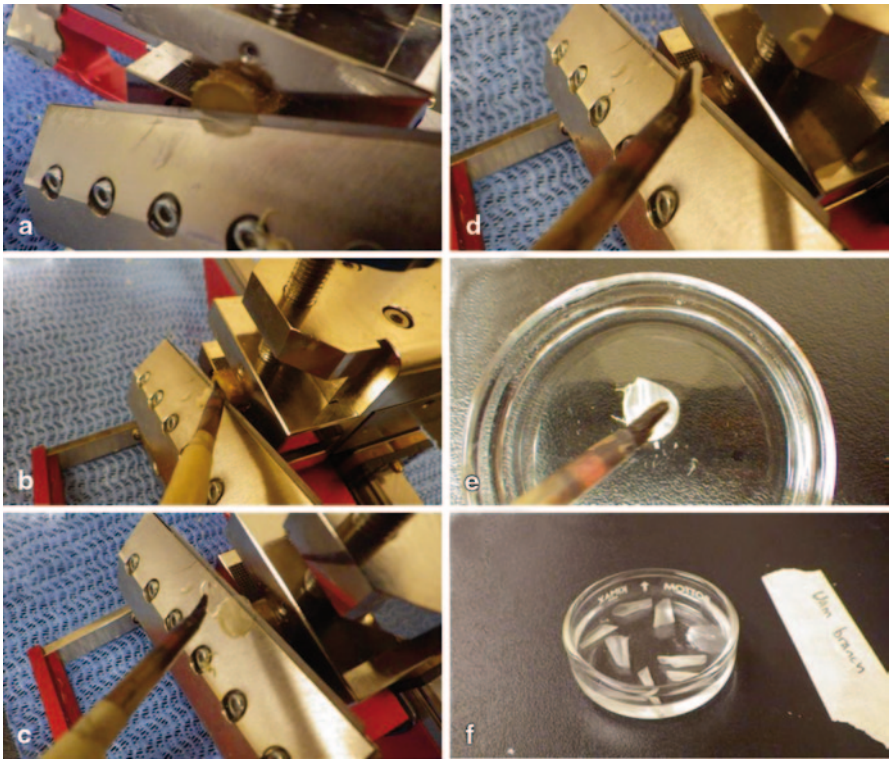

Fig. 22.5 Sectioning of a stem with the GLS1 microtome (**a**–**f**). The thin section is floated on the blade with a soft brush and deposited in a Petri dish filled with distilled water. A label is affixed to the dish using masking tape and will identify the thin sections from staining to mounting

22.4.4.1 Cutting Fluid

One basic sectioning procedure that applies no matter the microtome or the material being sectioned is the use of a cutting fluid. During sectioning, a cutting fluid is applied with a fine brush to constantly keep both the sample and the edge of the blade wet. The cutting fluid allows softening or hardening of the sample and also helps to float and slide the section over the blade when sectioning (Fig. 22.5). If sectioning is interrupted for a few minutes (e.g., pause santé), the sample should be kept impregnated with the cutting fluid [38]. If a waterproof piece of adhesive (scotch tape or spray bandage) is used to stabilize a fragile sample, only the blade may be wet to ease sectioning and section manipulation.

Cutting fluids usually include dH_2O or alcohol in combination with glycerin [45]. The mixture will vary according to the need to soften or harden the material being sectioned. Distilled water (or tap water if appropriate) should be used with all material as it increases cell wall flexibility preventing them from breaking during cutting [9]. In contrast, mixtures with alcohol may be preferred for hardening effects and may help prevent very soft thin sections from tearing. Alcohols 40–70 % have been commonly used. Stronger alcohol concentration may be used with very soft material [9]. Various mixtures of alcohol and glycerin have also been proposed with glycerin having been favored when curling is excessive. Both 50/50 mixture of alcohol and glycerin and a solution of 15 % glycerin and 95 % alcohol have been commonly used. Alternatively, water with added gelatin (0.1–0.5 % solutions in water) was advocated to allow sections to be easily unrolled with a fine brush when sectioning [98]. Adding gelatin has the effect of reducing surface tension with the solution spreading evenly keeping the blade moistened.

22.4.4.2 Clamping the Sample

Before proceeding with sectioning, the microtome should be firmly positioned on the worktable. If the lightweight GLS1 microtome is used, a nonslip rubber mat may be placed underneath it (Fig. 22.2 bottom). The practitioner should also make sure that the microtome is well lubricated and that all mobile parts are moving freely and well adjusted. The knife or removable blade should be properly installed (be cautious, these are extremely sharp...). It is important that the blade be firmly secured in its holder and that all clamping screws and clamping levers be tight.

Once a wood block is selected and decision has been made regarding the surface to be cut, it needs to be clamped on the sample holder assuring it will protrude just enough to assure optimal stability during sectioning (Fig. 22.5). The block must be clamped firmly enough so it does not move but not too tightly so it gets compressed and damaged. Adequate clearance between the holder and the knife is also needed to avoid damaging the blade. According to the clamp types, it may be advisable to hold the block between thin pieces of cork or rubber to avoid damaging the planes not being sectioned (radial, tangential or transversal). This procedure reduces damage to the block done by the clamp teeth when pressure is exerted. Alternatively, it may be desirable to use a different block for each section plane.

If transversal, radial, and tangential sections are needed, it may be advisable to first cut the transversal section followed by the two longitudinal ones. A new sharp blade may be used to cut the transverse section which has the greatest resistance to cutting. In contrast, it was also suggested to start with the longitudinal cuts [42]. For longitudinal cuts, the sample does not need to be clamped as tight thus reducing potential damages to the other surfaces. At times, it may also be necessary when doing the longitudinal cuts to slightly tilt the block (if feasible) because radial and tangential sections in porous species are liable to split along the vessels and it may be necessary to make a slightly oblique cut if splitting is a problem [40].

22.4.4.3 Block Orientation and Angles

More than a century ago, it was written that part of the success of working a microtome depends in regulating the angles at which the knife cuts, and in orienting the surface of the block in accordance with the hardness and structure of the wood [43]. This remains true today. Depending on the microtome type, it may be feasible to adjust the position of the sample, the blade angle, and the cutting angle (slicing effect). Determining the optimum setting for these parameters is largely a matter of trial-and-error. As a general statement, a blade in an excessively flat position (low inclination) will cause mechanical damages such as compression and cell wall dislocation whereas a blade with an excessively steep inclination (high inclination) may make the edge of the knife to vibrate causing the section to chatter and scratches to be observed (e.g. [89, 94]). Too low of an angle may also damage the surface of the sample during the return stroke whereas too steep of an angle can increase curling. A balance must thus be found between these extremes. Depending on the material (soft or hard wood, transverse, radial, or tangential cut) and the desired section thickness, the angles may also need to be modified for optimal results.

Starting with the sample position, various recommendations have been made regarding the optimal positioning in regard to the cutting blade. If samples have bark (e.g., small fresh stem), the transverse cut should start from the bark toward the inside to prevent the bark from breaking off. It was also recommended that when sectioning soft wood the knife should first meet the earlywood whereas for hard wood it should first meet the latewood [37, 45, 67]. It was recommended that when making transversal sections, the sample should be clamped so that the wood rays run parallel to the direction of blade travel for best results [30, 37]. In contrast with difficult material, it may be advantageous to orient the block with the rays running perpendicular to the direction of blade travel for best results [48]. For longitudinal sections, it was suggested that the block should be placed so the fibers lie perpendicular to the direction of cut with a cutting angle of about 15° [98]. In our laboratory, we will often start a project by making various systematic trials in which the blade will cut the sample from various transversal directions (i.e., earlywood to latewood, latewood to earlywood, sideways perpendicular to the rays or obliquely) and varying both the blade and the cutting angle (slicing effect). The best orientation may vary given the specificity (density, cells arrangement, etc.) of the material

being sectioned and the practitioner should experiment to determine the optimal positioning of the sample. It should also be noted that the block size will influence sectioning and that reducing the width of the transversal section will ease sectioning while allowing to keep the full length of the sample.

Similarly, the practitioner should start by experimenting with varying the blade angle and the cutting angle (slicing effect) if the microtome allows for it. Using a sliding microtome, it was recommended to start by setting these angles at 10–15° [30], at 15–20° [52], or at 10° (e.g. [29, 36, 37]) and to modify them, if necessary, to prevent sectioning problems like uneven section thicknesses and excessive curling. The GLS1 microtome was developed with a fixed blade angle set at 20° which proved after many years of practice to be the optimized angle for cutting wood and other plant materials [11]. In general, the cutting angle should be set so that as much of the cutting edge is used to get a full slicing action. The practitioner should not be afraid to experiment with blade and cutting angles if the microtome allows as it will provide a sense of what may be the optimal settings for any given material. Depending on the blade and blade holder used (standard, S35 Feather type, or NT-cutter knife blades), these angles will also vary.

22.4.4.4 Trimming to a Flat Surface

Once the wood block is positioned and ready to be sectioned, it will be trimmed to a flat surface. The blade and cutting angles should be adjusted and the blade holder should be moved to the far end of the slide way. It may be advantageous to spare the microtome blade by trimming using (i) an older blade or (ii) a specific portion of the blade (identified with indelible pen). Different portion of the blade can also be used for tangential, radial, and transversal sections [42].

At this point, the sample holder should be visually moved until the surface of the wood block barely touches the blade edge. Make sure all screws are tightly secured to avoid vibrating parts during sectioning and set the microtome screw to a thickness of 15–30 μm. The sample and the blade are wet with the cutting fluid using a fine brush and the block is trimmed until the full wood block surface area is exposed. The partial thin sections originating from trimming will usually be discarded. It is preferable to make numerous thin trimming cuts rather than few very thick cuts to avoid dulling the blade and/or tearing/damaging the sample.

After trimming the sample to a flat surface, the section thickness is reset if needed and the blade is moved to the sharper area (or replaced) for optimal results. A section thickness between 10 and 20 μm is usually satisfactory but it may vary according to the investigation. We usually use 15–20 μm as a starting point. Alternatively, several thin sections of varying thicknesses (6–16 μm) could be made to offer a range for analysis [99]. The practitioner should not be afraid to vary the section thickness as it may often resolve sectioning issues. Thicker sections may tend to curl more whereas thinner sections may show more imperfections. It may also be added that one usually aims at preparing the thinnest section whereas there are instances when a thicker section may be fully advisable [28]. Sections that are

30 µm may be entirely satisfying. At the beginning of a new project, we will often determine the section thickness as part of the practical sessions. Adjust the feed mechanism to give the desired thickness.

22.4.4.5 Making Thin Sections

Following trimming, one or two initial sliding strokes may be done before a complete slice is obtained (especially if section thickness has been modified after trimming). These first sections will normally be discarded. One needs to remember that it takes two consecutive flawless sectioning strokes (cuts) to make one high-quality section [12]. A high-quality section must have flawlessly cut surfaces on both sides. The preliminary surfacing cut is thus no less important than the removal cut.

Using a fine brush in one hand both the sample and blade are wet with the cutting fluid (or non-Newtonian fluid is applied to sample only) and the brush is left lightly resting on the surface of the block while sliding the blade (e.g., Euromex sliding microtome) or the block (e.g., GLS1 microtome) with the other hand (Fig. 22.5). The brush is held on the surface of the block during the cutting strike to prevent curling of the section. The sliding motion should be done with a steady movement. A slow, steady stroke generally gives the best results. Depending on the microtome type (e.g., Euromex sliding microtome), you may need to put a downward pressure on the carrier to assure steadiness in the movement during sectioning. Compared to older sliding microtomes, the WSL-microtomes offer the advantage of having a fixed-sledge guidance system with no internal play allowing great stability when sectioning hard material. The practitioner should exercise until skill is gained at controlling the cutting stroke and manipulating the brush. After the cut, if the wood block is not automatically raised (e.g., GLS1 microtome) it will be raised manually. After each stroke, the wood block and blade are wet again with the cutting fluid.

Once a section is cut, it is floated (glided) with the fine brush to the edge of the blade and transferred to a Petri dish (watch glass or slide) filled with dH_2O (Fig. 22.5e). The same fluid as the cutting fluid should be used. If the non-Newtonian CWG fluid is being used, the sections should be transferred to dH_2O, gently washed to remove starch grains, and then transferred to another Petri dish filled with dH_2O. In order to avoid dulling the blade, wood section should also never be removed from the sharp edge of the blade. Depending on the microtome, the thin section may be slide off the blade with a down movement (upward with GLS1 microtome) to avoid damaging the blade and the brush hair (Fig. 22.5). Tweezers (forceps or needles) should never be used to remove a section from the blade to avoid damaging the section and/or the blade edge.

Sectioning is continued until a few thin sections of good quality are produced. Early in the process the sections should be examined with a stereoscopic microscope to detect any problems and also verify that the sections are really transversal, radial, or tangential. For example, a stereoscopic microscope will allow to quickly assess blade sharpness as a dull blade will produce blade marks and poor-quality sections (Fig. 22.6a and b). If the sections are too thin or too thick the proper adjust-

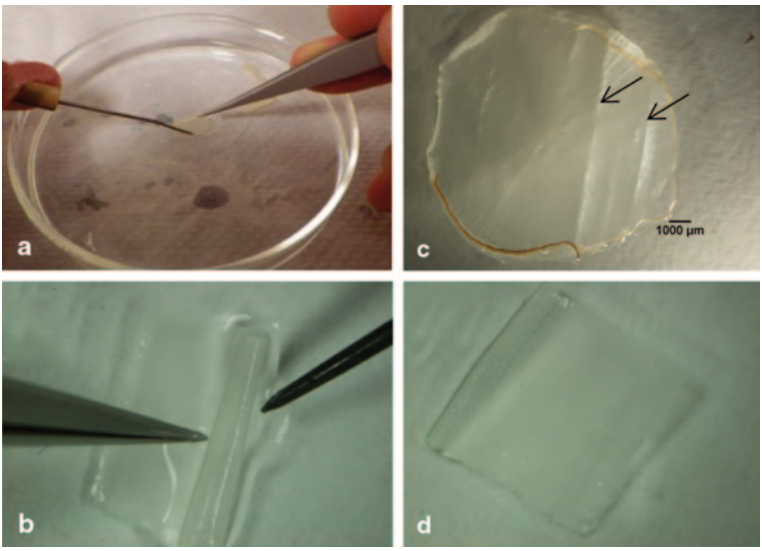

Fig. 22.6 Examining thin sections to assess their quality (**a** and **b**) and uncurling a section under a stereoscopic microscope (**c** and **d**). The section in picture **b** shows numerous blade marks and depending on the project it could be kept or discarded. The curled section **c** is carefully unfolded using a pair of fine-point tweezers and an angled dissecting needle

ment can also be made. A portable lamp that reflects off the top of the block may also be used to help quickly assess thin section quality and imperfections such as scratches [38].

As the thin sections are cut, it is crucial to assure their proper identification and it should be carried at all steps until final labeling of the mounted preparation on a slide. Once an adequate number of sections are made from a given wood block and plane (usually 4–5 good sections or more if several staining procedures are to be tested), they are stored in a Petri dish. A stereoscopic microscope can be used to select the best sections which can also be placed in small strainer baskets (3–4 sections) for subsequent treatment [47]. If sections are directly deposited on a slide where the staining, dehydrating, clearing, and mounting steps will be performed, a label can be added to the slide (white border of frosted glass) with an indelible pen or a soft pencil. It is important that the label not be dissolved during staining and dehydration.

In our laboratory, we commonly use a small piece of masking tape to identify the Petri dish in which the thin sections are deposited (Fig. 22.5f). Sections can then be set aside for a certain period or directly stained and/or mounted. Transverse, radial, and tangential sections from a given sample can easily be recognized and stored together until staining. We will often make thin sections in a given day and stain them the next day. The work flow will however vary according to the project being conducted. A few drops of alcohol 70 % or a few drops of Javex may be added to the dH_2O if sections are put aside for more than a few hours. Alternatively, sections can

be left in a glycerin–water (1:1) solution for hours or days until staining. A coverslip may also be dropped onto the sections and they can remain even for months before getting stained (H. Gärtner, personal comm. 2014).

22.4.4.6 Curling and Other Artifacts

When making thin sections of wood, a small amount of curling of the section is expected (Figs. 22.5f, 22.6c). Curling may however at times become an issue. Excessive curling may indicate inadequate softening, an inadequate blade angle, or it may relate to the position of the block in relation to the blade [45]. Thin sections from dense wood exhibit a definite tendency to curl and if not adequately softened they may roll up tightly when placed in water [54]. If the sections curl like springs it usually indicates inadequate softening [46]. Sometimes changing the orientation of the block surface in relation to the blade may help reducing the problem.

If curling is not excessive, one of the first steps may be to simply modify the cutting fluid. For example, if alcohol is used it could be replaced by a 50/50 mixture of alcohol and glycerin to provide further softening. Softly holding a well-moistened fine brush tip on the upper surface of the block during the cutting stroke may also help to reduce curling. A thin section will usually start curling at the beginning of the cut and holding it flat on the blade with the brush may be helpful. Cutting at a slower pace (speed) may also ease controlling the section while it is being sectioned. Interrupting the cutting action and uncurling the section prior to finishing the cut may also be helpful [46]. To alleviate the curling problem, it was also suggested that an incomplete thick cut (60–100 μm and solely attached by a corner) be made followed by a normal one allowing the thin section to remain attached to the thicker section until final mounting [38].

Once cut, curled sections may be uncurled and flattened with the fine brush, wet with glycerin and allowed to remain on the upper blade portion for a minute or so before transferring to the Petri dish with dH_2O. Alternatively they may also be straightened out by dragging them over the edge of a slide partly immersed in 50 % alcohol. It was also proposed that curled sections be transferred onto a finger and folded at right angles to the rays before being transferred to a Petri dish [40] or curl face down on a slide flooded with a thin film of glycerin–alcohol [39]. Once stacked, the uncurled sections are pressed down with another slide and the slides transferred in a Petri dish filled with dH_2O with a lead weight on top [39]. Uncurled sections may also be placed between two glass slides and left drying before staining [19]. It was also suggested to place several uncurled sections between glass slides held by paper clips and to heat the bundle in dH_2O for several hours [54]. Round sections with bark (e.g., roots, small branches, stems of shrubs, or herbs) always tend to curl because the bark exerts tension. On such sections, a razor blade could be used to cut the bark (scar it, not cut it off) at about three locations. This procedure will allow the section to widen again, the curling to disappear, and the potentially covered cambium to become visible again (H. Gärtner, personal comm. 2014).

During the dehydration process that follows staining (*see* Sect. 22.5.3), thin sections may start to curl again as water is removed. Thin sections may also become more brittle and unrolling them without breaking them may be a difficult task. It was proposed to put previously uncurled thin sections between wired mesh screen slides during staining and dehydrating [100]. In our laboratory, if sections curl, they will be stored in dH_2O with glycerin to allow them to soften more. At the staining stage, we will gently slide the sections in a watch glass and uncurl (unroll) them with a pair of fine tweezers using a stereoscopic microscope (Fig. 22.6c and d). Once the sections are uncurled they can stay flat onto the watch glass until they are stained as long as they remain wet (not soaking).

When a large number of sections are being cut, it is advisable to examine them under the microscope and only select the best ones for the staining process. Generally, thin sections that show irregularities or are of poor quality can often be traced back to the sample quality or uneven softening, a dull blade, blade imperfections, the blade angle, or the position of the sample. For example, a dull blade may simply disintegrate a friable sample during sectioning. Scratches on a thin section may reflect the nicks on the blade (Fig. 22.6b). Many of the references provided at the end of this chapter provide information on how to deal with curling sections and other sectioning problems (e.g., [3, 9, 34, 39, 45, 46, 49]).

22.5 Staining of Thin Sections

The purpose of staining thin sections is to enhance the contrast (differentiate) among various cell types and/or cell constituents—each stain being specific to a special feature. The number of stains and staining recipes that exist for botanical material is enormous. Despite this variability, the staining procedure is probably the easiest stage in the preparation for light microscopy. Among the available stains safranin O, astra blue, Fast Green FCF, and aniline blue are among the most important for wood anatomists. These widely utilized stains also call for minimum reagents and techniques. In many situations, safranin O may be the only stain required (Fig. 22.7) to enhance contrast prior to image acquisition and measurement of various anatomical features [63, 64, 92, 99]. It should be noted that when preparing a staining solution, proper safety protocols must always be followed as powders may be inhaled and stain solutions may be irritating to the skin [9]. We also suggest that staining solutions always be filtered to avoid the precipitate contaminating the sections (Fig. 22.8a–c).

Staining thin sections is a relatively simple procedure. A few approaches are generally used. Either staining is carried directly on the mounting slide, in a watch glass, or the sections are transferred from one solution to the next. Staining can be done directly on a slide with the staining solutions being directly trickled onto the section and the runoff collected in a Petri dish [31]. A pipette filled with the stain solution is used, followed by dehydration using pipettes filled with various grade

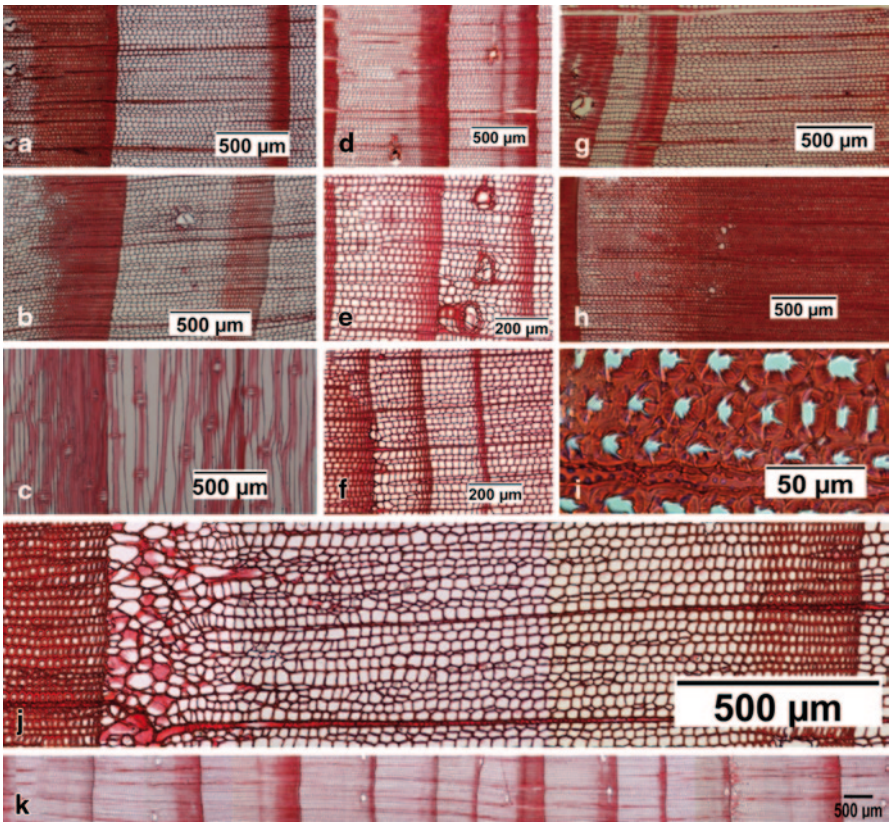

Fig. 22.7 Coniferous thin sections showing tree-rings with or without anomalies and stained in aqueous safranin only. **a** thin latewood, **b** and **c** pale latewood in both transversal and radial view, **d** false ring, **e** resin ducts, **f** frost ring followed by a white earlywood ring, **g** white earlywood ring, **h** and **i** compression wood, **j** frost ring, and **k** thin section containing numerous tree rings

of alcohol [9]. Glass dishes may be preferable to plastic ones depending on the reagents used to prepare the stain, dehydrate, and clear the sections. This approach is convenient when thin sections are cut and directly processed to final mounting [99]. In contrast, if thin sections are not to be immediately mounted, we will carry out the same procedures in a single watch glass to also avoid unnecessary handling of the sections and minimize transfer from one solution to the next (Fig. 22.8d and e). The thin sections will only be transferred to a slide at the mounting stage.

Staining of thin sections will normally be integrated with dehydration, clearing, and mounting. Staining and dehydrating bottles as well as the clearing solvent and mounting medium should be properly labeled and logically disposed in the work area to facilitate the work flow. Having access to a stereoscopic microscope will help to quickly assess the quality of the previously cut sections and to select those to be stained (Fig. 22.6a). It will also be used to assess staining uniformity and proper

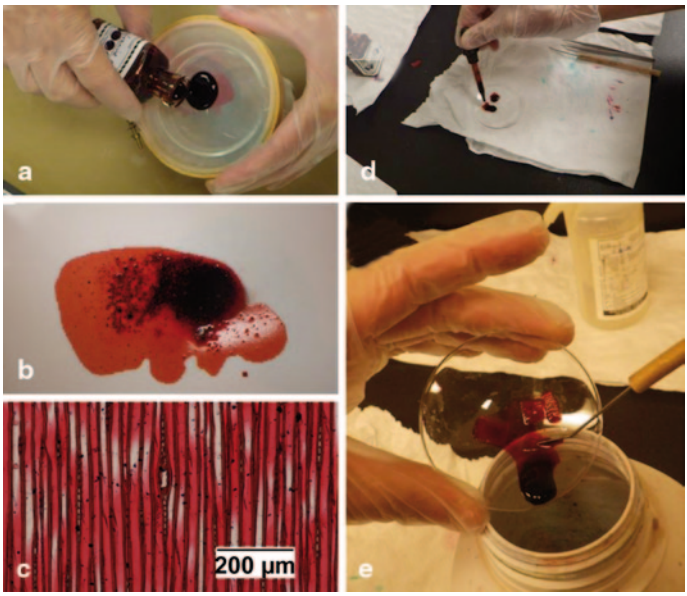

Fig. 22.8 Filtering a 1 % aqueous safranin O solution to remove precipitate (**a** and **b**). A 160 µm mesh is used. The bottom picture (**c**) illustrates a section stained with unfiltered solution and safranin precipitates can be observed. Staining multiple thin sections with aqueous safranin (**d**) and discarding excess stain from the watch glass (**e**)

rinsing. Usually many sections (those to be mounted together) are stained at once. Once the staining procedures are mastered, one will develop their own routine to optimize time and effort. During the staining, dehydrating, and clearing procedures, the sections may be manipulated using a fine brush. Low-quality tweezers and forceps should not be used as they may break or damage the sections. In our laboratory, we stain, dehydrate, and clear the sections directly in a watch glass (and we use the angled portion of a dissecting angled needle to gently hold the sections (Fig. 22.8d and e). We will then use Dumoxel fine tip tweezers (11231–30 by Dumont) to delicately transfer the sections and precisely place them on a slide.

The practitioner should master the main processes involved in staining thin sections after reading this section. He/she is encouraged to experiment to determine what variations may be possible and which may improve results. It requires trial and error to get the proper timing of each stain [51]. In the case of safranin, the staining time in the literature may vary from a few minutes up to 24 h. The practitioner with specific needs may refer to botanical microtechnique publications for more information (e.g., [3, 9, 17, 30, 34, 35, 45, 46, 48, 51]). Specific staining procedures associated with xylogenesis can also be found in Chap. 24.

22.5.1 Bleaching of Sections

Prior to staining, thin sections may need to go through a short bleaching treatment. This procedure may be used to remove cell contents like phenolic substances, gums, resins, and tyloses that may hinder the observation under the microscope and measurement after image acquisition. Bleaching was reported to increase section brightness [47] and cell wall contrast [9]. Thin sections (previously stored in dH_2O or another solution) are transferred to a watch glass (that will be used for staining) and immersed in household bleach (sodium hypochlorite or potassium hypochlorite). The time may vary but usually 5 min is sufficient. The sections are then washed in dH_2O until the odor disappears. Care should be taken not to ruin the brush by getting it into the bleach. Immersion should also not be prolonged (seconds to ~5 min) to avoid denaturing the tissues. Household bleach will remove some lignin whereas alcohol will help to dissolve the abundant resin in species such as pine. In our laboratory, we seldom use bleaching as it tends to degrade already delicate sections, especially if the bleaching treatment is too long.

22.5.2 Safranin O, Astra Blue, Fast Green FCF, and Aniline Blue

Safranin O is a classic stain used in wood anatomy (Fig. 22.7). It is a red dye soluble in either water or alcohol and routinely used to stain lignified cells and structures. Aqueous safranin solution may be reused several times and should be filtered (Fig. 22.8) to remove debris prior to reuse. Safranin is a regressive dye. When using a regressive dye, the sections can be left for long periods in a strong stain solution until an excess of dye has been taken. This excess will be later extracted [3, 7]. After staining with aqueous (alcoholic) safranin, excess stain will need to be removed by rinsing with dH_2O (alcohol). In our laboratory, we routinely use a 1 % aqueous solution of safranin O (1 g of powdered safranin in 100 mL of dH_2O). Other solutions may also be used (0.8 g of safranin O in 100 mL of dH_2O [9], or 1 g of safranin in 50 mL of 95 % alcohol and 50 mL of water [44]). Using a 0.1 % solution of safranin O in 60 % alcohol was also advocated to avoid over-staining and reduce de-staining time [101].

In numerous situations, a counterstain to safranin O may be used (Figs. 22.9 a–c, 22.10). Depending on the project, it may be useful to differentiate between lignified and un-lignified tissues. Among the most commonly used counterstains are astra blue and Fast Green FCF [102]. Astra Blue is a regressive dye that has affinity to cellulose. It is soluble in either water or alcohol. As a counterstain to safranin O it has been used to identify early stages of delignification in wood. Safranin will stain lignin regardless of whether cellulose is present whereas astra blue will stain cellulose only in the absence of lignin [103]. Both stains have also been used in aqueous

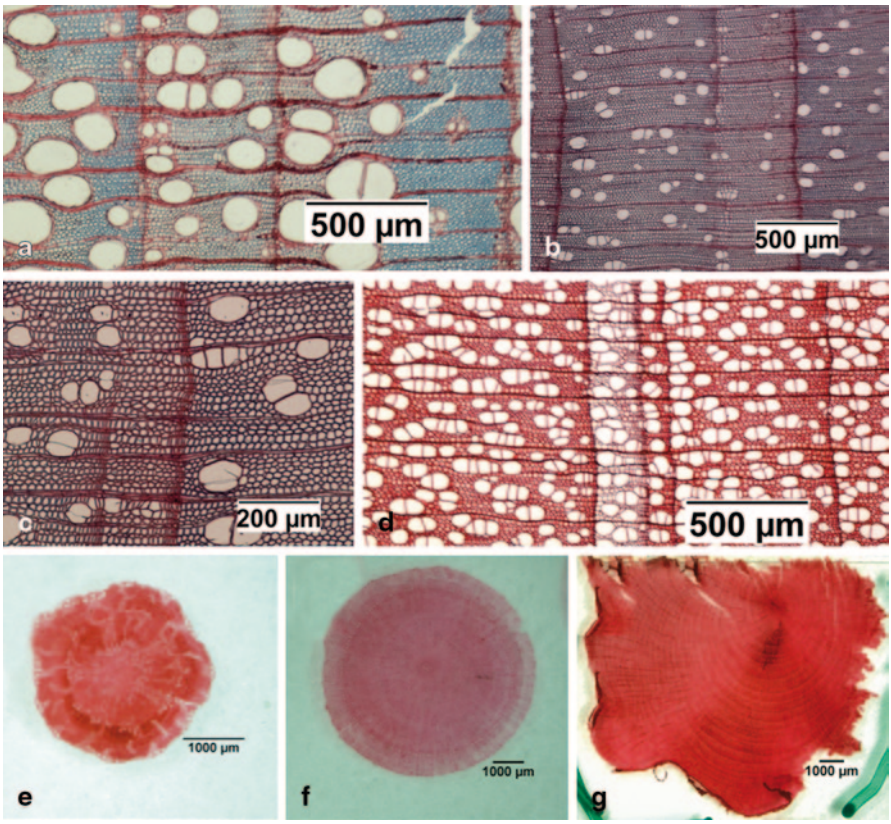

Fig. 22.9 Deciduous thin sections showing various tree-ring with or whitout anomalies. **a** *Fraxinus nigra*, **b**–**c** *Betula papyrifera* with a false ring, **d** *Populus tremuloides* with a white ring showing a lack of lignification as indicated by reduced safranin staining, **e** *Quercus macrocarpa*, **f** *Fraxinus pennsylvanica* and **g** *Dryas integrifolia*

[96] and alcoholic [104] solutions to visualize the degree of lignification in woody tissues. This double staining was also used to document cell wall lignification in vessels of trembling aspen (*Populus tremuloides* Michx.) trees in relation to insect defoliation [66]. Tension wood can also be nicely observed with lignified walls being colored red and the gelatinous layer (G-layer) rich in cellulose being colored blue (Fig. 22.10a and b).

In our laboratory, we routinely use a 1% aqueous solution of astra blue (1 g of powdered astra blue in 100 mL of dH_2O + 2 mL of tartaric acid). In this recipe, acetic acid can be substituted for tartaric acid [31]. Slight variations in the preparation or in the application of astra blue solution exist. For example, it was proposed to apply a drop of astra blue solution (0.5 g astra blue in 100 mL of aqueous 2% (w/w) tartaric acid) to thin sections followed by heat for 10 s at ~60 °C before washing the excess stain [51].

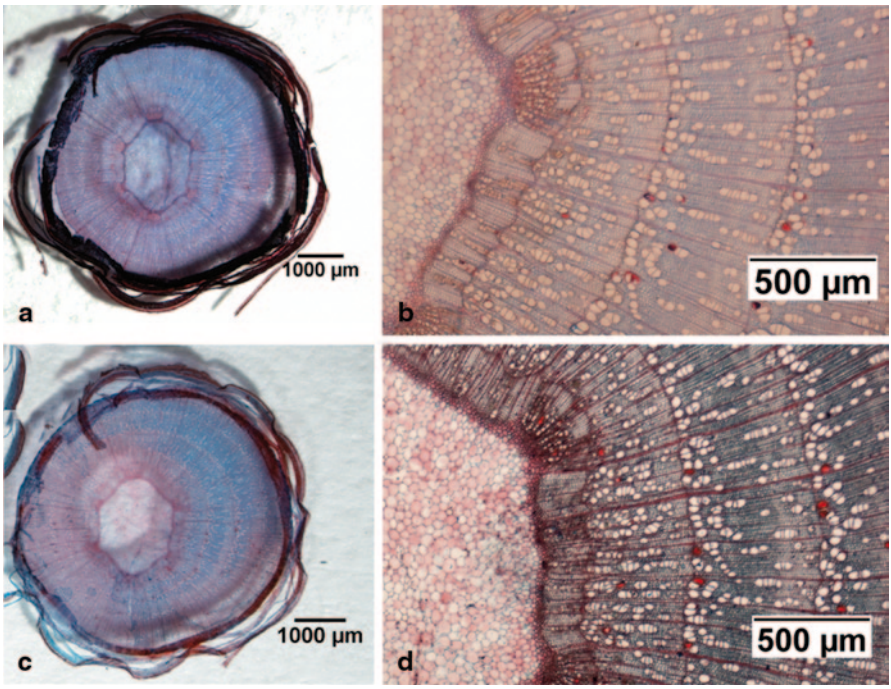

Fig. 22.10 Transverse thin sections of *Acer negundo* showing tension wood as revealed by both astra blue (**a** and **b**) and Fast Green (**c** and **d**)

To ease the staining procedures, simultaneous staining of the thin sections can be done by combining both aqueous safranin and astra blue solutions in equal proportion with a staining time of 3–5 min [31]. We commonly use this approach and mix together 1 % aqueous safranin and 1 % aqueous astra blue solutions in equal proportion. Others have proposed a safranin–astra blue mixture (40 mg safranin and 150 mg astra blue to a solution of 100 mL demineralized water with 2 mL acetic acid) with a 10 min staining time [105]. Simultaneous staining with astra blue was also used [106] but safranin O was replaced by Basacryl Brillant Rot BG (10 mg Basacryl-Brillant-Rot BG (C.I. basic violet 16), from BASF, 100 mg astra blue FM and 100 mL dH_2O). The authors claimed that contrary to safranin, Basacryl Brillant Rot BG did not form a precipitate and that it allowed for the use of fluorescence microscopy.

Fast Green FCF is another commonly used counterstain to safranin O (Fig. 22.10c and d). It is also soluble in either water or alcohol. Contrary to the previous two stains, Fast Green is a progressive dye. In progressive staining, the section is immersed in the staining solution (usually a dilute solution) until they are stained to the desired intensity and removed [7]. It is thus recommended [35] to make a weak Fast Green solution (1 % aqueous or 0.1 % alcoholic). In our laboratory, we routinely use a filtered 0.5 % alcoholic solution of Fast Green (0.5 g of powdered Fast Green FCF in a total volume of 100 mL of 95 % alcohol). Extra care should be taken

when staining with Fast Green as the stain may seep when exposed to the clearing solvent leading to uneven staining (Fig. 22.4i and j). A double staining procedure using both alcoholic safranin O and Fast Green solutions may also be used [101]. Both safranin and Fast Green FCF were also combined to differentiate hyphae in tissues of 13 species of soft and hard woods [107]. The authors prepared the stain by dissolving with shaking 0.5 g Fast Green FCF and 1.5 g of safranin O in 200 mL of 60% alcohol with the addition of two drops of concentrated hydrochloric acid. Staining time was 3 min or more. Combining both stains was also used to identify mycelium in bark tissues [108] with the authors modifying the Gram and Jorgenson procedure [107] by omitting the hydrochloric acid and increasing the staining time to 5 min.

Aniline blue is another stain that is often used as a counterstain to safranin O. It was used to differentiate rust fungi in pines [109]. In a study of lignification in balsam fir (*Abies balsamea* (L.) Mill.) and testing over ten staining solutions, very good results were reported using safranin O and aniline blue [110]. A double staining schedule with 1% aqueous safranin O followed by picro aniline blue was used to successfully stain cell walls and both hyphae and bacterial cells [32, 58]. The authors reported that while safranin O and Fast Green were very satisfactory for the identification of wood structure, it performed less well when observing hyphae of decaying fungi. The use of picric-aniline blue was also proposed in the study of xylogenesis and to visualize organic cell contents [9]. The authors also used aniline blue in combination with safranin O-astra blue to study cell lignification and also to stain hyphae, bacteria, and decomposed cell walls.

22.5.3 Staining Schedule Including Dehydration and Clearing

Various staining protocols/schedules have been published. No uniformed one-size-fits recipe exists and the practitioner will need to adapt/modify a specific procedure and make it their own. We describe below the staining, dehydrating, and clearing procedures that we routinely used in our laboratory (Table 22.2). These procedures usually involve processing numerous thin sections in a single day and keeping track of the procedures and of the properly labeled thin sections. When permanently mounting thin sections on slide, they need to be dehydrated. The dehydration procedures contribute not only to eliminate excess stain but are mainly intended to remove water which has a lower refraction index than glass. Water will ultimately be replaced by a mounting medium having a proper refraction index [9].

Dehydration is accomplished by rinsing/washing (or immersing) the stained thin sections through a graded series of alcohols (low concentration to absolute alcohol) and the number and duration of rinsing/washing steps vary with publications. For example, when working with delicate sections it may be necessary, instead of directly starting with 70% alcohol, to add a 50% (or even a 30%) alcohol rinse/wash to avoid drastic shrinkage and distortion of the section. We routinely start with 50% alcohol. Gradually replacing water through a gradual series of increasing

Table 22.2 General staining and dehydrating procedures using safranin O and Fast Green FCF. If a combined staining with an aqueous solution of safranin O-astra blue is used, simply avoid the Fast Green FCF procedure described in step #4

Steps	Procedures
1)	Transfer thin sections to be mounted together into a watch glass and drain-off excess liquid with an absorbent paper or a syringe
2)	Add a few drops of 1 % aqueous safranin O solution to cover the sections and leave for 1–5 min gently agitating the solution to make sure all sections are properly stained. Remove excess stain by gently rinsing the thin sections with dH_2O until the water become colorless
3)	Dehydrate (gently rinse and agitate) the thin sections using a progressive alcohol series (50, 70, and 95 %) for about 1–2 min each
4)	Add a few drops of 0.5 % alcoholic Fast Green FCF solution to cover the sections and leave for 1–3 min gently agitating the solution. Remove excess stain by rinsing with 95 % alcohol for 1 min. We will usually repeat this entire sequence twice. Fast Green is a progressive dye and both its concentration and section exposure should be tested to determine best results [3]
5)	Complete the dehydration of the thin sections by rinsing/agitating the sections in two changes of absolute alcohol for 1–2 min each
6)	If Euparal is the mounting medium, it is possible to directly mount the sections on a clean slide. If using alcoholic Fast Green as a counterstain directly mount the sections at this step. If a combined aqueous safranin O—astra blue solution is used, proceed to the clearing procedure
7)	Rinse/agitate the thin sections in two changes of clearing solvent (such as d—limonene) for 1–2 min prior to mounting. The liquid should run clear. If cloudiness is observed dehydration is incomplete and repeat from step 5
8)	Carefully transfer the sections on a clean slide, drain the excess clearing solvent with an absorbent paper and mount the sections using the preferred mounting media (e.g., Canada balsam, euparal, permount)

concentrations of alcohol to absolute alcohol reduces plasmolysis and distortion of the cells [32]. It was stated that the need to wash the samples in numerous absolute alcohol baths for complete dehydration may have been overemphasized [35]. Both money and time may be saved by using less, so room for experimentation is available. If sections are transferred from one bath to the next, care should be taken not to contaminate the alcohol with stain and water, especially the absolute alcohol. Contamination can easily be avoided by carrying all staining and dehydration procedures directly on a slide or in a watch glass. It was recommended to replace the very hydrophilic absolute alcohol by an anhydrous ethanol solution made of 96 % alcohol mixed with 2,2-dimethoxypropane (95 mL of alcohol 96 % and 5 mL of 2,2-dimethoxypropane [9] or a 10 % solution (90 mL of alcohol 96 % and 10 mL of 2,2-dimethoxypropane; H. Gärtner, personal comm. 2014)). Dehydration is successful when no fogginess (milkiness) is observed when adding the clearing solvent to the dehydrated sections. Fogginess indicates incomplete dehydration and the sections should be taken back to absolute alcohol. It may indicate (i) that samples were not left long enough in absolute alcohol, (ii) that the alcohol is contaminated with water, and/or (iii) that the clearing solvent (e.g., xylene) is old [48].

After dehydration and before permanently mounting a thin section, it will be washed (immersed) in a clearing solvent. This solvent will remove alcohol which does not mix with natural resin such as Canada balsam and other synthetic resins. The clearing solvent may also be used to thin the mounting medium which tends to harden and dry with time. It is important that the clearing solvent be compatible with the mounting medium. Numerous mounting media made of natural and synthetic resins exist and can be used [3, 35]. Canada balsam is one of the most widely used media. It is a natural resin obtained from balsam fir (*Abies balsamea* (L.) Mill.). Numerous other resins such as Euparal, Permount, DPX, Eukit, and Caedax are also used.

The choice of the mounting medium can be emotionally challenging and it bears similarity to the passionate debate regarding the sound quality of tubes versus transistors amplifiers [111]. A journal editor once asked that we eliminate the name of the mounting medium from the methods because it was not Canada balsam. Many researchers consider Canada balsam to be the best and the most permanent mounting resin. One downside of using Canada balsam outside its cost may be that the clearing solvent recommended often xylene. Xylene constitutes a potential occupational hazard [3, 112]—direct skin contact and breathing its vapors should be avoided. Less toxic alternatives to xylene exist, and both clove oil followed by cedar oil have been used instead of xylene to clear woody sections [37, 44]. In contrast to Canada balsam, Euparal resin is often considered a safer alternative as it does not necessitate the use of a clearing solvent. Thin sections can be directly mounted in Euparal from absolute alcohol. In our laboratory we routinely use d-limonene as a clearing solvent prior to mounting. D-limonene is a constituent of citrus peel oils. However it may not be compatible with all mounting media [112] and the authors provide an overview of the pros and cons of d-limonene as a replacement for xylene. In all cases, clearing and mounting procedures should be carried out in a fume hood.

22.6 Mounting, Drying, Labeling, and Storing

22.6.1 Mounting of Sections

Stained, dehydrated, and cleared thin sections will be permanently mounted using undamaged and clean, dry slides and coverslips. It was recommended that slides and coverslips be cleaned before usage even though they look spotless [34]. Decisions on the slide layout, the number of slides per wood sample, and the number of sections to be mounted on each slide should be made before starting the procedure. We usually mount the best 2–3 sections per slide from the initial pool of thin sections to assure that backup sections are available. Both the slide and coverslip sizes will be determined and various dimensions are available. The coverslip used should be of generous size but not of wasteful dimension. It is also not advantageous to try mounting more sections than the coverslip will allow for.

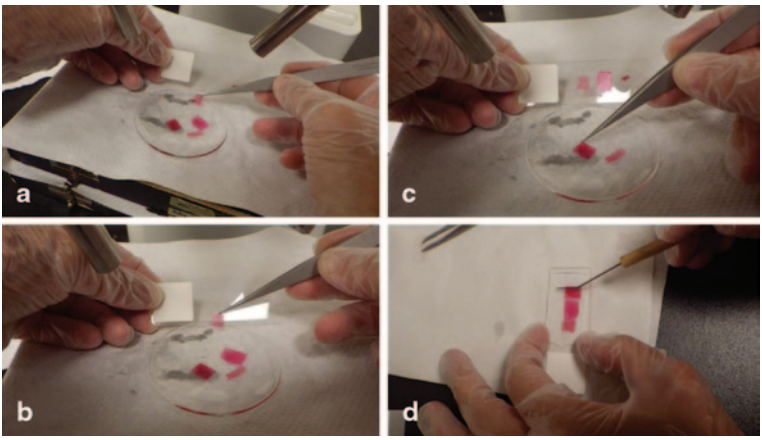

Fig. 22.11 Mounting of thin sections (**a**–**d**). The sections after having been cleared are one by one gently transferred on a slide and positioned after which a few drops of mounting medium will be added and the coverslip affixed

If transversal, radial, and tangential sections are to be mounted on a single slide, the stained sections are transferred from the watch glass to the mounting slide immediately after the clearing stage (Fig. 22.11). A fine brush may be used to transfer the section but we prefer using fine tip Dumoxel tweezers. Alternatively, the mounting procedure may follow clearing of the sections which has taken place directly on a slide. The excess clearing solvent (e.g., xylene, d-limonene) is drained using absorbent paper to avoid unnecessary thinning of the mounting medium. At this stage, the practitioner should work quickly (and with confidence) to avoid letting the sections dry on the slide prior to applying the preferred mounting medium. If the medium is Euparal, the sections may be directly transferred from the watch glass to a slide after dehydration in absolute alcohol and immediately mounted. Euparal has clearing properties and can tolerate traces of water [36].

After draining the excess of clearing solvent, a slight amount of mounting medium is deposited on the sections. Some suggested first adding a minimum quantity of medium directly to the slide to ease positioning the thin sections [29, 46]. With the tip of a glass rod (or a pipette), a few drops of the medium are applied in the center of the sections and allowed to spread a moment. In general 1–3 drops (or a thin line) are sufficient. It was also proposed to apply the mounting medium directly on the coverslip in the form of a small streak or a tick trail according to the surface to be covered [29, 49]. Extra medium may be used with thick sections to reduce the risk of air entering under the coverslip as the preparation dries. Quantity will also be adapted according to the coverslip size and form (rectangular, square, or circular). It is worth mentioning that for very small stem sections a specific technique in which thin sections are stained, dehydrated, and cleared on a small piece of tissue (e.g., Tyvec made by Dupont) and deposited directly on the medium was described [9, 57].

It is also important to assure that the medium maintains optimal viscosity as it will normally slowly dry in the course of the day. Excess thinning by adding clearing solvent will accentuate the formation of air bubbles during the drying process. With a little experience the practitioner will be able to gauge the right amount of medium needed for a given preparation and also to assess when it needs to be thinned and to what degree. Experimenting with mounting medium of various viscosities may be a part of the initial practical work needed to develop skills in preparing stem sections for microscopic examination.

Once the mounting medium is applied, the sections are quickly covered with a coverslip. If the sections curl, they should carefully be straightened with fine tip tweezers in one hand and the angled portion of an angled dissecting needle in the other. To affix the coverslip, the latter is held obliquely (angle of ~30°) and gently lowered so the lower side touches the slide and the other side is slowly lowered until it nearly touches the slide and dropped. Applied in sufficient quantity the mounting medium should spread and form a narrow rim around the edge of the coverslip allowing for some shrinkage to occur when drying. No excess medium should occur around or over the coverslip. In insufficient quantity, an imperfect seal will be created allowing air pockets or bubbles to form and be drawn under the coverslip (Fig. 22.4f) as the solvent evaporates [7, 49]. With the help of a dissecting angled needle (i.e., small wooden handle, eraser head of a pencil), gentle pressure is applied on the coverslip with the angled portion (Fig. 22.11d). This operation is carried out smoothly to help flatten the sections, to allow the medium to spread evenly, and to push air bubbles outside the coverslip. Avoid using the tip of tweezers or dissecting needles as coverslips are fragile and can easily be broken. A drop of mounting medium can also be deposited on the edge of the coverslip if it is in insufficient quantity. If needed, slightly warming the slide will also ease expelling the air bubbles [7, 40]. We usually use a stereoscopic microscope to remove air bubbles and assess the mounting quality. A black background may also help when removing air bubbles [34]. Depending on the project, a few air bubbles may be acceptable whereas in others it may not. It may also be easier to un-cover and re-cover a preparation earlier than later in the mounting process [39]. If air bubbles cannot be expelled, it was suggested to immerse the slide in the clearing solvent until the coverslip and the sections can be slid off and the sections remounted. Procedures on dissolving the mounting medium to salvage or repair broken slides or coverslips have also been described [9].

22.6.2 Drying of Slide

Once mounted, the excess mounting medium should be removed to avoid later cleanup problems. Great care is required when manipulating a freshly prepared slide because the coverslip can easily be dislodged and the tissue damaged. Given that mounted sections may be of uneven thickness it may be necessary to flatten them by placing on the coverslip one or two small lead weights (about 25 g each)

Fig. 22.12 Drying mounted sections on a slide warming plate (**a**) and in a drying stove (**b**). Lightweights or small magnets are deposited on the coverslip during the drying process (**c**). Example of various mounted thin sections and slides labeling (**d**)

or using a weak magnet and an iron plate (Fig. 22.12). This procedure also helps prevent air bubbles forming under the coverslip as the preparation dries. Wooden clothes pegs [36, 37, 40] and modified paper clips [49] have also been used. Excessive compression should be avoided as it may squeeze out too much of the mounting medium favoring the formation of air bubbles underneath the coverslip as the solvent evaporates (Fig. 22.4f).

Once mounted, the slide can be left to dry at room temperature for weeks to months. The process can be hastened by placing the slides at 40–60 °C in a drying oven or on a slide warming plate for many hours, days, or weeks. The recommended drying time as for many of the procedures already presented is highly variable from one publication to the next. Mounting media will dry at different speeds. Placing the slides between two layers of heat resistant plastic prior to drying them on a warming plate was also recommended to avoid gluing the slides onto the warming plate [9]. In our laboratory, we will usually remove the obvious excess mounting medium with absorbent paper after setting the coverslip and wait 1–2 days before we carefully (the medium is not yet fully dried) clean the slide to assure that the lead weights or magnets will not get glued to the coverslip. After this verification, the slides are put back on the warming plate until they are fully dry.

In any cases, the mounting medium should be thoroughly hardened for safe handling of the slides. It is essential to initially store all slides in a horizontal position until the medium is properly hardened. There is nothing worse than finding out many weeks after drying that the coverslip has moved and the storing box is soiled with mounting medium. Extreme precautions should be taken if freshly mounted slides are to be examined under a compound microscope as traces of mounting medium on an objective may be difficult to remove.

22.6.3 Labeling and Storing

Once the slides are properly dried, they are removed from the warming plate or drying oven. They are allowed to cool and only then will the lead weight, magnet, clothes peg, or modified paper clips be carefully removed. At this stage, the mounting medium in warm slides may still be liquid and it remains easy to ruin a slide preparation [9]. Avoid pulling on the small weights (magnets, etc.) as they may be partly glued to the slide and it may draw air into the preparation, break the coverslip, and damage the preparation. A little bit of clearing solvent may be used to detach the weights if they are really hard to be detached, otherwise just gently turn them left to right to detach them from the coverslip. After removing them, proceed with care and scrape the excess dried mounting medium with a razor blade. After removal of the excess medium, we usually clean the slide with a piece of absorbent paper lightly moistened with clearing solvent. We then finally carefully wipe the slide and coverslip with a piece of absorbent paper dampened with alcohol to polish the slide. It was suggested however that chemicals should not be used to remove excess dried mounting media [9, 31].

Once slides are dried and cleaned, a proper label should be applied to each one. Basic labeling should have been carried during all procedures from the sample preparation to the mounting and drying procedures. Proper labeling is crucial and it cannot be stressed enough that thin sections from various origins cannot be mixed-up. At this point, a self-adhesive label may be placed on the left side of the slide. Alternatively permanent ink can be used. In our laboratory, we will usually do the final labeling using polypaper labels one or two days after the slides have been mounted and are drying on the warming plate. Excess mounting medium should have been removed as it may ruin the label. We will also carry the labeling using masking tape pieces fixed beside the slide warmer with explicit information on the position of each of the slides (Fig. 22.12a).

The information provided on each slide label may include a sample ID, collection date, species name, exceptional feature in the preparation (may be marked on the underside of the slide with ink in case an immersion lens should be used), as well as other pertinent details regarding the sample and the investigation (Fig. 22.12d). For example, if a cross-dated tree-ring sequence is mounted, the date of the first and last complete rings will be indicated. Information on digital classification of slides is also available [9]. Once properly labeled, the slide will be stored usually in specific slide boxes.

22.7 Conclusion

At the risk of being redundant, making high-quality thin sections of stem material remains a skill that develops with practice and the best “cook book” on techniques can only be a starting point. The practitioner needs to become skilled with basic procedures and techniques. With patience and curiosity, expertise develops as one

proceeds openly with experimentation. Many of the procedures presented in this chapter can and will be adapted according to the project pursued. At the start of a new project, the practitioner should spend time experimenting and practicing until he/she has gone through all of the procedures and become familiar with the procedures and techniques until successfully mounting good quality thin sections. This initial time will not be lost. It is a time filled with trial and errors. The practitioner should not be afraid to (i) purposely make deliberate errors as part of the learning process and (ii) test alternative procedures so the various outcomes can be compared and the technique corrected. This practical time will also allow organizing the work flow and establishing routine procedures. It should not be random practice but systematic. With good note keeping habits, one can critically examine and compare slides so the best procedures are secured for the best final results. Good note keeping is crucial so alternative sample preparations, sectioning, and staining/dehydrating protocols may be compared and evaluated. Keep written notes of everything you do... . This is particularly important at the beginning of a new project or until a specific procedure is mastered and routine work habits have been established. Our experience indicates that no time is gained by trying to rush the slide making process. Finally, do not get discouraged if the first trials are inconclusive. Various elements need to be taken into account and the practitioner needs to get over the initial overwhelming stage. Hopefully this chapter has provided sufficient detailed information and specific references so the practitioner may overcome the initial difficulties associated with the making and mounting of thin sections. We also hope that the references provided will also trigger the curiosity to test less widely used methods and to improve the techniques currently employed in the preparation of wood/stem material for microscopic examination.

Acknowledgments We thank Dr. H. Gärtner for providing constructive comments on an earlier draft of this chapter. We are also thankful to the numerous undergraduate students who participated over the years in the dendrochronology course at the University of Winnipeg and also to both undergraduate and graduate students who participated in research or completed a thesis related to anatomical characteristics of tree rings. We thank Dr. F. Schweingruber for communicating his passion about wood/stem anatomy. We also thank the biology department technicians L. Buchanan and B. Van Dekerkhove for providing assistance and the librarians of the University of Winnipeg for tracking down rare books and journals, and securing them through inter-library loan.

References

1. Gärtner H, Heinrich I (2010) Anatomie des cernes chez les plantes ligneuses en régions tempérées et tropicales. In: Payette S, Filion L (eds) La Dendrochronologie: Principes, méthodes et applications. Presses de l'Université Laval, Québec, pp 33–60
2. James J (1976) Light microscopic techniques in biology and medicine. Martinus Nijhoff Medical Division, The Hague
3. Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, New York
4. Bracegirdle B (1986) A history of microtechnique: the evolution of the microtome and the development of tissue preparation. History of microscopy series. Science Heritage Ltd, Lincolnwood

5. Smith GM (1915) The development of botanical microtechnique. Trans Am Microsc Soc 34:71–129
6. American Optical Corporation (1975) Effective use and proper care of the microtome. American Optical Co, Scientific Instrument Division, New York
7. Galigher AE, Kozloff EN (1971) Essentials of practical microtechnique. Lea & Febiger, Philadelphia
8. Gärtner H, Nievergelt D (2010) The core-microtome: a new tool for surface preparation on cores and time series analysis of varying cell parameters. Dendrochronologia 28:85–92
9. Gärtner H, Schweingruber FH (2013) Microscopic preparation techniques for plant stem analysis. Kessel Publishing House, Germany
10. Schneider L, Gärtner H (2013) The advantage of using non-Newtonian fluids to prepare micro sections. Dendrochronologia 31:175–178
11. Gärtner H, Lucchinetti S, Schweingruber FH (2014) New perspectives for wood anatomical analysis in dendrosciences: the GSL1-microtome. Dendrochronologia 32:47–51
12. Hoadley RB (1990) Identifying wood: accurate results with simple tools. The Taunton Press, Connecticut
13. Fish J (1966) The identification of wood fragments. J Forensic Sci Soc 6:67–75
14. Smith FH, Gannon BL (1972) Sectioning of charcoals and dry ancient woods. Am Antiq 38:468–472
15. Figueiral I (1999) Lignified and charcoalified fossil wood. In Jones TP, Rowe NP (eds), Fossil plants and spores: modern techniques. The Geological Society, London, pp 92–96
16. Green OR (2001) A manual of practical laboratory and field techniques in palaeobiology. Kluwer Academic, Dordrecht
17. Cutler DF, Botha CEJ, Stevenson DW (2008) Plant anatomy: an applied approach. Blackwell, Oxford
18. Timar MC, Gurau L, Cionca M et al (2010) Wood species for the Biedermeier furniture—a microscopic characterisation for scientific conservation. Int J Conserv Sci 1:3–12
19. Brown FBH (1919) The preparation and treatment of woods for microscopic study. Bull Torrey Bot Club 46:127–150
20. Wimmer R (2002) Wood anatomical features in tree-rings as indicators of environmental change. Dendrochronologia 20:21–36
21. Wheeler EA, Baas P (1998) Wood identification—a review. IAWA J 19:241–264
22. Barefoot AC, Hankins FW (1982) Identification of modem and tertiary woods. Oxford University Press, Oxford
23. Panshin AJ, de Zeeuw C (1980) Textbook of wood technology, 4th edn. McGraw-Hill, New York
24. IAWA Committee (1989) IAWA list of microscopic features for hardwood identification. IAWA Bull 10:219–332
25. IAWA Committee (2004) IAWA list of microscopic features for softwood identification. IAWA J 25:1–70
26. Schweingruber FH (2007) Wood structure and environment. Springer, Berlin
27. Schweingruber FH (1990) Microscopic wood anatomy. Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf
28. Carlquist S (1988) Comparative wood anatomy: systematic, ecological, and evolutionary aspects of dicotyledon wood. Springer, London
29. Jane FW (1970) The structure of wood, 2nd edn. A & C Black, London
30. Tsoumis G (1968) Wood as raw material. Pergamon Press, Oxford
31. Schweingruber FH, Börner A, Schulze ED (2006) Atlas of woody plant stems: evolution, structure, and environmental modifications. Springer, Berlin
32. Rawlins TE (1933) Phytopathological and botanical research methods. Wiley, London
33. Kryn JM (1953) Annotated list of references on the preparation of wood for microscopic study. USDA Forest Service, Forest Product Laboratory, Report No 1939. University of Wisconsin

34. Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. The Iowa State University Press, Iowa
35. Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York
36. TRDA (Timber Research and Development Association) (1971) Examination of Timbers (Teaching Aid No. 7). High Wycombe, Buckinghamshire, England
37. Anonymous (1956) The preparation of wood for microscopic examination. British Forest Products Research Laboratory, Leaflet No 40, Department of Scientific and Industrial Research. HMSO, London
38. Garland H (1935) Notes on wood section microtechnique. J For 33:142–145
39. Sass JE (1958) Botanical microtechnique. The Iowa State University Press, Iowa
40. Desch HE (1950) The preparation of microscope slides. Wood 15:459–461
41. Averell JL (1926) Suggestions to beginners on cutting and mounting wood sections for microscopic examination. J For 24:791–801
42. Barker CS (1926) The anatomy of New Zealand wood. Part 1: laboratory technique. New Zealand J For 2:10–15
43. Bailey IW (1910) Microtechnique for woody structures. Bot Gaz 49:57–58
44. Koehler A, Gerry E, Weinstein AI (1927) Preparing woody tissues for making microscopic mounts. USDA Forest Service, Forest Products Laboratory, Madison
45. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi Pty Ltd, Melbourne
46. Ives E (2001) A guide to wood microtomy; making quality microslides of wood sections. Ernie Ives, Suffolk
47. Jansen S, Kitin P, De Pauw H et al (1998) Preparation of wood specimens for transmitted light microscopy and scanning electron microscopy. Belg J Bot 131:41–49
48. Purvis MJ, Collier DC, Walls D (1966) Laboratory techniques in botany. Butterworth & Co, Ltd., London
49. Sanderson JB (1994) Biological microtechniques. Royal Society Microscopy Handbooks, 28. Bios Scientific Publishers, Oxford
50. Barbosa ACF, Pace MR, Witovisk L et al (2010) A new method to obtain good anatomical slides of heterogeneous plant parts. IAWA J 31:373–383
51. Chaffey NJ (2002) Wood formation in trees: cell and molecular biology techniques. Taylor & Francis, New York
52. Amparado RF Jr, Beeckman H, Lemeur R (1990) Thin section preparation of poplar tree-rings for computer-aided image analysis in dendrochronological investigations. Silva Gandavensis 55:81–85
53. Schweingruber FH (2012) Microtome sectioning of small plant stems without embedding. IAWA J 33:457–460
54. Kukachka BF (1977) Sectioning refractory woods for anatomical studies (USDA Forest Service Research Note FLP-0236). Department of Agriculture, Forest Service, Forest Products Laboratory, Madison
55. Prakash N (1986) Methods in plant microtechnique, 2nd edn. University of New England, Armidale
56. Tardif J (2003) Wood anatomy of tree rings—report from an international course. IAWA J 24:86
57. Schweingruber FH, Büntgen U (2013) What is 'wood'—An anatomical re-definition. Dendrochronologia 31:187–191
58. Wilcox WW (1964) Some methods used in studying microbiological deterioration of wood, (U.S. Forest Service Research Note FPL-063). USDA, Madison
59. Xing M, Du Y, Wang X et al (2010) A simplified paraffin embedding method for small botanical samples. Biotech Histochem 85:241–246
60. Rossi S, Anfodillo T, Menardi R (2006) Trephor: a new tool for sampling microcores from tree stems. IAWA J 27:89–97
61. Storke HE (1940) Sections for microscopic study from increment boring. Trop Woods 64:40–41

62. Wegner L, Sass-Klaassen U, Eilmann B et al (2013) Micro-core processing—a time and cost efficient protocol. Wageningen University. https://www.wageningenur.nl/upload_mm/1/8/2/e3c369be-137d-4215-808e-51699334635c_protocol_%20for_%20microcore_%20processing.pdf. Accessed 24 June 2014
63. Jones B, Tardif J, Westwood R (2004) Weekly xylem production in trembling aspen (*Populus tremuloides*) in response to artificial defoliation. Can J Bot 82:590–597
64. Heinrichs D K, Tardif JC, Bergeron Y (2007) Xylem production in six tree species growing on an island in the boreal forest region of western Quebec, Canada. Can J Bot 85:518–525
65. Franklin GL (1946) A rapid method of softening wood for microtome sectioning. Trop Woods 88:35–36
66. Sutton A, Tardif J (2005) Distribution and anatomical characteristics of white rings in *Populus tremuloides*. IAWA J 26:221–238
67. Abbott ML, Barefoot AC, Hankins FW (1982) Appendix 2, Specimen preparation. In: Barefoot AC, Hankins FW (eds) Identification of modern and tertiary woods. Oxford University Press, Oxford, pp 165–172
68. Crowell IH (1930) Cutting microscopic sections of wood without previous treatment in hydrofluoric acid. Stain Tech 5:149–150
69. Davis GE, Stover EL (1936) A simple apparatus for the steam method of softening woods for microscope sections. Trans Ill Acad Sci 28:87
70. Harlow WM (1944) The chemical softening of wood for microtome sectioning. Tech Publ No 63, Bull New York State College of Forestry at Syracuse University 17:5–16
71. Burkart LF (1966) Triethylene glycol as a woody tissue softener in preparation for microtome sectioning. Stain Tech 41:255–259
72. Carlquist S (1982) The use of ethylenediamine in softening hard plant structures for paraffin sectioning. Stain Tech 57:311–317
73. Ohori N (1988) Wood softening methods for preparing microscopic samples. Forest Research Centre (FRC Publ No 48, Sabah), Malaysia
74. Cohen WE, Harris EE (1937) Pretreatment of wood with hot dilute acid. Ind Eng Chem Anal Ed 9:234–235
75. Kollman F (1939) Vorgänge und änderungen von holzeigenschaften beim dämpfen. Holz Roh Werkstoff 2:1–11
76. Kisser J (1931) Neue erfahrungen auf dem gebiete des schneidens harter objekte. Zts Wis Mikr 48:320–342
77. Kisser J (1928) Der heutige stand botanisch-mikrotechnischer schneidemethoden. Emil Haim-and Co, Leipzig
78. Varga D, van der Zee ME (2008) Influence of steaming on selected wood properties of four hardwood species. Holz Roh Werkst 66:11–18
79. Limback JP (1941) New woody tissue techniques. MSc Thesis, University of Wisconsin
80. Plowman AB (1904) The celloidin method with hard tissues. Bot Gaz 37:456–461
81. Langdon La Dema M (1920) Sectioning hard woody tissues. Bot Gaz 70:82–84
82. Jeffrey EC (1928) Improved method of softening hard tissues. Bot Gaz 86:456–458
83. Chowdhury KA (1934) An improved method of softening hard woody tissues in hydrofluoric acid under pressure. Ann Bot 48:308–310
84. Kerr T (1934) Action of hydrofluoric acid in softening wood. Trop Woods 40:37–42
85. Barker JA (2005) A prototype interactive identification tool to fragmentary wood from eastern central Australia, and its application to Aboriginal Australian ethnographic artefacts. PhD Thesis, School of Earth and Environmental Sciences. Adelaide University, http://digital.library.adelaide.edu.au/dspace/handle/2440/37793
86. Serdar B, Mazlum R (2014) An alternative approach in sectioning of archeological woods: the case of *Quercus pontica*. Turkish J Bot 38:623–626
87. Williamson HS (1921) A new method of preparing sections of hard vegetable structures. Ann Bot OS-35:139
88. Weinstein AI (1926) Methods for cutting and differential staining of microscopic section of hardwood glue joints. Colloid Symp Monogr 4:270–280

89. Dinwoodie JM (1966) Induction of cell wall dislocations (slip planes) during the preparation of microscope sections of wood. Nature 212:525–527
90. Dempster WT (1942) Distortions due to the sliding microtome. Anat Record 84:269–274
91. Kuĉera LJ (1981) Cutting wood specimens for observation in the scanning electron microscope. J Microsc 124:319–325
92. Waito J, Conciatori F, Tardif JC (2013) Frost rings and white earlywood rings in *Picea mariana* trees from the boreal plains, Central Canada. IAWA J 34:71–87
93. Levy JF (1953) A method of embedding brittle specimens of wood for sectioning. Nature 171:984
94. Walter F, Schmitt W (1980) The microtome: manual of the technique of preparation and of section cutting. Ernst Leitz Wetzlar, Germany
95. Bonga JM (1961) A method of sectioning plant material using cellulose tape. Can J Bot 39:729–730
96. Srebotnik E, Messner K (1994) A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. Appl Environ Microbiol 60:1383–1386
97. Fuji T (2003) Application of the NT-Cutter knife blade to microtome sectioning of wood. IAWA J 24:241–245
98. Kisser J, Anderson DB (1928) A method of preparing thin cross and longitudinal sections of cotton fibers, and its importance in cell-wall research. Am J Bot 15:437–441
99. Au R, Tardif J (2007) Allometric relationships and radial growth of the dwarf shrub *Dryas integrifolia* near Churchill, subarctic Manitoba. Can J Bot 85:585–597
100. Hyland F (1940) Preventing the curling of wood sections. Trop Woods 64:141–143
101. Reeve RM (1948) An adaptable staining schedule for plant tissues. Biotech Histochem 23:13–15
102. De Micco V, Aronne G (2007) Combined histochemistry and autofluorescence for identifying lignin distribution in cell walls. Biotech Histochem 82:209–216
103. Schwarze FWMR (2007) Wood decay under the microscope. Fungal Biol Rev 21:133–170
104. Vazquez-Cooz I, Meyer RW (2002) A differential staining method to identify lignified and unlignified tissues. Biothech Histochem 77:277–282
105. van der Werf GW, Sass-Klaassen U, Mohren GMJ (2007) The impact of the 2003 summer drought on the intra-annual growth pattern of beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) on a dry site in the Netherlands. Dendrochronologia 25:103–112
106. Rapp AO, Behrmann K (1998) Preparation of wood for microscopic analysis after decay testing. Holz als Roh und Werkstoff 56:277–278
107. Gram K, Jorgensen E (1952) An easy, rapid and efficient method of counter-staining plant tissues and hyphae in wood-sections by means of fast green or light green and safranin. Friesia 4:262–266
108. Rauter RW, Zufa L (1972) A rapid technique for the determination of *Cronartium ribicola mycelium* in white pine bark tissues. In: Biology of rust resistance in forest trees. Proc of a NATO-IUFRO Advanced Study Institute, USDA Forest Service, (Miscellaneous Publ No 1221), Washington DC, pp 387–392
109. Jewell FF (1958) Stain technique for rapid diagnosis of rust in southern pines. For Sci 4:42–44
110. Kutscha NP, Gray JR (1972) The suitability of certain stains for studying lignification in balsam fir *Abies balsamea* (L.) Mill. Tech Bull 72. Life Sciences and Agriculture Experimental Station, University of Maine
111. Bussey W, Haigler R (1981) Tubes versus transistors in electric guitar amplifiers. Acoustics, speech, and signal processing. IEEE International Conference on ICASSP 6:800–803
112. Kandyala R, Raghavendra SPC, Rajasekharan ST (2010) Xylene: an overview of its health hazards and preventive measures. J Oral Maxillofac Pathol 14:1–5

Chapter 23
Collecting and Processing Wood Microcores for Monitoring Xylogenesis

Annie Deslauriers, Sergio Rossi and Eryuan Liang

23.1 Introduction

Wood production is the result of a gradual accumulation of xylem that plants produce in order to renew their transport systems, store substances and ensure mechanical support [1]. At the basis of growth and wood formation, there is a meristem, the cambium. Through a cyclical alternation of periods of activity and rest, the cambium produces conducting elements during the more favourable periods, leaving indelible traces constituted of tree rings. A tree ring is the product of an entire season of cambium activity that corresponds to the period between spring and autumn in cold and temperate zones [2].

Intra-annual analyses of wood formation divide the vegetative season into short periods through sampling at weekly to monthly intervals. This provides images of the situation at the moment of sampling. Reporting all these images along a temporal axis, the wood formation can be visualized and analyzed. To do this, wood samples are extracted as small cores, called microcores. The samples contain the preceding 4–10 tree rings and the developing annual layer with the cambial zone and adjacent phloem tissue. This chapter focuses on the technical procedure for describing wood formation, but a similar procedure can be applied for phloem [3, 4].

The samples should be as small as possible, in order to perform repeated samplings on the same individuals by reducing the effects on the remaining tissues [5]. Microcores exhibit a heterogeneous consistency with a soft side in correspondence to phloem, cambium and recently differentiating tissues, and a hard or very hard side, the xylem [6]. The size, combined with the difference in consistency and

A. Deslauriers (✉) · S. Rossi
Département des Sciences Fondamentales, Université du Québec à Chicoutimi,
555 boulevard de l'Université, Chicoutimi, Québec G7H 2B1, Canada
e-mail: Annie_Deslauriers@uqac.ca

E. Liang
Key Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau Research,
Academy of Sciences, Beijing, China

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_23

density of the microcore, requires appropriate handling, stable fixation and careful cutting to prepare histological sections for microscopic analyses. Although hand cutting is possible, it is challenging for novices and is time-consuming when numerous samples have to be processed. Because of the round shape, numerous cuts are necessary to expose a sufficient part of the tissues and collect the definitive sections. Thus, it is more effective if the tissues of the sample are embedded and blocked in paraffin or resin. Embedding fills all the cavities with a matrix allowing cutting to be performed efficiently and quickly. The embedding techniques used for studying cambial activity mainly use paraffin [6] or polyethylene glycol solution [7]; the former requires less time to embed the samples and is potentially less of an irritant for users. For additional information on specimen preparation, see Chap. 22 of this book.

Intra-annual analysis of wood formation is used in many fields of plant science aiming at linking wood formation with ecology [8–17], plant physiology [18–22], environmental effects [23–29] and climate change [30–32]. In this chapter, the most recent and widely used procedure for monitoring xylogenesis is described, identifying all the methodological steps involved in microcore extraction, sample preparation, paraffin embedding, cutting and microscopic observations of the tissues.

23.2 Materials

23.2.1 Microcore Extraction

1. Trephor, or another tool for microcore extraction such as increment puncher, surgical bone needle, Trapsystem needle (Fig. 23.1a, b).
2. Blade or cutter.
3. Eppendorf microtubes (2.5 mL) filled with an ethanol solution (10–50% in water) or a formalin-ethanol-acetic acid solution (FAA).
4. Teflon hammer.

23.2.2 Embedding

1. Leica TP 1020 or a similar automatic tissue processor *(see Note 1)*.
2. Leica EG 1150 or a similar tissue embedding system *(see Note 2)*.
3. Embedding cassettes.
4. Stainless-steel base moulds.
5. Ethanol (70, 90, 95 and 100%).
6. Safeclear II (tissue clearing agent) or a similar solvent for deparafinization. It can be substituted with (the more toxic) xylene.

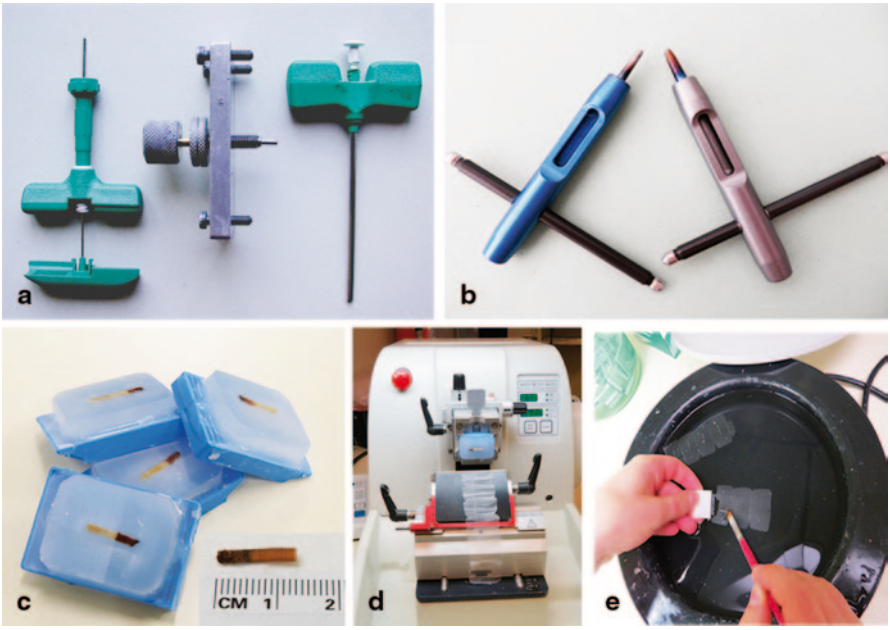

Fig. 23.1 **a** Surgical bone needle, increment puncher and Trapsystem needle. **b** Two Trephor tools with sampling heads of different size. **c** Trimmed paraffin blocks with microcores. **d** Paraffin strip with microcore sections obtained by rotary microtome. **e** Paraffin strip plunged in water to stretch the sections

7. TissuePrep® paraffin (melting point at 56–57 °C, Cat. No. T565, Fisher Scientific) or a similar paraffin for routine tissue embedding.
8. Histology water bath for tissue floating.
9. Glass staining dishes and slide racks.

23.2.3 Section Preparation

1. Leica RM 2255 or a similar rotary microtome.
2. Disposable microtome blades for routine and hard tissues (Feather Microtome blades A35 or N35 or similar blades for rotary microtome).
3. Slides and cover glass.
4. Colorants: cresyl violet acetate, safranin and astra blue.
5. Distilled water.
6. Brushes with different sizes.
7. Permount (toluene solution, Fisher Scientific) or a similar histological mounting medium.

23.2.4 Observations

1. Stereomicroscope attaining magnifications of 40×.
2. Optical microscope equipped with normal and polarized light attaining magnifications of 500×.
3. Entomological needles.

23.3 Methods

23.3.1 Microcore Extraction

1. Select a sampling point on the stem as far as possible from living or dead branches and at least 5 cm from any previous sampling points to avoid anatomical malformation or getting resin ducts on adjacent cores. Avoid sampling close to the root collar because of the anatomical modifications of the tissues in this part of the stem *(see Note 3)*.
2. In species with thick bark or old trees, remove the bark with a knife until the phloem is exposed. This step is not required in young trees or species with thin bark to minimize the damage to the stem.
3. Hold the piercing head of Trephor against the sampling position with three fingers, checking that the asymmetric arms do not touch the hand.
4. Hammer the cutting tube into the wood.
5. Separate the microcore from the xylem by holding the asymmetric arms and rotating the tool like a corkscrew, thus extracting the cutting tube with movements coaxial to the main element.
6. Hold Trephor in the palm of the hand with the blade facing the fingers and the longer asymmetric arm towards the outside; separate the sample from the cutting tube with a delicate pressure. The microcore slides along the inside of the cutting tube and falls directly onto the hand from the back of the piercing head. The overall time spent for sampling is < 1 min.
7. Place the microcore in Eppendorf microtubes, with the ethanol or FAA solution, and store at 5 °C to avoid tissue deterioration. Storage can last several years for anatomical analyses *(see Note 4)*.

23.3.2 Embedding

1. Put the microcores in embedding cassettes. Remove any old tree rings in hardwood species; this reduces the sample length and makes the cutting at the microtome easier.

2. Dehydrate the microcores with successive immersions in ethanol at increasing concentrations for a total of 11.5 h *(see Note 5)*.
3. Immerse the microcores in three successive baths with Safeclear II for a total of 4.5 h *(see Note 5)*.
4. Immerse the microcores in two successive baths with liquid paraffin at 65 °C for a total of 4 h *(see Note 5)*. The paraffin penetrates deeply into the wood and fills the cell lumens of all tissues.
5. Fix the microcores on their embedding cassettes using stainless-steel base moulds by forming paraffin blocks (Fig. 23.1c). Orientate the microcores for cross cuttings.

23.3.3 Section Preparation

1. Remove excess paraffin from the embedding tissues by trimming the paraffin blocks at 20 μm until the wood is exposed (Fig. 23.1c).
2. Put the sample in fresh water at room temperature. The immersion rehydrates cell walls, softens the wood tissues and lubricates the sample. The immersion can last from 1 h to 2 days depending on the sample size and hardness of the wood.
3. Block the sample in the microtome clamp and cut it to produce a series of sections of thickness 6–8 μm. In each run, the clamp advances towards the knife and generates a paraffin film containing the sections (Fig. 23.1d). After 5–10 runs, remove the continuous paraffin strip and plunge it in water at 45–55 °C to stretch the sections (Fig. 23.1e).
4. Put the film containing the sections on the microscope slides (Fig. 23.1e) *(see Note 6)*.
5. Dry the sections in an oven for 1 h at 50 °C or at room temperature for 3 h.
6. Remove the residual paraffin by immersing the slides in two baths with Safeclear II and two baths with ethanol for a total of 40 min [6].
7. Stain the sections with single *(see Note 7)* or double *(see Note 8)* staining. Orientate the sections in the staining dish with the cambium side upwards.

23.3.4 Observations

1. Observe the sections under the optical microscope and identify the various phases of differentiation of the developing xylem. Cell counting *(see Note 9)* or measurement *(see Note 10)* can be performed along three or more radial files. Select the files with the largest cells, which are more likely cut near the centre of the longitudinal length.
2. Identify the cambial cells, characterized by thin cell walls and small radial diameters (Fig. 23.2). Because of the alternation between growth and rest, the number of cambial cells alters during the year. In autumn and winter, when there is no cell

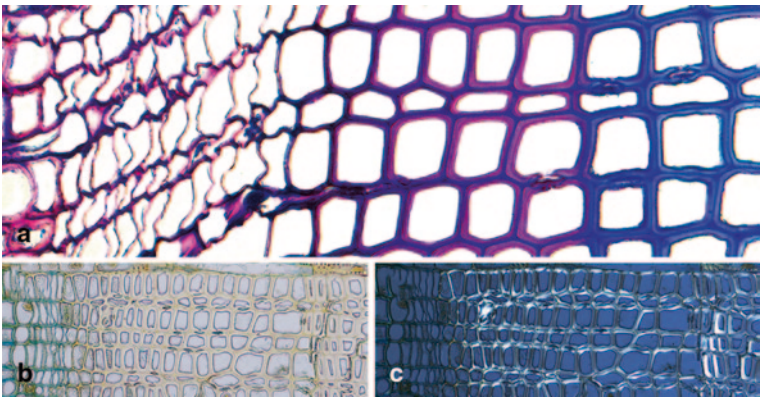

Fig. 23.2 Developing xylem of *Picea mariana* stained with cresyl violet acetate (**a**) and *Abies georgei* var. smithii observed under normal and polarized light (**b, c**). Cambial zone, cells in enlargement and secondary wall formation, and mature cells are shown

production, the cambial zone constitutes 2–8 cells. In spring, cambial cells swell and expand radially, the radial cell walls grow thin, cytoplasm assumes shinier and less densely granulate features and the nuclei enlarge [1]. The cambium cell number can increase up to 10–13 during the period of maximum growth activity. Once the annual activity ends, the cambium stops dividing and the number of cells in the cambial zone reduces to a minimum value corresponding to quiescence conditions.

3. Identify the enlarging cells with radial diameter at least twice that of a cambial cell and consisting of a protoplast still enclosed in the thin and elastic primary wall (Fig. 23.2). Following positive turgor increase by water movement into the vacuoles, the cell wall stretches, increasing the radial diameter of the tracheid and, consequently, the lumen area. This process occurs despite the strong compression, the cells being enclosed between xylem tissues and bark and deformed files of tracheids with collapsed cells can be observed *(see Note 11)*. No shining is observed under polarized light.
4. Identify the cells in secondary wall formation (Fig. 23.2), which shine when observed under polarized light because of the arrangement of the cellulose microfibrils accumulated in the secondary wall [33, 34]. Observe the process of lignification detected by the reaction of unlignified and lignified secondary cell walls with cresyl violet acetate or safranin–astra blue. Lignification is shown by a colour change from violet to blue (cresyl violet acetate) or from blue to red (safranin–astra blue). At first, lignin deposition is observed at the cell corners on the primary wall, then extending along the middle lamella and primary walls and, finally, on the secondary wall.
5. Identify mature cells that shine under polarized light and exhibit a blue or red colour over the whole cell wall when stained with cresyl violet acetate or safranin–astra blue, respectively (Fig. 23.2). Complete maturation involves the ending of cell differentiation and autolysis of the protoplast.

23.4 Notes

1. Embedding could be performed without an automatic tissue embedding system. However, the procedure can become time-consuming and complex when many samples are done manually.
2. The modular tissue embedding system can be replaced by a wax dispenser, a hot plate and an ice block.
3. Sampling can also be performed on branches or roots. In roots, microcores should be collected following a sinuous pattern, beginning about 25 cm from the stump and only in the upper parts because of their eccentric growth [35].
4. Storage in Eppendorf microtubes is safe, even during transportation, and microcores very rarely break. In cold climate, the microcore extraction cannot be done in winter or early spring, when the stems are still frozen, because the wood samples frequently break into little parts corresponding to the different tree rings. In early spring, at the beginning of growth resumption, the microcores easily split into two, in correspondence to the cambial zone; thus, extreme care is required when sampling in this period of the year.
5. The periods of immersion are suggested according to Anderson and Bancroft [36] and Rossi, Anfodillo and Menardi [6] for microcores embedded without a pressure system. They can be modified according to the sample size and reduced in the case of tissue processors equipped with a pressurized system. Some research teams do not embed microcores before cutting [17].
6. For a greater adhesion of the sections, the slides have to be previously treated with an albumin fixative (Albumin Glycerol Fixative ES796-8-CA, Azer Scientific, Morgantown, PA). Put 1–2 drops of albumin on the slide and spread it with a fingertip for 2–4 s to form a very thin layer. Albumin can stain, so remove all excess from the slide. Leave the albumin on the slide to dry at room temperature for 10 min. Poly-L-Lysine-coated slides can also be used to create an electrostatic coupling between samples and glass, with the advantage that Poly-L-Lysine does not stain.
7. Single staining is performed with cresyl violet acetate (0.16% in water) for 5 min. The sections are observed immediately after staining.
8. Double staining is performed with a safranin–astra blue mixture prepared by adding 40 mg safranin and 150 mg astra blue to a solution of 100 mL demineralized water with 2 mL acetic acid [37]. The samples are stained for 10 min. The sections can be kept in glycerol after measuring or permanently fixed with a histological mounting medium.
9. Cell counting is generally performed on conifers [8, 38], which have a homogeneous xylem. Counting of cell numbers along radial files, for each developmental zone and mature state, gives more useful information about the current activity of cambium, as cells represent the most important unit to have quantitative data on [39].
10. Measurement of the radial width of the differentiation zones is performed on young, fast-growing trees or broadleaves [19, 40], where, because of the presence of both vessels and fibres, measuring is more appropriate and easier to

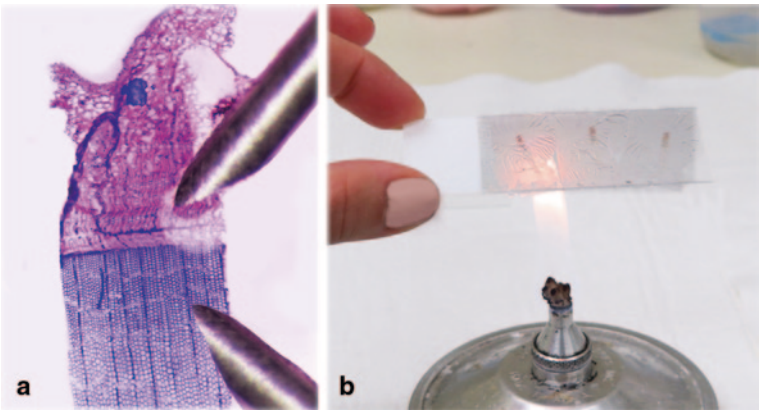

Fig. 23.3 Procedure for stretching the developing xylem tissues using two needles under the stereomicroscope (**a**) or by warming up the slide with the sections still embedded in paraffin over the flame of a Bunsen burner (**b**)

do. Measurements can be performed directly with a micrometer eyepiece or on photographs taken with a digital camera fixed on the microscope.

11. Collapsed cambial and enlarging cells can occur in some species and during certain periods of the year, more commonly when cambial growth is vigorous, preventing observations being carried out appropriately. To make the analysis easier, the developing xylem tissues can be delicately stretched under the stereomicroscope with entomological needles (Fig. 23.3a). This procedure is performed directly on sections freshly stained with cresyl violet acetate. Another solution is to stretch the tissues by warming the slide with the sections still embedded in paraffin over the flame of a Bunsen burner for 1–2 s (Fig. 23.3b).

23.5 Interpretations and Conclusions

During development, the cambial derivatives alter both morphologically and physiologically, gradually assuming definite features differentiating into the specific elements of the stem tissues. Figure 23.4 represents the phases of xylem formation in *Picea mariana* observed at a magnification of 200× on sections stained with the safranin–astra blue mixture. The cycle is composed of a dormant phase, in this species covering more than half the year, and a growth period (from day of year (DOY) 165 to 240). The description of the different cell developmental phases starts from top left at DOY 140 and proceeds clockwise along the cycles. During growth, the phases of production and differentiation of each cell are separated in space and time according to an overlapping and partial delay (Fig. 23.4). After division, the new cells produced by cambium are chronologically disposed along radial files, where they gradually undergo enlargement and cell wall formation in the same order as their production, following the rule of "first in—first out".

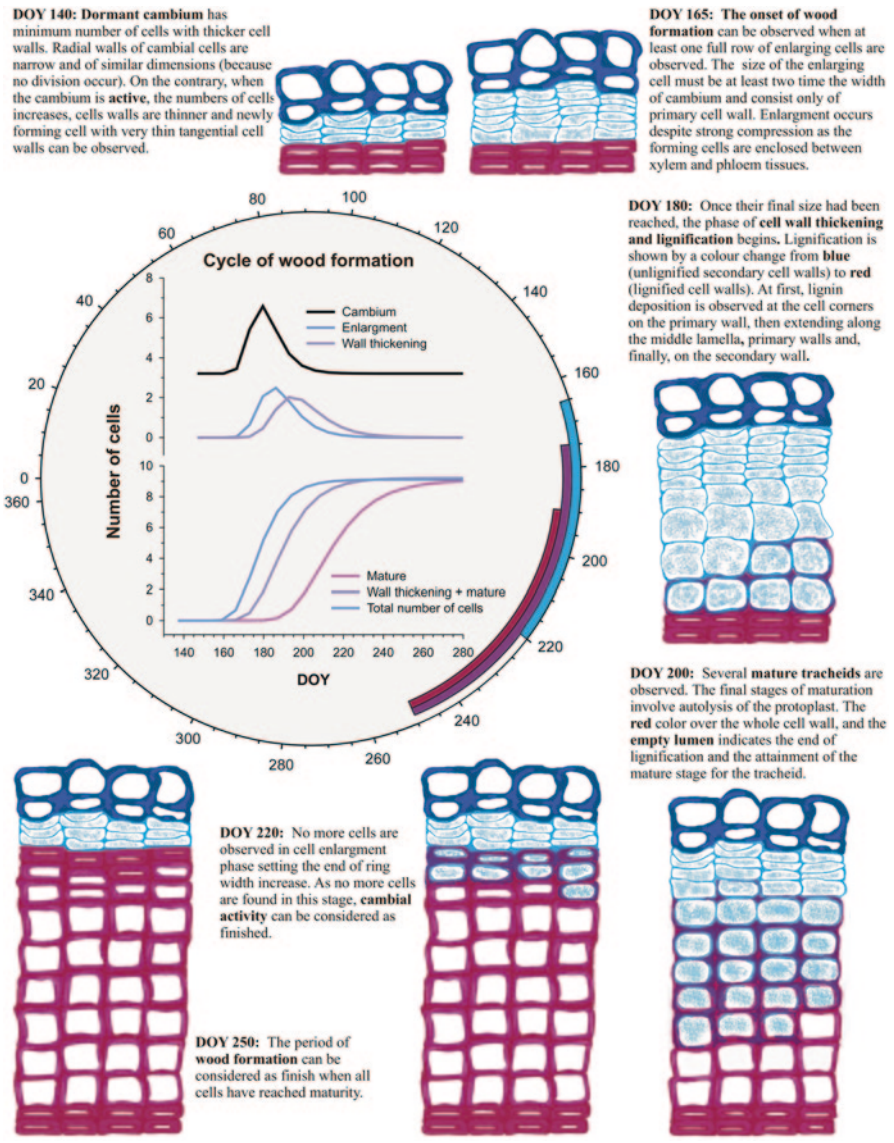

Fig. 23.4 Growth dynamics of xylem in *black spruce* (*Picea mariana* (Mill.) BSP) observed on sections stained with safranin and astra blue under a light microscope at magnifications of 200×. The cycle is composed of a dormant phase, in this case covering more than half the year, and a growth phase (from DOY 165 to 240). The description of the different cell developmental phases starts from *top left* at DOY 140 and proceeds clockwise. Intra-annual variations in rate of cell division result in a typical annual pattern consisting of three delayed *bell-shaped curves* (cambial, cell enlargement and cell wall thickening) and three *S-shaped curves* (total number of cells, cell wall thickening + mature cells, and mature cells)

Intra-annual variations in rate of cell division and differentiation determine variations among the cell queues, resulting in a typical annual pattern consisting of three delayed bell-shaped curves (cambial, cell enlargement and cell wall thickening) and one S-shaped curve (mature cells). The bell-shaped patterns are connected with the number of cells undergoing each differentiation phase, whereas the sigmoid curve is associated with the gradual accumulation of cells in xylem. Cold and temperate biomes lacking a distinct summer drought are characterized by a unimodal S-shaped pattern [10, 12, 41], while Mediterranean and tropical climates exhibit uni- to multimodal growths [42–45].

At the end of a growth cycle, the raw data collected at different moments throughout the year, and displayed in Fig. 23.4, can be processed according to Rossi et al. [46], Cuny et al. [47] or Rathgeber et al. [48] in order to calculate rates and duration of all the measured phases. Timing, duration and rate of growth affect the anatomical features of xylem cells along a tree ring [19, 28, 49]. A precise assessment of the kinetics of cell development is therefore crucial to obtain a mechanistic understanding of how environmental constraints influence wood growth and anatomy.

The analysis of wood formation is based on periodic samplings at short time intervals to provide information on xylem phenology, which is represented by the different developmental stages of the xylem cells and the timings of their occurrence. This type of monitoring requires specific techniques and tools for extracting and processing the samples containing the developing xylem. In this chapter, a recent and widely used procedure for monitoring xylogenesis is described, identifying all methodological steps involved in microcore extraction, sample preparation and cutting, and microscopic observations of the tissues. The procedure has several advantages. Sampling and sample preparation can be accomplished rapidly and easily, even by unexperienced students. More than 80 samples can be prepared and observed in 2 days. All substances used in the laboratory are classified as having no or reduced toxicity. The procedure is mastered quickly and in a few days novices can be ready to perform all the steps efficiently under limited supervision. However, despite the low cost of the materials, the laboratory should be equipped with several expensive machines and microscopes. Microscopic observations of the tissues necessarily require a training period to learn the common criteria for identification of the phenological phases and to ensure that data will be collected with a homogeneous measurement protocol.

Acknowledgements This work was funded by grants from Consortium de Recherche sur la Forêt Boréale Commerciale, Fonds de Recherche sur la Nature et les Technologies du Québec, National Sciences and Engineering Research Council of Canada, Canada Foundation for Innovation, and Forêt d'Enseignement et de Recherche Simoncouche. The author thanks E. Gallo, R. Menardi, C. Soucy for their technical recommendations and A. Garside for editing the English text.

References

1. Fahn A, Werker E (1990) Seasonal cambial activity. In: Iqbal M (ed) The vascular cambium. Research Studies Press, Taunton, pp 139–157
2. Fritts HC (1976) Tree rings and climate. Academic, London
3. Gricar J, Cufar K (2008) Seasonal dynamics of phloem and xylem formation in silver fir and Norway spruce as affected by drought. Rus J Plant Physiol 55:538–543
4. Cufar K, Cherubini M, Gricar J et al (2011) Xylem and phloem formation in chestnut (Castanea sativa Mill.) during the 2008 growing season. Dendrochronologia 29:127–134
5. Forster T, Schweingruber FH, Denneler B (2000) Increment puncher—a tool for extracting small cores of wood and bark from living trees. IAWA J 21:169–180
6. Rossi S, Anfodillo T, Menardi R (2006) Trephor: a new tool for sampling microcores from tree stems. IAWA J 27:89–97
7. Seo J-W, Eckstein D, Schmitt U (2007) The pinning method: from pinning to data preparation. Dendrochronologia 25:79–86
8. Deslauriers A, Morin H, Begin Y (2003) Cellular phenology of annual ring formation of Abies balsamea in Quebec boreal forest (Canada). Can J For Res 33:190–200
9. Mäkinen H, Nöjd P, Saranpää P (2003) Seasonal changes in stem radius and production of new tracheids in Norway spruce. Tree Physiol 23:959–968
10. Jyske T, Mäkinen H, Kalliokoski T et al (2014) Intra-annual tracheid production of Norway spruce and scots pine across a latitudinal gradient in Finland. Agric For Meteorol 194:241–254
11. Cuny HE, Rathgeber CBK, Lebourgeois F et al (2012) Life strategies in intra-annual dynamics of wood formation: example of three conifer species in a temperate forest in north-east France. Tree Physiol 32:612–625
12. Rossi S, Deslauriers A, Griçar J et al (2008) Critical temperatures for xylogenesis in conifers of cold climates. Global Ecol Biogeog 17:696–707
13. Deslauriers A, Rossi S, Anfodillo T et al (2008) Cambial phenology, wood formation and temperature thresholds in two contrasting years at high altitude in southern Italy. Tree Physiol 28:863–871
14. Rossi S, Deslauriers A, Anfodillo T et al (2007) Evidence of threshold temperatures for xylogenesis in conifers at high altitudes. Oecologia 152:1–12
15. Cufar K, Prislan P, de Luis M et al (2008) Tree-ring variation, wood formation and phenology of beech (*Fagus sylvatica*) from a representative site in Slovenia, SE Central Europe. Trees 22:749–758
16. Heinrichs DK, Tardif JC, Bergeron Y (2007) Xylem production in six tree species growing on an island in the boreal forest region of western Quebec, Canada. Can J Bot 85:518–525
17. Moser L, Fonti P, Buntgen U et al (2010) Timing and duration of European larch growing season along altitudinal gradients in the Swiss Alps. Tree Physiol 30:225–233
18. Deslauriers A, Beaulieu M, Balducci L et al (2014) Impact of warming and drought on carbon balance related to wood formation in black spruce. Ann Bot 114:335–345
19. Deslauriers A, Giovannelli A, Rossi S et al (2009) Intra-annual cambial activity and carbon availability in stem of poplar. Tree Physiol 29:1223–1235
20. Gruber A, Pirkebner D, Florian C et al (2012) No evidence for depletion of carbohydrate pools in Scots pine (*Pinus sylvestris* L.) under drought stress. Plant Biol 14:142–148
21. Simard S, Giovannelli A, Treydte K et al (2013) Intra-annual dynamics of non-structural carbohydrates in the cambium of mature conifer trees reflects radial growth demands. Tree Physiol 33:913–923
22. Michelot A, Simard S, Rathgeber C et al (2012) Comparing the intra-annual wood formation of three European species (*Fagus sylvatica, Quercus petraea* and *Pinus sylvestris*) as related to leaf phenology and non-structural carbohydrate dynamics. Tree Physiol 32:1033–1045
23. Lupi C, Morin H, Deslauriers A et al (2012) Xylogenesis in black spruce: does soil temperature matter? Tree Physiol 32:74–82

24. Gričar J, Čufar K, Oven P et al (2005) Differentiation of terminal latewood tracheids in silver fir trees during autumn. Ann Bot 95:959–965
25. Gricar J, Zupancic M, Cufar K et al (2006) Effect of local heating and cooling on cambial activity and cell differentiation in the stem of Norway spruce (*Picea abies*). Ann Bot 97:943–951
26. Gruber A, Baumgartner D, Zimmermann J et al (2009) Temporal dynamic of wood formation in *Pinus cembra* along the alpine treeline ecotone and the effect of climate variables. Tree—Struct Funct 23:623–635
27. Gruber A, Strobl S, Veit B et al (2010) Impact of drought on the temporal dynamics of wood formation in *Pinus sylvestris*. Tree Physiol 30:490–501
28. Balducci L, Deslauriers A, Giovannelli A et al (2013) Effects of temperature and water deficit on cambial activity and woody ring features in *Picea mariana* saplings. Tree Physiol 33:1006–1017
29. Pasho E, Camarero JJ, Vicente-Serrano SM (2012) Climatic impacts and drought control of radial growth and seasonal wood formation in *Pinus halepensis*. Tree—Struct Funct 26:1875–1886
30. Rossi S, Morin H, Deslauriers A et al (2011) Predicting xylem phenology in black spruce under climate warming. Global Change Biol 17:614–625
31. Rossi S, Girard MJ, Morin H (2014) Lengthening of the duration of xylogenesis engenders disproportionate increases in xylem production. Global Change Biol 20:2261–2271
32. Lugo JB, Deslauriers A, Rossi S (2012) Duration of xylogenesis in black spruce lengthened between 1950 and 2010. Ann Bot 110:1099–1108
33. Donaldson LA (1991) Seasonal changes in lignin distribution during tracheid development in *Pinus radiata* D. Don. Wood Sci Tech 25:15–24
34. Abe H, Funada R, Ohtani J et al (1997) Changes in the arrangement of cellulose microfibrils associated with the cessation of cell expansion in tracheids. Trees 11:328–332
35. Thibeault-Martel M, Krause C, Morin H et al (2008) Cambial activity and intra-annual xylem formation in roots and stems of *Abies balsamea* and *Picea mariana*. Ann Bot 102:667–674
36. Anderson G, Bancroft JD (2002) Tissue processing and microtomy including frozen. In: Bancroft JD, Gamble M (eds) Theory and practice of histological techniques. Churchill Livingstone, London, pp 85–107
37. van der Werf GW, Sass-Klaassen UGW, Mohren GMJ (2007) The impact of the 2003 summer drought on the intra-annual growth pattern of beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) on a dry site in the Netherlands. Dendrochronologia 25:103–112
38. Rossi S, Deslauriers A, Gricar J et al (2008) Critical temperatures for xylogenesis in conifers of clod climates. Glob Ecol Biogeog 17:696–707
39. Savidge RA (2000) Biochemistry of seasonal cambial growth and wood formation—an overview of the challenges. In: Savidge RA, Barnett JR, Napier R (eds) Cell and molecular biology of wood formation. BIOS Scientific Ltd, Oxford, pp 1–30
40. Marion L, Gricar J, Oven P (2007) Wood formation in urban Norway maple trees studied by the micro-coring method. Dendrochronologia 25:97–102
41. Rossi S, Deslauriers A, Anfodillo T et al (2006) Conifers in cold environments synchronize maximum growth rate of tree-ring formation with day length. New Phytol 170:301–310
42. Vieira J, Rossi S, Campelo F et al (2014) Are neighbouring trees in tune? Eur J Forest Res 133:41–50
43. de Luis M, Novak K, Raventos J et al (2011) Climate factors promoting intra-annual density fluctuations in Aleppo pine (*Pinus halepensis*) from semiarid sites. Dendrochronologia 29:163–169
44. Camarero JJ, Olano JM, Parras A (2010) Plastic bimodal xylogenesis in conifers from continental Mediterranean climates. New Phytol 185:471–480
45. Krepkowski J, Brauning A, Gebrekirstos A et al (2011) Cambial growth dynamics and climatic control of different tree life forms in tropical mountain forest in Ethiopia. Trees 25:59–70
46. Rossi S, Deslauriers A, Morin H (2003) Application of the Gompertz equation for the study of xylem cell development. Dendrochronologia 21:33–39

47. Cuny HE, Rathgeber CBK, Kiesse TS et al (2013) Generalized additive models reveal the intrinsic complexity of wood formation dynamics. J Exp Bot 64:1983–1994
48. Rathgeber CBK, Longuetaud F, Mothe F et al (2011) Phenology of wood formation: Data processing, analysis and visualisation using R (package CAVIAR). Dendrochronologia 29:139–149
49. Cuny HE, Rathgeber CBK, Frank D et al (2014) Kinetics of tracheid development explain conifer tree-ring structure. New Phytol 203:1231–1241

Chapter 24
Three-Dimensional Imaging of Cambium and Secondary Xylem Cells by Confocal Laser Scanning Microscopy

Satoshi Nakaba, Peter Kitin, Yusuke Yamagishi, Shahanara Begum, Kayo Kudo, Widyanto Dwi Nugroho and Ryo Funada

24.1 Introduction

Wood has been used as an important renewable resource for thousands of years as a raw material for timber, furniture, pulp and paper, chemicals, and fuels. In addition, wood has been recently used as a resource of bioethanol. Moreover, since wood is a major carbon sink of photosynthesis derived from trees, it is expected to play an important role in removing excess atmospheric carbon dioxide that is generated by the burning of fossil fuels. Therefore, there is still great demand for wood as biomaterial and a source of bioenergy.

Wood is produced by the vascular cambium (cambium) [1–4]. Secondary xylem derived from cambium is a complex tissue composed of four basic types of cells: fibers, tracheids, vessel elements, and parenchyma cells. Each cell type is classified into several subtypes depending on their morphology and corresponding function [5]. The structure of wood, in particular the proportions between fibrous and parenchymatic cells, the morphology of fibers and vessels, and the porosity of wood (cross-sectional diameter and distribution of vessels), strongly influence the properties and the commercial value of wood. Wood density is also an important variable for estimating the commercial value of wood as well as tree growth and the carbon stock in forests. Therefore, methods for the precise anatomical analyses of the struc-

R. Funada (✉) · S. Nakaba · Y. Yamagishi
Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-Tokyo, Japan
e-mail: funada@cc.tuat.ac.jp

P. Kitin
Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, USA

S. Begum
Faculty of Agriculture, Bangladesh Agricultural University, Mymensingh, Bangladesh

K. Kudo
Institute of Wood Technology, Akita Prefectural University, Noshiro-Akita, Japan

W. D. Nugroho
Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_24

ture of wood are important in forest ecology and forest management, as well as research for predicting wood properties. Furthermore, vessels, composed of vessel members, are the major water-conducting elements in angiosperms, which provide efficient water transport in the plant body [6]. Studying the structure of vessels and their three-dimensional (3D) network is essential for understanding the mechanism of the ascent and distribution of sap in plants [7].

Since differences in wood quality are largely due to the differences in wood structure, wood structure is one of the most important targets in the attempt to control wood quality. Wood structure is determined by the process of secondary xylem differentiation from cambial derivatives involving cell enlargement, cell wall thickening, and cell wall sculpturing (formation of modified structure). The secondary xylem cells, such as tracheids, wood fibers, and vessel elements, have secondary cell walls with a highly organized structure. Continuous deposition of cell wall materials, such as cellulose microfibrils (MFs), increases the thickness of the wall. The thick cell wall supports the heavy weight of the tree itself, which can sometimes reach more than 100 m in height and functions in the transport of water from roots to leaves.

The direction of the orientation of cellulose MFs changes progressively with the changing speed of rotation during the formation of the secondary cell wall resulting in a secondary wall consisting of multilayers [4]. The texture of the secondary wall, in particular the orientation of cellulose MFs of the secondary wall, is closely related to the physical properties of secondary xylem cells. Cellulose is synthesized by enzyme complexes (terminal complexes) in the plasma membrane. Observations in a wide variety of lower and higher plant cells [4, 8] have revealed that cortical microtubules (MTs) play an important role in the orientation of newly deposited cellulose MFs. It has been postulated that cortical MTs that are co-aligned with the plasma membrane guide the movement of the terminal complexes. In addition, localized cortical MTs might control the localization of terminal complexes in the plasma membrane and the localized deposition of cellulose MFs in cell wall including modified structures such as pits and helical thickenings in secondary xylem cells. Therefore, visualization of cortical MTs in cambium and differentiating xylem provides important information on the mechanism of xylem differentiation.

With the onset of secondary wall deposition, lignification begins at the intercellular layer, then the primary cell wall, and eventually to the secondary cell wall. When lignification has been completed, cell death occurs immediately in short-lived tracheary elements such as tracheids and vessel elements [4, 8]. This process of cell death might resemble the programmed cell death that occurs in differentiating tracheary elements derived from single cells isolated from the mesophyll of *Zinnia elegans* [9, 10]. By contrast, ray parenchyma cells are long-lived cells, derived from ray cambial cells, that remain alive for several years or more without immediate autolysis of cell organelles [11].

Cell death plays an important role in the functioning of secondary xylem cells in trees. Vessel elements and tracheids lose their organelles immediately after their differentiation, and play a critical role in the transport of water. In contrast, ray parenchyma cells retain their organelles for several years after maturation [4], and play

important roles in the transport of nutrients and storage materials [12–14]. In addition, ray parenchyma cells, prior to their deaths, synthesize heartwood substances, such as polyphenols, which contribute to the enhancement of the resistance of the tree trunk to decay and contribute to the coloration of the heartwood in some species [15–19].

In this chapter, we illustrate the application of confocal laser scanning microscopy (CLSM) for the visualization of (1) the structure of cambium and secondary xylem cells, (2) cortical MTs in cambium and differentiating secondary xylem cells, and (3) cell organelles during cell death of secondary xylem cells. The CLSM offers significant advantages over conventional microscopy techniques. The CLSM detects only information that originates from the focal plane because all other unfocused light is removed by a small pinhole in front of the image detector. Therefore, the confocal mode of the microscope with the pinhole yields a sharp and high contrast optical image in the *x*–*y* plane with a very low depth of focus. In addition, since laser beams can penetrate beneath the surface of specimens, the ability to see inside thick specimens and collect serial optical sections reveals the 3D complexity of plant cells and tissues [20–24]. The position of the focal plane is changed with a computer-controlled motor and optical sections from the interior of a thick sample can be recorded in series along the *z*-axis at user-defined intervals. Each digital image obtained via a photomultiplier can be stored automatically with a computer. Therefore, the CLSM allows easy 3D reconstruction of large and relatively thick specimens using the CLSM software. These techniques provide detailed cellular and molecular 3D information on the process of wood formation in trees.

24.2 CLSM to Visualize 3D Structure of Cambium and Secondary Xylem

Understanding the complex architecture and function of wood requires 3D imaging in order to appreciate the networks for long-distance transport throughout the plant body. Anatomical studies of xylem are routinely performed using histological sections showing three different planes relative to the axis of the stem, namely, transverse, longitudinal-radial, and longitudinal-tangential sections. Historically, 3D reconstructions of xylem were performed using a series of mechanical sections and conventional or video light microscopy (for reviews, *see* [6, 7, 25]). More recently, 3D reconstructions of xylem or cambium were performed using series of either thick mechanical sections and fluorescence microscopy or optical sections by CLSM [7, 23, 26, 27].

X-ray computer tomography (μCT) is also a very useful technique where large area 3D reconstruction of xylem structure is desired [25]. However, the resolution of cell microstructure by μCT is still less than what can be practically achieved by CLSM. CLSM can easily reveal in submicron detail the 3D structure of pit connections and cellular protuberances of xylem cells [28] or cellulose MFs in living cells [29–32].

Cambial cells have cellulosic primary walls in contrast to lignified secondary walls of xylem cells. Traditional stains for conventional light microscopy of cambium or cellulosic walls are astra blue, fast green or gentian violet [33, 34]. The use of fluorescent agents in morphological studies of cambium has been reported relatively infrequently. Kitin et al. [26, 35, 36] used safranin, calcofluor white, and acridine orange (AO) for visualization of cambium by CLSM. Other fluorescent dyes, such as Congo red or pontamine fast scarlet 4B with affinity to cellulose have been reported for visualization of non-lignified cells of xylem (Table 24.1). In addition to AO or safranin, any other periodic acid-Schiff's reagent dye could work for staining cambium (*see* Table 11.4 in [37]). AO and safranin have no specificity to cellulosic walls and produce weaker fluorescence signals from cambium when compared to xylem, which may require adjacent cambium and xylem to be imaged separately after adjustments of laser power. On the other hand, calcofluor white and Congo red have the potential to produce high contrast images for distinguishing lignified and non-lignified cell walls depending on the available fluorescence excitation/emission configuration [38, 39]. Calcofluor white was traditionally used as a fluorescent brightener in paper and textile industries as well as for studying cell walls of fungi and bacteria [40, 41]. Congo red is a diazo compound which is commonly used to stain amyloid in muscle or nerve tissue sections. Because of its specificity for cellulose and chitin, Congo red also permits the detection of fungal elements for medical diagnostics [42]. Cell walls stained with Congo red or pontamine fast scarlet 4B show bifluorescence when rotated on the microscope stage, which has been useful for measurement of MF orientation in conifer tracheids by CLSM (*see* review in [43]). Pontamine fast scarlet 4B was found to have superior bifluorescent properties and stronger specificity for cellulose compared to Congo red and Calcofluor [29, 43]. However, Congo red could be the preferred choice for achieving a higher contrast between cambium and xylem because of the following features: (1) Congo red is fluorescent only when bound to cell walls therefore dye leaching from the section and background fluorescence do not constitute a problem for prolonged imaging; (2) removal of excess dye and clearing of the sample are less critical for the quality of imaging compared to other dyes; (3) the excitation/emission maxima of Congo red (561 nm/630 nm) compared to

Table 24.1 Fluorescent dyes for cellulosic walls

Names	Recommended working solution	Excitation/emission (can vary depending on conditions and equipment)	Toxicity	References
Calcofluor white M2R	0.01 % in water	UV/blue (λ_{max} = 460 nm)	Irritating to the eyes, respiratory system, and skin	[37, 38]
Congo red	0.1 % in 50 % ethanol	Green/red (λ_{max} = 630 nm)	Possible carcinogen	[37, 38, 44]
Pontamine fast scarlet 4B	0.1 % in water	Blue-green/yellow-red, image with laser lines 488 or 561 nm (λ_{max} = 580 nm)	Possible carcinogen	[29, 32, 43]

the excitation/emission maximum of Pontamine fast scarlet 4B (488 nm/580 nm or 561 nm/580 nm), allows for red/green two-channel imaging with a less cross-talk between Congo red-stained cellulosic walls and the green autofluorescence of lignified walls or AO-staining of wood.

In thin sections for conventional light microscopy, cambial cells with their non-birefringent cell walls can be easily differentiated from secondary walls of xylem and phloem by using polarized-light optics or with traditional double staining, such as safranin/fast green, safranin/astra blue or safranin/gentian violet [35, 36, 45–47]. However, thick sections for CLSM allow for larger area 3D reconstructions and also provide less artifacts and less preparation time compared with thin sectioning of epoxy-embedded material [7, 23, 26, 48]. Furthermore, the deposition of secondary walls and lignification of cambial derivatives can be studied using autofluorescence or fluorescent probes with affinity to lignin (Table 24.2). Safranin is the most common dye used for fluorescence microscopy of xylem and its properties and applications are characterized in detail by Bond et al. [49]. Other useful dyes for the study of xylem morphology and cell lignification include AO [50], acriflavin, and toluidine blue O [38]. All of these xylem dyes are cationic and bind to negatively charged groups in lignified cell walls producing a sharp contrast to calcofluor- or Congo red-stained non-lignified walls. Safranin and AO will also stain non-lignified walls but differentiation between cambium and xylem is possible because of a considerably less fluorescence intensity of the non-lignified walls [36] (Figs. 24.1 and 24.2). The fluorescence intensity of AO or safranin staining depends on the quantity of lignin in the cell walls [49, 50].

Table 24.2 Fluorescent dyes for lignified walls

Names	Recommended working solution	Excitation/emission (can vary depending on conditions and equipment)	Toxicity	References
Acridine orange (AO)	0.001 % in water	Blue/green-orange, monomer fluorescence peaks at 487/510 (λ_{max} = 510 nm)	Possible mutagen	[36, 37, 50]
Acriflavine	0.0025 % in water	Blue/yellow (λ_{max} = 520 nm)	Possible mutagen	[38]
Basic fuchsin (pararosanilin)	0.001 % in water	Green/red, peaks at 530/640 (λ_{max} = 640 nm)	Possible carcinogen	[37, 38]
Safranin O	0.1 % in water	Blue/green-red green/red, wide ranges of absorbance and fluorescence (λ_{max} = 590 nm)	Irritating to the eyes, respiratory system, and skin	[37, 49]
Toluidine blue O (TBO)	0.005 % in water	Orange/red, absorbance and fluorescence peaks nearly overlap, Image with Ex 563 nm/LP 590 nm (λ_{max} = 610 nm)	Irritating to the eyes, respiratory system, and skin	[38]
Autofluorescence		UV/blue blue/green		[51, 52]

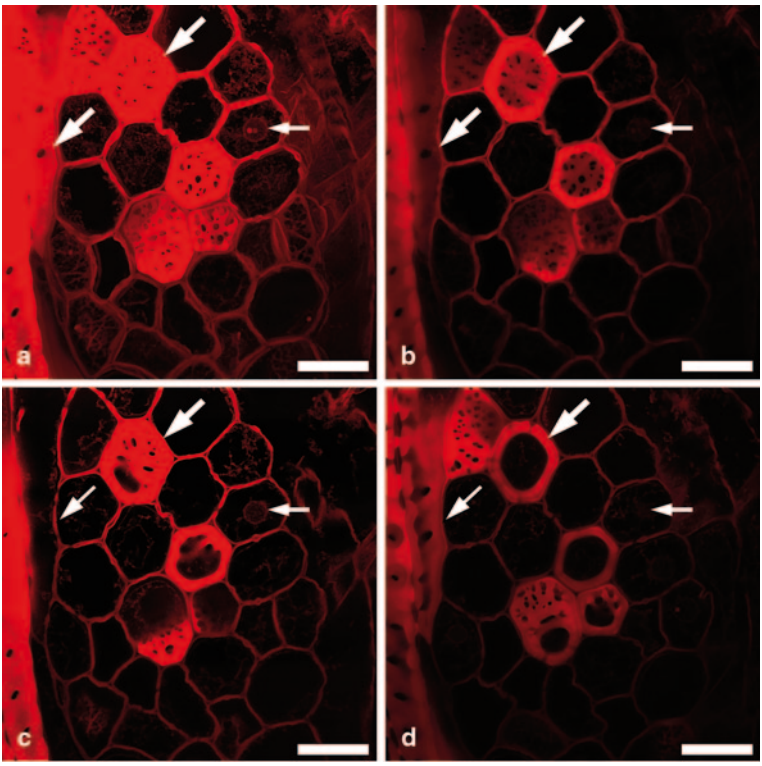

Fig. 24.1 Comparison between different methods of 3D visualization by CLSM. The images show tangential interface between cambium and xylem in *Kalopanax pictus*. **a** Maximum intensity projection, 3D rendering from a series of 26 optical sections at 1.04 μm step. **b** Average intensity projection of the same series. **c** An optical section from the same series collected at 14.6 μm beneath the surface of the section. **d** An optical section at 25 μm beneath the surface of the section. *Large arrows* point to identical xylem cells in (**a**) through (**d**). The rounded cells are ray cells and the elongated cells at the *left* are xylem fibers. Small *horizontal arrows* show an identical cambial ray cell. Note that the fluorescence intensity of the lignified xylem walls is stronger than that of cambial cells. In the maximum intensity projection (**a**), the images of xylem walls are saturated with pixels while at the same time cambial cell walls and nuclei are well visualized. In the average intensity projection (**b**), there is no pixel saturation and xylem walls are visualized with details of the cell wall pits. However, compared to (**a**) cell nuclei and protoplasts in cambial cells are not seen in (**b**). The optical sections in (**c**) and (**d**) reveal details of the cellular structure depending on the *z*-position. Note that small intercellular spaces and cell wall pits are visualized in cambial ray cells. The sample is observed with excitation from an argon ion laser (514 nm) and a long-pass filter (LP 585 nm). The sample is a hand-cut section that was stained with safranin, cleared and dehydrated in acetone, and immersed in immersion oil. The objective lens was an Olympus PlanApo 100x/0.85 (oil immersion), Kalman averaging is performed on 11 frames. *Bars* = 20 μm

Lignins and secondary compounds in wood exhibit a wide range of autofluorescence (indigenous fluorescence) which has been used for imaging the structure and degree of delignification of wood [39, 51, 53–56]. The fluorescence of AO has been found effective for imaging fungal decay of wood [57] as well as for assessment

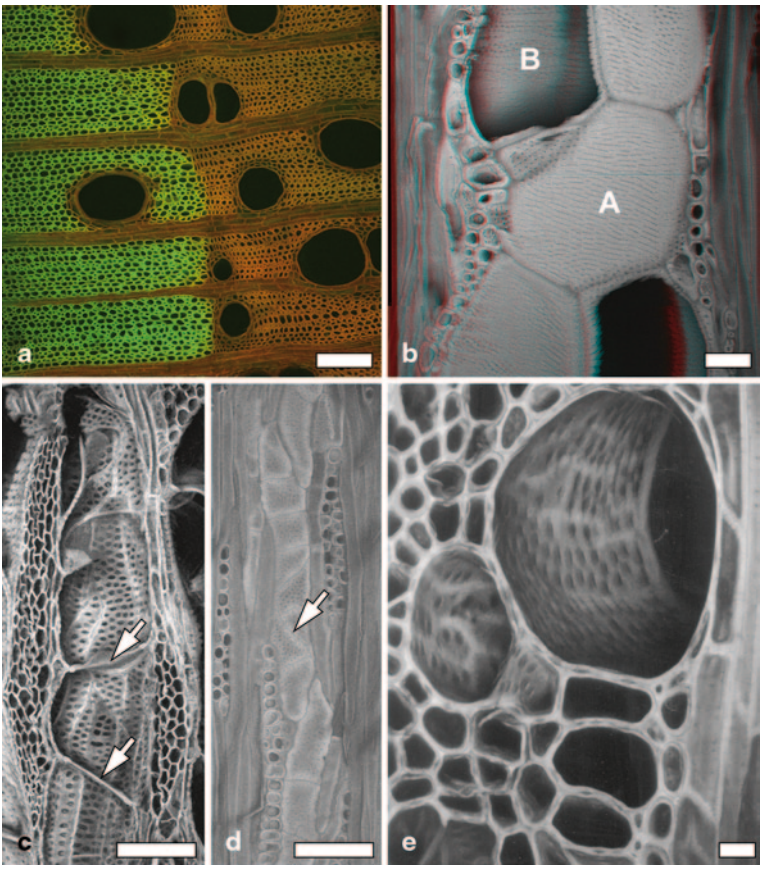

Fig. 24.2 Imaging of wood by CLSM after safranin staining. **a** Transverse section of differentiating wood in teak (*Tectona grandis* L.f) [47]. *Red* color indicates lignified mature cells and green color shows developing cell walls. Two corresponding images were collected in the *red* and *green* channels of the confocal system and the two images were merged (Ch1: Ex 488 nm/BP nm/BP 500–550 nm; Ch2: Ex 543 nm/LP 560 nm). The real *fluorescence* color was assigned to each channel. Single optical section, Plan-Neofluar 10x/0.3, Kalman averaging on eight frames. *Bar* = 200 µm. **b** Earlywood vessels in *Fraxinus lanuginosa* [7]. Portions of the inside walls of the two adjacent vessels that have twisted around each other are shown. Xylem vessels can deviate from the axial direction along their long courses. Maximum projection, 3D rendering from 61 tangential optical sections at 1 µm intervals. *Bar* = 50 µm. **c** Free-hand tangential section through differentiating secondary xylem of *Kalopanax pictus* [69]. 3D rendering from 40 optical sections obtained at 1.5 µm interval. *Arrows* point to membranes at perforation plates of developing vessel elements. *Bar* = 100 µm. **d** Tangential section of secondary xylem of *Fraxinus lanuginosa* [69]. Maximum projection, 3D rendering from 34 optical sections obtained at 1.5 µm interval. *Arrow* points to axial parenchyma cells. *Bar* = 100 µm. **e** Transverse thick section of wood of *Kalopanax pictus* [69]. Maximum projection, 3D rendering from 51 optical sections obtained at 3 µm interval. *Bar* = 20 µm. The images in (**b**) through (**e**) are obtained with excitation with a helium neon laser (543 nm) and a long-pass filter (LP 590 nm)

of the delignification of pulp fibers [50]. Optical sectioning by CLSM of safranin or AO-stained pulp fibers has also been used for measurements of the diameters of fibers [58, 59]. In addition, some fixatives can induce fluorescence in plant tissues, in particular cells with protoplasts, which can be useful for fluorescence microscopy [37, 60]. Glutaraldehyde-induced fluorescence was successfully used by Singh et al. [61] to image wood-degrading fungi.

In this section, we present protocols for the analysis of the 3D structure of cambium and secondary xylem cells in trees by CLSM.

24.2.1 Materials

1. Sample collection and fixation: sharp knives, chisel, hammer, razor blades, screw cap storage bottles, glassware, gloves and eye protection, ventilated hood, 50% ethanol, glycerol, phosphate buffered saline (PBS) buffer pH 7.3 (0.137 M NaCl and 0.05 M NaH_2PO_4), Formalin-acetic acid- alcohol (FAA; ethanol: glacial acetic acid: 37% formaldehyde: water/50: 5: 10: 35), 4% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.3), liquid nitrogen, freezer.
2. Sectioning: sliding microtome, sharp knife, disposable stainless-steel blades, glass cups, distilled water, and glycerol.
3. Cryo-sectioning: freezing stage at −20 °C (or a cold room at −20 °C), sliding microtome, sharp knife, and disposable stainless-steel blades.
4. Staining: fluorescent dyes for cambium* (Calcofluor white M2R (syn. fluorescent brightener 28), Congo red), fluorescent dyes for xylem* (AO, safranin O), glass cups, small Petri dishes or six-well-cultured plates.

(*) The choice of the dye would depend on the objectives of study and on the available fluorescence excitation and emission filter configurations (*see* Tables 24.1 and 24.2 and *Notes*).

5. Preparation of microscope slides: distilled water, ethanol, glycerol, bioleit or euparal (optional, for preparation of permanent slides), slide glasses and coverslips.
6. Observation: fluorescence microscope, CLSM.

24.2.2 Methods

24.2.2.1 Sample Collection and Fixation for Observation of Cell Walls and 3D Structure of Cambium and Adjacent Phloem and Xylem

1. Excise small blocks ($30 \times 30 \times 30$ mm^3) containing secondary phloem, cambium and secondary xylem from main stem using a sharp knife, a chisel and a hammer (*see Note 1*).

2. Depending on the purpose of observation, fix the sample blocks with (a) water/ethanol/glycerol mix (in the ratio of 45 mL/45 mL/30 mL, respectively), or (b) FAA (*see Note 2*), or (c), glutaraldehyde (*see Note 3*). The samples are inserted in bottles with the fixative and quickly brought to the lab. Fix again for about 10 min under a vacuum for removal of air from samples. Then change to fresh fixative solution for overnight fixation at room temperature.

24.2.2.2 Preparation of Thick Sections or Planed Surfaces of Cambium, Phloem, and Xylem for Wide-Field Fluorescence or Confocal Microscopy

1. Freehand sections in different planes (transverse, longitudinal-tangential, and longitudinal-radial) are prepared with a razor blade, a sharp knife or a scalpel (*see Note 4*).
2. Thick sections varying from 20 to more than 100 μm or planed surfaces of specimens are prepared with a sliding microtome (*see Note 5*).

24.2.2.3 Sample Collection and Cryo-Fixation for Observation of Cell Extractives by Wide-Field Fluorescence or Confocal Microscopy

1. Collect small blocks of tissues from main stem as described in Sect. 24.2.2.1.
2. Snap-freeze the blocks in liquid nitrogen.
3. Store in a freezer at −12 °C or below.
4. Cut frozen sections at −20 °C and immediately insert sections in liquid nitrogen (*see Note 6*).
5. Observe the autofluorescence of frozen sections using a fluorescence microscope equipped with a cryo-stage (*see Note 7*).
6. Alternatively, the cryo-fixed sections can be freeze-dried and observed with a wide-field fluorescence microscope or a CLSM at room temperature (*see Note 8*).

24.2.2.4 Congo Red Staining

1. Wash the fixed sections in running tap water for 30 min.
2. Stain with a 0.1 % Congo red in 50 % ethanol for 5–10 min at room temperature.
3. Wash two times with distilled water.
4. Place sections on a glass slide, apply a drop of aqueous glycerol, and mount the sections with coverslips.
5. Observe with a wide-field fluorescence or a CLSM with a dual channel excitation Ex 488 nm/BP 510 nm and Ex 561 nm/LP 590 nm (*see Note 9*).

24.2.2.5 Acridine Orange/Congo Red Staining for Differentiation of Cambium and Xylem

1. Wash the fixed sections in running tap water for 30 min.
2. Immerse in 0.001 % aqueous AO for 5 min at room temperature.
3. Remove excess dye in an ethanol series (25, 50, 70, and 95 %) for 30 min with several washes at each concentration (*see Note 10*).
4. Return to water through an ethanol series (70, 50, and 25 %) for 30 min at each concentration.
5. Follow steps 2 through 4 from Sect. 2.2.4 (*see Note 11*).
6. Observe with a fluorescence microscope using excitation/emission combination for AO (BP 465–505 nm/BP 515–565 nm) and for Congo red (BP 534–558 nm/LP 590 nm, or with a CLSM with a dual channel excitation/emission Ex 488 nm/BP 510 nm and EX 561 nm/LP 590 nm (*see Note 12*).

24.2.2.6 Calcofluor Staining

1. Wash the fixed sections in running tap water for 30 min.
2. Immerse in 0.01 % wt/vol aqueous Calcofluor white M2R for 5 min at room temperature.
3. Rinse two times with distilled water.
4. Put sections on a glass slide, apply a drop of aqueous glycerol, and mount the sections with coverslips.
5. Observe with a wide-field fluorescence microscope at 340–380 nm excitation and a long pass emission filter (LP 420) or with a CLSM with Ex 405 nm/LP 420 nm (*see Note 13*).

24.2.2.7 Calcofluor/Safranin Staining for Differentiation of Cambium and Xylem

1. Wash the fixed sections in running tap water for 30 min.
2. Immerse in 0.1 % aqueous safranin for 5 min at room temperature.
3. Remove excess dye in an ethanol series (25, 50, 70, and 95 %) for 30 min with several washes at each concentration until no leach of dye is visible (*see Note 14*).
4. Return to water through an ethanol series (70, 50, and 25 %) for 30 min at each concentration.
5. Follow steps 2 and 3 from Sect. 24.2.2.6.
6. Place sections on a glass slide, apply a drop of water, and mount the sections with coverslips.

7. Observe with a wide-field fluorescence microscope at 340–380 nm excitation and a long pass emission filter (LP 420 nm) or with a CLSM with a dual channel excitation/emission combinations for calcofluor Ex 405 nm/BP 410–485 nm and for safranin Ex 488 nm/BP 515–565 nm or Ex 541 nm/LP 590 nm (*see Note 15*).

24.2.2.8 Staining of Cambium with Safranin

1. Follow steps 1 and 2 from Sect. 24.2.2.7.
2. Remove excess dye in an acetone series (25, 50, 70, and 100 %) for 30 min with several washes at each concentration until no leach of dye is visible (acetone removes safranin from cellulosic walls less than ethanol while at the same clears the background staining (Fig. 24.1).
3. Put sections on a slide, blot the acetone and mount in immersion oil (*see Note 16*).
4. Alternatively, after step 2 return to water through an acetone series (70, 50, and 25 %) for 30 min at each concentration.
5. Put sections on a slide, apply a drop of water, and mount the sections with coverslips.
6. Observe with a CLSM with excitation/emission for safranin Ex 488 nm/BP 515–565 nm or Ex 541 nm/LP 590 nm (Fig. 24.2).

24.2.2.9 Mounting Sections in Immersion Oil or in Resin

1. Stain sections with calcofluor, Congo red, safranin, or AO as indicated above.
2. Remove excess dye in an ethanol series (25, 50, 70, 95, and 100 %) for 30 min with several washes at each concentration until no leach of dye is visible.
3. Rinse in xylene.
4. Mount in euparal or bioleit (not appropriate with calcofluor-stained specimens because of blue background autofluorescence of the resins). Sections can be mounted in euparal directly after dehydration in the ethanol series as ethanol is soluble in euparal.
5. Alternatively, sections can be mounted in immersion oil after dehydration in acetone or after freeze-drying (*see Note 16* and Fig. 24.1).
6. Observe with the appropriate excitation/emission filters.

24.2.3 Notes for Subheading 24.2

1. It is accepted as a standard to sample wood at breast height (1.30 m above ground) of mature tree stems. Since the activity of cambium as well as the wood

structure may vary depending on the position in different parts of the tree, indicate the position of the probes (height of stem, age of branches or roots). With a chisel and a hammer remove the outer layers of bark. Then, using a sharp knife cut notches deep into the phloem and wood in the form of a square with the desired dimensions of the wood probe, usually about 3 × 3 cm tangential face. Then, insert the chisel into the notch with the help of the hammer and cutout a block of cambium and the adjacent xylem and phloem. In our experience, this procedure ensures the best preservation of the integrity of the cambial layer and the files of differentiating cells. The cambial and differentiating xylem cells are very fragile and special care has to be taken for their preservation, particularly during periods of active growth when the cambial zone contains several layers of vacuolated cells with thin walls. An increment puncher for quick and little-destructive sampling of cambium and wood has been used [62]. This device was found useful for multiple samplings, for assessments of the cambial activity and for DNA or chemical analyses of cambium.

2. The water/ethanol/glycerol mix provides an adequate preservation of cell walls and nuclei when samples are stored at 4 °C. The green-yellow fluorescence of xylem cell walls can be useful for morphological observation by fluorescence microscopy [7, 63]. FAA is a well-studied aldehyde fixative of plant tissues for preservation of cell wall structure and cell organelles (*see* [37, 60]). FAA has to be well washed out from the specimens before the staining or mounting on slides for microscopy. We rinse FAA-fixed samples for at least 30 min in running tap water before cutting sections for microscopy.
3. Glutaraldehyde is a well-characterized fixative for preservation of cell organelles for light or electron microscopy (*see* also *Note 3.3. 1*). The infiltration of plant tissues with glutaraldehyde is slow and requires longer time of fixation. We further cut the samples of cambium and wood into 2–3-mm-thin longitudinal slivers and infiltrate with 1 % glutaraldehyde under vacuum [35, 36]. Glutaraldehyde induces fluorescence in plant tissues, which can be used for fluorescence studies without the need for staining [61].
4. For fluorescence microscopy or CLSM, the thickness of section is not critically important for the quality of images [26, 36]. It is more important to have evenly planed surfaces of the specimens. Use new sharp blades for achieving smooth cuts and better preservation of cell shapes.
5. Sliding microtomes are great tools for achieving evenly cut surface of specimens, as well as series of sequential sections with controlled thickness. Kitin et al. [7] were able to cut and reconstruct 21.2-mm-long series of sequential 200-μm-thick sections. Sectioning artifacts such as distortions of shapes or damages of cell walls are less common in thick sections than in thin ones. For better preservation of cambium and bark structure during sectioning with a sliding microtome, some authors employ embedding of samples in PEG [46, 64, 65].
6. Commercial cryotomes may not always be suitable for cutting specimens of wood. Sano et al. [66], Utsumi et al. [67, 68], and Kitin et al. [39] cut frozen xylem tissue with a sliding microtome or by freehand with a razor blade in a cold room (walk-in-freezer) at −20 °C.

7. As an alternative to the commercial cryo-stages for microscopy, Kitin et al. [39] and Barnard et al. [63] used an in-house built cryo-system for observation of frozen wood specimens with a fluorescence microscope.
8. Cryo-fixation followed by freeze-drying preserves the content and original position of secondary metabolites or tracer dyes within cell lumens. Then, freeze dried specimens can be observed with a wide-field fluorescence microscope or a CLSM at room temperature (*see* [39, 63]).
9. Congo red can be visualized by CLSM with excitation/emission Ex 561 nm/LP 590 nm. The second channel at Ex 488 nm/BP 510 nm reveals the autofluorescence from lignified xylem cell walls, which combined with the red signal of Congo red produces a good contrast between lignified xylem and cambium. Variations in the intensity of Congo red should be considered with caution because of the bifluorescent properties of this dye. The fluorescence intensity of bifluorescent dyes (Congo red, Pontamine fast scarlet 4B) depends on the orientation of cellulose MFs in the image plane [29, 43].
10. Rinsing in an ethanol series will remove excess dye and nonspecific staining. Sections should not be stored in solutions of alcohol, even after destaining, because AO dye will continue to slowly leach out of the sections. For prolonged storage, AO or safranin stained sections can be kept in xylene.
11. AO stains both cellulosic and lignified walls but the staining of cellulose is relatively weak. Aqueous glycerol can be used as a mounting medium with a higher refractive index for observations with glycerol or immersion oil objectives [36]. However, AO tends to leach out of specimens stored for long time in glycerol. The leach is mainly from cellulosic walls and not from lignified walls where AO is strongly attached. While the leach will increase the contrast between xylem and cambium, it will also result in unwanted background fluorescence, therefore, the mounting medium has to be refreshed right before imaging.
12. AO can fluoresce from green to red depending on the concentration of the dye. The peak of fluorescence excitation/emission of the pure monomer form of AO, when the dye is at low concentrations, is around Ex 490 nm/Em 525 nm. A similar peak of fluorescence is observed in wood sections stained with strongly diluted AO. The fluorescence of the dimer found in AO solutions at higher concentrations is around Ex 460 nm/Em 650 nm but the dimer signal disappears or is very weak after the destaining step via ethanol series. Two channels excitation/emission Ex 488 nm/BP 515–565 nm for AO and Ex 561 nm/LP 590 nm for Congo red-stained samples yields images with high contrast between cambium (red) and lignified xylem (green).
13. Calcofluor dissolves better in water at higher pH, therefore, adding few drops of 1 N NaOH is recommended [37, 38]. However, we achieved strong staining of cambium with low concentrations of calcofluor without adding NaOH. Observation with a wide-field fluorescence microscope at 340–380 nm excitation and a long pass emission filter (LP 420 nm) or by CLSM with a dual-channel Ex 405 nm/LP 420 nm and Ex 488 nm/LP 510 nm results in blue images of cambial cell walls and green-yellow images of lignified xylem.

14. Safranin will slowly but continuously leach out when sections are left in ethanol. Safranin-stained sections can be stored for a prolonged period of time in xylene without much losing the intensity of safranin-staining, however, the calcofluor-staining may have to be refreshed after prolonged storage.
15. The results are strong blue fluorescence from cambium and cellulosic walls and strong green-red fluorescence from lignified walls. The intensity of the green varies in relation to variations in the amount of lignin [49]. After the destaining steps 3 and 4, which remove excess dye, sections can be mounted in aqueous glycerol for observation with glycerol or immersion oil objective lens [23, 36]. However, slides must be prepared immediately before observation because safranin will continue to slowly leach out and produce fluorescent background in glycerol mounting medium.
16. In immersion oil, these dyes will not noticeably leach out from the specimen and the higher refractive index of immersion oil improves the quality of optical sectioning by CLSM [38]. Freeze-drying is usually appropriate for preparation of samples of xylem but may cause deformations in the cambial tissue. We have achieved good results for observing cell walls by dehydrating safranin-stained samples of cambium in an acetone series (*see* Sect. 2.2.8) and mounting in immersion oil (Fig. 24.1).

24.3 CLSM to Visualize Cortical Microtubules

The successful introduction of indirect immunofluorescence microscopy made it possible to visualize MTs over large areas within plant tissues [70]. In addition, CLSM with immunofluorescence staining made it possible to construct 3D images of MTs in cambium and differentiating secondary xylem or phloem cells of several trees such as *Abies sachalinensis, Abies firma, Pinus densiflora, Taxus cuspidata, Larix leptolepis, Aesculus hippocastanum*, and *Populus tremula* x *P. tremuloides* [3, 4, 8]. This method provides a powerful tool to follow the dynamics of MTs.

Cortical MTs have been observed in active and dormant cambial cells and their derivatives of trees by immunofluorescence microscopy [13, 71–88]. Random arrays of cortical MTs have been demonstrated in fusiform cambial cells [72, 73, 75–81]. A careful study of cortical MTs in differentiating tracheids and wood fibers has revealed that the predominant orientation of cortical MTs is longitudinal with respect to the axis of the cell at the early stage of cell expansion [72, 73, 77, 80, 81]. The predominant orientation changes progressively from longitudinal to transverse during the radial expansion of cells. Finally, ordered and transversely oriented cortical MTs are observed in tracheids at subsequent stages of differentiation when radial expansion ceases. These observations indicate that the orientation and organization of cortical MTs in differentiating tracheids and wood fibers changes successively during formation of the primary wall.

Successive changes in the orientation of cortical MTs can be observed in differentiating tracheids or wood fibers during the formation of secondary walls [72, 74, 80, 82, 87]. The orientation of cortical MTs changed by clockwise rotation (when viewed from the lumen side) from a flat S-helix to a steep Z-helix. Then, the cortical MTs are oriented in a steep Z-helix at almost the same angle in tracheids or wood fibers of the radial file. After further differentiation, the orientation of cortical MTs returns from the steep Z-helix to a flat S-helix in tracheids or wood fibers. During the formation of the secondary wall, newly deposited cellulose MFs changes successively their orientation from a flat S-helix to a steep Z-helix and then to a flat S-helix [89]. These observations provide evidence for the hypothesis that the orientation of cortical MTs changes progressively in a similar manner to the changes in the orientation of newly deposited cellulose MFs during the formation of the secondary wall. Thus, the cortical MTs might control the ordered orientation of cellulose MFs in tracheids or wood fibers in trees.

Heterogeneous thickenings of the secondary wall are normal features of the cell wall in the secondary xylem cells. These modifications in structure, such as pits, helical thickenings, and perforation plates, are formed by localized deposition of cellulose MFs. During the formation of pits, helical thickenings, and perforation plates, the localization of cortical MTs is found in differentiating secondary xylem cells [73, 75, 79–81, 83, 90]. In addition, the application of an MT-depolymerizing agent, such as colchicine, disrupts some cortical MTs in differentiating tracheids of some species such as *Taxus cuspidata*, in which helical thickenings are generally formed [90]. Such tracheids have no helical thickenings. Therefore, localized cortical MTs control the localized deposition of cellulose MFs in the cell wall, thereby the formation of modified structure.

These results using immunofluorescence microscopy provide strong evidence that the dynamics of cortical MTs are closely related to the orientation and localization of newly deposited cellulose MFs in the differentiating secondary xylem cells. The dynamics of cortical MTs control the orientation and localization of newly deposited cellulose MFs in the cell wall, thereby, controlling the structure and function of secondary xylem cells in gymnosperm and angiosperm trees [3, 4, 8, 70, 91].

In this section, we present protocols for visualizing the orientation and localization of cortical MTs in cambium and differentiating secondary xylem cells in trees by CLSM.

24.3.1 Materials

1. Sample collection: sharp knives, scalpels, razor blades, and Piperazine-*N′,N′*-bis(2-ethanesulfonic acid) (PIPES) buffer.
2. Fixation: glass vials, razor blades, deep freezer at −80 °C, *p*-formaldehyde, glutaraldehyde, dimethyl sulphoxide, Nonidet P-40, PIPES buffer, ethylene glycol-*bis*(b-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), $MgSO_4$, NaOH, distilled water, liquid nitrogen.

3. Cryo-sectioning: freezing stage at −20 °C, sliding microtome, disposable stainless-steel blades, distilled water, NaCl, KCl, KH_2PO_4, and Na_2HPO_4.
4. Immunostaining of cortical MTs: Parafilm™, Petri dish, pipettes, drying oven at 30 °C, deep freezer at −80 °C, refrigerated centrifuge, glass slides, coverslips, filter papers, glass vials with top, aluminum foil, freezer at −20 °C, PBS, glycerol, *p*-phenylene diamine, NaN_3, bovine serum albumin, anti-a-tubulin antibody, fluorescein *iso*thiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibody.
5. Observation: CLSM.

24.3.2 Methods

24.3.2.1 Sample Collection

1. Take blocks from a 30 mm x 30 mm area containing outer bark, mature and differentiating phloem, cambial zone cells and mature and differentiating xylem from the stem of trees using a sharp knife and a scalpel.
2. Cut longitudinal slivers, with dimensions approximately 30 mm (longitudinal) x 5–10 mm (radial) x 2 mm (tangential), from the block with a razor blade under 50 mM PIPES buffer. The pH of the buffer is adjusted to approximately 7.0 with 1 M NaOH.
3. Remove the outer bark.

24.3.2.2 Fixation for Immunostaining

1. Fix longitudinal slivers for about 20 min under a vacuum for removal of air from intercellular spaces in vials in a mixture of 3.6 % *p*-formaldehyde and 0.2 % glutaraldehyde that contains 10 % DMSO and 0.1 % Nonidet P-40 in 50 mM PIPES buffer, pH 7.0, supplemented with 5 mM EGTA and 10 mM $MgSO_4$ (*see Notes 1* and *2*).
2. Fix again in fresh fixative overnight at room temperature (*see Note 3*).
3. Remove the terminal 5 mm ends of the slivers with a razor blade to eliminate damaged regions. Since fusiform cambial cells and differentiating tracheids generally reach up to 4 mm in length, cells at the terminal ends might be expected to have lost their cytoplasm during sampling.
4. Trim the slivers into small blocks of 5–10 mm (longitudinal) x 5–10 mm (radial) x 2 mm (tangential).
5. Wash briefly in distilled water.
6. Freeze in liquid nitrogen. The frozen samples can be stored in a freezer at −80 °C for at least 6 months.

24.3.2.3 Cryosectioning

1. Put each of small blocks in a drop of distilled water on a freezing stage of sliding microtome at −20 °C.
2. Cut longitudinal radial sections at a thickness of approximately 50 μm with a disposable stainless-steel blade.
3. Wash three times for 3 min each with PBS in a polystyrene microplate.

24.3.2.4 Immunostaining of Microtubules

1. Place sections on a sheet of Parafilm™ in a Petri dish, and replace lid.
2. Apply the primary mouse-raised monoclonal antibodies (approximately 20 μL per section) against chicken brain α-tubulin with a pipette and incubate for 60–120 min at 30 °C in darkness in the drying oven (*see Notes 4* and *5*).
3. Wash three times for 3 min each with PBS.
4. Apply the FITC-conjugated secondary antibodies (approximately 20 μL per section) with a pipette and incubate for 60–120 min at 30 °C in darkness. These antibodies are diluted 1:10 in PBSB and the solution of diluted antibodies should be stored at 2–8 °C in darkness.
5. Wash three times for 3 min each with PBS in darkness. The sections can be stored in PBS in darkness for 2–3 days at room temperature.
6. Mount sections stored in PBS on glass slides in 50 % glycerol in PBS that has been supplemented with an antioxidant, such as 0.1 % *p*-phenylene diamine with coverslips to reduce photo-bleaching. In particular, antifade mounting agents should be used for FITC-stained sections to protect against photo-bleaching because this dye is much more stable at or above pH 8 than at pH 7. The diluted mounting agent should be stored in darkness (e.g., in vials wrapped in aluminum foil) in a freezer at −20 °C (*see Note 6*).
7. Wipe off excess mounting medium with a filter paper.

24.3.2.5 Optical Sectioning by CLSM

Several companies such as Carl Zeiss, Leica, Olympus, Nikon, etc., have produced confocal laser scanning microscopes. Detailed operation procedures are different between microscopes. Therefore, we need to follow the manual of manufacturers operation of each microscope. In this chapter, we observed MTs with a confocal laser scanning microscopes from Carl Zeiss (LSM-310, LSM-410, and LSM-710; Carl Zeiss Co.). An outline of the operating instructions for LSM-310 and LSM-410 is given below.

1. Place a section on the stage of the microscope. An objective lens at low magnification, such as 10×, with conventional transmitted light, with reflected light or in the fluorescence mode, is used to select the area to be examined.

2. Select a wavelength of lasers and an emission filter appropriate to the investigation. Since the peak wavelengths for excitation and emission of conjugated-FITC are 496 and 518 nm, respectively, an argon ion laser (488 nm, blue) or a krypton–argon ion laser (488 nm) is used to excite FITC.
3. Select a bandpass filter (510–525, 515–540, or 515–565 nm) as the emission filter for detection of excited FITC. In the case of LSM-710, select the name of dye used (e.g., FITC) and a wavelength of lasers and region of emission are automatically selected. Lignified cell walls of secondary xylem cells have autofluorescence. An adequate combination of a wavelength of lasers and an emission filter reduces effectively the background due to autofluorescence.
4. Select the objective lens (e.g., 40 or 63×), the wavelength of laser beam (e.g., 364, 488, 543, or 568 nm), the scan field size (e.g., 512 × 512, 1024 × 1024 or 2048 × 2048 (for LSM-710) pixels), the intensity of laser light (choice of optical attenuating filters), electron bandwidth (choice of low-pass filters), scan time, zoom factor, pinhole size, and region of interest on the sample, on the control panel. The pixel size (μm/pixel), which is adjusted by zoom factor, should be determined by width of field-views, optimum objective resolution and photo-bleaching. A high zoom factor (small pixel size), scans over small areas of the specimen. Thus, it produces a high magnification in the final image on the display but increases the photo-bleaching. By contrast, a low zoom factor (large pixel size) increases the field of view and reduces the photo-bleaching. In the case of LSM-710, push "Optimal" button to select the most adequate pixel size by the wavelength of lasers and the numerical aperture (NA) of lens. In addition, push "1AU" button to select the adequate pinhole size according to the objective lens, NA of lens and wavelength of lasers. An objective dry lens 40× (NA 0.75 or 0.95) is often used for the wide field views of cortical MTs, although the confocal depth becomes smaller than the objective lens with a higher NA (*see Note 7*).
5. Start the laser beam scanning.
6. Adjust the position of pinhole. This is one of the most important steps and it is a pre-requisite for enhancement of the image from only a single focal plane. In the case of LSM-710, the position of pinhole is automatically adjusted.
7. Adjust alternately contrast and brightness. The contrast function controls the high voltage at the photo-multiplier and the electronic pre-amplification factor. The over-saturation of contrast should be avoided. The brightness function controls the electron offset, which determines the overall brightness of the image. However, the setting of high brightness increases background noise. Thus, a compromise must be found. In the case of LSM-710, push "Auto Exposure" to adjust Master Gain (contrast and brightness).
8. Produce a single optical image. Numbers for averaging images in a line or a frame are selected to eliminate effectively the noise. Four- or eight-times averaging is usually used, but at the expense of photo-bleaching.
9. Obtain a series of optical sections along the *z*-axis (referred to as *z*-sectioning, *z*-series, or *z*-stack). The starting position for optical sectioning, the distance between planes (e.g., 0.5 or 1 μm along the *z*-axis), the number of sections, the refractive index of the objective, and the refractive index of the immersion fluid

must all be selected. Fifteen to thirty optical sections along the *z*-axis at 1 μm intervals are usually obtained for the cytoskeleton in differentiating tracheids and ray parenchyma cells (*see Note 8*).
10. Store automatically the sequence of digital images in the host computer's memory on a hard disk or a CD-disk. In general, the Tag Image File Format (TIFF) is used.
11. Obtain images with transmitted light for observations of the structure of cells. Other optical imaging techniques, such as differential interference contrast (DIC), phase contrast, polarisation contrast, and dark-field contrast can also be used.

24.3.2.6 Construction of 3D Images

Each series of confocal images can be processed for 3D reconstruction of the sample with the software of the CLSM. The following methods can be used for depiction of 3D information; the choice depends on the particular CLSM.

1. Gallery: the simultaneous display of several images.
2. Projection: a single projection image or a series of projections after rotation for an animation.
3. Animation: scrolling of a series of images, resembling a film.
4. Depth coding: a colored projection for visualization of depth information.
5. Stereo imaging: a stereo image that can be observed with red/green spectacles (an anaglyph) or a pair of stereo images (split stereo images).
6. Orthogonal sections: sections from a 3D packet with borders that parallel the principal planes, namely, *x–y, x–z*, and *y–z*.
7. Overlay: superimposed images of two or three images of the same area, for example, a fluorescent image and a transmitted image. Pseudocolors are used to mimic the original fluorochromes (e.g., green for FITC).

The 3D images of cortical MTs in differentiating ray parenchyma cells and tracheids are shown in Fig. 24.3a, b, c, d, e. Since ray parenchyma cells or tracheids derived from ray cambial cells or fusiform cambial cells are aligned in a radial direction, successive aspects of differentiation of secondary xylem cells can be observed in a radial file within a single longitudinal radial section. Therefore, cambial derivatives are a suitable system to follow the process of xylogenesis in situ.

24.3.3 Notes for Subheading 24.3

1. *p*-Formaldehyde, which has monoaldehyde groups, forms bridges via amino and peptide linkages between neighboring peptide chains. Glutaraldehyde, which is a dialdehyde, increases the potential for the cross-linking of peptide chains. Therefore, glutaraldehyde provides better preservation of cell structure and cell contents than *p*-formaldehyde, but it may eliminate the recognition of

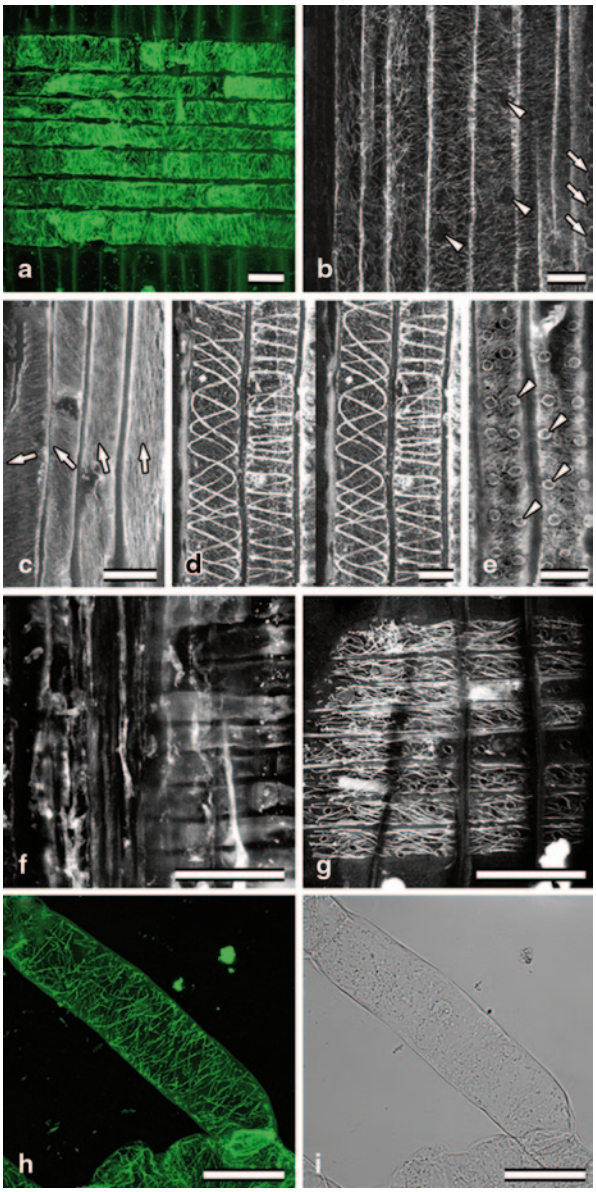

Fig. 24.3 Immunofluorescence images (projection images), obtained by CLSM, in differentiating ray parenchyma cells, tracheids and cultured cells. **a** Cortical MTs in xylem ray parenchyma cells of *Taxus cuspidata*. The orientation of cortical MTs is transverse or oblique with respect to the cell axis during radial elongation of cells. *Bar*=25 μm. **b** Cortical MTs during formation of the primary wall in differentiating tracheids of *Abies sachalinensis*. Cortical MTs disappear locally (*arrowheads*) at sites of future intertracheal *bordered pits* and *circular bands* of cortical MTs (*arrows*) are visible around the edges of developing bordered pits. *Bar*=25 μm. **c** Successive changes in the orientation of cortical MTs, viewed from the lumen side, in differentiating tracheids of *Abies sachalinensis*. Changes in the orientation of cortical MTs (*arrows*) from a flat S-helix to a steep

proteins by antibodies. The addition of small amounts of glutaraldehyde, for example, 0.1–0.2 %, to *p*-formaldehyde can improve the quality of fixation for immunostaining.

2. Detergents, such as Nonidet P-40, facilitate penetration of sections by antibodies, in particular, when sections are relatively thick (e.g., 50 μm).
3. Since MTs tend to depolymerize at low temperatures, it is necessary to avoid low temperatures during the fixation of samples. However, cold stability of MTs in cambium and differentiating secondary xylem cells shows seasonal variations ([87, 88]; Fig. 24.3f and g). The presence of secondary wall of differentiating cells also affects cold stability of MTs.
4. The antibodies (aliquots) should be stored in a freezer at −80 °C. Each antibody is diluted 1:500 in PBS that contains 0.1 % NaN_3 and 1 mg mL^{-1} BSA (PBSB). After dilution, the antibody solution is clarified at 12,000× g for 5 min at 0 °C with a refrigerated centrifuge. The resultant solution should be stored at 2–8 °C and not frozen. Repeated freezing and thawing can denature antibodies.
5. When we visualize MTs in calli, prior to immunofluorescence staining, fixed and washed cells with PBS are placed on coverslips coated with polylysine and then treated with a solution of 1 % cellulase and 0.05 % pectolyase. Then, immunofluorescence staining is performed with rat monoclonal antibodies against α-tubulin that has been diluted 1:10 in PBSB for 60 min at 30–35 °C ([92]; Fig. 24.3h and i).
6. Autofluorescence of tissues can be reduced by lightly staining sections with 0.01 % (w/v) Toluidine Blue in PBS before mounting in anti-fade mountant (*p*-phenylene diamine in glycerol) [76, 79].
7. An objective lens with a high NA is needed for high resolution. However, the working distance of the lens becomes the factor that limits the depth of optical sectioning. The confocal depth is also determined by the refractive index of the immersion fluid. The refractive index of water and oil is 1.33 and 1.52, respectively. Therefore, a water immersion lens has higher resolution than an oil immersion lens when the NA is the same.
8. The confocal depth (resolution on the *z*-axis) is determined by the NA of lens, the wavelength of the exciting laser and the size of the pinhole. For example, the confocal depth is approximately 0.5 μm when an oil immersion lens with an NA of 1.4 is used with an exciting laser at 488 nm. The use of longer wavelengths

---

Z-helix during formation of the secondary wall. *Bar* = 25 μm. **d** The localization of cortical MTs (a pair of stereo images) at the final stage of formation of the secondary wall in differentiating tracheids of *Taxus cuspidata*. Bands of helically oriented cortical MTs are visible during formation of helical thickening. Bar = 25 μm. **e** Circular bands of cortical MTs (*arrowheads*) are visible around the edges of pit borders of differentiating tracheids in the cross-field (between tracheids and ray parenchyma cells) of *Taxus cuspidata*. *Bar* = 25 μm. **f** MTs are not visible in differentiating xylem ray parenchyma cells of *Abies firma* due to depolymerization after low-temperature fixation (2–3 °C) in May (during season of active cambium). *Bar* = 25 μm. **g** MTs remain visible in xylem ray parenchyma cells of *Abies firma* after low-temperature fixation (2–3 °C) in December (during season of dormant cambium). *Bar* = 25 μm. **h** Cortical MTs in suspension-cultured cells of *Cryptomeria japonica*. Transverse MTs with respect to the long axis of the cell are visible. *Bar* = 50 μm. **i** Bright-field micrograph of the same field as in **h** showing an elongating cultured cell. *Bar* = 50 μm

of the exciting laser such as a krypton–argon ion laser (647 nm) provides lower confocal depth than the use of shorter wavelengths such as an argon ion laser (364 or 488 nm). Therefore, if we need the same confocal depth at a different wavelength, we must adjust the size of the pinhole.

24.4 CLSM to Visualize Cell Organelles During the Process of Cell Death

Morphological changes and the timing of disappearance of individual organelles during cell death provide important clues to the mechanism of cell death. In previous studies, we used short-lived ray tracheids and long-lived ray parenchyma cells as model systems for the analysis of the deaths of secondary xylem cells in situ [11, 85, 86, 93–95]. We revealed the timing of disappearance of organelles, such as nuclei and vacuoles in the process of the death of secondary xylem cells by conventional light or fluorescence microscopy and CLSM [85, 86]. In addition, visualization of heartwood substances is important for understanding the process of biosynthesis and the diffusion pathway of heartwood substances in the process of formation of heartwood. Imaging systems combined with chemical analysis, such as ultraviolet (UV) microspectrophotometry and time-of-flight secondary ion mass spectrometry (TOF-SIMS) are effective tools for investigation of localization of heartwood substances at cellular level [96–100]. In a previous study, we observed changes in autofluorescence of cell walls due to lignin and deposition of heartwood substances during heartwood formation in *Robinia pseudoacacia* var. *inermis* by CLSM with fluorescence spectrum analysis [101]. This method is useful for detecting the changes in cell wall components.

In this section, we introduce techniques for the visualization of nuclei and vacuoles, the measurement of fluorescence spectra of autofluorescence in cell walls by CLSM and the detection of nuclear DNA fragmentation in secondary xylem cells in trees. These methods provide useful information for understanding the mechanisms of differentiation and death of secondary xylem cells in trees.

24.4.1 Materials

1. Sample collection and fixation for visualization of nuclei and detection of fluorescence spectra of cell walls: incremental borer, incremental core, 4% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.3).
2. Sample collection and fixation for visualization of vacuoles in living cells or nuclear DNA fragmentation: small blocks containing cambium, phloem and xylem, sharp knife, chisel, hammer, 0.2 M aqueous sucrose, 4% paraformaldehyde solution in PBS.

3. Resin embedding: Technovit 7100 kit, razor blades, screw vial, ethanol series (30, 50, 70, 90, and 100 %), disposable paper cup, micropipette, gelatin capsule, tweezers.
4. Sectioning, staining, and observation: razor blades, sliding microtome, disposable stainless-steel blades, freezing stage at −20 °C, wood block (10 × 10 × 10 mm^3), superglue, ultramicrotome, glass knife.
5. Staining and observation: 4′,6-diamidino-2-phenylindole (DAPI), permeabilisation solution (0.1 % Triton-X in 0.1 % sodium citrate, freshly prepared), fluorescein diacetate (FDA), 0.1 M phosphate buffer (pH 7.3), TdT-mediated dUTP nick end labeling (TUNEL) assay kit (e.g., in situ cell death detection kit, fluorescein, Roche diagnostics), screw vial, aluminum foil, distilled water, Petri dish, watch glass, aqueous glycerol, slide glass, coverslip, fluorescence microscope, and CLSM.

24.4.2 Methods

24.4.2.1 Sample Collection and Fixation for Visualization of Nuclei and Detection of Fluorescence Spectra of Cell Walls

1. Take incremental cores (diameter: approximately 5 mm) containing cambium, sapwood and heartwood from main stem using an incremental borer.
2. Fix incremental cores for about 10 min under a vacuum for removal of air from samples in a centrifuge tube in a 4 % glutaraldehyde solution in phosphate buffer (pH 7.3).
3. Fix again in fresh fixative solution overnight at room temperature (*see Note 1*).

24.4.2.2 Sample Collection for Visualization of Vacuoles in Living Cells

1. Take small blocks (30 × 30 × 20 mm^3) containing secondary phloem, cambium and secondary xylem from main stem using a sharp knife, a chisel, and a hammer.
2. Immerse collected blocks into 0.2 M aqueous sucrose to avoid desiccation.
3. Cut slivers from collected blocks at 2 mm thickness in tangential direction.
4. Cut small pieces (10 × 10 × 2 mm^3) from slivers with a fresh razor blade.

24.4.2.3 Sample Collection and Fixation for Detection of Nuclear DNA Fragmentation

1. Take small wood blocks (30 × 30 × 20 mm^3) from main stem as described in Sect. 24.4.2.2.
2. Cut slivers from collected blocks at 1 mm thickness in tangential direction.

3. Cut small pieces ($10 \times 10 \times 1$ mm^3) from slivers with a fresh razor blade.
4. Fix small pieces for about 10 min under a vacuum for removal of air from samples in screw vials in a 4% paraformaldehyde solution in PBS.

24.4.2.4 Resin Embedding for Detection of Nuclear DNA Fragmentation

1. Wash small pieces with PBS two times.
2. Cut small blocks (3×2 x 1 mm^3) from small pieces with a fresh razor blade.
3. Wash small blocks with PBS three times.
4. Dehydrate small blocks with graded ethanol series, 30, 50, 70 for 15 min, 90% for 30 min, respectively.
5. Dehydrate small blocks absolutely with 100% ethanol for 60 min three times.
6. Prepare Technovit 7100 resin according to manufacturer's manual.
7. Infiltrate Technovit 7100 resin and ethanol mixture (1:5 v/v), (1:3 v/v), (1:1 v/v), (3:1 v/v), and (5:1 v/v) for 1 h each.
8. Infiltrate Technovit 7100 resin (100%) with rotator overnight.
9. Set up gelatin capsules on stand.
10. Set samples in gelatin capsules with tweezers and add label of each sample.
11. Prepare Technovit 7100 resin with hardener according to manufacturer's manual (*see Note 2*).
12. Fill gelatin capsules with Technovit 7100 resin with hardener.
13. Adjust direction of samples with tweezers.
14. Put the top on gelatin capsules.
15. Keep gelatin capsules in refrigerator for 3 days.
16. Store samples in desiccator with silica gel until use.

24.4.2.5 Sectioning for Visualization of Nuclei and Detection of Fluorescence Spectra of Cell Walls

1. Put each small block in a drop of distilled water on a freezing stage (MA-101; Komatsu Electronics Inc.) at −20 °C.
2. Cut radial sections of approximately 40 μm thickness with a sliding microtome equipped with a disposable stainless-steel blade.
3. Wash two times with phosphate buffer (pH 7.3) using Petri dishes.

24.4.2.6 Sectioning for Visualization of Vacuoles in Living Cells

1. Affix each small block ($10 \times 10 \times 2$ mm^3) to a wood block with superglue.
2. Cut radial sections of approximately 40 μm thickness with a sliding microtome equipped with a disposable stainless-steel blade (*see Note 3*).
3. Wash two times with 0.2 M aqueous sucrose using Petri dishes.

24.4.2.7 Sectioning for Detection of Nuclear DNA Fragmentation

1. Place drops of distilled water on a glass slide.
2. Cut radial sections of approximately 1–2 μm thickness with an ultramicrotome equipped with a glass knife.
3. Place the sections on a drop of distilled water.
4. Dry out the sections in drying oven at 60 °C for several minutes.

24.4.2.8 DAPI Staining

1. Place sections on a watch glass.
2. Infiltrate the sections with permeabilization solution (0.1% Triton-X in 0.1% sodium citrate, freshly prepared) for 15 min at room temperature.
3. Apply 0.0001% DAPI solution and stain for 15 min at room temperature.
4. Wash two times with distilled water.
5. Place sections on a slide and apply a drop of aqueous glycerol.
6. Mount the sections with coverslips.

24.4.2.9 FDA Staining

1. Place sections in a screw vial protected from light by aluminum foil.
2. Apply 0.0002% of FDA solution in 0.2 M aqueous sucrose and incubate for 30 min at 25 °C (*see Note 4*).
3. Wash two times with 0.2 M aqueous sucrose.
4. Place sections on a slide and apply a drop of 0.2 M aqueous sucrose.
5. Mount the sections with coverslips.

24.4.2.10 Making Prepared Slides for Detection of Fluorescence Spectra of Cell Walls

1. Place sections on a slide and apply a drop of aqueous glycerol.
2. Mount the sections with coverslips.

24.4.2.11 Apply TUNEL Assay

1. Place permeabilization solution on sections and incubate for 15 min at room temperature.
2. For making positive control, apply 0.2 U/mL DNase I (TaKaRa Bio, Tokyo Japan) on section and incubate for 30 min at 37 °C.

3. Prepare TUNEL reaction mix according to manufacturer's manual.
4. Apply TUNEL reaction mix on sections and incubate for 60 min at 30 °C.
5. For making negative control, apply TUNEL reaction buffer on section and incubate for 60 min at 30 °C.
6. Stain with 0.0005 % of propidium iodide for 5 min at room temperature for examination of nuclei.
7. Wash with PBS two times.
8. Mount the sections with coverslips.

24.4.2.12 Observation of Nuclei by CLSM

In this section, we introduce the general operation to obtain a single optical section, a series of optical sections, and 3D projection images and fluorescence spectra using an LSM-710 (Carl Zeiss).

1. Place a prepared slide on the stage of the microscope. Select the area to be examined with fluorescence mode using objective lens at low magnification, such as 10×.
2. Select objective lens with higher magnification (40× or 63×) for observation of distribution, shape of nuclei and vacuoles.
3. Choose acquisition mode.
4. Select laser for excitation of fluorochrome and the range of wavelength to detect fluorescence signals (excitation/emission combination for DAPI: Ex 405 nm/BP 410–585 nm and for FDA: Ex 488 nm/BP 515–565 nm).
5. Select the resolution of image (512 × 512, 1024 × 1024 pixels, etc.).
6. Set the size of pinhole as 1 airy unit.
7. Start laser scan and acquisition of images.
8. Adjust laser power, master gain, digital offset, angle of image, scan speed and zoom.
9. Set number of averaging images to eliminate digital noise. Usually, two or four times of averaging is used.
10. Obtain an image of single optical plane (Fig. 24.4).
11. Store an image by file export. TIFF is a desirable file format.

24.4.2.13 Obtain a Series of Optical Sections along the *z*-Axis to Construct 3D Images

1. Choose *Z*-stack mode.
2. Set start and end point of scanning for taking sequences of optical sections.
3. Set interval between pictures or number of optical sections.
4. Enter the refractive index of mounting solution for correction of aberration.
5. Set number of averaging images to eliminate digital noise.
6. Obtain a series of optical sections along the *z*-axis.

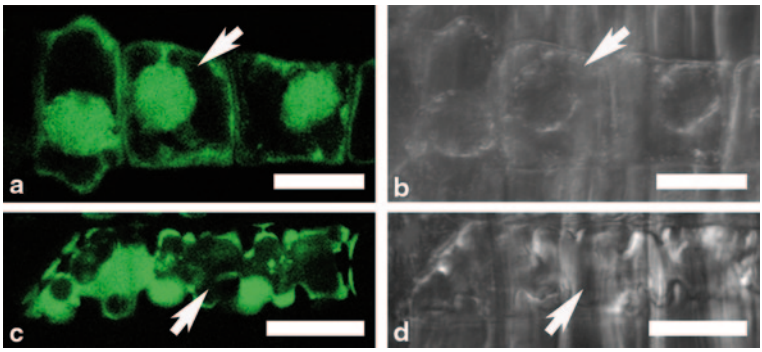

Fig. 24.4 Vacuoles (*arrows*) appearing as dark regions on a field of bright fluorescence in differentiating ray tracheids in *Pinus densiflora*. **a** Vacuoles in ray tracheids that have only a thin cell wall. **b** Differential interference contrast (*DIC*) image of section in (**a**). **c** Vacuoles in ray tracheids that are forming dentate thickenings. **d** *DIC* image of section in (**c**). *Bars* = 25 μm

24.4.2.14 Construction of 3D Projection Images

1. Select a series of optical sections to construct a 3D projection image.
2. Choose "Processing" mode.
3. Select maximum intensity projection and construct a 3D projection image (Fig. 24.5).
4. Store an image by file export. TIFF is the desirable file format.

24.4.2.15 Obtain Fluorescence Spectra of Autofluorescence of Cell Walls

1. Place a prepared slide on the stage of the microscope. Select the area to be examined with fluorescence mode using objective lens at low magnification, such as 10× (Fig. 24.6a and b).
2. Select objective lens with higher magnification.

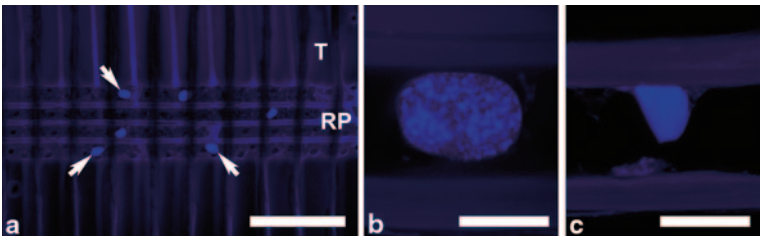

Fig. 24.5 Images obtained by CLSM of radial sections, stained with DAPI, showing nuclei (*arrows*) in ray parenchyma cells in *Cryptomeria japonica*. **a** Distribution of nuclei in ray parenchyma. **b** Elliptical nucleus. **c** Deformed nucleus. The *left side* of micrographs corresponds to the outer side of the tree. *RP* ray parenchyma cell, *T* tracheid. *Bars* = 100 μm (**a**) and = 10 μm (**b** and **c**)

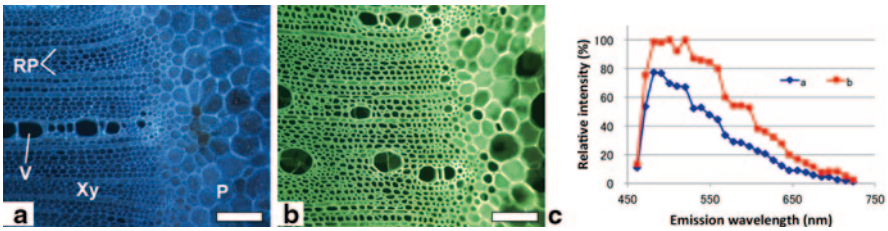

Fig. 24.6 Autofluorescence micrographs obtained by fluorescence microscopy (**a, b**) and emission spectra obtained by CLSM (**c**) of transverse sections of tissue around the pith of branches of *Robinia pseudoacacia* var. *inermis*. **a** A transverse section from near the apical region of a branch without *dark colored* heartwood. **b** A transverse section, from around the pith, of tissue with *dark colored* heartwood. **c** Averaged emission spectra of autofluorescence under fluorescence excitation at 405 nm from cell walls around the pith, namely, from sections in (**a**) and (**b**). The left side of micrographs corresponds to the outer side of the tree. *P* pith, *RP* ray parenchyma cell, *V* vessel element, *Xy* xylem. *Bars* = 100 μm

3. Choose Lambda mode.
4. Select excitation laser, such as 405, 488, 561, and 633 nm.
5. Set range and interval of the spectrum.
6. Start laser scan and acquisition of images. Adjust laser power, gain, and digital offset.
7. It is important to avoid saturation of signals.
8. Start acquisition of spectrum data.
9. Choose "Unmixing" mode.
10. Select a spot, a line or an area on obtained image to display a data of spectrum.
11. Set *y*-axis as relative value or absolute value in a spectrum graph. If you want to compare several samples, you should choose *y*-axis as the absolute value.
12. Store data as text file via file export.
13. Make graphs of spectra from obtained data by a spreadsheet program such as Microsoft Excel (Fig. 24.6c).

24.4.2.16 Observation of Nuclear DNA Fragmentation by Fluorescence Microscopy

1. Observe with a fluorescence microscope (Axioskop) under fluorescence illumination (excitation/emission combination for TUNEL, BP 465–505 nm/BP 515–565 nm and for propidium iodide, BP 534–558 nm/LP 590 nm).
2. Confirm reliability of TUNEL assay by observation of positive and negative control.
3. Detect nuclear DNA fragmentation during cell death (Fig. 24.7).

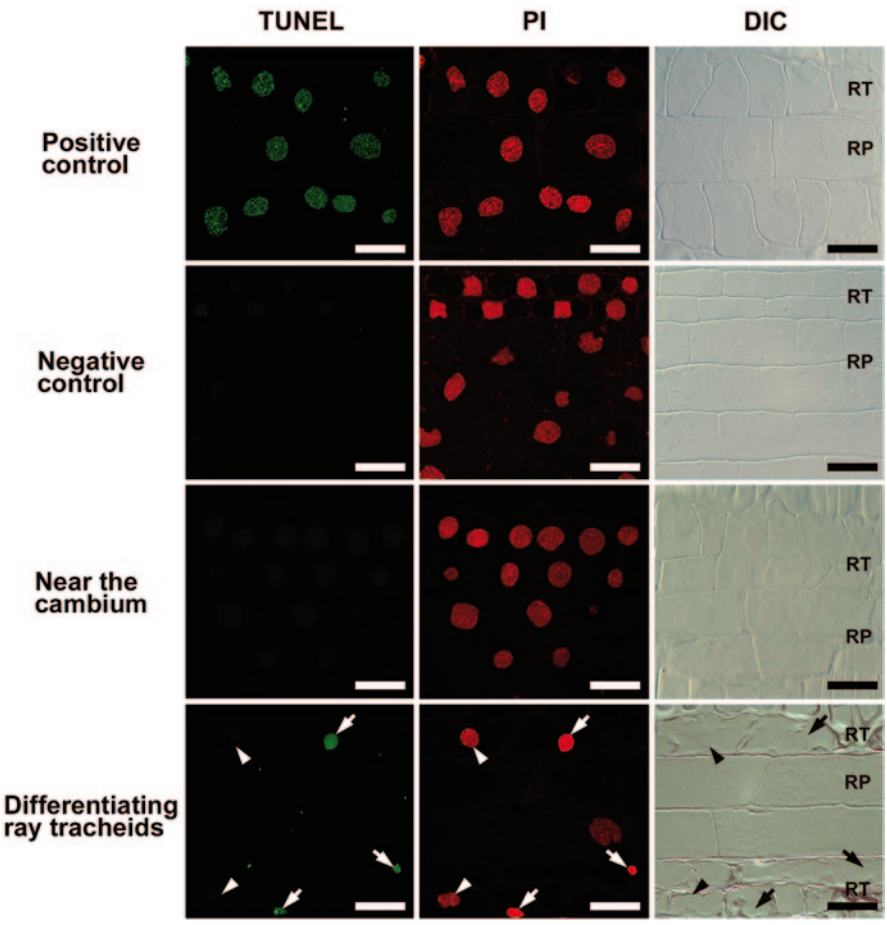

Fig. 24.7 DNA fragmentation detected by TdT-mediated dUTP nick end labeling (*TUNEL*) assay in positive and negative control, near the cambium and differentiating ray tracheids in *Pinus densiflora*. Columns show fluorescence due to *TUNEL* (*left*), fluorescence due to propidium iodide (*PI*) (*middle*) and differential interference contrast (*DIC*) images (*right*). *Arrows* indicate nuclei with DNA fragmentation in ray tracheids. *Arrowheads* indicate nuclei without DNA fragmentation. The *left* side of micrographs corresponds to the outer side of the tree. *RP* ray parenchyma cell, *RT* ray tracheid. *Bars* = 30 μm

24.4.3 Notes for Subheading 24.4

1. For observation of autofluorescence of cell walls in heartwood, analysis should be performed as soon as possible after sample collection. Long-term preservation of samples in solution induces removal and diffusion of extractives.
2. Technovit 7100 resin with hardener become hardened quickly. If you are making many embedded samples, we recommend preparing small amounts of resin several times.

3. For the visualization method of vacuoles in living cells, put aqueous sucrose on the sample with a brush to avoid desiccation during sectioning with the sliding microtome.
4. Make 0.2% FDA in DMSO (stock solution) and store in a freezer at −20 °C. Dilute by adding 1000 times of 0.2 M aqueous sucrose to stock solution just before use. For example, to make 10 mL of working solution, 10 μL of stock solution of FDA is added to 10 mL of 0.2 M aqueous sucrose.

Acknowledgments This work was supported, in part, by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (nos. 19580183, 20120009, 21380107, 22· 00104, 23380105, 24380090, 24880016, 24· 2976, 25850121, 15H04527 and 15K07508) and by Scholarship and Education Funds from Nisshin Sugar Manufacturing Co., Ltd.

References

1. Catesson AM (1994) Cambial ultrastructure and biochemistry: changes in relation to vascular tissue differentiation and the seasonal cycle. Int J Plant Sci 155:251–261
2. Larson PR (1994) The vascular cambium: development and structure. Springer-Verlag, Heidelberg
3. Funada R (2002) Immunolocalisation and visualisation of the cytoskeleton in gymnosperms using confocal laser scanning microscopy (CLSM). In: Chaffey N (ed) Wood formation in trees: cell and molecular biology techniques. Taylor and Francis, London, pp 143–157
4. Funada R (2008) Microtubules and the control of wood formation. In: Nick P (ed) Plant microtubules. Springer, Berlin, pp 83–119
5. Carlquist S (2001) Comparative wood anatomy: systematic, ecological, and evolutionary aspects of dicotyledon wood, 2nd edn. Springer-Verlag, Berlin
6. Tyree M, Zimmermann M (2002) Xylem structure and the ascent of sap. Springer Verlag, Berlin
7. Kitin P, Fujii T, Abe H, Funada R (2004) Anatomy of the vessel network within and between tree rings of *Fraxinus lanuginosa* (Oleaceae). Am J Bot 91:779–788
8. Funada R (2000) Control of wood structure. In: Nick P (ed) Plant microtubules. Springer, Berlin, pp 51–81
9. Fukuda H (1996) Xylogenesis: initiation, progression, and cell death. Annu Rev Plant Physiol Plant Mol Biol 47:299–325
10. Fukuda H (2004) Signals that control plant vascular cell differentiation. Nat Mol Cell Biol 5:379–391
11. Nakaba S, Sano Y, Kubo T, Funada R (2006) The positional distribution of cell death of ray parenchyma in a conifer, *Abies sachalinensis*. Plant Cell Rep 25:1143–1148
12. Catesson AM (1990) Cambial cytology and biochemistry. In: Iqbal M (ed) The vascular cambium. Research Studies Press, Taunton, pp 63–112
13. Chaffey N, Barlow P (2001) The cytoskeleton facilitates a three-dimensional symplasmic continuum in the long-lived ray and axial parenchyma cells of angiosperm trees. Planta 213:811–823
14. Sauter JJ (2000) Photosynthate allocation to the vascular cambium: facts and problems. In: Savidge R, Barnett J, Napier R (eds) Molecular and cell biology of wood formation. BIOS Scientific Publishers, Oxford, pp 71–83
15. Bamber RK, Fukazawa K (1985) Sapwood and heartwood: a review. For Abstract 46:567–580

16. Hillis WE (1987) Heartwood and tree exudates. Springer-Verlag, New York
17. Magel E (2000) Biochemistry and physiology of heartwood formation. In: Savidge R, Barnett J, Napier R (eds) Molecular and cell biology of wood formation. BIOS Scientific Publishers, Oxford, pp 363–376
18. Taylor A, Gartner BL, Morrell JJ (2002) Heartwood formation and natural durability—a review. Wood Fiber Sci 34:587–611
19. Spicer R (2005) Senescence in secondary xylem: heartwood formation as an active developmental program. In: Holbrook NM, Zwieniecki MA (eds) Vascular transport in plants. Elsevier Academic Press, Amsterdam, pp 457–475
20. Knebel W, Schnepf E (1991) Confocal laser scanning microscopy of fluorescently stained wood cells: a new method for three-dimensional imaging of xylem elements. Trees 5:1–4
21. Running MP, Meyerowitz EM (1996) Using confocal microscopy in the study of plant structure and development. Aliso 14:263–270
22. Hepler PK, Gunning BES (1998) Confocal fluorescence microscopy of plant cells. Protoplasma 201:121–157
23. Kitin P, Sano Y, Funada R (2003) Three-dimensional imaging and analysis of differentiating secondary xylem by confocal microscopy. IAWA J 24:211–222
24. Cheng PC (2006) Interaction of light with botanical specimens. In: Pawley JB (ed) Handbook of biological confocal microscopy, 3rd edn. Springer, New York, pp 414–441
25. Brodersen C (2013) Visualizing wood anatomy in three dimensions with high-resolution X-ray micro-tomography (μCT)—a review. IAWA J 34:408–424
26. Kitin P, Sano Y, Funada R (2002) Fusiform cells in the cambium of *Kalopanax pictus* are exclusively mononucleate. J Exp Bot 53:483–488
27. Yahya R, Koze K, Sugiyama J (2011) Fibre length in relation to the distance from vessels and contact with rays in *Acacia mangium*. IAWA J 32:341–350
28. Kitin P, Beeckman H, Fujii T, Funada R, Noshiro S, Abe H (2009) What is disjunctive xylem parenchyma? A case study of the African tropical hardwood *Okoubaka aubrevillei* (Santalaceae). Am J Bot 96:1399–1408
29. Anderson CT, Carroll A, Akhmetova L, Somerville C (2010) Real-time imaging of cellulose reorientation during cell wall expansion in Arabidopsis roots. Plant Physiol 152:787–796
30. Suslov D, Verbelen JP, Vissenberg K (2009) Onion epidermis as a new model system to study the control of growth anisotropy in higher plants. J Exp Bot 60:4175–4187
31. Wiltshire EJ, Collings DA (2009) New dynamics in an old friend: dynamic tubular vacuoles radiate through the cortical cytoplasm of red onion epidermal cells. Plant Cell Physiol 50:1826–1839
32. Liesche J, Ziomkiewicz I, Schulz A (2013) Super-resolution imaging with Pontamine fast scarlet 4BS enables direct visualization of cellulose orientation and cell connection architecture in onion epidermis cells. BMC Plant Biol 13:226. doi:10.1186/1471-2229-13-226
33. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termacarphi Pty Ltd, Melbourne
34. Chaffey NJ (2002) Wood formation in trees: cell and molecular biology techniques. Taylor and Francis, London
35. Kitin P, Funada R, Sano Y, Beeckman H, Ohtani J (1999) Variations in the lengths of fusiform cambial cells and vessel elements in *Kalopanax pictus*. Ann Bot 84:621–632
36. Kitin P, Funada R, Sano Y, Ohtani J (2000) Analysis by confocal microscopy of the structure of cambium in the hardwood *Kalopanax pictus*. Ann Bot 86:1109–1117
37. Ruzin JE (1999) Plant microtechnique and microscopy. Oxford University Press, Oxford
38. Donaldson LA, Bond J (2005) Fluorescence microscopy of wood (CD ROM). Scion, Rotorua, New Zealand
39. Kitin P, Voelker SL, Meinzer FC, Beeckman H, Strauss SH, Lachenbruch B (2010) Tyloses and phenolic deposits in xylem vessels impede water transport in low-lignin transgenic poplars: a study by cryo-fluorescence microscopy. Plant Physiol 154:874–886

40. Hughes J, McCully ME (1975) The use of an optical brightener in the study of plant structure. Stain Technol 50:319–329
41. Galbraith DW (1981) Microfluorimetric quantitation of cellulose biosynthesis by plant protoplasts using Calcofluor White. Physiol Plant 53:111–116
42. Slifkin M, Cumbie R (1988) Congo red as a fluorochrome for the rapid detection of fungi. J Clin Microbiol 26:827–830
43. Thomas J, Manfred I, Nair H, Chauhan SS, Collings DA (2013) Pontamine fast scarlet 4B: a new fluorescent dye for visualising cell wall organisation in radiata pine tracheids. Wood Sci Technol 47:59–75
44. Verbelen JP, Kerstens S (2000) Polarization confocal microscopy and Congo Red fluorescence: a simple and rapid method to determine the mean cellulose fibril orientation in plants. J Microsc 198:101–107
45. Srebotnik E, Messner K (1994) A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white-rot fungi. Appl Environ Microbiol 60:1383–1386
46. Schmitz N, Robert EMR, Verheyden A, Kairo JG, Beeckman H, Koedam N (2008) A patchy growth via successive and simultaneous cambia: key to success of the most widespread mangrove species *Avicennia marina*? Ann Bot 101:49–58
47. Dié A, Kitin P, Kouamé FNG, Van den Bulcke J, Van Acker J, Beeckman H (2012) Fluctuations of cambial activity in relation to precipitation result in annual rings and intra-annual growth zones of xylem and phloem in teak (*Tectona grandis*) in Ivory Coast. Ann Bot 110:861–873
48. Kitin P, Fujii T, Abe H, Takata K (2009) Anatomical features that facilitate radial flow across growth rings and from xylem to cambium in *Cryptomeria japonica*. Ann Bot 103:1145–1157
49. Bond J, Donaldson L, Hill S, Hitchcock K (2008) Safranine fluorescent staining of wood cell walls. Biotech Histochem 83:161–171
50. Liu Y, Gustafson R, Callis J, McKean W (1999) Microspectroscopic analysis and kappa determination of single pulp fibres stained with acridine orange. J Pulp Pap Sci 25:351–355
51. Hutzler P, Fischbach R, Heller W, Jungblut TP, Reuber S, Schmitz R, Veit M, Weissenböck G, Schnitzler J-P (1998) Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J Exp Bot 49:953–965
52. Donaldson LA (2013) Softwood and hardwood lignin fluorescence spectra of wood cell walls in different mounting media. IAWA J 34:3–19
53. Kim YS, Singh AP (1999) Micromorphological characteristics of compression wood degradation in waterlogged archaeological pine wood. Holzforschung 53:381–385
54. Donaldson LA (2002) Abnormal lignin distribution in wood from severely drought stressed *Pinus radiata* trees. IAWA J 23:161–178
55. Donaldson LA, Singh AP, Yoshinaga A, Takabe K (1999) Lignin distribution in mild compression wood of *Pinus radiata* D. Don. Can J Bot 77:41–50
56. Donaldson LA, Radotić K, Kalauzi A, Djikanović D, Jeremić M (2010) Quantification of compression wood severity in tracheids of *Pinus radiata* D. Don using confocal fluorescence imaging and spectral deconvolution. J Struct Biol 169:106–115
57. Yamashita Y, Fukazawa K, Ishida S (1978) Histochemical observation of wood attacked by white rot fungi, *Coriolus versicolor* and *Cryptoderma yamanoi*. Res Bull College Exp Hokkaido Univ 35:109–121
58. Jang HF, Robertson AG, Seth RS (1991) Optical sectioning of pulp fibers using confocal scanning laser microscopy. TAPPI J 74:217–219
59. Xu L, Parker I (1999) Correction of fluorescence attenuation with depth in fibre and paper images collected by confocal laser scanning microscopy. Appita 52:41–44
60. Kiernan JA (2000) Formaldehyde, paraformaldehyde and glutaraldehyde: what they are and what they do. Micros Today 98:4–14
61. Singh A, Xiao Y, Wakeling R (1997) Glutaraldehyde fluorescence useful in confocal studies of fungi. Micros Today 97:8–16

62. Rossi S, Deslauriers A, Anfodillo T, Carrer M (2008) Age-dependent xylogenesis in timberline conifers. New Phytol 177:199–208
63. Barnard DM, Lachenbruch B, McCulloh KA, Kitin P, Meinzer FC (2013) Do ray cells provide a pathway for radial water movement in the stems of conifer trees? Am J Bot 100:322–331
64. Schmitz N, Verheyden A, Kairo JG, Beeckman H, Koedam N (2007) Successive cambia development in *Avicennia marina* (Forssk.) Vierh. is not climatically driven in the seasonal climate at Gazi Bay, Kenya. Dendrochronologia 25:87–96
65. Delvaux C, Sinsin B, Van Damme P, Beeckman H (2013) Size of conducting phloem: The "key" factor for bark recovery of 12 tropical medicinal tree species. Flora 208:111–117
66. Sano Y, Fujikawa S, Fukazawa K (1995) Detection and features of wetwood in *Quercus mongolica* var. *grosseserrata*. Trees 9:261–268
67. Utsumi Y, Sano Y, Fujikawa S, Ohtani J (1996) Seasonal changes in the distribution of water in the outer growth rings of *Fraxinus mandshurica* var. *japonica*, a study by cryo-scanning electron microscopy. IAWA J 17:113–124
68. Utsumi Y, Sano Y, Funada R, Ohtani J, Fujikawa S (2003) Seasonal and perennial changes in the distribution of water in the sapwood of conifers in a sub-frigid zone. Plant Physiol 131:1826–1833
69. Kitin P, Abe H, Fujii T (2005) Characterization of wood structure by confocal microscopy and SEM. In: Tanaka R, Cheng LH (eds) Lignocellulose: materials for the future from the tropics. Japan International Center for Agricultural Sciences (JIRCAS) Working Report No. 39, pp 285–287
70. Chaffey NJ (2002) Immunolocalisation of the cytoskeleton in the secondary vascular system of angiosperm trees and its visualisation using epifluorescence microscopy. In: Chaffey NJ (ed) Wood formation in trees: cell and molecular biology techniques. Taylor and Francis, London, pp 133–142
71. Uehara K, Hogetsu T (1993) Arrangement of cortical microtubules during formation of bordered pit in the tracheids of *Taxus*. Protoplasma 172:145–153
72. Abe H, Funada R, Imaizumi H, Ohtani J, Fukazawa K (1995) Dynamic changes in the arrangement of cortical microtubules in conifer tracheids during differentiation. Planta 197:418–421
73. Abe H, Funada R, Ohtani J, Fukazawa K (1995) Changes in the arrangement of microtubules and microfibrils in differentiating conifer tracheids during the expansion of cells. Ann Bot 75:305–310
74. Prodhan AKMA, Funada R, Ohtani J, Abe H, Fukazawa K (1995) Orientation of microfibrils and microtubules in developing tension-wood fibers of Japanese ash (*Fraxinus mandshurica* var. *japonica*). Planta 196:577–585
75. Chaffey N, Barlow P, Barnett J (1997) Cortical microtubules rearrange during differentiation of vascular cambial derivatives, microfilaments do not. Trees 11:333–341
76. Chaffey NJ, Barnett JR, Barlow PW (1997) Cortical microtubule involvement in bordered pit formation in secondary xylem vessel elements of *Aesculus hippocastanum* L. (Hippocastanaceae): a correlative study using electron microscopy and indirect immunofluorescence microscopy. Protoplasma 197:64–75
77. Chaffey NJ, Barnett JR, Barlow PW (1997) Visualization of the cytoskeleton within the secondary vascular system of hardwood species. J Microsc 187:77–84
78. Chaffey NJ, Barlow PW, Barnett JR (1998) A seasonal cycle of cell wall structure is accompanied by a cyclical rearrangement of cortical microtubules in fusiform cambial cells within taproots of *Aesculus hippocastanum* L. (Hippocastanaceae). New Phytol 139:623–635
79. Chaffey N, Barnett J, Barlow P (1999) A cytoskeletal basis for wood formation in angiosperm trees: the involvement of cortical microtubules. Planta 208:19–30
80. Chaffey N, Barlow P, Sundberg B (2002) Understanding the role of the cytoskeleton in wood formation in angiosperm trees: hybrid aspen (*Populus tremula* x *P. tremuloides*) as the model species. Tree Physiol 22:239–249

81. Funada R, Abe H, Furusawa O, Imaizumi H, Fukazawa K, Ohtani J (1997) The orientation and localization of cortical microtubules in differentiating conifer tracheids during cell expansion. Plant Cell Physiol 38:210–212
82. Furusawa O, Funada R, Murakami Y, Ohtani J (1998) Arrangement of cortical microtubules in compression wood tracheids of *Taxus cuspidata* visualized by confocal laser microscopy. J Wood Sci 44:230–233
83. Chaffey N, Barlow P (2002) Myosin, microtubules, and microfilaments: co-operation between cytoskeletal components during cambial cell division and secondary vascular differentiation in trees. Planta 214:526–536
84. Oribe Y, Funada R, Shibagaki M, Kubo T (2001) Cambial reactivation in locally heated stems of the evergreen conifer *Abies sachalinensis* (Schmidt) Masters. Planta 212:684–691
85. Nakaba S, Yoshimoto J, Kubo T, Funada R (2008) Morphological changes in the cytoskeleton, nuclei and vacuoles during the cell death of short-lived ray tracheids in the conifer *Pinus densiflora*. J Wood Sci 54:509–514
86. Nakaba S, Sano Y, Funada R (2013) Disappearance of microtubules, nuclei and starch during cell death of ray parenchyma in *Abies sachalinensis*. IAWA J 34:135–146
87. Begum S, Shibagaki M, Furusawa O, Nakaba S, Yamagishi Y, Yoshimoto J, Jin HO, Sano Y, Funada R (2012) Cold stability of microtubules in wood-forming tissues of conifers during seasons of active and dormant cambium. Planta 235:165–179
88. Begum S, Nakaba S, Yamagishi Y, Oribe Y, Funada R (2013) Regulation of cambial activity in relation to environmental conditions: understanding the role of temperature in wood formation of trees. Physiol Plant 147:46–54
89. Abe H, Funada R (2005) The orientation of cellulose microfibrils in the cell walls of tracheids in conifer: a model based on observations by field emission-scanning electron microscopy. IAWA J 26:161–174
90. Funada R, Miura H, Shibagaki M, Furusawa O, Miura T, Fukatsu E, Kitin P (2001) Involvement of localized cortical microtubules in the formation of modified structure of wood. J Plant Res 114:491–497
91. Funada R, Furusawa O, Shibagaki M, Miura H, Miura T, Abe H, Ohtani J (2000) The role of cytoskeleton in secondary xylem differentiation in conifers. In: Savidge RA, Barnett JR, Napier R (eds) Cell and molecular biology of wood formation. BIOS Scientific Publishers, Oxford, pp 255–264
92. Yamagishi Y, Uchiyama H, Sato T, Kitamura K, Yoshimoto J, Watanabe U, Nakaba S, Funada R (2015) In vitro induction of the formation of tracheary elements from suspension-cultured cells of the conifer *Cryptomeria japonica*. Trees 29:1283–1289
93. Nakaba S, Kubo T, Funada R (2008) Differences in patterns of cell death between ray parenchyma cells and ray tracheids in the conifers *Pinus densiflora* and *Pinus rigida*. Trees 22:623–630
94. Nakaba S, Kubo T, Funada R (2011) Nuclear DNA fragmentation during cell death of short-lived ray tracheids in the conifer *Pinus densiflora*. J Plant Res 124:379–384
95. Nakaba S, Begum S, Yamagishi Y, Jin HO, Kubo T, Funada R (2012) Differences in the timing of cell death, differentiation and function among three different types of ray parenchyma cells in the hardwood *Populus sieboldii* x *P. grandidentata*. Trees 26:743–750
96. Dünisch O, Richter HG, Koch G (2010) Wood properties of juvenile and mature heartwood in *Robinia pseudoacacia* L. Wood Sci Technol 44:301–313
97. Imai T, Tanabe K, Kato T, Fukushima K (2005) Localization of ferruginol, a diterpene phenol, in *Cryptomeria japonica* heartwood by time-of-flight secondary ion mass spectrometry. Planta 221:549–556
98. Kuroda K, Fujiwara T, Hashida K, Imai T, Kushi M, Saito K, Fukushima K (2014) The accumulation pattern of ferruginol in the heartwood-forming *Cryptomeria japonica* xylem as determined by time-of-flight secondary ion mass spectrometry and quantity analysis. Ann Bot 113:1029–1036

99. Kuroda H, Shimaji K (1983) Distribution of coloring substances in sugi heartwood. Holzforschung 37:225–230
100. Saito K, Mitsutani T, Imai T, Matsushita Y, Fukushima K (2008) Discriminating the indistinguishable sapwood from heartwood in discolored ancient wood by direct molecular mapping of specific extractives using time-of-flight secondary ion mass spectrometry. Anal Chem 80:1552–1557
101. Nakaba S, Yamagishi Y, Sano Y, Funada R (2012) Temporally and spatially controlled death of parenchyma cells is involved in heartwood formation in pith regions of branches of *Robinia pseudoacacia* var. *inermis*. J Wood Sci 58:69–76

Part IV
Botanical Techniques and Protocols for Archeology and Herbarium Collection

Chapter 25
Collection of Plant Remains from Archaeological Contexts

Alessandra Celant, Donatella Magri and Francesca Romana Stasolla

25.1 Introduction

Plant remains may be found preserved in various types of deposits of natural or human origin. Archaeological contexts are especially useful to study the relationships between ancient human populations and plants, and provide information on the exploitation of natural resources for shelter, fuel, and food for humans and animals [1].

Archaeobotany (or paleoethnobotany) is the field of research dealing with the study of plant remains from archaeological sites. It was extensively developed during the last century, but only since the 1960s standard protocols were established for systematic sampling and analysis, together with reference collections [2].

Plant remains from archaeological contexts include wood, fruits and seeds, leaves, fibers, fungi, pollen, spores, algae, and phytoliths. On the whole, they constitute a variety of materials that were used by humans in different ways and for different purposes. Thus, they may indicate different types of human activities (agriculture, pasture, foraging, clearance, trade, building, and so on). They may be preserved in different ways, depending on the chemical composition of the tissues and the processes of post-depositional modification. Different types of plants remains and of preservation states are often related to specific archaeological structures: for example, fragments of charred wood are expected to be found in hearths whereas waterlogged seeds and fruits will be easily recovered from cesspits.

Careful consideration of the type of plant remains, preservation modes, and archaeological contexts is therefore mandatory to properly plan a sampling strategy. In fact, they may strongly affect the results and inevitably influence the outcome of the research and the final interpretation of the data.

A. Celant (✉) · D. Magri
Department of Environmental Biology, Sapienza University, Piazzale Aldo Moro, 5, 00185 Rome, Italy
e-mail: alessandra.celant@uniroma1.it

F. R. Stasolla
Department of Humanities, Sapienza University, Piazzale Aldo Moro, 5, 00185 Rome, Italy

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_25

25.1.1 Plant Remains

Plant remains are commonly subdivided in macrofossils and microfossils, depending on the possibility to isolate them with the naked eye [3–6]. In practice, plant macroremains have average sizes >0.1–0.2 mm [7]. However, it must be considered that in most cases macrofossils are also studied by microscopic analysis. For example, even a large wood fragment must be sectioned and observed under the microscope (*see* Chap. 26). Macrofossils include wood, roots, fruits and seeds, flowers, leaves, fibers, and fungi among others. Plant microfossils include remains that are not visible to the naked eye, such as pollen, spores, non-pollen palynomorphs (NPPs), microcharcoal, diatoms, and phytoliths. Their size is usually in the range 10–200 μm. Besides, vegetal biomolecules such as ancient DNA and starch may be found in organic residues in connection with artifacts and various archaeological structures.

The most common plant remains are related to archaeological deposits as follows:

- Woods may range from large trunks to small twigs. The most frequent use of archaeological wood is fuel. Besides, in archaeological contexts it is not unusual to find wooden posts, beams, planks, roofing material, fences, weapons (e.g., bow, arrows, and lance shaft), wheels, statues, coffins, saddles, handles, ploughs, spades, and domestic tools (e.g., combs, boxes, bowls, turnery, shoes, spoons, baskets, and barrels) [8, 9].
- Roots and tubers are often not properly identified because of the parenchymatous structure of their tissues, especially in tubers. However, they were commonly used as food and are in principle abundant in archaeological sites [3, 10, 11].
- Seeds and fruits include edible plants and weeds. Cereals, pulses, and oil crops were the most important domesticated taxa since the Neolithic. Cereal macroremains include fruits (caryopses), glumes, rachis parts, awns, and chaff remains [3, 5, 11, 12]. Pulses are generally found as seeds [11, 12]. Oil crops are represented by seeds and fruits (e.g., flax and olives) that are often found crushed [11, 12]. Seeds and fruits may also belong to vegetable plants (e.g., watermelon, onion, carrot, and celery), fruit trees (e.g., olive, grapefruit, plum, cherry, apple, fig, walnut, and hazelnut), and condiments (e.g., coriander, anise, dill, and cumin) [11, 12]. Weeds include edible plants, used in the diet of humans and animals [13], arable weeds that have been evolving within agro-ecosystems since agriculture began [14], and synanthropic ruderal plants [15].
- Flower remains are rare because of their delicate consistency. However, they have been found in dung [3, 16] and occasionally in human settlements [17].
- Leaves are not very common in archaeological contexts; however, they may also be identified from cuticle fragments and impressions on pottery, often in tombs and cesspits [18].
- Fibers are found in fragments of cordage, ropes, nets, baskets, textiles, shoes, cloths, and sacks, even if they often consist of only residual threads twisted

together. They belong to either cultivated plants, such as flax and hemp, or wild plants, such as nettle, willow, and various species of grasses, reeds, and sedges [3, 8, 19].

- Fungi [3] and mosses [20] may also be occasionally found, as they were used for different purposes as food and drugs.
- Pollen and spores are usually found in fine-grained sediments and peat. In natural contexts they are generally collected from lakes and peat bogs. In archaeological contexts they may be found in wells, cesspits, and caves, as well as in dung and resins. They provide information on the natural vegetation dynamics around the deposits, on human-induced changes in the vegetational landscape, including clearance, agricultural, pastoral activities, as well as domestic, economic, and cult practices [21–23].
- NPPs are remains of a great variety of organisms, including microscopic algae, cyanobacteria, fungi, insects, other invertebrates, and cormophytes that are found in the organic residue prepared for pollen analysis. They can be sensitive to various ecological parameters and human presence [24–26].
- Microcharcoal in sediments is used to reconstruct long-term variations in fire occurrence, complementing paleoenvironmental and archaeological records. In most cases, microcharcoal is studied from the same samples used for pollen analysis to examine the linkages between climate, vegetation, fire, and human activity [27].
- Diatoms are the most frequently studied algae from archaeological sites. They may contribute to questions of pottery typology and provide information about the absence, level, pollution, productivity, acidity, or salinity of a water body on or near to an excavation [28–30].
- Phytoliths are a major component of the microscopic archaeological record in many sites, as they are durable in soils and sedimentary environments due to their mineral composition. In addition to sediments from lakes and human occupations, stone tools, dental remains, and ceramics can also include phytoliths [31].
- Biomolecules, including starch, oils, and DNA among others, are organic residues that are increasingly studied from archaeological contexts. They can provide key insights into many prominent archaeological research questions, including processes of domestication, past subsistence strategies, and human interactions with the environment [32, 33].

25.1.2 Preservation Modes

Archaeological deposits do not always present optimal conditions for the preservation of plant materials, because aerobic conditions result in rapid decomposition of plant materials [2, 8]. Besides, human activity often reworked the sediment layers through use and reuse of structures, provoking selection or destruction of materials

of vegetal origin. However, it also constituted structures where preservation of fossil and subfossil material was especially favored (*see* Sect. 25.1.3).

Vegetative tissues, once separated from the living plants, usually deteriorate rapidly, unless the deposition environment ensures their preservation. Plant remains are preserved when the decomposing action of fungi, bacteria, and other microorganisms is prevented. In practice, this may happen in anaerobic (poorly oxygenated) waterlogged environments, in extremely dry conditions, and after charring or inclusion in mineral matter. In addition, traces of ancient plants may be found as impressions in fine-grained materials (e.g., clay, travertine, pottery). A combination of preservation modes is also possible.

- Waterlogged plant remains generally occur in bogs, bottom of lakes, submerged coastal areas, wells, canals, and cesspits. All plant remains such as wood, seeds and fruits, part of flowers, leaves and cuticles, stems, roots, fibers, fungi, pollen and spores, microcharcoal, diatoms, and phytoliths may be well preserved. When waterlogged preservation conditions are good, most plant remains look fresh and even the most delicate features may be retained. These remains can be identified easily using modern reference collections [3]. However, in some cases, plant remains may be eroded, partly decomposed, or damaged by swelling. In general, deposits containing waterlogged plant remains may be considered fairly representative of the surrounding environment and of the human activities carried out in the vicinity of the site, as little selective preservation occurs and a large variety of plant remains may be found. However, in some archaeological structures with a specific function, overrepresentation of some plant types may occur. For example, in cesspits waterlogged plant remains, mostly coming from feces, will especially represent elements of the human diet related to cultivations, collection, and trade of edible plants. It should also be considered that a few types of plant remains may be missing in waterlogged conditions. For example, caryopses of cereals are usually dissolved in water because of their starch-abundant chemical composition. Tracing the presence of cereals in waterlogged conditions will be possible only if phytoliths, pollen, awns, glumes, rachis parts, or charred caryopses are found. A particular case is the preservation (mummified) of stomach content, where plant materials may be preserved by acidic conditions that prevent bacterial activity [11, 34].
- Dried plant remains are usually found in arid regions (dried clay, caves, and sheltered places out of the reach of water), but also in temperate areas in sealed containers (e.g., offerings in vases and tombs) [3, 11], and as filling and insulation material in buildings [35]. These finds include soft parts of vegetables (seeds, fruits, flowers, leaves), wood, fibers, and phytoliths. In many cases, desiccated materials show an excellent preservation state [36], even if some anatomical structures (e.g., wood tissues) may be difficult to identify because of distortion of the cell walls during the drying process. Besides, dried plant remains are often extremely friable and may undergo selective preservation [8, 35].
- Charred remains are the most frequent plant materials in archaeological contexts, as they are resistant to biological decomposition, even if water-rich and delicate tissues and organs (e.g., leaves, flowers, small twigs, and pollen) do not survive

charring. Plants may have been intentionally charred by humans (e.g., fuel and offensive activities), or accidentally charred during domestic activities and crafts (e.g., food preparation, firing of pottery, and rituals). The majority of charred plant materials represent parts of buildings, ships, wooden objects, and food remains, but also plants used for funerary offerings, fodder, dyeing, and drugs [2]. The most common charred remains are wood, hard or dry fruits, and seeds (e.g., caryopses, pulses, nuts, and fruit stones). Parenchymatous tissues (e.g., tubers) and fleshy fruits may be preserved if desiccated before combustion, or slowly and mildly charred. Morphological and anatomical features may be extremely well preserved, allowing identification of the charred plant remains. However, charred remains may be potentially fragile, so they are rare in superficial layers of archaeological sites. Besides, a considerable reduction in volume occurs during charring, the intensity of deformation depending on the amount of humidity present in tissues, the spread of heating, and the temperatures reached [11]. Swelling and cracking may also occur. In cereals charring provokes reduction in the length of grains and a slight increase in breadth and thickness [37].

- Mineralized plant remains are found when silica, calcium carbonate, and salts dissolved in water may fill the cell cavities of vegetal tissues and mineralize. In humid situations, bronze, silver, and iron may produce metal oxides, highly toxic to bacteria and fungi, blocking decomposition [11]. Conditions for this preservation type are often met in tombs containing metal goods and offerings, and in pits and wells used as garbage dumps, where plant remains were deposited in calcareous sediments below the water table, or in latrines and wells sanitized by caustic lime. In these contexts, it is not unusual to find combinations of preservation states, for example, mixed charred and mineralized plant remains from domestic hearths and food scraps. Wood, fruits and seeds, leaves and fibers (textile fragments) may be found mineralized, with little selective preservation, although some corrosion may occur [3].
- Impressions of plants occur on fine-grained material, such as silt and clay, when it is still soft and moldable because it is saturated with water. In archaeological contexts, this preservation mode is often found in daub, bricks, and pottery [12]. Chaff remains, straw, ear fragments, and stems were often added to clay to act as tempering elements [11]. Seeds, fruits, cereals, and leaves were accidentally included within the pottery clay, or intentionally used for ornamental purposes on the external surface of potsherd [3]. On the whole this preservation mode is rather selective, but may provide valuable information on crop plants, especially if no charred material is available.

25.1.3 Archaeological Structures

In archaeological sites, human frequentation and activity act as vectors for the dispersal and accumulation of plant remains: storage of plants, elimination of organic waste in latrines, pits, tanks, and wells, operations related to cult activities (e.g., votive offers in burials), preparation of food, trade, construction of dwellings, etc.

The recognition and distinction of the origin of the plant materials are fundamental for a correct reconstruction of the surrounding plant landscape and of the human activities onsite.

The analysis of the presence of the plant remains also allows to date the beginning of the vegetable resources exploitation, the diffusion of some alimentary species and their selection. In some cases, the presence of seeds or woods has clarified the dynamics of exchanges among different cultures and/or among areas communicating in ways that we would not be able to understand. Interdisciplinary studies connect the domestication and the acquisition of the techniques for the exploitation of the vegetable resources with the formation of a specific lexicon in different languages [38].

In archaeobotanical deposits abundant finds may be recovered [3, 12]:

- Artificial wet environments (cisterns, wells, fountains, canals) collect pollen, microcharcoal, seeds, and fruits from the surrounding vegetation, and provide evidence of the land use, for example, crops and related arable weeds, pasture, fires, and forest clearance. Natural wet environments could be managed by humans, for instance, through reclamation works.
- Garbage dumps include either structures specifically destined to that purpose or disused structures secondarily engaged as rubbish dumps. Among the first, it is worth mentioning holes/ditches dug in the ground and masonry basins, usually under the ground level. Among the latter, there are wells, tanks, water troughs, etc. Depending on their location in the archaeological site, they contain food scraps, home garbage, kitchen refuse, cleaning residues from hearths, courtyards, and vegetable gardens, and other plant debris.
- Sewers and cesspits contain plant remains predominantly related to defecation. Dung of both human and animal origin may contain abundant plant remains, including seeds, fruits, leaves, cuticles, pollen, and spores of coprophilous fungi. Typical indicators of latrine are carpological remains of fig, strawberry, grapevine pips, blackberry, etc. The particular nature of these deposits makes them especially useful in providing information on diet and subsistence economy. In areas where wood is absent (e.g., steppe areas), dung may help reconstruct the natural environment, as it was collected, stored, and used for fuel, often mixed with herbaceous plants [12].
- Food storage rooms/facilities, such as barns, silos, warehouses, jars, and courtyards with accumulations of food plant, often provide large amounts of remains of edible plants, both cultivated and wild.
- Floors/decking provide evidence for plants connected to bedding and litter, meal preparation, or production processes, including threshing floors.
- Furnaces, ovens, and fireplaces usually contain charred remains, directly or indirectly connected to crafting or food cooking, and traces of vegetal fuel material.
- Tombs, burials, and votive wells preserve plant materials used for offerings and funeral banquets, funerary objects, coffins, and charred remains connected to cremation practices.
- Potsherds and mud bricks may keep imprints accidentally formed during the processing operations (e.g., chaff remains), or deliberately impressed for orna-

mental purposes. They can also contain vegetable elements, for instance straw, used to alter the plasticity of the clay.
- Buildings, huts, pile-dwellings built with wooden posts, beams, planks, roofing materials, fences, and wooden furniture are also frequently found. Scaffoldings and structures for the house building, such as balconies and galleries, are for instance very diffused in different epochs; inside the masonries it is often possible to recover the rests of the poles sawn during the dismantlement of the structures.
- Ships, boats, and canoes were generally constructed with various types of wood and frequently transported foodstuffs, spices, wooden objects, and other products of plant origin for trading purposes.
- Wooden containers such as barrels, vegetable fibers, or textile packings were also used for long distance transports.
- Kitchen tools, farming implements, figurines, votive objects, weapons, and their hilts are made with different woods.
- Writing tools: papyrus; stylus; and wooden writing tablets covered with wax, birch bark, and paper were used for writing documents.

This list of archaeological structures, although not exhaustive, provides an idea of the complexity of the situations in which plant remains can be found, and suggests which contexts should preferably be chosen for an efficient, nondestructive, and informative sampling.

25.2 Materials

1. Safety footwear (rubber boots), gloves, and safety helmet
2. Trowels, hammer, spatulas, and brushes
3. Plastic bags and rigid containers (various sizes)
4. Adhesive tape (masking tape can be also used as label)
5. Aluminum foil, paper towels, and a tank of water (to clean the tools)
6. Waterproof pens
7. Notebook
8. Camera
9. Hand lens magnifier
10. Metal sieves with meshes of 4, 2, 1, and 0.5 mm
11. Corer for pollen analysis (*see* Sect. 25.3.4, point number 7)

25.3 Methods

The first step for any collection of plant remains is a preliminary survey of the archaeological site, to establish the best sampling strategy and to plan times and modes of collection, also in view of the target and expected goals of the ongoing

research. Different collection techniques can be applied depending on the types of plant remains, as macrofossils, microfossils, and biomolecules require different techniques [39], partly related also to preservation modes and type of archaeological structures where the plant remains are found.

25.3.1 Preliminary Survey

1. Wear safety footwear (rubber boots if necessary), gloves, and a safety helmet when you visit the excavation site and collect the samples.
2. Visit the excavation site together with the leading archaeologist and a geologist/soil scientist, to obtain an insight of the archaeological and paleoenvironmental context and to discuss the main research questions addressed.
3. Consider the extension of the site, the spatial distribution of the different functional areas and structures within the excavation, the chronological range, and the phases of human frequentation involved.
4. Identify the archaeological structures present in the excavations that may potentially contain plant remains (e.g., tombs, fireplaces, cesspits, pots, or closed containers).
5. Examine the available sediments, paying special attention to grain size, wetness, and consistency (*see Note 1*).
6. Take note of the accessibility of the area, weather conditions, and any possible logistic problem.
7. Take time for a survey around the area, to check if any pond/lake/marsh is found in the vicinity of the archaeological site that may be used for microfossil analysis.
8. Describe the plants growing on and around the site (*see Note 2*).
9. Decide whether sampling will involve both macro- and microfossils, so as to prepare the necessary equipment.
10. Choose the best sampling strategy. Sediment sampling for archaeobotanical research may be total, random, statistic, or judgmental [2, 40]. Total sampling (or "blanket" sampling [6]) implies the practical problem of transporting, storing, and analyzing a huge volume of sediment. It is the basis for a thorough research, but it is rather unpractical, unless large amounts of soils are floated in the field (*see* Sect. 25.3.2, point number 6). Random and statistic samplings are limited by the heterogeneous nature of archaeological sites. In practice, judgmental sampling, which means sampling only the archaeological structures or layers where plant remains are apparently abundant, is often preferred, even if it involves the fundamental methodological bias of neglecting sediments with sparse remains, which may provide information on different paleoenvironmental or paleoethnobotanical aspects [41]. For this reason, judgmental sampling is often combined with some random or interval sampling as a control [2] (*see Note 3*).
11. Examine both the spatial distribution and the chronological relationships among archaeological layers and consider how to collect samples both horizontally and vertically [31, 42]. Horizontal sampling of coeval layers in different

areas will provide information on the different use of the structures within the site (synchronic analysis). Vertical sampling, that is collection of sediment columns from exposed sections or from sediment cores, will provide information on vegetation changes and related human activities through time (diachronic analysis).

25.3.2 Collecting Large Macrofossils

1. If large wood fragments are available (e.g., beams, planks, and poles), sample a piece of a few centimeter from each wood, making sure to take the whole sequence of annual rings for possible dendrochronological analyses. Place the samples in plastic bags/rigid containers and immediately label them (*see Note 4*), recording date, site, layer, and archaeological unit. Record and sketch in your notebook also any other detail that may turn useful (e.g., preservation state, location in the archaeological structure, and dimensions). Take photographs of the entire remain and of the sampled fragment.
2. If wooden objects are present (e.g., the sheath of a sword or a sickle), sample them in the least destructive way, according to the instructions of an archaeologist and a restorer.
3. If large seeds and fruits (e.g., pine cone, nut) are visible, collect them in plastic bags/rigid containers. Immediately label the sample.
4. If you find impressions on pottery (or daub and bricks) take photographs and collect samples of pottery, wrap them in paper (e.g., newspaper), and bring them to the archaeobotanical laboratory. Label the sample.
5. If there are mineralized fragments (e.g., from a tomb) collect them carefully and place them in a rigid container. Label the container.
6. If the plant remains are wet (e.g., waterlogged), pay attention to keep them wet and place them in plastic bags or rigid boxes. Immediately label the samples.
7. If the plant remains are immersed in water, place the samples in a plastic box with water. Label the container with a waterproof marker.
8. If the finds are charred, they are usually very fragile. Take care not to fragment them. Wrap them in aluminum foil and place them in a rigid container. Label the container (Fig. 25.1a).
9. Take photographs before and during the sampling operations and record all the details of sampling procedures in your notebook.

25.3.3 Collecting Small Macrofossils

1. If small plant remains (<1 cm), including charred and uncharred wood, seeds, fruits, and leaves, are interspersed in the sediments, take samples of bulk material (1–2 kg) from stratigraphical layers and store them in plastic bags. Label the bags.

Fig. 25.1. Sampling of plant remains from archaeological contexts: **a** collection of large anthracological remains from a hearth of late Bronze age; **b** sediment sampling for macro- and microfossil analysis from a stratigraphical section; **c** scraping an encrustation of organic matter from an Eneolithic pottery

2. If plant remains are found in a small vase, collect all the material from within the vase and put it in plastic bags/rigid containers. If you have a large vase filled with sediments, it is best to sample the bottom sediments, where the risk of contamination is minimal.
3. If plant remains are from a silos or a warehouse, collect samples of a standard size from all layers (statistical sampling).
4. If a stratigraphical section is exposed and profiled, it is convenient to sample the whole sediment column [6, 31]. A sampling interval of 5 cm may be reasonable in most cases (*see Note 5*). The exposed surface of the section must be carefully cleaned before sampling, to avoid contamination with fresh plant material (Fig. 25.1b). Take the samples using spatulas and trowels, and place them in plastic bags. Label the bags. Alternatively, press metal or plastic boxes into the profile to collect the vertical series [12].
5. If you have planned a total/statistical sampling strategy from a large excavation or archaeological structure, the amount of sampled sediment may be massive. In these cases, it is convenient to extract the plant macroremains directly in the field using flotation or sieving (wet or dry) procedures, or a combination of methods, depending on the nature of the sediments. Choose the extraction method that is more convenient in order to recover the highest possible number

of plant remains. You may also choose to use more than one method [43]. In any case, it is important to treat all samples of the same weight (or volume), to compare the concentrations of plant remains in the soil.

6. Flotation is used to concentrate plant remains floating in water, e.g., charcoal fragments. Flotation can be carried out in buckets or in flotation machines, depending on the amount of soil to be treated (*see Note 6*). Soil must be loosened by water movements, so that plant remains can move to the surface. The water with floating debris is poured over a sieve to retain the plant remains (*see Note 7*). Sieving allows the elimination of the mineral component through the sieves.
7. Wet sieving is preferred with compact, clayey, and waterlogged sediment (*see Note 8*). It is generally carried out in combination with flotation, but soil samples may be directly wet-sieved by pouring water on a set of stacked sieves with decreasing meshes (e.g., 4-2-1-0.5 mm) gently using a sprayer nozzle on the hose (washing-over [44]), or stirring the sieves under water. In some cases only two or three sieves may be used (e.g., 2 and 0.5 mm). Light material floating on water may be gently skimmed with a small brush (*see Note 9*).
8. After floating or wet sieving the samples, dry the residue by spreading it on a cloth or on paper (e.g., newspaper) in the shadow and far from air currents (*see Note 10*).
9. Dry-sieving is especially convenient to isolate macroremains from loose sediments with heterogeneous grain size, which are often found in arid environments. A set of stacked sieves with decreasing meshes (e.g., 4-2-1-0.5 mm) may be gently used, paying attention not to break or polish the plant remains.
10. Once sieved, dry plant remains should be stored in containers and immediately labeled. Record original sample size (weight or volume), mesh size, and separation procedure in your notebook and on the labels, in addition to date, sample code, and archaeological layer.
11. Take photographs before and during the sampling operations and record all the details of the sampling procedures in your notebook.

25.3.4 Collecting Microfossils

1. Sampling for microfossils is carried out in contexts with fine sediments (e.g., clay, silt, peat, organic matter, dung, and gut), without knowing if they contain any plant remain.
2. The most important precaution at all stages of microfossils sampling is to avoid contamination with modern material and with remains from adjacent sediment layers. For that purpose, wear new starch-free plastic gloves, carefully clean the trowels and digging tools immediately before collecting each new sample, sample one sediment layer at a time, use new sterile containers or plastic bags to store the samples, and open the samples only in a sterile laboratory.

3. After collection, immediately label the samples (*see Note 4*) and record date, archaeological unit, stratigraphical layer, and depth of the sample in your notebook.
4. Sediment samples for microfossil analysis may be rather small (e.g., 100–200 g). In case of resins, encrustations, portions of gut or stomach, material from mummies, artifacts, etc., the material available for sampling may be much less (e.g., 0.2–1 g). In these cases, which may also involve ancient DNA and other biomolecules analysis, carefully collect all the available material by scraping it from pottery or utensils, and place it in plastic bags (Fig. 25.1c).
5. Within the archaeological excavation site, take several sparse samples from synchronic horizontal layers as soon as they are uncovered, so as to reconstruct the different usage of different areas (*see Note 11*).
6. If exposed stratigraphical profiles are available, take sediment columns, by either pressing the sediment in clean metal or plastic boxes and wrapping the samples in plastic sheets, or removing the sediment layer by layer with clean tools and placing the sample in plastic bags (*see Note 12*).
7. If lakes or ponds are present in the vicinity of the archaeological site, they may be sampled for pollen and NPPs, diatoms, phytoliths, and microcharcoal to obtain valuable complementary information to on-site analyses. Sampling in lake deposits is carried out through sediment coring (*see Note 13*). Different types of coring equipments can be used, all of which must be designed to collect undisturbed and uncontaminated sediment samples. Equipment and coring techniques for pollen and other microfossil analysis are the subject of several specialized texts to which we refer, as drilling operations are normally undertaken by specialists [21, 45, 46]. Commercial coring devices are available from https://en.eijkelkamp.com/products/augering-soil-sampling-equipment/.
8. When sediments are very stiff, as in the case of dried out lacustrine basins, industrial truck-mounted, hydraulic drilling devices can also be rented, using bottom-filling samplers that collect the sediment in plastic tubes within the corer chamber.
9. Sediment cores must be immediately placed into labeled plastic sleeves to prevent contamination, and opened only in the laboratory for subsequent inspection, subsampling, and analysis.

25.4 Notes

1. When first visiting the archaeological site, it is a good practice to collect a few test samples and check the potential "fertility" of the sediment and modify the subsequent sampling activities accordingly. In particular, it is important to test the presence of microfossils that are not visible to the naked eyes and to choose the best stratigraphical sections to be studied.
2. Although in human-disturbed environments the modern vegetation may be significantly different from ancient patterns, observing the composition of local

and regional vegetation provides information on the relationships between climate, soil type, and anthropogenic influences, and may identify plant taxa that can potentially be present in the store [6, 31].

3. The strategy of sampling must also take into account the available budget, the expected working time, and the number of people necessary for field work, so as to maximize the results, minimizing cost and labor [41].
4. It is most important that the samples are double labeled in a permanent way. By experience it is better to use both waterproof markers and labels.
5. The sampling interval depends on the sediment accumulation rate and the desired detail of the analysis. A fast accumulated deposit (e.g., a cesspit) may be sampled at larger intervals than a deposit where sediments accumulate slowly (e.g., a well).
6. Different kinds of flotation machines have been designed. See [6] for a thorough review. The advantage of these machines is that they are capable to treat large amounts of sediments. A disadvantage is the quantity of water that is needed. To avoid contamination, clean water should be used, or recycled water should be filtered through a fine sieve. Besides, experimental data indicate that flotation techniques can affect the yield of charred plant remains, and thus in some cases alter the results of analysis [47].
7. Sieves of small mesh size (e.g., 0.5–0.2 mm) should be used if seeds of wild plants have to be collected. However, such small mesh size tends to silt up easily during the flotation process and needs to be cleaned frequently [12].
8. When collecting latrine sediments take precautions against infections by using gloves, masks, and having tetanus protection [6].
9. Waterlogged materials may not disaggregate easily in water [6]. Thus, they may be soaked in water for a few days and frequently hand stirred before wet sieving them. Using hot water and a few drops of hydrogen peroxide (H_2O_2) may help disaggregating the sediment.
10. It is important to let the wet-sieved material dry away from any direct heat to avoid breakage and/or deformation of plant remains. Rapid drying under hot sunlight causes damages to seeds and charcoal [6]. Gently handle the samples after drying to avoid deterioration.
11. The number of horizontal samples and the extent of sampled area largely depend on the goals of the research; site function, technology or intra-site variability may require larger sampling areas than detection of food remains or burial procedures.
12. Always choose the profiles that have the clearest stratigraphy and the least amount of disturbance [31]. It is also desirable that a chronological control (e.g., radiocarbon dates or tephra layers) is available from the same profile. When possible, collect samples from several sections, as the plant microfossil content may significantly vary within short distances. It is a good practice to collect some samples also from sterile soils at the top and bottom of the profile as a control for possible contaminations.
13. Replicate sediment cores may be very useful to ensure the comparability between adjacent cores and to store additional material that may be needed for further interdisciplinary analyses.

25.5 Interpretation and Conclusions

Although it is clear that no sampling technique of plant remains is 100% effective, extreme attention must be paid to all phases of collection, as this will determine the final results and interpretation of archaeobotanical analyses [2, 6, 31]. How much material must be sampled and analyzed remains an open question, as different laboratories use different strategies, according to their experience and the features of the study sites. Thus, archaeobotanists from the New World generally stress the importance of complete sampling [6, 41], while in the Old World there are mostly indications of random and judgmental sampling [2]. This is probably because of the abundance of remains in many contexts of Europe and the Near and Middle East. In our experience, which is mostly from extraordinarily rich contexts in Italy, the best results are obtained through careful and detailed laboratory analysis of small amounts of sediments collected in specific well-studied and well-dated archaeological structures, rather than from large amounts of plant remains extracted in the field.

In many cases, plant remains from archaeological sites are unique specimens, for which no repeatable sampling is possible. It is therefore advisable to collect material in abundance, and store part (e.g., 1/10) of the original, intact samples for future analyses, especially if they yield rich and interesting plant assemblages. Storage conditions are also important for a proper conservation of the sampled materials, which may be subjected to losses and breakages. In general, it is advisable to maintain the plant remains in the same conditions they were at retrieval: waterlogged materials should be kept wet; dry materials must be kept dry; charred and mineralized materials may be very fragile and are best stored in rigid containers to prevent crumbling.

One point that is never stressed enough is the importance of avoiding contamination with fresh material or with plant remains from adjacent layers, especially when microfossils are involved. When plant remains are found in aerobic sites, they may be especially problematic, because of possible contamination by older fossil-bearing sediment brought on to the site, or through percolation from biological activities in the surface. For this reason, it is recommended that sampling is carried out by archaeobotanists themselves, with the advice of archaeologists. In this way, archaeobotanists will also have a direct perception of the site, which is also important to address the right questions when interpreting the botanical data.

When studying archaeobotanical remains, it is of utmost importance to sample and analyze different types of plant remains from the same layers, including both macro- and microfossils, complementing each other in the reconstruction of different aspects of human activities, past climate, and environmental changes [48]. However, a thorough understanding of the relationships between humans and plants in the past can only be reached when sampling is planned in the context of multidisciplinary studies, involving archaeologists, soil scientists, geochronologists, archaeobotanists, archaeozoologists, and paleoanthropologists, so that the results of different disciplines are conveyed toward common interpretations in environmental archaeology.

References

1. van Zeist W (1991) Economic aspects. In: van Zeist W, Wasylikowa K, Behre K-E (eds) Progress in old world palaeoethnobotany. Balkema, Rotterdam, pp 109–130
2. Figueiral I, Willcox G (1999) Archaeobotany: collecting and analytical techniques for subfossils. In: Jones TP, Rowe NP (eds) Fossil plants and spores: modern techniques. Geological Society, London, pp 290–294
3. Körber-Grohne U (1991) Identification methods. In: van Zeist W, Wasylikowa K, Behre K-E (eds) Progress in old world palaeoethnobotany. Balkema, Rotterdam, pp 3–24
4. Hastorf CA (1999) Recent research in paleoethnobotany. J Archaeol Res 7:55–103
5. Jacomet S, Kreuz A (1999) Archäobotanik: Aufgaben, Methoden und Ergebnisse vegetations-und agrargeschichtlicher Forschung. Ulmer, Stuttgart
6. Pearsall DM (2000) Paleoethnobotany. A handbook of procedures. Academic, San Diego
7. Jacomet S (2007) Use in environmental archaeology. Encyclopedia of quaternary science. Elsevier, Oxford, pp 2384–2412
8. Gale R, Cutler D (2000) Plants in archaeology. Identification manual of vegetative plant materials used in Europe and the southern Mediterranean to c. 1500. Westbury Publishing and Royal Botanic Gardens, Kew, pp 1–13
9. Behre K-E, Jacomet S (1991) The ecological interpretation of archaeobotanical data. In: van Zeist W, Wasylikowa K, Behre K-E (eds) Progress in old world palaeoethnobotany. Balkema, Rotterdam, pp 81–108
10. Hather JG (1993) An archaeobotanical guide to root and tuber identification. Oxbow Books, Oxford
11. Zohary D, Hopf M, Weiss E (2012) Domestication of plants in the old world. Oxford University Press, Oxford
12. Cappers RT, Neef R (2012) Handbook of plant palaeoecology. Barkhuis, Groningen
13. Duke JA (1992) CRC handbook of edible weeds. CRC Press, Boca Raton
14. Willcox G (2012) Searching for the origins of arable weeds in the Near East. Veg Hist Archaeobot 21:163–167
15. Latalowa M, Badura M, Jarosińska J (2003) Archaeobotanical samples from non-specific urban contexts as a tool for reconstructing environmental conditions (examples from Elbląg and Kołobrzeg, northern Poland). Veg Hist Archaeobot 12:93–104
16. Wilson DG (1979) Horse dung from Roman Lancaster: a botanical report. Archaeo-physika 8:331–350
17. van Zeist W, Waterbolk-Van Rooijen W (1992) Two interesting floral finds from third millennium BC Tell Hammam et-Turkman, northern Syria. Veg Hist Archaeobot 1:157–161
18. Wadley L, Sievers C, Bamford M, Goldberg P, Berna F, Miller C (2011) Middle Stone Age bedding construction and settlement patterns at Sibudu, South Africa. Science 334:1388–1391
19. Wild JP (2003) Textiles in archaeology. Osprey Publishing, Oxford
20. Dickson JH, Bortenschlager S, Oeggl K, Porley R, McMullen A (1996) Mosses and the Tyrolean Iceman's southern provenance. Proc Royal Soc London (Series B: Biol Sci) 263:567–571
21. Faegri K, Kaland PE, Krzywinski K (1989) Textbook of pollen analysis, 4th edn. Wiley, Chichester, pp 1–328
22. Bennett KD, Willis KJ (2001) Pollen. In: Smol JP, Birks HJB, Last WM (eds) Tracking environmental change using lake sediments, vol. 3 (Terrestrial, algal, and siliceous indicators). Kluwer Academic Publishers, Dordrecht, pp 5–32
23. Camacho CN, Carrión JS, Navarro J, Munuera M, Prieto AR (2000) An experimental approach to the palynology of cave deposits. J Quat Sci 15:603–619
24. Van Geel B (2001) Non-pollen palynomorphs. In: Smol JP, Birks HJB, Last WM (eds) Tracking Environmental change using lake sediments, vol 3 (Terrestrial, algal, and siliceous indicators). Kluwer Academic Publishers, Dordrecht, pp 99–119

25. Gauthier E, Bichet V, Massa C, Petit C, Vannière B, Richard H (2010) Pollen and non-pollen palynomorph evidence of medieval farming activities in southwestern Greenland. Veg Hist Archaeobot 19:427–438
26. Miola A (2012) Tools for non-pollen palynomorphs (NPPs) analysis: a list of quaternary NPP types and reference literature in English language (1972–2011). Rev Palaeob Palynol 186:142–161
27. Whitlock C, Larsen C (2001) Charcoal as a fire proxy. In: Smol JP, Birks HJB, Last WM (eds) Tracking environmental change using lake sediments, vol 3 (Terrestrial, algal, and siliceous indicators). Kluwer Academic Publishers, Dordrecht, pp 75–97
28. Battarbee RW, Jones VJ, Flower RJ, Cameron NG, Bennion H, Carvalho L, Juggins S (2001) Diatoms. In: Smol JP, Birks HJB, Last WM (eds) Tracking environmental change using lake sediments, vol 3 (Terrestrial, algal, and siliceous indicators). Kluwer Academic Publishers, Dordrecht, pp 155–202
29. Battarbee RW (1988) The use of diatom analysis in archaeology: a review. J Archaeol Sci 15:621–644
30. Reitz E, Shackley M (2012) Environmental archaeology. Springer, New York
31. Piperno DR (2006) Phytoliths: a comprehensive guide for archaeologists and paleoecologists. AltaMira Press, Lanham
32. Brown TA, Brown K (2011) Biomolecular archaeology: an introduction. Wiley, Chichester
33. Wales N, Andersen K, Cappellini E, Ávila-Arcos MC, Gilbert MTP (2014) Optimization of DNA recovery and amplification from non-carbonized archaeobotanical remains. PLos One 9:e86827
34. Oeggl K, Kofler W, Schimidl A, Dickson JH, Egarter-Vigl E, Gaber O (2007) The reconstruction of the last itinerary of Ötzi, the Neolithic Iceman, by pollen analyses from sequentially sampled gut extracts. Quat Sci Rev 26:853–861
35. Ernst M, Jacomet S (2006) The value of the archaeobotanical analysis of desiccated plant remains from old buildings: methodological aspects and interpretation of crop weed assemblages. Veg Hist Archaeobot 15:45–56
36. Van der Veen M (2007) Formation processes of desiccated and carbonized plant remains—the identification of routine practice. J Archaeol Sci 34:968–990
37. Renfrew JM (1973) Palaeoethnobotany: the prehistoric food plants of the Near East and Europe. Columbia University Press, New York
38. Rowley-Conwy P (2011) Westward Ho! The spread of agriculture from Central Europe to the Atlantic. Curr Anthropol 52:S431–S451
39. Wright PJ (2010) Methodological issues in Paleoethnobotany: a consideration of issues, methods, and cases. In: Van Derwarker AM, Peres TM (eds) Integrating zooarchaeology and paleoethnobotany: a consideration of issues, methods, and case. Springer, New York, pp 37–64
40. Jones MK (1991) Sampling in palaeoethnobotany. In: van Zeist W, Wasylikowa K, Behre K-E (eds) Progress in old world palaeoethnobotany. Balkema, Rotterdam, pp 53–62
41. Lennstrom HA, Hastorf CA (1995) Interpretation in context: sampling and analysis in Paleoethnobotany. Am Antiquity 60:701–721
42. Bandini Mazzanti M, Bosi G, Mercuri AM (2005) Semi, frutti e annessi fiorali: l'archeocarpologia. In: Caneva G (ed) La Biologia Vegetale per i Beni Culturali. Vol. II. (Conoscenza e valorizzazione). Nardini Editore, Firenze, pp 46–56
43. Hageman JB, Goldstein DJ (2009) An integrated assessment of archaeobotanical recovery methods in the Neotropical rainforest of northern Belize: flotation and dry screening. J Archaeol Sci 36:2841–2852
44. Tolar T, Jacomet S, Velušček A, Čufar K (2010) Recovery techniques for waterlogged archaeological sediments: a comparison of different treatment methods for samples from Neolithic lake shore settlements. Veg Hist Archaeobot 19:53–67
45. Aaby B, Digerfeldt G (1985) Sampling techniques for lakes and bogs. In: Berglund B (ed) Handbook of Holocene palaeoecology and palaeohydrology. Wiley, Chichester, pp 181–194

46. Glew JR, Smol JP, Last WM (2001) Sediment core collection and extrusion. In: Last WM, Smol JP (eds) Tracking environmental change using lake sediments, vol 1 (Basin analysis, coring, and chronological Techniques). Kluwer Academic Publishers, Dordrecht, pp 73–105
47. Wright PJ (2005) Flotation samples and some paleoethnobotanical implications. J Archaeol Sci 32:19–26
48. García-Granero JJ, Lancelotti C, Madella M (2015) A tale of multi-proxies: integrating macro-and microbotanical remains to understand subsistence strategies. Veg Hist Archaeobot 24:121–133

Chapter 26
Archaeological Wood Preparation

Alessandra Celant and Gemma Coccolini

26.1 Introduction

The preparation of wood samples from archaeological contexts largely depends on the preservation type and on the chemical and physical state of the material, which may vary from near normal to highly degraded [1]. Compared to fresh wood, archaeological materials have generally undergone hydrolysis and/or degradation through the action of fungi, bacteria, and other soil microorganisms, especially in oxygen-rich environments [2, 3]. Archaeological wood may be waterlogged, dried, charred, or mineralized. It can also present a combination of preservation types (e.g., waterlogged and mineralized [4]). As such, different portions of the same wood fragment may require different treatments. For this reason, while several attempts have been made to standardize the preparation methods for archaeological wood [1, 5–8], it is a common experience that each case study may need some adjustment after the application of standard procedures.

The first step to study archaeological wood is to define how it is preserved, so as to apply the most suitable procedure (*see* Chap. 25).

Waterlogged wood is found in permanently submerged and poorly oxygenated soils, where plant tissues may maintain their anatomical structure and original shape, especially when they are kept wet, cool, and dark [9]. Once the wood is exposed to air, the cell walls will collapse and the wood will shrink and warp, so it is necessary to examine the specimen immediately, before any distortion occurs.

Desiccated wood is recovered from dry areas and generally appears intact. It may be rather difficult to prepare and identify, as it is often very light and extremely friable [7]. It should be kept in dry and rigid containers to avoid disintegration and humidity variations.

A. Celant (✉) · G. Coccolini
Department of Environmental Biology, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy
e-mail: alessandra.celant@uniroma1.it

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_26

Charred or carbonized wood (anthracological remains) is the product of more or less prolong and incomplete combustion by fire. It is chemically highly inert and not subjected to the degrading action of microorganisms, so it may be recovered from soil horizons and archaeological contexts where uncharred plant materials cannot be preserved [10]. However, it undergoes a significant reduction in volume and can be extremely fragile. It should be stored in dry and rigid containers, to avoid fragmentation and crumbling. Charred wood may present well-preserved anatomical structures, allowing easy identification [11].

Mineralized wood may form when tissues are immersed in water containing a high concentration of dissolved minerals [12]. Dissolved carbonates, silicates, and phosphates, as well as metal oxides may precipitate around and within the wood fragment, filling the cell cavities. Iron-, silver-, and copper-oxides may be effective preservatives for wood material, as they are toxic to bacteria and fungi [13]. Mineralized woods may be very hard and do not require any particular care for conservation. In some cases, however, they may be fragile and crumbly, when only their outer surface is covered by a thin layer of oxides (Fig. 26.1).

Archaeological wood is sometimes found in large amounts, but in many cases only small fragments are available. They may be very delicate or may be part of priceless artefacts, so it is important to handle them very carefully, to use the minimum possible surface for identification, and to keep them stored in the same environmental conditions of their retrieval. In general, preparing archaeological wood is simple and allows processing of a large number of samples in a relatively short time [14].

Here we describe two different procedures in relation to different preservation states. Sectioning is usefully applied to waterlogged or desiccated wood. Fracturing is used with charcoal and mineralized wood. We do not describe the embedding procedures because in our experience they are complicated and unnecessary, besides being lengthy and wasteful of material [14].

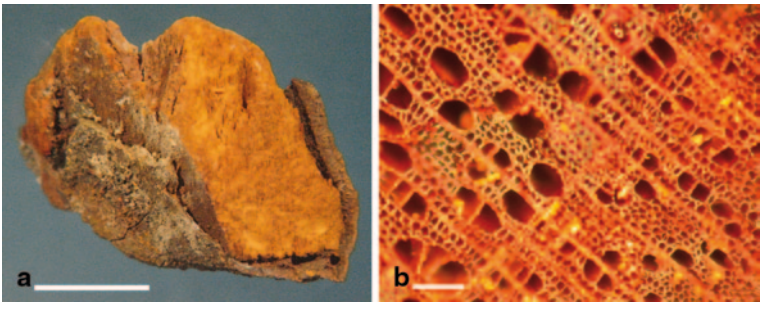

Fig. 26.1 **a** Mineralized fragment of poplar wood (*Populus* sp.) surrounded by leather, forming part of the sheath of a lance (Roman Imperial Age, Palatine Hill of Rome, Italy). Scale bar = 0.5 cm. **b** The transverse surface of the wood fragment, to the left, obtained by fracturing and observed under a stereomicroscope. Scale bar = 100 μm

26.2 Materials

26.2.1 Sectioning

1. Waterlogged or desiccated wood fragment.
2. A soft brush and/or a scalpel.
3. Double-sided razor blade in a safety holder.
4. Freezer (if the waterlogged sample is very soft or spongy).
5. Hot water (if the desiccated sample is hard).
6. Bleach (if the sample is dark) in a watch glass.
7. Seventy percent glycerol.
8. Forceps.
9. Microscope slides and coverslips.
10. Stereomicroscope.
11. Transmitted light microscope.

26.2.2 Fracturing

1. Charred, mineralized, or desiccated wood fragment.
2. A soft brush and/or a scalpel.
3. A small hammer (if the sample is very hard).
4. Forceps with flexible tips.
5. Plasticine or wax (or dried and clean poppy seeds).
6. Glass slide or shallow disk.
7. Stereomicroscope with objectives × 5, × 10, × 20, × 50, possibly with Nomarski differential interference contrast equipment.
8. Scanning electron microscope (SEM) stubs, double-sided conductive tape, and environmental scanning electron microscope (ESEM; if examination under the stereoscopic microscope is not satisfactory).

26.3 Methods

26.3.1 Sectioning

1. Define the preservation state of the wood sample, whether it is waterlogged or desiccated (*see Note 1*).
2. Clean the surface of the sample with a soft brush and/or a scalpel to eliminate the embedding sediment.

3. If the sample is dry, soften it through boiling in water, until it sinks. Alternatively, it can be soaked in water for hours or days until it is soft enough to be hand cut by a razor blade.
4. If the sample is very soft or spongy, freeze it, or air dry it in the laboratory, avoiding vicinity to heat sources and ventilation. It is essential to frequently check the drying process in order to avoid rapid and excessive desiccation.
5. If the sample was frozen, ensure that the equipment for sectioning is ready beforehand.
6. Trim away the outer surface of the wood sample using a razor blade under a stereomicroscope, to obtain a clean surface along the transverse plane (*see Note 2*).
7. Hand cut sections from the transverse plane using a new razor blade (*see Note 3*). When sectioning the sample, the blade should form an angle of 10–20° from the horizontal. If enough study material is available, different cutting angles can be tried, until a suitable section thickness is obtained. A section 15–20 μm thick would be optimal.
8. If the sections are too dark for observation under the light microscope, soak them in a watch glass with a drop of bleach for a few minutes, and then rinse them in a watch glass with water.
9. Delicately transfer the transversal sections onto a microscope slide using forceps and mount them in 70% glycerol. Cover with a coverslip.
10. Trim away the outer surface of the wood sample along the longitudinal (radial and tangential) planes (*see Note 4*).
11. If the sections are too dark for observation under the light microscope, soak them in a watch glass with a drop of bleach for a few minutes, and then rinse them in a watch glass with water.
12. Delicately transfer the radial and tangential sections to a microscope slide using forceps and mount them in 70% glycerol. Cover with a coverslip.
13. Observe the samples under the transmitted light microscope for identification.

26.3.2 Fracturing

1. Define the preservation state of the wood sample, whether it is charred, mineralized, or desiccated (*see Note 1*).
2. Clean the surface of the sample with a soft brush and/or a scalpel to eliminate the embedding sediment (*see Note 2*).
3. Examine the sample, if necessary under the stereomicroscope, to find the anatomical orientation.
4. Obtain a transversal surface of the sample by snapping it using the thumbs and index fingers of both hands (Fig. 26.1; *see Note 5*).
5. If the sample is very hard (e.g., mineralized), it may be necessary to fracture it using a scalpel and/or a small hammer (*see Note 6*).
6. If the specimen is extremely delicate or very small, it is better to manipulate it as little as possible and observe it under the microscope without fracturing it (*see Note 7*).

7. Use forceps with highly flexible tips to delicately transfer the prepared fragment to a microscope slide having a bit of plasticine or wax, or to a shallow disk containing dried and clean poppy seeds, which serve to support the sample and facilitate its correct orientation under the microscope.
8. Radial and tangential longitudinal surfaces will be prepared following the same procedure of the transversal section (steps 3–7).
9. Observe the samples under a stereomicroscope with objectives × 5, × 10, × 20, × 50, possibly with Nomarski differential interference contrast equipment (*see Note 8*).
10. If examination under the stereoscopic microscope is not satisfactory because the obtained surface is not sufficiently plain, the wood fragment is very small, or the anatomical diagnostic features cannot be recognized, the fractured samples can be mounted on SEM stubs using a double-sided conductive tape and observed under an ESEM (*see Note 9*).

26.4 Notes

1. In some cases, extremely desiccated wood cannot be prepared for sectioning, as they dissolve in water. In these cases, they may be fractured using the same procedure of charcoal.
2. When cleaning the samples and trimming away the outer surface of the wood fragment, take care not to sacrifice too much sample, especially if it is small and useful characteristics might be lost.
3. It is important to use new and very sharp blades to obtain very thin sections. Double-sided blades (to be used in a blade holder) are most convenient because they are sharper than the single-edged type and can be bend between the fingers to find the right cutting angle. Always cut away from the body and face, using sharp blades. Blunt blades are a safety hazard because they require more force to make the cut and can slip out of the hand, procuring injuries [7]. Fine sections obtained by hand cutting are generally sufficient for observation under the light microscope. However, the use of a sliding microtome is recommended if good photographs are needed [15].
4. The radial plane is easily found parallel to the ray cells as observed in the transversal section. The tangential plane should be cut perpendicular to the ray cells. A properly trimmed wood fragment should present three planes at right angles to each other [16].
5. Using a razor blade to obtain plain surfaces may be useful in some cases, but there is also the risk of obliterating the diagnostic plane with the very fine powder produced by the blade [11].
6. Obtaining longitudinal surfaces in the required orientation may be rather difficult when only small fragments of mineralized wood are available, as they can be very hard and the use of hammer and scalpel may reduce control over the line of fracture [7].

7. In cases of particularly friable charcoal fragments, if fracturing along the desired plane observation is not successful, epoxy resin embedding may be considered. A description of embedding techniques is reported by Figueiral [11].
8. Observation of archaeological wood under Nomarski differential interference contrast microscope is the most convenient and fast technique because it requires minimum preparation procedures and has excellent resolving power and contrast. It is also appropriate for routine analyses with large amounts of samples [17]. It can also used with small and very fragile wood fragments.
9. Usage of an ESEM instead of an SEM is preferable for the speed of the procedure and for the lower cost, as samples do not require coating with gold. Besides, in the SEM vacuum chamber, the samples may be easily fragmented to the point of not being identified.

26.5 Interpretation and Conclusion

Archaeological contexts can provide large quantities of wood fragments. Thus, it is very important that preparation techniques for wood and charcoal are fast and effective. In this respect, sectioning and fracturing appear as very convenient methods, which have only the main disadvantage of being destructive techniques, producing further fragmentation and loss of study material. Particular attention is needed with crumbly charred wood: When quali–quantitative analyses are performed, it is important to maintain the unity of the sample in order to avoid wrong interpretation based on counts of the wood fragments.

Obtaining sections from wooden artefacts and decorated or carved fragments is often impossible and identification must proceed only with the observation of the available exposed surfaces under a stereomicroscope [7]. In these cases, no repeatable observations are possible, except for taxonomic revisions or reexaminations of the samples. If decorated archaeological wood has been treated with supporting media, such as polyethylene glycol (PEG), the analysis of the exposed surfaces may be further complicated by the reflection that the consolidating material produces under the stereomicroscope [7].

When the available wood fragments are very small or require examination at high magnifications, it may be necessary to study them by SEM (or ESEM). In these cases, preparation and identification are definitely more time consuming and expensive. This technique should be kept to a minimum if large amounts of wood are accessible.

Compared to fresh wood, archaeological wood is more delicate and requires some manual skill for the preparation of adequate diagnostic sections and planes. In particular, evaluating when the degree of desiccation is right for sectioning soft or spongy woods is a matter of knowledge of the material, which can only be acquired through experience. The same is true for the degree of softening desiccated samples and of bleaching dark woods. The main problem is that once the right degree of desiccation, or softening, or bleaching, is exceeded, permanent changes may have

occurred in the wood structure that may not be recovered. In these cases, and if only small wood fragments are available, the possibility of identifying them may be lost forever.

It is, therefore, recommended that sectioning and fractioning archaeological woods are always carried out with caution not to produce irreparable damage.

References

1. Florian ME (1990) Scope and history of archaeological wood. In: Rowell RM, Barbour RJ (eds) Archaeological wood properties, chemistry, and preservation. American Chemical Society, Washington, pp 3–32
2. Huisman DJ, Manders MR, Kretschmar EI, Klaassen RKWM, Lamersdorf N (2008) Burial conditions and wood degradation at archaeological sites in the Netherlands. Int Biodeter Biodegr 6:33–44
3. Björdal CG (2012) Microbial degradation of waterlogged archaeological wood. J Cult Herit 13:S118–S122
4. Remazeilles C, Tran K, Guilminot E, Conforto E, Refait P (2013) Study of Fe(II) sulphides in waterlogged archaeological wood. Stud Conserv 58:297–307
5. Jones TP, Rowe NP (1999) Locating and collecting. In: Jones TP, Rowe NP (eds) Fossil plants and spores: modern techniques. Geological Society, London, pp 5–8
6. Grattan DW, Clark RW (1987) Conservation of marine archaeological objects. Butterworths, London, pp 164–206
7. Gale R, Cutler D (2000) Plants in archaeology. Identification manual of vegetative plant materials used in Europe and the southern Mediterranean to c. 1500. Westbury Publishing and Royal Botanic Gardens, Kew, pp 1–13
8. Pearsall DM (2000) Paleoethnobotany. A handbook of procedures. Academic, San Diego, pp 11–97
9. Holden J, West LJ, Howard AJ, Maxfield E, Panter I, Oxley J (2006) Hydrological controls of in situ preservation of waterlogged archaeological deposits. Earth-Sci Rev 8:59–83
10. Thompson GB (1994) Wood Charcoals from tropical sites: a contribution to methodology and interpretation. In: Hather JG (ed) Tropical archaeobotany. Applications and new developments. Routledge, New York, pp 9–23
11. Figueiral I (1999) Lignified and charcoalified fossil wood. In: Jones TP, Rowe NP (eds) Fossil plants and spores: modern techniques. Geological Society, London, pp 92–96
12. Taylor NT, Taylor EL, Krings M (2009) Paleobotany: the biology and evolution of fossil plants. Academic, Burlington, pp 1–42
13. Zohary D, Hopf M, Weiss E (2012) Domestication of plants in the old world. Oxford University Press, Oxford, pp 9–13
14. Vernet JL (1973) Etude sur l'histoire de la végétation du sud-est de la France au Quaternaire d'après les charbons de bois principalement. Paléobiologie Continentale 4:1–90
15. Schweingruber FH (1978) Mikroskopische Holzanatomie. Swiss Federal Institute for Forest, Birmensdorf, pp 211–212
16. Jansen S, Kitin P, De Pauw H, Idris M, Beeckman H, Smets E (1998) Preparation of wood specimens for transmitted light microscopy and scanning electron microscopy. Belg J Bot 131:41–49
17. Thinon M (1990) Utilisation de la microscopie épiscopique interférentielle pour l'identification botanique des charbons de bois. In: Hackens T, Munaut AV, Till C (eds) Bois et Archéologie, 1st European Conference, Louvain-la-Neuve, 1987, PACT 22:179–188

Chapter 27
Archaeopalynological Preparation Techniques

Donatella Magri and Federico Di Rita

27.1 Introduction

Extraction of pollen, spores and other non-pollen palynomorphs from sediments consists of removal of organic and inorganic matter from a sample, with the aim of rendering the grains visible under the microscope and enabling easy identification and counting [1–7].

The preparation procedure used to extract pollen from the enclosing sediment is based on the possibility to use chemical breakdown to eliminate various sediment components without significantly affecting chemical-resistant pollen grains, to separate palynomorphs from water by means of centrifugation, to eliminate the sediment fraction coarser than 200 μm and finer than 8 μm by sieving without losing pollen.

Ideally the same techniques can be used regardless of provenance, type, or age of the samples, but in practice several variations can be applied depending on the nature of the sediment involved and on procedural details found in the literature. Hardly two laboratories process samples in exactly the same way [8]. In all cases, some laboratory skill is necessary to obtain "clean" samples easy to study under the microscope and representative of the original pollen content in the sediment. Great care at this stage of the analysis is critical if we want the results of pollen analysis to reflect natural differences in the samples and not differences in the pollen preparation procedures.

Most palynological laboratories reserve a room for the preparation process, as accurately clean conditions are necessary to prevent contamination of the samples from the atmosphere, water or other sediment samples. To that purpose, a filtered air inflow is often provided by air conditioning and use of distilled or microfiltered water must be ensured in all the phases of the preparation. A second reason for reserving a preparation room for pollen analysis is that strict safety requirements are

D. Magri (✉) · F. Di Rita
Department of Environmental Biology, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy
e-mail: donatella.magri@uniroma1.it

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_27

necessary, as hazardous protocols with strong acids are used to remove inorganic particles from the sediment [8]. Apart from these recommendations, no special equipment is required.

The pollen preparation technique consists of a sequence of steps that may or may not be included in the protocol, depending on the features of the sediment under treatment. It is, however, desirable that all the samples from a specific site or stratigraphic sequence be processed by the same laboratory technician using the same procedure to avoid any difference in preparation techniques that may affect the final results and interpretation of pollen data. Especially when pollen grains in the sediment are scarce, often the case for archaeological samples, the extraction process by chemicals must be as extensive as possible without breaking down the pollen grains themselves. A good rule is to avoid chemical treatments that are unnecessary and to find a good balance in the protocol steps, so as to obtain a final residue easily observable under the microscope without exceeding in applying protocols that may produce loss or degradation of pollen grains [3].

Here we describe the standard minimum procedure necessary to extract pollen from archaeological sediments and a number of modules that can be additionally applied (sieving, deflocculation, gravity separation, and acetolysis) depending on the composition of the analyzed material. Sieving will be used if the sample is composed of a large fraction of sediment coarser than 200 μm or finer than 8 μm. Deflocculation will be applied if the sediment is high in clay. Gravity separation is convenient when samples are poor in pollen and contain very high amounts of mineral fragments. Acetolysis is the last step of the pollen preparation if the samples still contain significant amounts of undissolved organic matter that may affect pollen identification and counting.

27.2 Materials

27.2.1 Equipment and Supplies for Standard Procedure

1. Protective personal equipment including laboratory coat, chemical-resistant apron, full face mask, chemical-resistant rubber gloves, and closed shoes
2. Waterproof pen, labels, and notebook
3. Glassware: 50 and 1000 mL graduated cylinders, 250 and 500 mL beakers (500 or 1000 mL) for mixing solutions, microscope slides, and coverslips
4. 50 mL (100 mL) polypropylene boiling tubes with caps and tube holder for 8–16 tubes
5. Litmus paper, polypropylene and glass rods, scalpels
6. Fume cupboard resistant to hydrofluoric acid (HF) fumes
7. A swing head centrifuge that can spin 8–16 tubes to 3000 rpm
8. Hot water bath that can contain 8–16 tubes (50–100 mL)
9. A vortex mixer to stir samples in tubes

10. Distilled or microfiltered water
11. *Lycopodium* tablets (available from Department of Quaternary Geology in Lund; pollentab@geol.lu.se)
12. 37 % hydrochloric acid (HCl)
13. 50 % hydrofluoric acid (HF)
14. Solution of sodium carbonate (Na_2CO_3) available inside the fume cupboard to wipe up HF drips and contaminated equipment
15. An adequate supply of 2.5 % calcium gluconate gel, an HF antidote, to be kept refrigerated and renewed regularly
16. Sodium hydroxide (NaOH) or potassium hydroxide (KOH) in granules
17. Glycerol
18. Disposal for acidic (including HF) waste marked with completed hazardous waste labels, according to local regulations
19. Disposal for alkaline waste marked with completed hazardous waste labels, according to local regulations

27.2.2 Sieving

1. Sieve with 180 µm (or 200 µm) mesh for coarse sieving

27.2.3 Deflocculation

1. Sodium pyrophosphate ($Na_4P_2O_7$)
2. Sieve with 8 µm (or 10 µm) mesh for fine sieving

27.2.4 Gravity Separation

1. Zinc chloride ($ZnCl_2$; specific gravity 1.96)

27.2.5 Acetolysis

1. Glacial acetic acid (CH_3CO_2H)
2. Acetic anhydride ($(CH_3CO)_2O$)
3. Sulphuric acid (H_2SO_4)

27.3 Methods

The standard procedure is the minimum treatment generally used to extract pollen grains. Many palynologists apply only this procedure. Additional treatments (sieving, deflocculation, gravity separation, and acetolysis) can be performed at various stages of the standard procedure, as indicated in the following protocol (Fig. 27.1).

27.3.1 Standard Procedure

1. Wash laboratory benches and glassware before the treatment in order to limit pollen contamination. Laboratory and glassware must always be kept very clean.
2. Use clean scalpels to take subsamples of approximately 1 g sediment each (*see Note 1*) and to include them in boiling tubes labelled with appropriate codes. Make sure the tube labels remain legible, as several chemicals can remove waterproof ink. Take note of the date in the laboratory notebook, and record each step of the procedure, including any errors and anything you note of importance about the samples. For each sample take note of the site name and location, sample code, depth, volume or weight, spike amount, and batch. It is a good practice to double check that the label matches the notes in the laboratory logbook.
3. Wear chemical protective gloves and clothes.
4. Add 30 mL of 37% HCl, in order to remove the carbonate fraction of the sediment, and let it sit until effervescence stops (*see Note 2*).
5. Add 1–2 tablets of *Lycopodium* to allow calculation of the pollen concentration (*see Note 3*).
6. Balance samples, centrifuge at 3000 rpm for 10 min, preferably using a centrifuge able to spin all processed samples at once. Carefully decant the supernatant liquid (pour away the liquid from the tube, retaining the residue) in the disposal for acidic waste under the fume cupboard (*see Note 4*). Stir each sample with a vortex mixer.
7. Add 30 mL of 50% HF and leave overnight, in order to remove silica and silicates from the sediment (*see Note 5*). Do not use glass tubes with HF.
8. Balance, centrifuge, decant in the disposal for acidic waste under the fume cupboard, and stir the samples.
9. Add 30 mL of 37% HCl. This process is required to remove colloidal silica and silicofluorides.
10. Balance, centrifuge, decant in the disposal for acidic waste under the fume cupboard, and stir the samples. If the samples contain a lot of silica and silicates steps 9 and 10 should be repeated until the supernatant liquid appears colourless or light yellowish.
11. Add 25 mL of 10% NaOH (or KOH) and leave for 10 min in boiling water bath, in order to remove humic acids that will be brought into solution. Frequently stir the samples with alkali resistant sticks, in order to enhance the sediment disaggregation and facilitate the dissolution of humic acids.

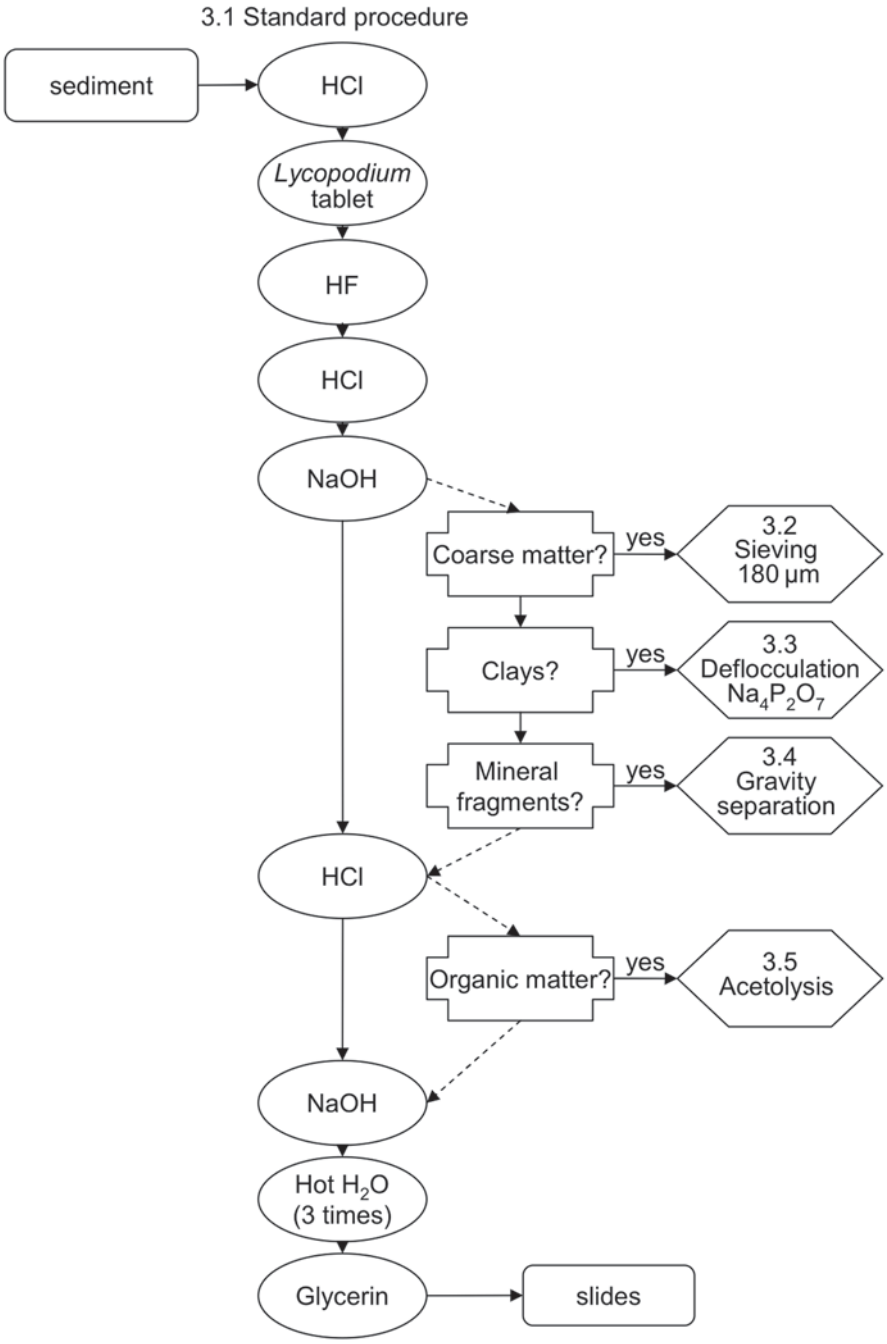

Fig. 27.1 Flowchart showing the laboratory procedure for pollen extraction. Standard procedure and additional methods refer to the respective Sects. 3.1–3.5

12. Balance, centrifuge, decant in the disposal for alkaline waste, and stir the samples.
13. If wood or other organic fragments float in the supernatant liquid, or coarse mineral matter is still present, a sieving operation is desirable to eliminate them: go to Sect. 27.3.2.
14. If samples contain clays, deflocculation is useful to remove these particles: go to Sect. 27.3.3.
15. If samples contain very high amounts of mineral fragments, gravity separation and removal by heavy liquids may be attempted at this stage of the treatment: go to Sect. 27.3.4.
16. Add 20 mL of 37% HCl, in order to remove the residual carbonates possibly released during the sediment disaggregation of the preceding step in Sect. 27.3.1, point number 11.
17. Balance, centrifuge, decant in the disposal for acidic waste under the fume cupboard, and stir the samples.
18. If the samples still contain significant amounts of undissolved organic matter that may affect pollen identification and counting, it may be necessary to make an acetolysis, which dissolves polysaccharides, especially cellulose, by decomposing the long polymer chain into soluble monosaccharide units. Go to Sect. 27.3.5.
19. Add 10 mL of 10% NaOH (or KOH) and leave cold. This process is useful to raise the pH toward neutral pH 7.
20. Balance, centrifuge, decant in the disposal for alkaline waste, and stir the samples.
21. Wash repeatedly by adding 35 mL of hot water (60 ℃) and centrifuge at each step until neutral pH. Test the pH conditions of the supernatant with litmus paper.
22. Add 10–20 drops of glycerol and stir the samples with the vortex mixer *(see Note 6)*.
23. For each sample, put a drop of the suspension on a clean microscope slide, using a thin glass rod. Spread out and add a coverslip. The slide may be sealed by using nail polish.
24. At the end of the chemical treatment, countertops, shelves, glassware and all laboratory items in use must be cleaned and arranged accurately according to standard safety rules for chemicals products and glassware, with the purpose of avoiding future contamination of fossils and fresh pollen. Glassware and boiling tubes should be cleaned up with lab detergent and/or bleach, then rinsed several times with tap water followed by distilled or microfiltered water. Gloves and plastic apron are recommended when washing glassware and boiling tubes. When dry, glassware and boiling tubes must be ranged in filtered storage cabinets.

27.3.2 Sieving

1. To remove coarse particles, pour the samples through 180 μm (or 200 μm) mesh sieves and wash them with distilled or microfiltered water into a second set of tubes. Eliminate the coarse particles from the sieves and retain macrofossils *(see Note 7)*.
2. Balance the sieved samples, centrifuge, decant, and stir with a vortex mixer.
3. Proceed with Sect. 27.3.1, point number 14.

27.3.3 Deflocculation

1. To remove clay particles, add 20 mL of 10 % $Na_4P_2O_7$. Stir energetically with a stick and leave for 15 min in a hot water bath.
2. Pour the samples through 8 μm (or 10 μm) mesh sieves and wash them with distilled or microfiltered water into a second set of tubes until the filtrate becomes clear *(see Note 8)*.
3. Balance, centrifuge, decant, and stir.
4. Proceed with Sect. 27.3.1, point number 15.

27.3.4 Gravity Separation

1. Add 25 mL of a saturated solution of $ZnCl_2$ (specific gravity: 1.96). Stir energetically with a stick *(see Note 9)*.
2. Balance and centrifuge.
3. Decant the supernatant into a solution of distilled water with a few drops of HCl in order to avoid precipitation of zinc hydroxide.
4. Proceed with Sect. 27.3.1, point number 16.

27.3.5 Acetolysis

1. Completely dehydrate the sample by adding 20 mL of CH_3CO_2H *(see Note 10)*.
2. Prepare the acetolysis mixture in a measuring cylinder by accurately mixing up nine parts of $(CH_3CO)_2O$ and one part of concentrated (95–98 %) H_2SO_4. The acetolysis mixture appears as a pale yellow liquid *(see Note 11)*.
3. Immediately, add 15 mL of acetolysis mixture to each sample. Put the samples into a boiling water bath (90 ℃) and leave for 3 min continuously stirring with thin glass rods. Do not extend past 3 min.
4. Rapidly add 25 mL of CH_3CO_2H.

5. Balance, centrifuge, decant, and stir.
6. Wash with distilled water to remove the CH_3CO_2H. Repeat steps 5 and 6 until neutral pH. Test the pH of the supernatant with litmus paper.
7. Proceed with Sect. 27.3.1, point number 22.

27.4 Notes

1. The right amount of processed material depends on the pollen concentration in sediments. Barber selects small quantity samples of 0.5 cm^3 of peat [9], Bennett and Willis suggest volumes of 0.5–1 cm^3 sediment [4], Litt et al. treated 2 cm^3 of calcitic marls, aragonite, and gypsum from an arid environment [10], Kaniewski et al. processed 15–20 g sediment from a cave [11], Horowitz suggests treatment of 50 g of archaeological soils from arid lands [3]. In some cases, only very small quantities of material are available from rare and/or valuable archaeological finds. For example, the processed weight of material from the gut of "Ötzi", the Neolithic Iceman, ranged between 0.04 and 0.39 g and the chemical treatment was reduced to 10 min boiling in 10% NaOH [12].
2. If the samples are calcareous, HCl should be added cautiously to avoid strong chemical reactions that may cause tube breakage and loss of sediment. Strong reactions can be controlled by adding some drops of ethyl alcohol to the samples. It is a good practice to use a bottle top dispenser to dispense all dangerous reagents. It is also important not to touch the tubes with the tip of the dispenser to prevent sample contamination.
3. Tablets with a known number of spores of an exotic species of *Lycopodium* are produced and sold by the Department of Quaternary Geology in Lund. They are now prepared in a slightly different way compared to that described by Stockmarr 1971 and 1973 [13, 14], being based mainly on Na_2CO_3 together with polyethylene glycol. Although *Lycopodium* may also be found native in some regions of Europe, the exotic spores employed appear differently coloured and darker than native ones, mostly due to the acetylation undergone during tablet preparation [15]. They can be dissolved in HCl at the beginning of the preparation and treated together with the sediment sample. Take note of the mean number of *Lycopodium* spores contained in each tablet for the batch in use. Pollen concentration will then be calculated using the following function: pollen concentration per gram of sediment treated = [(exotic spore added × pollen counted)/(exotic spore counted)]/grams of sediment treated. An alternative to *Lycopodium* tablets may be a fixed volume of a calibrated suspension of exotic pollen (e.g. *Alnus* in the southern hemisphere or *Eucalyptus* in the northern hemisphere), stirred overnight before the laboratory treatment [4].
4. Take care to decant with one single smooth movement to avoid loss of sediment. If pouring is too slow, you may get suspension of sediment. If pouring is too fast you may lose material. Skill comes from practice. If there is any sign of movement of material in the tube, stop immediately and do not restart pouring.

Instead, add liquid (usually water or the chemical product in use), and centrifuge again.

5. HF is an extremely dangerous colourless gas, or a fuming liquid, with a strong irritating odour, whose handling requires special training. When working with HF it is important to understand the health hazards posed by this substance. The toxicity of HF is related to both the initial caustic skin injury, which is not usually significant unless concentrations are greater than 20 %, and the HF moiety that can pass through cell membranes due to the high permeability of this acid [16]. HF must be handled wearing HF-resistant gloves as well as face and body protection, such as face shields and specific aprons. A solution of Na_2CO_3 should always be available inside the fume cupboard to wipe up drips and HF contaminated equipment. Some treatment protocols include hot and highly concentrated HF [3]. However this practice is very dangerous, with increased risk of fatal exposures, because the high concentrations and hot temperature dramatically increase the evaporation of HF, whose boiling point (19.4 °C) is below room temperature. Notice that in the case of highly organic samples, the HF treatment can be omitted [15].
6. Staining the samples before storing them is largely a matter of preference. Acetolysis usually darkens the pollen grains, but it is also possible to stain the samples with basic fuchsin (1 % in ethanol) or Safranin O (1 % aqueous solution; [1, 17]). Our experience is that in most cases staining the samples is unnecessary and time consuming. The easiest method to store the treated samples and mount them on microscope slides is to add a few drops of glycerol to the residue and pour them into small collection containers. The relatively low viscosity of glycerol makes it easy to turn the pollen grains during the analysis under the microscope by gently pressing the coverslip with a needle or the tip of a pencil [1]. An alternative storage medium is glycerine jelly, which can be used after melting in a water bath. Put a drop of the suspension on a warm microscope slide and add a coverslip. A third possibility is mounting in silicone oil diluted by tertiary butyl alcohol. This technique has the advantage of not altering pollen grain size compared to glycerol and glycerine jelly. Besides, the slides prepared with silicone oil never dry out and do not need sealing. In this case the procedure includes: washing in tertiary butyl alcohol, centrifuging, and decanting. Silicone oil is then added to the residue and stirred with a small disposable rod. The samples are left for at least 24 h to allow tertiary butyl alcohol to evaporate. Place a drop of the suspension on a microscope slide, add the coverslip and press gently [4].
7. Sieving removes larger silt- and sand-sized particles, and helps eliminate rootlets or leaf fragments. It is important to disaggregate the sediment lumps before coarse sieving to avoid loss of trapped pollen [18]. It is good practice to use a sieve for each sample and to clean the sieves accurately after use, to avoid possible contamination.
8. Nylon sieves of 8 μm (10 μm) mesh can be conveniently used. If washing with distilled water does not proceed satisfactorily, it is possible to enhance the process of sieving using an ultrasonic bath to stir the sample through vibration.

This technique is commonly used in marine sediments to concentrate pollen grains and spores. However, it may force some small pollen grains to pass through the sieve mesh and may cause damage to pollen grains [8]. A membrane microsieve procedure under water pressure has also proven effective in microdebris filtering and clay removal [19].

9. The basic principle of the method is that pollen and other palynomorphs have a specific gravity of 1.4, while mineral particles have a specific gravity higher than 2.5. As the density of a heavy liquid will be reduced when added to a wet or damp sample, it may be convenient to repeat the treatment after the addition of new heavy liquid [18]. Traverse [8] suggests to centrifuge for 30 min at 500 rpm and then 10 min at 2000 rpm to avoid minerals dragging pollen down. Zinc bromide ($ZnBr_2$), mixed with water or HCl, is a less expensive, but more toxic heavy liquid that is sometimes preferred to $ZnCl_2$ [8, 18]. Disposal and recycling considerations should also be considered when choosing a heavy liquid.
10. A complete dehydration is essential for personal safety and for the final success of the reaction, since adding the acetolysis mixture to a sample with some water residue may cause explosion and loss of material.
11. Calculate how much acetolysis mixture is needed to process the samples to avoid preparing excess. Any excess amounts cannot be stored and must be disposed of in the acidic waste. As the mixing reaction is exothermic, add the H_2SO_4 to the $(CH_3CO)_2O$ a little at a time.

27.5 Interpretation and Conclusion

Pollen preparation techniques have been developed, tested, and proposed for nearly a century, and a number of standard procedures have been fixed. Even so, new experimental methods continue to be suggested, in relation to specific advancement of palynological research. This is the case for archaeopalynological studies, which are increasingly of interest by archaeologists for the important paleoenvironmental and paleoclimatic information they provide. In particular, methods for pollen extractions in dry archaeological contexts have been discussed by Fish [20] and Horowitz [3]. They have shown that in spite of low pollen concentration and selective preservation, pollen analysis from arid sites may provide evidence for paleoenvironmental changes in most interesting climate-sensitive areas.

In general, keeping the mechanical and chemical treatment to a minimum is a good practice, because it minimizes the loss of pollen grains and the risk of affecting the final interpretation of the data. Besides, laboratory protocols can affect the residue of pollen preparation, which may be used for further analyses. For example, when HF is not used and only gravity separation and sieving are applied, siliceous microfossils such as diatoms, sponge spicules and phytoliths can be counted in addition to pollen and other palynomorphs from the same slides.

Pure pollen samples can also be used for accelerator mass spectrometry (AMS) dating, provided that carbon-based chemicals are not used during the treatment. A combination of deflocculation, bleaching, and sieving techniques are generally used for that purpose [21, 22].

In recent years, it has been shown that $\delta^{13}C$ records may be obtained from fossil pollen, used together with conventional palynology to assess plant responses to environmental changes [23, 24]. It has also been shown that preparation of pollen for stable carbon isotope analyses must avoid acetolysis, which may provide a significant source of carbon isotopic contamination, affecting subsequent $\delta^{13}C$ measurements on acetylated pollen [23].

Preparation of pollen samples may also cause shifting of fluorescence wavelengths of pollen and spores towards the red end of the spectrum. In applying UV-fluorescence microscopy to archaeopalynological studies, Yeloff and Hunt [25] obtained a minimal shift in the fluorescence spectra of pollen grains by simply boiling the samples in KOH and sieving them.

These examples suggest that new frontiers in palynology, related to organic compounds or isotopes from pollen grains [26], may need different preparation techniques that are still to be explored.

References

1. Faegri K, Kaland PE, Krzywinski K, (1989) Textbook of pollen analysis, 4th edn. Wiley, Chichester, pp 1–328
2. Pearsall DM (2000) Paleoethnobotany. A handbook of procedures, 2nd edn. Academic, San Diego, pp 249–353
3. Horowitz A (1992) Palynology of arid lands. Elsevier, Amsterdam, pp 169–201
4. Bennett KD, Willis KJ (2001) Pollen. In: Smol JP, Birks HJB, Last WM (eds) Tracking environmental change using lake sediments, vol 3-terrestrial, algal, and siliceous indicators. Kluwer Academic Publishers, Dordrecht, pp 5–32
5. Berglund BE, Ralska-Jasiewiczowa M (1986) Pollen analysis and pollen diagrams. In: Berglund BE (ed) Handbook of Holocene palaeoecology and palaeohydrology. Wiley, Chichester, pp 455–484
6. Batten D (1999) Small palynomorphs. In: Jones TP, Rowe NP (eds) Fossil plants and spores: modern techniques. The Geological Society, London, pp 15–19
7. Wood DG, Gabriel AM, Lawson JC (1996) Palynological techniques-processing and microscopy. In: Jansonius J, McGregor DC (eds) Palynology: principles and applications, vol 1. American Association of Stratigraphic Palynologists Foundation, Dallas, pp 29–150
8. Traverse AT (2008) Paleopalynology, 2nd edn. Springer, Dordrecht, pp 615–667
9. Barber KE (1976) History of vegetation. In: Chapman SB (ed) Methods in plant ecology. Blackwell, Oxford, pp 5–83
10. Litt T, Ohlwein C, Neumann FH, Hense A, Stein M (2012) Holocene climate variability in the Levant from the Dead Sea pollen record. Quat Sci Rev 49:95–105
11. Kaniewski D, Renault-Miskovsky J, Lumley H de (2005) Palaeovegetation from a *Homo neanderthalensis* occupation in Western Liguria: archaeopalynology of Madonna dell'Arma (San Remo, Italy). J Archaeol Sci 32:827–840

12. Oeggl K, Kofler W, Schimidl A, Dickson JH, Egarter-Vigl E, Gaber O (2007) The reconstruction of the last itinerary of Ötzi, the Neolithic Iceman, by pollen analyses from sequentially sampled gut extracts. Quat Sci Rev 26:853–861
13. Stockmarr J (1971) Tablets with spores used in absolute pollen analysis. Pollen Spores 13:615–621
14. Stockmarr J (1973) Determination of spore concentration with an electronic particle counter. Danmarks Geologiske Undersøgelse Ärbog 1972:87–89
15. Chambers FM, van Geel B, van der Linden M (2011) Considerations for the preparation of peat samples for palynology, and for the counting of pollen and non-pollen palynomorphs. Mires Peat 7:1–14
16. Sheridan RL, Ryan CM, Quinby WC Jr, Blair J, Tompkins RG, Burke JF (1995) Emergency management of major hydrofluoric acid exposures. Burns 21:62–64
17. Jackson ST (1999) Techniques for analyzing unconsolidated lake sediments. In: Jones TP, Rowe NP (eds) Fossil plants and spores: modern techniques. Geological Society, London, pp 274–278
18. Coil J, Korstanje MA, Archer S, Hastorf C (2003) Laboratory goals and considerations for multiple microfossil extraction in archaeology. J Archaeol Sci 30:991–1008
19. Stephen A, Anupama K, Orukaimani K, Prasad S (2008) A new microsieving technique in pollen analysis. Palaeobotanist 57:551–557
20. Fish S (1994) Archaeological palynology of gardens and fields. In: Miller NF, Gleason KL (eds) The archaeology of garden and field. University of Pennsylvania Press, Philadelphia, pp 44–69
21. Richardson F, Hall VA (1994) Pollen concentrate preparation from highly organic Holocene peat and lake deposits for AMS dating. Radiocarbon 36:407
22. Eriksson JA, Possnert G, Aldahan AA, Lindström H, Ocklind G (1996) An improved method for preparing a pollen concentrate suitable for ^{14}C-dating. Grana 35:47–50
23. Loader NJ, Hemming DL (2000) Preparation of pollen for stable carbon isotope analyses. Chem Geol 165:339–344
24. Nelson DM, Hu FS, Scholes DR, Joshi N, Pearson A (2008) Using SPIRAL (single pollen isotope ratio analysis) to estimate C_3- and C_4-grass abundance in the paleorecord. Earth Planet Sci Lett 269:11–16
25. Yeloff D, Hunt C (2005) Fluorescence microscopy of pollen and spores: a tool for investigating environmental change. Rev Palaeob Palynol 133:203–219
26. Chambers FM, Booth RK, De Vleeschouwer F, Lamentowicz M, Roux G L, Mauquoy D, Nichols JE, van Geel B (2012) Development and refinement of proxy-climate indicators from peats. Quat Int 268:21–33

Chapter 28
Phytoliths: Preparation and Archaeological Extraction

Brian Kooyman

28.1 Introduction

Phytoliths are mineral deposits, primarily amorphous silica, in and around plant cells. Their deposition in conjunction with cells results in taking on the shape of these cells due to being casts and/or molds of the cells. Phytoliths (literally "plant stones") are found primarily not only in leaves but also to an extent in stems, flowers, and seeds. Many phytolith forms (Fig. 28.1) are the same or similar between species, genera, and families, but there are also many instances where they are specific to one taxon or another. To the extent that phytolith forms can be associated with particular taxa, they can be used to identify plants used by people and/or present in the environment. A significant advantage of phytolith analysis in archaeology, ecology, and geography is that phytoliths are extremely resistant to destruction in nature (though much less so than the silica in quartz) and survive many thousands of years in sediments and soils [1–3].

Preparation of modern comparative material basically involves destruction of the complete organic portion of the plants processed, leaving only the phytoliths. This is accomplished either by burning plant samples in a muffle furnace or by using strong oxidizing agents to chemically digest the organic portions [1, 2, 4]. The two approaches produce comparable phytolith extracts and neither jeopardizes use of the material in analysis [1, 2, 5]. Combustion must be undertaken at temperatures below 600 °C or else warping and form change may result, but the temperatures must be above 450 °C else some organic tissues are not removed. An advantage of combustion is that it has been found to better retain some aspects of phytolith form compared with at least some forms of chemical digestion [6]. Combustion also produces higher frequencies of the aggregated phytoliths associated with agricultural irrigation; hence it is a better method for study of these contexts, although the

B. Kooyman (✉)
Department of Anthropology and Archaeology, University of Calgary, 2500 University Drive NW., Calgary, Alberta T2N 1N4, Canada
e-mail: bkooyman@ucalgary.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_28

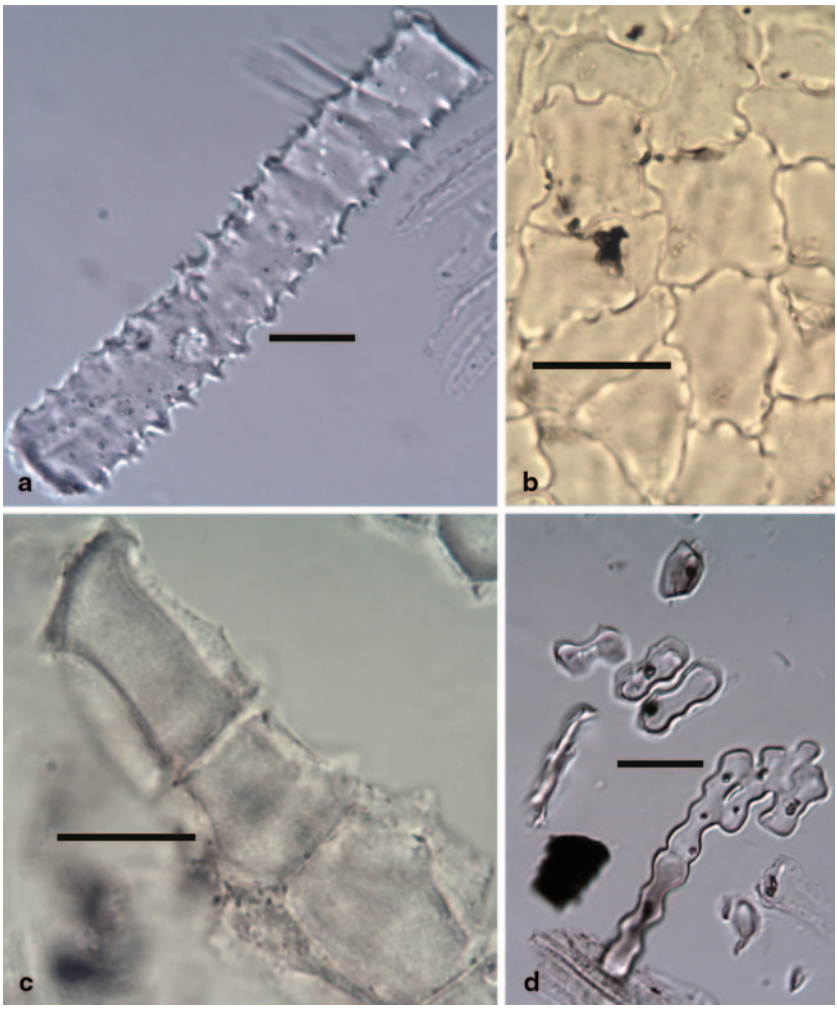

Fig. 28.1 A few examples of the many forms of phytoliths, including Baltic rush (*Juncus balticus*) (**a**), trembling aspen (*Populus tremuloides*) (**b**), white spruce (*Picea glauca*) (**c**), and green needle grass (*Nassella viridula*) (**d**). Scale bar = 20 μm

precise mechanism that produces these higher frequencies is not clear [6, 7]. Dry ashing (combustion) is the simpler and more widely used procedure, particularly as it is much safer for students and researchers with little background in chemistry and requires less specialized laboratory equipment. The basic procedure uses a muffle furnace to combust plant material in ceramic crucibles (or expedient aluminum foil boats [8]) at 500 °C for several hours, the resultant residue processed to remove some minerals such as calcium, and the phytoliths are then mounted on microscope slides for viewing. Wet ashing (chemical digestion) relies particularly on strong acids to digest organic material, similarly leaving a residue that is largely phytoliths.

Various reagents are used but the most common procedures use Schulze solution (concentrated nitric acid and potassium chlorate) as the main reagent.

Phytolith extraction from soils and sediments is generally accomplished using heavy density liquid flotation [1–3]. A variety of heavy density liquids have been used in the past, but recently this has usually been undertaken using sodium polytungstate (also called sodium metatungstate, $Na_6[H_2W_{12}O_{40}]$) because it is nontoxic, easy to use, and easily recycled for ongoing use. In sediment and soil processing various components of the matrix are removed by a series of processes so that the phytoliths can be floated from what remains in a final major step in the extraction. Phytoliths have a specific gravity of about 1.5–2.3, whereas the dominant mineral particles in soils have a specific gravity of about 2.6 [2], hence the specific gravity of the flotation liquid is adjusted to 2.3.

The sediment extraction process can be modified to separate and extract other plant remains as part of a single process, such as starch grains, generally by using two or more heavy liquid flotation steps using liquids of different specific gravities. Sediment extraction also extracts sponge spicules and diatom skeletons with the phytoliths due to their similar silica makeup and specific gravity [1, 2, 9]. Phytoliths, along with other plant remains such as starch granules and plant tissue fragments, can also be extracted from lithic and ceramic archaeological artifacts as well as from other contexts such as mammal tooth dental calculus [2, 9–11]. Of these other phytolith sources, only archaeological tool extraction procedures are discussed here.

Phytoliths are generally observed by mounting extracts on glass microscope slides and viewing at 200–630×, although scanning electron microscopy (SEM) has also been used to study their form and structure [2]. SEM is not practical for examination of the many phytoliths in archaeological sediment extractions. Differential interference contrast (DIC) microscopy does enhance visualization of form and has been used, with the caveat that as a transmitted light mechanism DIC is indicative of optical density and not texture and topography alone. Most work is done using liquid mounts so that individual phytoliths may be rotated for easy viewing of all aspects of three-dimensional form. Polarizing filters are useful particularly when viewing sediment extracts as phytoliths are composed of amorphous silica and hence extinguish under cross-polarization unlike most sediment grains, assisting in their rapid identification in scanning. Counting is done much as for palynology, and similarly a known quantity of *Lycopodium* spores, or glass, or plastic microspheres, are sometimes incorporated into the plant or sediment extraction procedure to allow absolute counts to be made. Each *Lycopodium* tablet used contains about 9600 spores (varies somewhat by batch); hence when added to a known weight of sediment or plant material the concentration of the spores acts as a standard for the phytoliths or other microbotanical remains under study. When adding microspheres or spores their specific gravity must be considered—for example, with a specific gravity of only about 1.2 [12], *Lycopodium* spores are better introduced after the last stage of sediment extraction (and certainly after the settling stage is completed in sediment extraction). Many palynologists also introduce spores or microspheres after the extraction process is complete [13–15]. Glass microspheres commonly

have a specific gravity of about 2 and hence act similarly to phytoliths in settling procedures, while polystyrene beads have a specific gravity of about 1 and so must be used only late in the sediment extraction process. Only glass beads will survive the ashing process used in extracting phytoliths from plant material.

28.2 Materials

28.2.1 Extraction from Modern Plants (Dry Ashing)

Equipment and laboratory supplies: muffle furnace, weigh scale, ultrasonic cleaner or Vortex mixer (optional), centrifuge, ceramic crucibles (or aluminum foil expedient "boats"), *Lycopodium* spore tablets or glass spheres (optional), disposable transfer pipettes, plastic centrifuge tubes (15 mL), hydrochloric acid 10 % (or 1 M) (270 mL distilled/deionized water, 30 mL concentrated HCl) (fill wash bottle), detergent, distilled or deionized water, liquid mounting medium (50/50 glycerol/water or similar), nail polish and disposable applicators (toothpicks or similar), slides, and coverslips.

28.2.2 Phytolith Extraction from Sediments

Equipment and laboratory supplies: hot water bath/hot plate (optional), ultrasonic cleaner or Vortex mixer (optional), 0.25 or 0.5 mm small geological sieve (or 4 mm tea strainer), 50 and 400 mL glass beakers, plastic transfer pipettes, 15 and/or 50 mL plastic centrifuge tubes, Kimwipes (or similar), toothbrush (compressed air optional), 30 % H_2O_2 (store in dark, opaque bottle; polyvinyl chloride (PVC) or polypropylene best), 0.1 % ethylenediaminetetraacetic acid (EDTA) (1 g Na_2H_2EDTA and mix to 1 L volume with distilled/deionized water) (fill wash bottle), 2.3 specific gravity sodium polytungstate (522 g in 200 mL distilled/deionized water) (best stored in nonglass container), glass funnel, 8 and 1 μm circular filter paper (VWR or similar supplier), and those listed in the section "Extraction from modern plants (dry ashing)."

28.2.3 Phytolith Extraction from Lithic Artifacts

Equipment and supplies required: ultrasonic cleaner, centrifuge, low-power microscope (10–40×), clean, single-use toothbrushes, plastic transfer pipettes (or pipette with disposable tips), 15 and/or 50 mL centrifuge tubes, plastic containers (centrifuge tubes, weigh boats, etc.), aluminum foil, 5 % ammonia (commercial) or ultrapure water, and supplies listed in the section "Extraction from modern plants (dry ashing)."

28.2.4 Phytolith Extraction from Carbonized Residues

Equipment and supplies required: centrifuge, weigh scale, orbital shaker or Vortex mixer (optional), homogenizer and low shear blades, drying oven, Vortex mixer, 50 mL centrifuge tubes, plastic transfer pipettes, 6% H_2O_2 (store in dark, opaque bottle; PVC or polypropylene best), sodium polytungstate with specific gravity of 1.3 (82 g in 200 mL distilled/deionized water) (best stored in nonglass container), 1.8 (for starch extraction) (244 g in 200 mL distilled/deionized water, *see Notes*) (best stored in nonglass container), and 2.3 (522 g in 200 mL distilled/deionized water) (stored in nonglass container), glass funnel, 8 and 1 μm circular filter paper, and supplies listed in the section "Extraction from modern plants (dry ashing)".

28.3 Methods

28.3.1 Extraction from Plant Material (Dry Ashing; see Note 1)

1. Soak plant material in water and detergent for 30 min, agitating, to remove surface dust and sediment. Best results are obtained by using an ultrasonic cleaner. Rinse thoroughly *(see Note 2)*.
2. Cut/break sample into small pieces and place in crucible. Use specific weighed amount if production quantification desired. Separate and process different plant portions (leaf, stem, etc.) individually if documenting phytoliths in different plant portions. Cover crucibles with crimped aluminum foil "cap" to prevent cross-contamination during ashing *(see Note 3)*.
3. Place samples in a room temperature muffle furnace and raise temperature to 500 °C; hold for several hours. In our laboratory we have found 7 h effective for all but highly lignified branch and stem material *(see Note 4)*. From room temperature, we ramp to 300 °C (3 °C/min for 100 min) and hold for 30 min, then ramp to 400 °C (3.3 °C/min for 30 min) and hold for 30 min, and then ramp to 500 °C in the final stage (3.3 °C/min for 30 min) when we hold the temperature for 7 h. Allow to cool.
4. Add a small amount of 10% (or 1 M) HCl to the crucible, to remove carbonates (if expedient foil ashing containers are used, transfer ash to a small beaker for the HCl treatment and then wash this into the centrifuge tube once HCl treatment is complete). If *Lycopodium* spores are to be added to obtain absolute phytolith production counts, they are added in this stage as they also require 10% HCl to dissociate the spores (if microspheres are used, they are likewise added here). Add only what is needed and stop once fizzing ceases (often there is little or no carbonate). Wash ash into a 15 mL centrifuge tube (or two tubes if more ash obtained) with distilled or deionized water and centrifuge at approximately 3000 rev/min

for 5 min. Decant and discard supernatant (pipette off if precipitate is loose). Top up with distilled/deionized water, centrifuge at 3000 rev/min, and decant twice more to ensure HCl has been removed. Allow to dry (may be directly mounted wet after centrifuging if mixed with pure glycerol as the mounting medium). Wash crucibles thoroughly to avoid cross-contamination in subsequent extractions (scrubbing and dilution via plentiful use of water is critical).

5. Liquid mount (50:50 glycerol/water or similar) *(see Note 5)* on microscope slide for viewing (use all or a weighed portion if quantifying phytolith production). Seal slide (nail polish or similar) so that phytoliths can be rotated in the mount. Extract should be thinly distributed over the area encompassed by the coverslip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best phytolith viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the coverslip. When sealing slide, to ensure no transfer of phytoliths from one sample to another, use disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard and use new applicator).

28.3.2 Extraction from Sediments

1. Sieve sediment sample to remove larger extraneous material such as roots. Small 0.25 mm or 0.5 mm geological sieve works well (latter if working at an agricultural site where larger, aggregate phytoliths may be present; good quality tea strainer (approximately 0.4 mm) can be used). Sieve onto tared paper or into 50 mL (tared) glass beaker. Sieved sample of about 2–3 g good, perhaps twice that if sediments are sandy and may contain fewer phytoliths per sediment weight (normally use same weight for each sample to ensure comparable sampling; 3 g has proven generally effective in our laboratory). Clean the sieve between samples to control cross-contamination *(see Note 6)*.
2. If the samples are especially high in organic content, particularly charcoal, treat with 30% hydrogen peroxide (H_2O_2), warming at 70 °C to improve and speed the digestion: add sample to a 50 mL beaker and add enough H_2O_2 to cover sample; top up as needed until organic matter is removed. Wash each sample into 15 or 50 mL centrifuge tube (depending on amount of H_2O_2 used), centrifuge (3000 rev/min for 5 min), and decant residual H_2O_2. Rinse and centrifuge precipitate twice in the same centrifuge tube *(see Note 7)*.
3. Pour the sieved sediment into a 50 mL beakers (if not already in them) and add 10% HCl to remove carbonates; add a small amount at first and stop when fizzing ceases (high carbonate sediments may take several HCl additions and several hours to complete this step). Once carbonates are removed by acid, wash the sediment (with distilled/deionized water), add HCl into 15 mL centrifuge tube, and centrifuge at about 3000 rev/min for 5 min to precipitate the sediment.

Decant and discard supernatant (pipette off if precipitate is loose), washing and centrifuging precipitate twice more with water to remove HCl. In our laboratory if we already know sediments have little or no carbonate, we often put the sieved sediment directly into the centrifuge tubes to minimize steps and loss of sediment.

4. Put 10 mL of 0.1% EDTA into each centrifuge tube and agitate well for about 5 min to disperse clays; use Vortex mixer or shake by hand about every 30–60 s. Pour the sediment and EDTA from centrifuge tube into 400 mL beaker that has had a line written on the side at 8 cm above the beaker base. Wash (distilled/deionized water) any sediment adhering to the centrifuge into the beaker, then top up to the 8 cm line. Stir vigorously and then allow to stand for 1 h, this time allowing all particles > 5 μm in size to settle. Clay particles, in particular, remain in suspension and these are removed by suctioning the supernatant from the beaker (to as close as feasible to the precipitate without losing any precipitate). This settling is repeated with distilled/deionized water a number of times (generally another 4–7) until the supernatant is essentially clear, indicating the clays have been removed *(see Note 8)*. Dry the final precipitate in the beaker (depending on how completely the final supernatant removal is, this may require a few days).
5. Mix the precipitate thoroughly in the beaker, breaking up any aggregates. Put 3–5 mL of sodium polytungstate with specific gravity of 2.3 (*see Note 9* for making up, storing, and recycling sodium polytungstate solutions) into a 15 mL centrifuge tube, to give a 2–3 cm fall distance, and add all (for quantitative work) or a portion of the beaker precipitate to the tube and shake well. Centrifuge at 3000 rev/min for 5 min. Sand and other dense material will precipitate out but phytoliths and other less dense material will remain in suspension. Pour off supernatant into new centrifuge tube (pipette off if precipitate is loose) and dilute with distilled/deionized water. Centrifuge (3000 rev/min for 5 min) this diluted solution and the phytoliths will precipitate out. Pour supernatant through 8 μm filter paper into flask to recycle sodium polytungstate (filter this a second time later, with 1 μm filter paper, to ensure all contaminants removed prior to reuse). Rinse and centrifuge (3000 rev/min for 5 min) precipitate twice in the same centrifuge tube to ensure that all sodium polytungstate is removed (these rinses may also be recycled, but there is little sodium polytungstate in them and it recovers little chemical). If *Lycopodium* spores or microspheres are to be added to obtain absolute phytolith productions counts (a defined amount of spores/microspheres added to the extraction results from a weighed amount of sediment), they are added in this stage (they have a specific gravity of about 1–2 and so will be suspended in the sodium polytungstate with the phytoliths). However, *Lycopodium* spore tablets also require 10% HCl to dissociate the spores, so that must also be added here; if microspheres are used, they are likewise added here and there is no need to add HCl.
6. Liquid mount (50:50 glycerol/water or similar) on microscope slide for viewing. Seal slide (nail polish or similar) so that phytoliths can be rotated in the mount. The specimen may be directly mounted wet, after centrifuging, if mixed with pure glycerol as the mounting medium. Extract should be thinly distributed over

the area encompassed by the coverslip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best phytolith viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the coverslip. When sealing slide, to ensure no transfer of phytoliths from one sample to another, use disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard and use new applicator).

28.3.3 Phytolith Extraction from Lithic Artifacts

1. Using a different clean *(see Note 10)* toothbrush for each artifact, gently dry brush surface sediment from artifact onto a plastic weigh boat, piece of aluminum foil, or comparable container. Store for future reference as needed (in our laboratory we often transfer this into a 15 mL centrifuge tube so that it can be easily sealed, labeled "dry brush", and stored).
2. Rinse artifact with distilled/deionized water by gently using the same toothbrush that is used to dry brush the artifact, to remove surface sediment as needed *(see Note 11)*. Rinse into a weigh boat or similar container, then transfer to a labeled "water rinse" centrifuge tube or similar sealable container for longer term storage, and subsequent examination if needed.
3. Optionally, based on surface examination macroscopically and/or with a low power microscope, undertake spot extractions of visible residues or likely residue traps such as cracks in the surface, vesicles or similar pores, and in deep flake scar corners or margins. It is common to omit this procedure and go directly to the next step. Use separate clean disposable pipettes or pipette tips for each artifact and transfer one to a few drops of distilled/deionized water, or ultrapure water, to the area *(see Note 12)*. The pipette tip may be used to agitate and loosen the residue in the extraction process, and the water also can be squirted into and out of the location a number of times in order to improve extraction. Pipette the water off the tool and onto a microscope slide and wet mount as in other extraction procedures *(see Note 13)*.
4. Place the tool in a new clean container and submerse all or a portion of the tool in distilled/deionized water, ultrapure water, or 5% ammonia (5% ammonia is most effective). Float the container with its tool in an ultrasonic bath for 1–45 min (30–45 min most effective). For smaller tools and full tool extraction, our laboratory generally uses 15 or 50 mL centrifuge tubes with the tops screwed on (in such cases we usually undertake some of the spot extractions as previously outlined in Step 3 to look at various potentially used areas) *(see Note 14)*. Transfer the extraction liquid to centrifuge tubes (if not already in them) and centrifuge at 3000 rev/min for 5 min; pipette or decant the liquid and discard it. If multiple centrifuge tubes are used for the liquid from a single artifact, use

distilled/deionized water to wash all the residue pellets into a single centrifuge tube and centrifuge at 3000 rev/min for 5 min, again discarding the water supernatant. If ammonia is used in the extraction, wash residue pellets twice with distilled/deionized water, centrifuging at 3000 rev/min for 5 min, to remove the water and remnant ammonia.
5. Wet mount a portion of the extract as outlined previously. This extraction procedure obtains all residues—starch granules, phytoliths, plant tissue fragments, hair fragments, and feather fragments—as a single extract. It is the first three residues that are most common.
6. Allow artifact to dry, rinsing first in water if ammonia was used in the extraction.

28.3.4 Phytolith Extraction from Carbonized Residues (Usually from Ceramic Sherds)

1. Carefully rinse (and dry brush as needed) sherds with adhering carbonized residue with deionized/distilled water to remove surface loose sediment (as in steps 1 and 2 in the section "Phytolith extraction from lithic artifacts," although commonly dry brushing is omitted as it can remove charred residue and so lose part of the sample). Remove carbonized residues with a clean stainless steel dental pick, dissecting needle, or scalpel (clean pick/needle/blade by flaming in Bunsen burner, by ultrasonic cleaner treatment using 5% ammonia, or by boiling in vinegar in a pressure cooker; *see Note 10*).
2. Weigh 0.1 g (more or less as sample dictates) of carbonized residue and place in a 50 mL plastic centrifuge tube with 5 mL of 6% H_2O_2. If desired, at this stage the sample may be homogenized with clean, low shear blades to enhance disaggregation of the carbonized material *(see Note 15)*. Place centrifuge tubes on orbital shaker on low (ca. 150 rev/min) for 10 min (or *see Note 16*).
3. Top up tubes to 50 mL mark with distilled/deionized water and centrifuge for 5 min at 3000 rev/min. Pipette off and discard the supernatant without disturbing the precipitate pellet. Repeat two more times (if there is a small amount of clay from archaeological sediment in the sample, the washing process usually removes this as well as the H_2O_2). Dry samples in drying oven set no higher than 40 °C (so that the starch is not gelatinized) or dry in fume hood with centrifuge tubes loosely covered with their lids or aluminum foil "caps" *(see Note 17)*. One may omit Steps 4–6 and proceed directly to Step 7 if it is acceptable to extract all potential residues together to mount on the same slide.
4. Add 5 mL of sodium polytungstate prepared to a specific gravity of 1.8 in the centrifuge tubes, vortex to mix well, and centrifuge at 2000 rev/min for 10 min (5 min at 3000 rev/min also works well). Pipette the supernatant, which contains the starch granules (starch granules have a specific gravity of ca. 1.5), into a new centrifuge tube labeled "starch extract". Repeat this step, again putting 5 mL of sodium polytungstate of 1.8 specific gravity into the original centrifuge tubes, vortex, centrifuge, and pipette the supernatant into the "starch extract"

tubes. This ensures all disaggregated starch has been extracted from the sample. Fill the "starch extract" tubes with distilled/deionized water, mix, and centrifuge at 3000 rev/min for 10 min. Pipette off and discard (recycle) about 15 mL of the supernatant, without disturbing the residue at the bottom of the centrifuge tube (pour supernatant through 8 μm filter paper into flask to recycle sodium polytungstate, filtering again later with 1 μm filter paper to ensure all contaminants have been removed prior to reuse). Top up the centrifuge tube again with distilled/deionized water, mix, again centrifuge at 3000 rev/min for 5 min, and this time pipette off (and recycle) about 20 mL of the supernatant. Top up with distilled/deionized water a last time, mix, centrifuge at 3000 rev/min for 5 min, and pipette off as much of the supernatant as possible without disturbing the pellet at the bottom of the centrifuge tube (this stepped procedure ensures that no starch granules are lost because of being suspended in the somewhat more dense supernatant near the residue pellet in the initial dilution steps) *(see Note 18)*.

5. Liquid mount (50:50 glycerol/water or similar) on microscope slide for viewing. Transfer some of the wet sample to a microscope slide, add mounting medium, cover with cover slip, and seal slide (nail polish or similar) so starch granules can be rotated in the mount. Extract should be thinly distributed over the area encompassed by the cover slip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best granule viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the cover slip. When sealing slide, to ensure no transfer of starch granules from one sample to another, use disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard and use new applicator).
6. Retain the original centrifuge tubes and label "phytolith extract" as this residue, with a specific gravity >1.8, contains any phytoliths in the sample. To remove any residual sodium polytungstate in this residue, top up the tubes with distilled/deionized water, vortex to mix, centrifuge at 3000 rev/min for 5 min, discard the supernatant, and dry the samples in a fume hood or drying oven for later processing.
7. To extract the phytoliths from the remaining residue, add 5 mL of sodium polytungstate, with specific gravity of 2.3, to the "phytolith extract" centrifuge tubes. Mix well and centrifuge at 3000 rev/min for 5 min. Pipette the supernatant, containing the phytoliths, into new centrifuge tubes and top up these new tubes with distilled/deionized water. Vortex to mix, centrifuge at 3000 rev/min for 5 min, and pipette the supernatant off to recycle the sodium polytungstate. Repeat the rinse with distilled/deionized water and centrifuge twice to remove all sodium polytungstate. The phytoliths will be in the residue pellet in the centrifuge tube.
8. Liquid mount the phytoliths as for starch granules (Step 5 above)

28.4 Notes

1. In the past most comparative collections were produced by a chemical, wet ashing technique to remove the organic portion of the plant specimens [e.g., 1, 2, 4]. Small plant samples (usually <0.2 g) were washed as in the dry ashing technique, then dried, and put in 50 mL glass centrifuge tubes or similar containers. The sample tubes were half-filled with Schulze solution (75 mL concentrated HNO_3, 25 mL distilled/deionized water, 2 g $KClO_3$) and heated in a hot water bath in a fume hood. The tubes were stirred to maintain the reaction and when the reaction ceased, a small amount of potassium chlorate ($KClO_3$) was added to initiate the reaction again, repeating this step until the reaction ceased completely. The samples were then cooled (1–2 h), centrifuged to remove the Schultz solution, concentrated nitric acid (HNO_3) was added to dissolve any remaining potassium chlorate, was centrifuged and rinsed with distilled/deionized water to remove the acid, 10% HCl was added to remove any calcium present, and then this too was washed and centrifuged out. The samples were dried and mounted. The dry ashing technique is much simpler and safer, and the resulting phytoliths are identical. As a result, few researchers currently use the wet ashing technique.
2. It is best to obtain samples from at least two different localities to be confident about variation in form. Plants collected should be rinsed in the field when collected and washed again in the laboratory (with detergent and/or a sonic bath). Wind-blown sediment particles make preparations more difficult to scan and large grains impede proper placing of the cover slip (large grains can usually be removed mechanically with a dissecting needle or tooth pick prior to covering with a coverslip).
3. In place of crucibles, boat-like containers of aluminum foil can be quickly made and the foil can be bent over and crimped to enclose the sample. This procedure eliminates the risk of cross-contamination from using rewashed crucibles. It is somewhat easier to remove the ashed sample from crucibles compared with the expedient foil "boats."
4. Some papers suggest holding the muffle furnace temperature at 500 °C for up to 3–4 days, but we have not found this necessary. Rapid temperature increase does not impact phytolith form, but some laboratories increase the temperature in stages as a precaution. We do this in our laboratory and it is this procedure that is outlined.
5. Phytoliths have a refractive index of 1.42 and so the mounting medium needs to have a sufficiently different refractive index that good contrast is provided. The 50:50 glycerol/water mounts that we commonly use have a refractive index of 1.397, provide good contrast, and pure glycerol (RI = 1.475) also works. Microscope lens immersion oils come in various refractive index values and we have used these extensively for phytolith mounting. Liquid mounts must be kept upright, to maintain the even distribution of the mounted material over the slide area, and are prone to leaking over time. They are needed, however, to be able to fully view and photograph all aspects of individual phytoliths. Due to the issue

of possible leakage during long-term storage, we prepare additional solid mount slides of the extracts as "voucher specimens."

6. The weigh paper used can be cutup pieces of clean smooth glossy magazine or poster paper, the smoothness ensuring that sediment is not lost when pouring. Sieving sediment results in a fairly wide scattering and so it is best if a small diameter sieve is used and sieving is onto a wider piece of paper that can be creased for pouring into a container such as a small, 50 mL beaker. The bowl shape of a tea strainer helps narrow the scattering, although we usually use geological sieves in our laboratory. Sieve cleaning minimally should include wiping out fine particulates (Kimwipes or similar) and dry brushing if particles are stuck in the sieve mesh (brushing one way, then diagonally to that, then at 90° to the first brushing). Normally our cleaning is dry brushing, then washing with running deionized or distilled water, then air-drying using the compressed air taps in the laboratory (sieve must be dry for each sample).
7. Alternatively, organic matter can be removed by burning the sample in a muffle furnace, although this can oxidize iron in the sediments and hence can create other issues. Chemical oxidation of samples, such as with Schulz solution, can also be used. Overall the use of H_2O_2 is simplest and very effective. If processing hearth or oven samples, much charcoal can be removed by an initial flotation of the sample and/or using a finer initial sieve. If floating charcoal from the sample, it is important to ensure that the sample is allowed to settle (for 1 h, or less if only the very upper portion of the water column—with floating charcoal—is removed by careful suction) and that only the upper portion of the water column is removed, so that none of the phytoliths are lost (some charcoal may sink during this time period). The specific gravity of charcoal varies significantly—from about 0.1 to 1.2 with an average of about 0.4—based on the original wood, the firing temperature, and the inclusion of material such as ash.
8. Alternatively, sediment may be agitated on an orbital shaker for 2 h on low to disperse clays. Commercial Calgon (50 gm/L) can be used in place of EDTA but often contains starch and so introduces contamination, although if phytoliths alone are being examined this contamination is inconsequential. If used, 25 mL is added in place of the EDTA.
9. One of the advantages of sodium polytungstate is that it is relatively easy to recycle. During extraction, we pour our used sodium polytungstate through 8 μm filter paper into an Erlenmeyer flask to remove most material still in suspension (this filters out most material and progresses sufficiently quickly that we can process multiple centrifuge tubes without the funnel and filter paper filling with liquid). Later we refilter this solution through 1 μm filter paper to remove very fine contaminants. We allow the twice filtered solution to evaporate (covering the flask opening loosely with aluminum foil, not using any heat) until it is again dense as seen in its golden color. It is best if all water does not evaporate leaving a precipitate, as this is more difficult to bring into solution than the original laboratory reagent powder. To ascertain if the evaporation has sufficiently concentrated the solution, we pipette 5 mL of solution into a small tared glass beaker and weigh it. Five mL of solution must weigh 11.5 g for a specific gravity of 2.3

(9.0 g for a specific gravity of 1.8, used to extract starch, and 6.5 g for a specific gravity of 1.3). Once the solution is known to be more dense than required, a small amount of distilled/deionized water is added and 5 mL are again weighed; this procedure is repeated until the correct weight is obtained, indicating the solution has reached the required weigh, and hence specific gravity (if the process again makes the solution less than the required density, it may be adjusted by adding a small amount of sodium polytungstate powder or by allowing partial evaporation of the solution again). If making solutions from powdered sodium polytungstate, approximately 522 g dissolved in 200 mL of water gives a solution of specific gravity 2.3; approximately 244 g dissolved in 200 mL distilled/deionized water gives a solution of specific gravity 1.8; approximately 82 g dissolved in 200 mL of water gives a solution of specific gravity 1.3 (the maximum attainable solution specific gravity for sodium polytungstate at room temperature is 3.1). Sodium polytungstate dissolves most readily if the powder is gradually added to the water. If calcium ions are present in the sediment floated, sodium polytungstate may form an insoluble compound with the calcium. Also note that it is best to store solution in plastic as it can also remove calcium from glass containers and again result in precipitation of an insoluble compound (in practice, we have not had this problem with glass storage containers but have sometimes had the issue in sediment extractions; pretreatment with dilute HCl usually eliminates this problem in phytolith extraction from sediments, but this cannot be used with starch granule extraction as HCl damages starch).

10. The toothbrushes used may be new, used once and then discarded after use, or may be cleaned in one of a number of manners and reused. Tool residues of all kinds are rare and the potential of losing this information because of contamination with fibers, hairs, starch granules, and other material from field and lab processing is a major concern. Circulated building air, laboratory supplies, and equipment are often contaminated by starch which is of particular concern during extraction of archaeological tool residues. In general, laboratory surfaces should be cleaned (e.g., with commercial ammonia) before each sampling, and sampling should be done on a disposable surface such as laboratory mats or stretch wrap that is changed between samples. Supplies, such as microscope slides, and reused equipment, such as forceps, should be thoroughly cleaned (even the glue from boxes containing supplies such as slides often contains starch). In our laboratory we use commercial ammonia, fill plastic containers with it and the supplies, and place the filled containers in an ultrasonic bath for 45 min to dislodge and remove starch and other contaminants; the ammonia is rinsed from the equipment after with distilled/deionized water. Boiling in vinegar (5% acetic acid) in a pressure cooker (and then rinsing in distilled/deionized water) for a minimum of 30 min can also be used, which gelatinizes contaminate starch and should remove other contaminants in the discarded liquid. Single-use gloves (or thoroughly washed hands) should be used to handle artifacts. Many powder-free gloves are contaminated with starch and each new lot must be tested to ensure the gloves are truly starch-free. As well, gloves

should generally be put on and then rinsed under distilled or deionized water to ensure surface starch is removed. We always wipe the surfaces of boxes and bags containing supplies with damp clothes before bringing them into the work space. If feasible, tool residue extraction should be undertaken in a dedicated space, if possible a space with HEPA filtration of the air and a slight positive pressure gradient to minimize the inflow of unfiltered air. We also wear disposable lab suits to minimize the introduction of contaminants such as hair, threads, and starch into the tool residue extracts.

11. A balance is needed between removing surface contamination and accidentally removing the ancient tool residues, hence dry brushing should not be too vigorous.
12. Ultrapure water is better than distilled/deionized water due to its greater ability to remove residues. Ammonia (5 %) can also be used, but cannot be mixed with many slide mounting media and so is not as good a choice for direct mounting of extracts as done in this procedure. If ammonia is used, it needs to be rinsed from the extracted residue before mounting, but this is difficult to do without losing the sample; so use of a microcentrifuge best accomplishes this, rinsing 2–3 times with distilled/deionized water, each time centrifuging for a few minutes and discarding the supernatant.
13. This stage, if included, is sometimes done before Step 2, if the Step 2 washing is vigorous [6]. More generally, Step 3 is done after washing to minimize the possibility of surface contamination of the extract.
14. For larger, heavy artifacts that may cause containers to sink to the bottom of the ultrasonic bath, we use larger plastic containers and rest the container on a small block (e.g., a piece of brick) in the ultrasonic bath, allowing as much of the container bottom surface as possible to be in contact with the bath water. For artifacts that are too large to fit in the ultrasonic bath, such as anvils and grinding stones, we used a plastic transfer pipette to squirt ammonia into and off a location on a surface for a period of 1–3 min, adding more liquid to replace that which is absorbed during the procedure if the rock is porous. We might do this on more than one location, depending on the size of the artifact. We can also use this procedure for rock types that may be too friable or fragile for ultrasonic treatment.
15. The carbonized residue is an aggregate that has phytoliths trapped inside it. The use of H_2O_2 gently oxidizes the matrix and breaks the aggregate apart. The use of a homogenizer also fragments the consolidated mass. Both of these actions release the embedded phytoliths and other trapped plant remains. This release of embedded remains can be successfully accomplished with the use of H_2O_2 alone if a homogenizer is not available but may be most complete with the use of a homogenizer.
16. There are alternative agitation methods that also work well to disperse the sediment particles if the laboratory does not have an orbital shaker. The sample may be agitated well for about 5 min using a Vortex mixer. It may also be shaken by hand about every 30–60 s over a period of 5 min. It is common in geology and geography laboratories to use Calgon as the dispersing agent; commercial Calgon often has starch in it and so it is best to use EDTA unless it is certain the Calgon contains no starch.

17. If the sample with H_2O_2 is still reacting after the initial water is placed into the centrifuge tube, due to high carbonate content, split the sample into 2 tubes, top up both with water, centrifuge, then recombine the split samples into one tube. Then do the 2 water rinses.
18. To make a somewhat cleaner extract preparation by removing extraneous less dense material, Step 4 may be preceded by a treatment using sodium polytungstate prepared to a specific gravity of 1.3. Add 8 mL of the sodium polytungstate with specific gravity of 1.3 to the sample in the centrifuge tube, mix well, centrifuge at 2500 rev/min for 12 min, and pipette off and discard (recycle) the supernatant without disturbing the residue pellet.

28.5 Interpretation and Conclusion

Reconstruction of paleoenvironments and ancient diet with phytoliths is based on relative and/or absolute frequencies based on counts of 100–500 phytoliths, in a similar manner to pollen grains and largely based on palynological methodology [2, 16]. Relative frequencies are based on simple counts, but absolute counts, often expressed as frequency per cc of sediment or residue, are determined by comparison to counts of foreign material (*Lycopodium* spores or plastic or glass microspheres) added to the extract in known quantities [1, 13–15, 17]. Most phytoliths are composed of amorphous silica and hence under cross-polarized light they extinguish; this is often useful in quickly distinguishing sediment grains from phytoliths in doing slide counts. Phytoliths also generally have a somewhat pink to purple-gray color in slide mounts, again often causing them to be readily apparent in the mix of material present on extraction slides.

Many of the interpretation issues in phytolith analysis are similar to those encountered in using starch granules and pollen grains. There is much redundancy in the form from one species to another, causing problems for species identification. Although there are patterns to form within larger taxonomic groups such as families [e.g., 18, 19], there are also inconsistencies which complicate identifications [e.g., 16]. It is clear that there are differences in phytolith production between species and higher taxa, as for example that grasses are generally prolific producers, but much work remains to be done in this area [e.g., 18, 19]. Differential survival is also certainly an issue, as seen for example in the much better preservation of spheres in tropical sediments, but again this has been little studied. The small size of phytoliths means that they may be displaced lower in sediments by being transported through drying cracks and pores in the soil or sediment, as is also the case with all microbotanical remains [20–22]. Comparing quantification with frequency interpretations derived from other botanical remains, such as pollen and seeds, is also a problem, but that is an issue for most paleobotanical samples and data [1].

Phytoliths are predominantly formed in leaves. Although this is not an issue for paleoenvironmental reconstructions, leaves are rarely the food portion of the plant in human diets and so phytoliths often do not provide direct information on the

portion of plants that human consumed or used for other purposes such as tools. On the other hand, other portions of some plants do produce phytoliths and these are often distinctive of the species and the portion (e.g., flower/seed glumes) [e.g., 23]. As phytoliths are essentially molds or casts of plant cells, it is often possible to specifically define which plant tissues are represented by phytoliths (e.g., epidermal tissue) (Fig. 28.2) [e.g., 16] and this can be useful in interpretation. On the other hand, cell form is often distorted by packing (Fig. 28.3) and crowding in structures and hence the associated phytoliths may have an incredible amount of natural minor

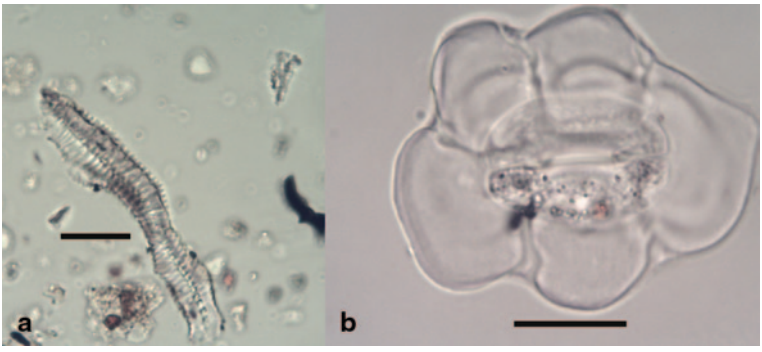

Fig. 28.2 **a** Phytoliths having the form of vascular tissue (snowberry, *Symphoricarpus occidentalis*). **b** A stoma (harebell, *Campanula rotundifolia*). Scale bars = 20 μm

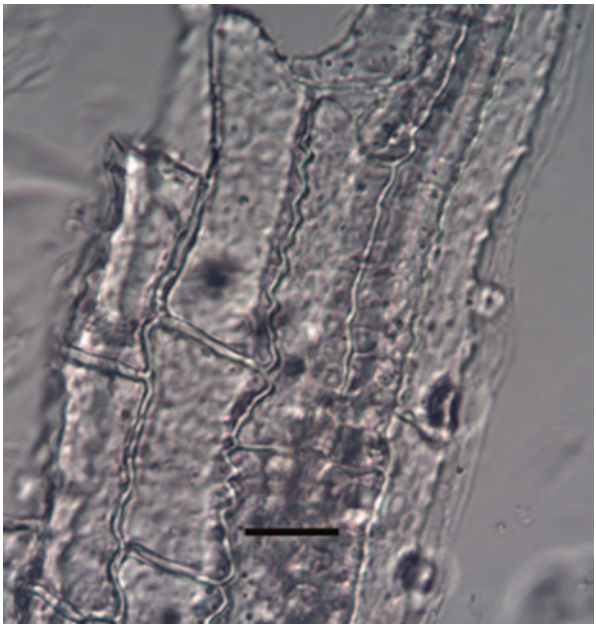

Fig. 28.3 Minor form variation in blue grama grass (*Bouteloua gracilis*) phytoliths resulting from epidermal cell crowding. Scale bar = 20 μm

variation that has little taxonomic relevance, confusing attempts to find and use distinctive forms in analysis.

Acknowledgments I have benefitted from discussions with a number of individuals over the years, but I particularly wish to thank Calla McNamee, Gerald Newlands, and Arlene Rosen.

References

1. Pearsall DM (2000) Paleoethnobotany: a handbook of procedures, 2nd edn. Academic, San Diego
2. Piperno DR (2006) Phytoliths: a comprehensive guide for archaeologists and paleoecologists. AltaMira Press, Oxford
3. Weiner S (2010) Microarchaeology: beyond the visible archaeological record. Cambridge University Press, Cambridge
4. Elbaum R, Weiner S, Alberta RM, Elbaum M (2003) Detection of burning of plant materials in the archaeological record by changes in the refractive indices of siliceous phytoliths. J Arch Sci 30:217–226
5. Parr JF, Lentfer CJ, Boyd WE (2001) A comparative analysis of wet and dry ashing techniques for the extraction of phytoliths from plant material. J Arch Sci 28:875–886
6. Wang X, Jiang H, Shang X, Wang T, Wu Y, Zhang P, Wang W, Wang C (2014) Comparison of dry ashing and wet oxidation methods for recovering articulated husk phytoliths of foxtail millet and common millet from archaeological soil. J Arch Sci 45:234–239
7. Jenkins E (2009) Phytolith taxonomy: a comparison of dry ashing and acid extraction on the breakdown of conjoined phytoliths formed in *Triticum durum*. J Arch Sci 36:2402–2407
8. Kondo R, Childs C, Atkinson I (1994) Opal phytoliths of New Zealand. Manaaki Whenua Press, Lincoln
9. Pearsall DM, Chandler-Ezell K, Zeidler JA (2004) Maize in ancient Ecuador: results of residue analysis of stone tools from the Real Alto site. J Arch Sci 31:423–442
10. Zarrillo S, Kooyman B (2006) Evidence for berry and maize processing on the Canadian plains from starch grain analysis. Amer Antiquity 71:473–499
11. Zarrillo S, Pearsall DM, Raymond JS, Tisdale MA, Quon DJ (2008) Directly dated starch residues document early formative maize (*Zea mays* L.) in tropical Ecuador. Proc Nat Acad Sci (USA) 105:5006–5011
12. Gregory PH (1961) The microbiology of the atmosphere. Leonard Hill Books, London
13. Bonny AP (1972) A method for determining absolute pollen frequencies in lake sediments. New Phytol 71:393–405
14. Craig AT (1972) Pollen influx to laminated sediments: a pollen diagram from northeastern Minnesota. Ecology 53:46–57
15. Peck RM (1974) A comparison of four absolute pollen preparation techniques. New Phytol 73:567–587
16. Caenelli AL, Theurillat J-P, Madella M (2004) Phytolith types and type-frequencies in subalpine–alpine plant species of the European Alps. Rev Palaeobot Palynology 129:39–65
17. Battarbee RW, Kneen MJ (1982) The use of electronically counted microspheres in absolute diatom analysis. Limnol Oceanogr 27:184–188
18. Wallis L (2003) An overview of leaf phytolith production patterns in selected northwest Australian flora. Rev Palaeobot Palynol 125:201–248
19. Morris LR, Baker FA, Morris C, Ryel RJ (2009) Phytolith types and type-frequencies in native and introduced species of the sagebrush steppe and pinyon-juniper woodlands of the Great Basin, USA. Rev Palaeobot Palynol 157:339–357

20. Horrocks M, Lawlor I (2006) Plant microfossil analysis of soils from Polynesian stonefields in South Auckland, New Zealand. J Archaeol Sci 33:200–217
21. Fishkis O, Ingwersen J, Streck T (2009) Phytolith transport in sandy sediment: experiments and modeling. Geoderma 151:168–178
22. Fishkis O, Ingwersen J, Lamers M, Denysenko D, Streck T (2010) Phytolith transport in soil: a field study using fluorescent labelling. Geoderma 157:27–36
23. Eichhorn B, Neumann K, Garnier A (2010) Seed phytoliths in West African Commelinaceae and their potential for palaeoecological studies. Palaeogeogr Palaeoclimatol Palaeoecol 298:300–310

Chapter 29
Starch Granules: Preparation and Archaeological Extraction

Brian Kooyman

29.1 Introduction

Starch granules (also referred to as grains) are produced as stored food in most plants and are basically found in roots, underground storage organs, and in seed endosperm and cotyledons [1]. It is the large storage starch granules (Fig. 29.1) that are the focus of archaeological study, particularly as they are directly indicative of the food value of plants used by ancient people. The granules are large, with many useful characteristics that allow identification to the level of family, genus, or species (Fig. 29.2). Transitory starch granules are also produced by plants but are generally small, undiagnostic of taxa, and little studied, although some small granules have been very useful (e.g., [2]). Many taxa produce stored food in the form of oils and non-starch complex carbohydrates, hence do not produce starch granules and are not amenable to study in this manner. Most comparative sample extraction techniques use simple mechanical means to remove starch granules from cut or macerated storage structure surfaces, whether seeds or root-like organs [3].

Starch is generally poorly preserved in sediments as it is quickly consumed by soil organisms. Starch is sometimes found in archaeological sediments where plant processing has occurred in the past, and starch granules are obtained from such sediments by heavy density flotation techniques similar to those used for phytolith extraction ([2, 4], also see Chap. 28). The most common location for preservation of archaeological starch is in the residues on stone tools and ceramic vessels [4–7], the latter particularly from the burned residues on vessel interiors [8]. Starch granules can be extracted along with phytoliths from both sediments and archaeological tool residues as part of a singled, staged extraction process [4, 6, 8, 9]. Other plant remains, such as tissue fragments, may also be so obtained (e.g., [10, 11]). Tool

B. Kooyman (✉)
Department of Anthropology and Archaeology, University of Calgary, 2500 University Drive NW., Calgary, Alberta T2N 1N4, Canada
e-mail: bkooyman@ucalgary.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_29

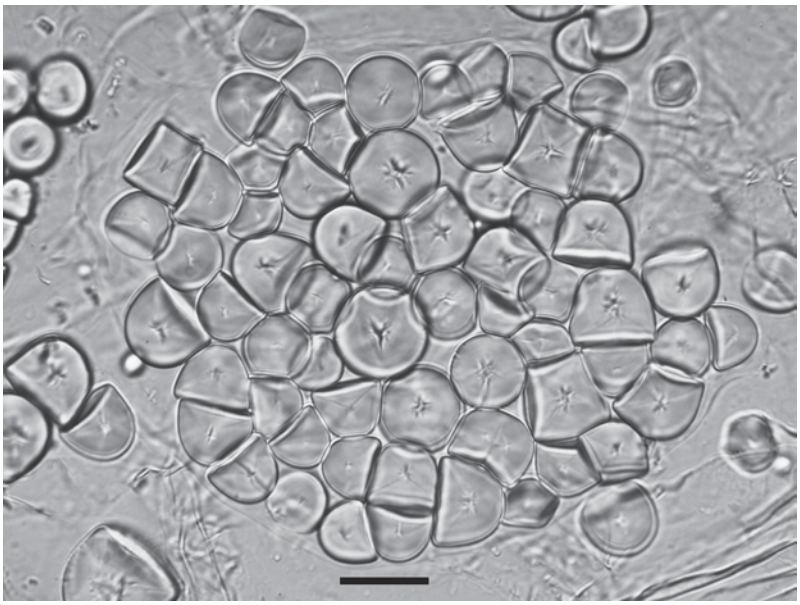

Fig. 29.1 Bitterroot (*Lewisia rediviva*) starch granules showing a variety of forms and features used in starch granule description. Scale bar = 20 μm

residues are commonly obtained by spot extractions from isolated high potential areas, and more generally with the use of an ultrasonic cleaner.

Archaeological tools commonly have archaeological sediment adhering to them, the result of burial in a site rather than tool use. These surface sediments are generally removed by dry brushing and rinsing with distilled/deionized water (e.g., [6]), the assumption being that ancient residues adhere strongly to the artifacts and so only recent "contamination" is removed by these procedures. Nonetheless, these initial extracts are kept for further examination where this is deemed relevant. Special procedures are required for extraction of starch grains from carbonized food residues [8]. In most instances, all tool residues contain very little plant material, and so strict protocols are employed to minimize the chance for modern contamination during handling and processing in the field and laboratory (e.g., [5, 8, 12]). These laboratory and handling protocols are only broadly discussed here, as aspects of the actual laboratory extraction procedures.

Starch granules are generally observed by mounting extracts on glass microscope slides and viewing at 200-630X, although SEM microscopy has also been used to study form and structure [13]. A scanning electron microscope (SEM) is not generally practical for examination of the many starch granules obtained in archaeological extractions. Differential interference contrast (DIC) microscopy does enhance

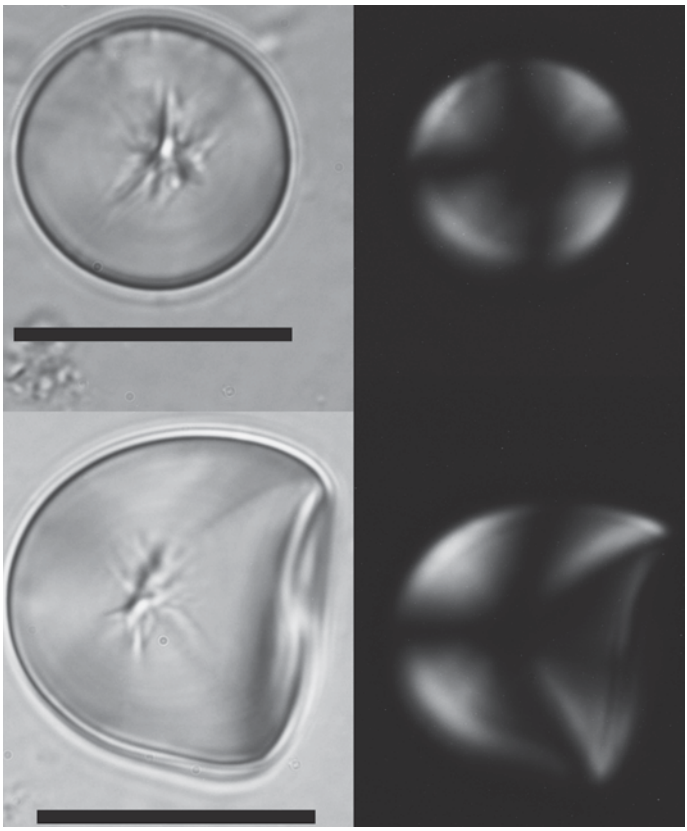

Fig. 29.2 Two bitterroot (*Lewisia rediviva*) starch granules showing different forms *(sphere above, bowl-shaped polygon below)*. Each image on the *left* is in normal light and that on the *right* in cross-polarized light showing the extinction cross and its variation in form with differences in granule shape and features such as fissures. Scale bar = 20 μm

visualization of form and has been used, although imaging is indicative of optical density and not texture per se. Most work is done using liquid mounts so that individual starch granules may be rotated for easy viewing of all aspects of the granule. Starch granules exhibit a distinctive extinction cross under cross polarization (Fig. 29.2), and so polarizing filters are important for identification and characterization.

Most of the extraction procedures outlined below are derived from a number of sources, including the procedures we employ in our laboratory. However, the extraction from carbonized remains derives from Zarrillo's work [8] and the extraction from sediments, mainly from Zarrillo and Kooyman [2]. This is also the case for extraction of phytoliths (see Chap. 28).

29.2 Materials

29.2.1 Extraction from Plant Material

Equipment and supplies required: mortar and pestle (or Petri dishes or watch glass, and dissecting needle), scalpel, transfer, or Pasteur pipette (or dissecting needle or plastic rod), Bunsen burner (optional), distilled or deionized water, liquid mounting medium (50/50 glycerol/water or similar), nail polish and disposable applicators (tooth picks or similar), slides and coverslips.

29.2.2 Extraction from Sediments

Equipment and supplies required: centrifuge, weigh scale, orbital shaker or vortex mixer (optional), 0.25 or 0.5 mm small geological sieve (or 4 mm tea strainer), 50 mL glass beakers, 50 mL centrifuge tubes, plastic transfer pipettes, Kimwipes (or similar), toothbrushes (compressed air optional), 0.1% EDTA in wash bottle (1 g Na_2H_2EDTA and mix to 1 L volume with distilled/deionized water), 6% H_2O_2 (store in dark, opaque bottle; PVC or polypropylene best), 1.8 specific gravity sodium polytungstate (approximately 244 g sodium polytungstate dissolved in 200 mL distilled/deionized water, best stored in non-glass container), glass funnel, 8 μm and 1 μm circular filter paper (VWR or similar supplier), and supplies listed in Sect. 29.2.1.

29.2.3 Extraction from Lithic Artifacts

Equipment and supplies required: ultrasonic cleaner, centrifuge, low power microscope (10–40X), clean, single-use toothbrushes, plastic transfer pipettes (or pipette with disposable tips), 15 mL and/or 50 mL centrifuge tubes, plastic containers (centrifuge tubes, weigh boats, etc.), aluminum foil, 5% ammonia (commercial) or ultrapure water, and supplies listed in Sect. 29.2.1.

29.2.4 Extraction from Carbonized Residues

Equipment and supplies required: centrifuge, weigh scale, orbital shaker or vortex mixer (optional), homogenizer and low shear blades, drying oven, vortex mixer, 50 mL centrifuge tubes, plastic transfer pipettes, 6% H_2O_2 (store in dark, opaque bottle; PVC or polypropylene best), 1.3 specific gravity sodium polytungstate (82 g in 200 mL distilled/deionized water; best stored in non-glass container), 1.8 specific gravity sodium polytungstate (244 g in 200 mL distilled/deionized water, best stored in non-glass container), 2.3 specific gravity sodium polytungstate (for

phytolith extraction; 522 g in 200 mL distilled/deionized water, store in non-glass container), glass funnel, 8 μm and 1 μm circular filter papers, and supplies listed in Sect. 29.2.1.

29.3 Methods

29.3.1 Extraction from Plant Material

1. For seeds, place a few seeds in a Petri dish, watch glass, or mortar *(see Note 1)*. Add 2–3 drops of distilled/deionized water and crush the seeds (not just the seed testa). For storage organs such as roots, cut a clean surface with a scalpel and scrape a small amount from the cut surface, placing it in a Petri dish or watch glass and add 1–2 drops of distilled/deionized water. To either of these preparations, add an equal amount of glycerol (2–5 drops generally, to make a roughly 50:50 glycerol/water mounting medium) and mix *(see Note 2)*.
2. Transfer 1–2 drops to a glass slide (with a dissecting needle, plastic rod, or Pasteur or transfer pipette), avoiding any plant tissue fragments *(see Note 3)*. The extract should be thinly distributed over the area encompassed by the coverslip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best granule viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the coverslip. Cover with a coverslip and seal slide (nail polish or similar) so the starch granules can be rotated in the mount. When sealing slide, to ensure no transfer of starch granules from one sample to another, use a disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard and use new applicator). Transfer implements should also be thoroughly washed and/or flamed in a Bunsen burner, or discarded, between samples to avoid cross contamination.

29.3.2 Extraction from Sediments

1. Sieve sediment sample to remove larger extraneous material such as roots. A small 0.25 mm or 0.5 mm geological sieve works well. Sieve onto tared paper or into a 50 mL (tared) glass beaker to give a sieved sample of 3 g (normally use same weight for each sample to ensure comparable sampling; *see Note 4)*. Clean sieve between samples to control cross-contamination. Cleaning minimally should include wiping out fine particulates (Kimwipes or similar) and dry brushing if particles are stuck in the sieve mesh (brushing one way, then diago-

nally to that, then at 90° to the first brushing). Normal cleaning in our laboratory comprises dry brushing, then washing with running deionized or distilled water, then air-drying using the compressed air jets in the laboratory (sieve must be dry for each sample).

2. Pour sieved sediment into 50 mL centrifuge tubes. Put 10 mL of 0.1% EDTA into each centrifuge tube and agitate on orbital shaker for 2 h on low to disperse clays *(see Note 5)*. Top up with distilled/deionized water, centrifuge at 3000 rpm for 2 min; pipette off and discard supernatant to 2 cm above sediment pellet without disturbing the sediment. Wash two more times with deionized water and centrifuge; this removes EDTA. On last removal of supernatant, pipette to as close to the sediment pellet as feasible without disturbing the pellet.
3. This step may be omitted if the sediment organic content is low; in this case, dry sediment at end of step 2 (above). To the centrifuge tube, add 10 mL of 6% H_2O_2, leaving for 10 min; this removes organic material. Top up with cold distilled/deionized water and centrifuge (2000 rpm for 2 min), then decant to within 2 cm of the sediment pellet. Rinse three more times with deionized water to remove H_2O_2, centrifuging at 2000 rpm for 2 min and decanting each time; as above, pipette off as much supernatant as possible on the last rinse. Dry in air or drying oven (drying oven maximum temperature 40 °C).
4. Add 5 mL of sodium polytungstate prepared to a specific gravity of 1.8 *(see Note 6)*, mix, and centrifuge at 2000 rpm for 5 min; pour/pipette supernatant (which contains starch) into new 50 mL centrifuge tubes. Repeat this step two more times, putting supernatant into the same 50 mL centrifuge tube. If desired, the remaining sediment can be processed to extract phytoliths (see Chap. 28).
5. Top up 50 mL tube with distilled/deionized water to decrease specific gravity so starch will precipitate out. Mix and centrifuge at 2000 rpm for 5 min to precipitate the starch; pipette off about 1/3 of the supernatant (and recycle the sodium polytungstate containing supernatant, *see Note 7*). Rinse precipitate twice more with water to remove residual sodium polytungstate from the starch extract, on the second rinse removing about 2/3 of the supernatant, and on the third as much as possible without disturbing the sediment pellet. The gradual, sequential staged pipette removal of the supernatant is done to ensure that no starch granules, with a specific gravity of about 1.5, are lost due to the initial dilution of the supernatant still remaining somewhat dense and granules remaining in suspension near the base of the supernatant column.
6. Liquid mount (50/50 glycerol/water or similar) on microscope slide for viewing. Most starch should be at the base of the sediment pellet (lighter material would be last to precipitate and hence is at the top of the pellet), hence remove material from the bottom of the pellet by pushing a plastic transfer pipette into the bottom to take the sample. Seal slide (nail polish or similar) so starch granules can be rotated in the mount. The extract should be thinly distributed over the area encompassed by the coverslip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best granule viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the

coverslip. When sealing slide, to ensure no transfer of starch granules from one sample to another, use a disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard, and use new applicator).

29.3.3 Extraction from Lithic Artifacts

1. Using a different clean *(see Note 8)* toothbrush for each artifact, gently dry brush surface sediment from artifact onto a plastic weigh boat, piece of aluminum foil, or comparable container. Store for future reference as needed (in our laboratory we often transfer this into a 15 mL centrifuge tube so it is easily sealed, labeled "dry brush," and stored).
2. Rinse artifact with distilled/deionized water, gently using the same toothbrush used to dry brush the artifact to remove surface sediment as needed *(see Note 9)*. Rinse into a weigh boat or similar container, then transfer to a labeled "water rinse" centrifuge tube or similar sealable container for longer term storage and subsequent examination as needed.
3. Optionally, based on surface examination macroscopically and/or with a low power microscope, undertake spot extractions of visible residues or likely residue traps such as cracks in the surface, vesicles or similar pores, and in deep flake scar corners or margins. It is common to omit this procedure and go directly to the next step. Use separate clean disposable pipettes or pipette tips for each artifact and transfer one to a few drops of distilled/deionized water, or ultrapure water, to the area *(see Note 10)*. The pipette tip may be used to agitate and loosen the residue in the extraction process, and the water can be squirted into and out of the location a number of times to also improve extraction. Pipette the water off the tool and onto a microscope slide and wet mount as in other extraction procedures *(see Note 11)*.
4. Place the tool in a new clean container and submerse all or a portion of the tool in distilled/deionized water, ultrapure water, or 5% ammonia (5% ammonia is most effective). Float the container with its tool in an ultrasonic bath for 1–45 min (30–45 min most effective). For smaller tools and full tool extraction, our laboratory generally uses 15 mL or 50 mL centrifuge tubes with the tops screwed on (in such cases, we usually undertake some of the spot extractions as previously outlined in step 3 to look at various potentially used areas) *(see Note 12)*. Transfer the extraction liquid to centrifuge tubes (if not already in them) and centrifuge at 3000 rpm for 5 min; pipette or decant the liquid and discard it. If multiple centrifuge tubes are used for the liquid from a single artifact, use distilled/deionized water to wash all the residue pellets into a single centrifuge tube and centrifuge at 3000 rpm for 5 min, again discarding the water supernatant. If ammonia is used in the extraction, wash residue pellets twice with distilled/deionized water, centrifuging at 3000 rpm for 5 min, to remove the water and remnant ammonia.

5. Wet mount a portion of the extract as outlined previously. This extraction procedure obtains all residues—starch granules, phytoliths, plant tissue fragments, hair fragments, and feather fragments—as a single extract. It is the first three that are most common.
6. Allow artifact to dry, rinsing first in water if ammonia was used in the extraction.

29.3.4 Extraction from Carbonized Residues (Usually from Ceramic Sherds)

1. Carefully rinse (and dry brush as needed) sherds with adhering carbonized residue with deionized/distilled water to remove surface loose sediment (as in steps 1 and 2 in Sect. 29.3.3, although commonly dry brushing is omitted as it can remove charred residue and so lose part of the sample). Remove carbonized residues with a clean stainless steel dental pick, dissecting needle, or scalpel (clean pick/needle/blade by flaming in Bunsen burner, by ultrasonic cleaner treatment using 5% ammonia, or by boiling in vinegar in a pressure cooker; *see Note 8*).
2. Weigh 0.1 g (more or less as sample dictates) of carbonized residue and place in a 50 mL plastic centrifuge tube with 5 mL of 6% H_2O_2. If desired, at this stage the sample may be homogenized with clean, low shear blades to enhance disaggregation of the carbonized material *(see Note 13)*. Place centrifuge tubes on orbital shaker on low (ca. 150 rpm) for 10 min (or *see Note 5)*.
3. Top up tubes to 50 mL mark with distilled/deionized water and centrifuge for 5 min at 3000 rpm. Pipette off and discard the supernatant without disturbing the precipitate pellet. Repeat two more times (if there is a small amount of clay from archaeological sediment in the sample, the washing process usually removes this as well as the H_2O_2). Dry samples in drying oven set no higher than 40 °C (so that the starch is not gelatinized) or dry in fume hood with centrifuge tubes loosely covered with their lids or aluminum foil "caps" *(see Note 14)*. One may omit steps 4–6 and proceed directly to step 7 if it is acceptable to extract all potential residues together to mount on the same slide.
4. Add 5 mL of sodium polytungstate prepared to a specific gravity of 1.8 to the centrifuge tubes, vortex to mix well, and centrifuge at 2000 rpm for 10 min (5 min at 3000 rpm also works well). Pipette the supernatant, which contains the starch granules (starch granules have a specific gravity of ca. 1.5), into new centrifuge tubes labeled "starch extract." Repeat this step, again putting 5 mL of 1.8 specific gravity sodium polytungstate into the original centrifuge tubes, vortex, centrifuge, and pipette the supernatant into the "starch extract" tubes. This ensures all disaggregated starch has been extracted from the sample. Fill the "starch extract" tubes with distilled/deionized water, mix, and centrifuge at 3000 rpm for 10 min. Pipette off and discard (recycle) about 15 mL of the supernatant, without disturbing the residue in the bottom of the centrifuge tube (*see Note 7*; pour supernatant through 8 μm filter paper into flask to recycle sodium polytungstate, filtering again later with 1 μm filter paper to ensure all contami-

nants have been removed prior to reuse). Top up the centrifuge tube again with distilled/deionized water, mix, again centrifuge at 3000 rpm for 5 min, and this time pipette off (and recycle) about 20 mL of the supernatant. Top up with distilled/deionized water a last time, mix, centrifuge at 3000 rpm for 5 min, and pipette off as much of the supernatant as possible without disturbing the pellet in the bottom of the centrifuge tube (this stepped procedure ensures that no starch granules are lost because of being suspended in the somewhat more dense supernatant near the residue pellet in the initial dilution steps; *see Note 15)*.

5. Liquid mount (50/50 glycerol/water or similar) on microscope slide for viewing. Transfer some of the wet sample to a microscope slide, add mounting medium, cover with coverslip, and seal slide (nail polish or similar) so starch granules can be rotated in the mount. The extract should be thinly distributed over the area encompassed by the coverslip and should not be allowed to dry before sealing as material dried to the slide often cannot be rotated; stir the material into the mounting medium to ensure best granule viewing if this can be accomplished while keeping the liquid within the area that will be encompassed by the coverslip. When sealing slide, to ensure no transfer of starch granules from one sample to another, use a disposable applicator (e.g., toothpicks) to apply nail polish and use a separate applicator for each line of nail polish (never dip applicator back into nail polish bottle after it has come into contact with the slide; discard and use new applicator).
6. Retain the original centrifuge tubes and label "phytolith extract" as this residue, with a specific gravity >1.8, contains any phytoliths in the sample. To remove any residual sodium polytungstate in this residue, top up the tubes with distilled/deionized water, vortex to mix, centrifuge at 3000 rpm for 5 min, discard the supernatant, and dry the samples in a fume hood or drying oven for later processing.
7. To extract the phytoliths from the remaining residue, add 5 mL of 2.3 specific gravity sodium polytungstate to the "phytolith extract" centrifuge tubes. Mix well and centrifuge at 3000 rpm for 5 min. Pipette the supernatant containing the phytoliths into new centrifuge tubes and top up these new tubes with distilled/deionized water. Vortex to mix, centrifuge at 3000 rpm for 5 min, and pipette the supernatant off to recycle the sodium polytungstate. Repeat the rinse with distilled/deionized water and centrifuging twice to remove all sodium polytungstate. The phytoliths will be in the residue pellet in the centrifuge tube.
8. Liquid mount the phytoliths as for starch granules (step 5 above).

29.4 Notes

1. It is best to obtain samples from at least two different localities to be confident about variation in form. Storage starch is not present in plants during all seasons and so it is important to collect specimens during an appropriate season. Unfor-

tunately, flowering season is often the best time to identify and collect material but it may be a poor time for starch content. Plant material should be rinsed in the field when collected and washed again in the laboratory (with detergent and/or a sonic bath) prior to extracting the starch, particularly root material which will have soil adhering to it. Sediment particles make preparations more difficult to scan and larger matrix grains can make it impossible to set a coverslip properly (such larger grains can usually be removed mechanically by moving them out of the mount with a dissecting needle or similar tool prior to placing the coverslip).

2. Starch has a refractive index of about 1.53 and so the mounting medium needs to have a sufficiently different refractive index that good contrast is provided. The 50:50 glycerol–water mounts we commonly use have a refractive index of 1.397 and so provide good contrast; pure glycerol (R.I. = 1.475) also works well. Microscope lens immersion oils come in various refractive index values and we have also used these. Liquid mounts must be kept upright and are prone to leaking over time, but are needed to be able to fully view and photograph all aspects of the granules. Due to the issue of long term storage, additional solid mount slides can be prepared as "voucher specimens."
3. Larger plant tissue fragments may be removed by sieving with cheese cloth or a sieve with a mesh size of about 0.4 mm, such as a tea strainer or small geological sieve. However, having a mounted sample with a variety of other plant tissue fragments included can be useful for identification of the variety of plant tissues that may be encountered in archaeological sediments and artifact residues. The inclusion of amyloplasts in particular can provide useful information on packing in these organelles, and the development of features such as pressure facets.
4. Sieving sediment results in a fairly wide scattering, and so it is best if a small diameter sieve is used and sieving is onto a wider piece of paper that can be creased, and the sieved sediment then poured into another container such as a small, 50 mL beaker. The bowl-shape of a tea strainer helps narrow the scattering, although we usually use geological sieves in our lab. We often use cut-up pieces of glossy posters that were never used as the paper pieces to initially catch the sieved sediment (the smooth surface ensures sediment is not trapped by the roughness of the paper).
5. There are alternative agitation methods that also work well to disperse the sediment particles if the laboratory does not have an orbital shaker. The sample may be agitated well for about 5 min using a vortex mixer. It may also be shaken by hand about every 30–60 s over a period of 5 min. It is common in geology and geography labs to use Calgon as the dispersing agent; commercial Calgon often has starch in it, and so it is best to use EDTA unless it is certain the Calgon contains no starch.
6. Starch has a specific gravity of approximately 1.5, and so the specific gravity of the extracting liquid must be greater than this, but close to it to ensure that many other materials are not extracted at the same time. Some labs use a heavy density liquid made up to 1.6, some to 1.7, and some to 1.8. In our lab, we employ 1.8 to ensure that the specific gravity of the liquid remains greater than 1.5, even if it is slightly diluted by moisture remaining in the sample after step 3.

7. One of the advantages of sodium polytungstate is that it is relatively easy to recycle. During extraction, we pour our used sodium polytungstate through 8 μm filter paper into an Erlenmeyer flask to remove most material still in suspension (this filters out most material and progresses sufficiently quickly that we can process multiple centrifuge tubes without the funnel and filter paper filling with liquid). Later, we refilter this solution through 1 μm filter paper to remove very fine contaminants, including very small starch granules. We allow the twice filtered solution to evaporate (covering the flask opening loosely with aluminum foil, not using any heat) until it is again dense as seen in its golden color. It is best if all water does not evaporate leaving a precipitate, as this is more difficult to bring into solution than the original laboratory reagent powder. To ascertain if the evaporation has sufficiently concentrated the solution, we pipette 5 mL of solution into a small tared glass beaker and weigh it. 5 mL of solution must weigh 9.0 g for a specific gravity of 1.8 (11.5 g for a specific gravity of 2.3, used to extract phytoliths, and 6.5 g for a specific gravity of 1.3). Once the solution is known to be more dense than required, a small amount of distilled/deionized water is added and 5 mL are again weighed; this procedure is repeated until the correct weight is obtained, indicating the solution has reached the required weight, and hence specific gravity (if the process again makes the solution less than the required density, it may be adjusted by adding a small amount of sodium polytungstate powder or by allowing partial evaporation of the solution again). If making solutions from powdered sodium polytungstate, approximately 244 g dissolved in 200 mL distilled/deionized water gives a solution of specific gravity 1.8; approximately 522 g dissolved in 200 mL of water gives a solution of specific gravity 2.3; approximately 82 g dissolved in 200 mL of water gives a solution of specific gravity 1.3 (the maximum attainable solution specific gravity for sodium polytungstate at room temperature is 3.1). Sodium polytungstate dissolves most readily if the powder is gradually added to the water. If calcium ions are present in the sediment floated, sodium polytungstate may form an insoluble compound with the calcium. Also note that it is best to store the solution in plastic as it can also remove calcium from glass containers and again result in precipitation of an insoluble compound (in practice, we have not had this problem with glass storage containers but have sometimes had the issue in sediment extractions; pre-treatment with dilute HCl usually eliminates this problem in phytolith extraction from sediments, but this cannot be employed with starch granule extraction as HCl damages starch).
8. The toothbrushes used may be new, used once, and then discarded after use, or may be cleaned in one of a number of manners and reused. Tool residues of all kinds are rare and the potential of losing this information because of contamination with fibers, hairs, starch granules, and other material from field and laboratory processing is a major concern. Circulated building air, laboratory supplies, and equipment are often contaminated by starch which is of particular concern during extraction of archaeological tool residues. In general, laboratory surfaces should be cleaned (e.g., with commercial ammonia) before each sampling, and sampling should be done on a disposable surface such as labora-

tory mats or stretch wrap that is changed between samples. Supplies such as microscope slides, and reused equipment such as forceps, should be thoroughly cleaned (even the glue from boxes containing supplies such as slides often contains starch). In our lab, we use commercial ammonia, filling plastic containers with it and the supplies, and placing the filled containers in an ultrasonic bath for 45 min to dislodge and remove starch and other contaminants; the ammonia is rinsed from the equipment afterwards with distilled/deionized water. Boiling in vinegar (5% acetic acid) in a pressure cooker (and then rinsing in distilled/deionized water) for a minimum of 30 min can also be employed, which gelatinizes contaminate starch and should remove other contaminants in the discarded liquid. Single-use gloves (or thoroughly washed hands) should be used to handle artifacts. Many powder-free gloves are contaminated with starch and each new lot must be tested to ensure the gloves are truly starch-free. As well, gloves should generally be put on and then rinsed under distilled or deionized water to ensure surface starch is removed. We always wipe the surfaces of boxes and bags containing supplies with damp clothes before bringing them into the work space. If feasible, tool residue extraction should be undertaken in a dedicated space, if possible a space with HEPA filtration of the air and a slight positive pressure gradient to minimize the inflow of unfiltered air. We also wear disposable laboratory suits to minimize introduction of contaminants such as hair, threads, and starch into the tool residue extracts.

9. A balance is needed between removing surface contamination and accidentally removing the ancient tool residues, hence dry brushing should not be too vigorous.
10. Ultrapure water is better than distilled/deionized water due to its greater ability to remove residues. 5% ammonia can also be used, but cannot be mixed with many slide mounting media and so is not as good a choice for direct mounting of extracts as done in this procedure. If ammonia is used, it needs to be rinsed from the extracted residue before mounting, but this is difficult to do without losing the sample; use of a microcentrifuge best accomplishes this, rinsing 2–3 times with distilled/deionized water, each time centrifuging for a few minutes and discarding the supernatant.
11. This stage, if included, is sometimes done before step 2, if the step 2 washing is vigorous [6]. More generally, step 3 is done after washing to minimize the possibility of surface contamination of the extract.
12. For larger, heavy artifacts that may cause containers to sink to the bottom of the ultrasonic bath, we use larger plastic containers and rest the container on a small block (e.g., a piece of brick) in the ultrasonic bath, allowing as much of the container bottom surface as possible to be in contact with the bath water. For artifacts that are too large to fit in the ultrasonic bath, such as anvils and grinding stones, we used a plastic transfer pipette to squirt ammonia into and off a location on a surface for a period of 1–3 min, adding more liquid to replace that which is absorbed during the procedure if the rock is porous. We might do this on more than on location, depending on the size of the artifact. We also use this procedure for rock types that may be too friable or fragile for ultrasonic treatment.

13. The use of H_2O_2 gently oxidizes the matrix and homogenization fragments the consolidated mass, together releasing the embedded starch granules; this can be successfully accomplished with use of H_2O_2 alone.
14. If the sample with H_2O_2 is still reacting after the initial water is placed into the centrifuge tube, due to high carbonate content, split the sample into two tubes, top up both with water, centrifuge, then recombine the spit samples into one tube. Then do the two water rinses.
15. To make a somewhat cleaner extract preparation by removing extraneous less dense material, step 4 may be preceded by a treatment using sodium polytungstate prepared to a specific gravity of 1.3. Add 8 mL of the 1.3 sodium polytungstate to the sample in the centrifuge tube, mix well, centrifuge at 2500 rpm for 12 min, and pipette off and discard (recycle) the supernatant without disturbing the residue pellet.

29.5 Interpretation and Conclusion

Interpretation of the importance of plant taxa in the environment, in the ancient diet, or in the use of archaeological tools is generally based on the relative or absolute abundance of starch granules in sediment and artifact extractions. Relative abundance is usually based on simple counts of 100–500 granules per sample. Estimates of absolute counts per mL of sediment or residue can also be obtained by adding and counting a known amount of a foreign material such as *Lycopodium* spores or artificial (e.g., polystyrene) microspheres [14–18] or by mounting and counting *all* granules from a weighed amount of plant material or archaeological sediment. Use of weight for modern plant samples is complicated by the fact that processed plants often contain an amount of soil and/or windblown sediment that is impossible to fully remove; hence, the plant material weight is inaccurate. The basic procedures for ecological reconstruction follow those developed in palynology.

Differential production and differential survival (taphonomy) of starch granules are factors that are still poorly known [19], and so the meaning of differences in frequencies in particular contexts (quantification) is also unknown. Does this mean we can only discuss presence versus absence, not relative frequency, or even just presence? Most researchers are not this pessimistic, but relative importance in the diet based on archaeological tool residues is usually interpreted conservatively. We also have only a poor idea of how well starch is preserved on artifacts versus in carbonized residues, and how those contexts relate in general to the usually poor preservation in soils [2]. We do not known how rapid and deep burial in sediments, and in specific archaeological contexts such as cooking ovens, may affect preservation. Ancient food preparation, including gelatinization in boiling and cooking, also likely affects preservation [20]. As a result, the issue is not just that it is unclear whether relative frequencies indicate relative frequencies of plant taxa, but whether even very large granule frequencies in residues can allow us to state that more of something was worked with the archaeological tools, or whether a particular plant

was more important in the diet of ancient people. Microbotanical remains of all types, including starch granules, have the potential to move from one sedimentary context or layer to another through the microscopic cracks and spaces present in sediments (e.g. [21, 22]). As a result of this potential movement, the question of the context of the remains is always important in interpretation.

Production and storage of starch granules varies over the seasons and so is an issue. This is an especially important consideration in making comparative collections, since plant identification is often easiest when plants are in flower, and hence this is when specimens are collected. The flowering season is not a time when plants are storing starch. This situation is likely not an issue for representation in archaeological contexts, since plants are generally used when they are most valuable as a food, hence when they contain abundant starch.

There are some structures in sediments that are not starch granules but that may look like them (conidia, bordered pits, etc.) [23]. Analysts must be aware of these structures and not include them in counts.

An outstanding question in interpretation concerns how to integrate starch data with data on presence (or absence) of plants that do not produce any, or much, starch—those plants that store energy as non-starch complex carbohydrates and oils. Integration of starch data with that of other paleobotanical remains, such as charcoal, seeds, and phytoliths, is also largely unexplored. This is a general problem in paleobotanical and paleoethnobotanical analyses [17].

Dehydration of granules changes the shape in both modern and archaeological material, and this must be kept in mind when looking at features and size as the basis for identification. Frequency distributions of some variables such as size can be distinctive of particular taxa, but in the mixed assemblages obtained in archaeological extractions, the combination of multiple taxa in a single assemblage may make it impossible to use this category of data.

Aspects of form often do not seem to vary with taxonomic proximity [24], so some similar forms are seen in quite different taxa, or are present rather randomly across taxa. For example, some grasses have small polygonal granules like rice, whereas other grasses have very large lenticular forms like wheat. Does this mean we cannot really use general family categories? Not always, as the two dominant forms in grasses just noted do clearly represent a taxonomically useful pattern. Much more study of this is required.

There has been much discussion about imaging starch granules and certainly not all aspects of form can be well understood with conventional light microscopy. Use of a number of complimentary techniques often best reveals the nature of features (e.g., [25). Unfortunately, it is not practical to employ scanning electron microscopy and other specialized imaging techniques in regular analysis of archaeological material comprising thousands of granules, although this may be feasible for study of comparative material and describing features of particular taxa [10]. However, it could be said that regardless of whether or not a feature is fully understood, if its existence is definable, then that may be all that is needed for identification (e.g., whether features are on the surface, or the interior, of a granule). Workers may be able to clarify the nature of such features in comparative and some archaeological

material with SEM and/or DIC microscopy, without having to use these methods routinely.

Acknowledgments I have benefitted from discussions with a number of individuals over the years, but I particularly wish to thank Gerald Newlands and Sonia Zarrillo.

References

1. Gott B, Barton B, Samuel D, Torrence R (2006) Biology of starch. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 35–45
2. Zarrillo S, Kooyman B (2006) Evidence for berry and maize processing on the Canadian plains from starch grain analysis. Amer Antiquity 71:473–499
3. Field J (2006) Reference collections. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 95–113
4. Torrence R (2006) Starch in sediments. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 145–176
5. Fullagar R (2006) Starch on artifacts. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 177–203
6. Pearsall DM, Chandler-Ezell K, Zeidler JA (2004) Maize in ancient Ecuador: results of residue analysis of stone tools from the Real Alto site. J Arch Sci 31:423–442
7. Perry L (2004) Starch analyses reveal the relationship between tool type and function: an example from the Orinoco valley of Venezuela. J Arch Sci 31:1069–1081
8. Zarrillo S, Pearsall DM, Raymond JS, Tisdale MA, Quon DJ (2008) Directly dated starch residues document early formative maize (*Zea mays L.*) in tropical Ecuador. Proc Natl Acad Sci (USA) 105:5006–5011
9. Horrocks M (2005) A combined procedure for recovering phytoliths and starch residues from soils, sedimentary deposits and similar materials. J Arch Sci 32:1169–1175
10. Briuer FL (1976) New clues to stone tool function: plant and animal residues. Amer Antiquity 41:478–484
11. Chichinadze M, Kvavadze E (2013) Pollen and non-pollen palynomorphs in organic residue from the hoard of ancient Vani (western Georgia). J Arch Sci 40:2237–2253
12. Crowther A, Haslam M, Oakden N, Walde D, Mercader J (2014) Documenting contamination in ancient starch laboratories. J Arch Sci 49:90–104
13. Barton H, Fullagar R (2006) Microscopy. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 47–52
14. Battarbee RW, Kneen MJ (1982) The use of electronically counted microspheres in absolute diatom analysis. Limnol Oceanogr 27:184–188
15. Bonny AP (1972) A method for determining absolute pollen frequencies in lake sediments. New Phytol 71:393–405
16. Craig AT (1972) Pollen influx to laminated sediments: a pollen diagram from northeastern Minnesota. Ecology 53:46–57
17. Pearsall DM (2000) Paleoethnobotany: a handbook of procedures, 2nd edn. Academic, San Diego
18. Peck RM (1974) A comparison of four absolute pollen preparation techniques. New Phytol 73:567–587
19. Barton H, Matthews PJ (2006) Taphonomy. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 75–94
20. Samuel D (2006) Modified starch. In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 205–216
21. Fishkis O, Ingwersen J, Lamers M, Denysenko D, Streck T (2010) Phytolith transport in soil: a field study using fluorescent labelling. Geoderma 157:27–36

22. Horrocks M, Lawlor I (2006) Plant microfossil analysis of soils from Polynesian stonefields in South Auckland, New Zealand. J Arch Sci 33:200–217
23. Loy T (2006) Optical properties of potential look-alikes (Box 7.3). In: Torrence R, Barton H (eds) Ancient starch research. Left Coast Press, Walnut Creek, pp 123–124
24. Gill LS, Nyawuame HGK, Aibangbee MI, Agho DA (1991) Nature of ergastic substances in some Mediterranean angiospermous seeds—VI. J Bot Taxon Geobot 102:613–628 (Feddes Repertorium)
25. Baldwin PM, Adler J, Dabies MC, Melia CD (1994) Holes in starch granules: confocal, SEM and light microscopy studies of starch granule structure. Starch/Stärke 46:341–346

Chapter 30
Plant Collection, Identification, and Herbarium Procedures

Bonnie Smith and C. C. Chinnappa

30.1 Collection of Plant Specimens

This chapter examines the methodologies of plant collection, processes for pressing and drying plant specimens, and identification of flower structures. Plant specimen collections are fundamental to taxonomic research because they reveal species variability; as a result, they are prime sources for floristic investigations. Because of their importance, collections must be created with careful attention to detail [1].

Plant specimens should be chosen on the basis of their value as scientific specimens as well as their suitability for particular herbaria. The reasons for the choice of specimen notwithstanding, care must be taken when plants are collected and preserved.

30.1.1 Ethics of Plant Collection

The collection of plants affects the environment in which the plants grow, and change may be minimal or significant. Collectors must therefore keep the consequences of their actions in mind [2]. The University of Alberta Herbarium [3] provides useful conservation guidelines:

1. Awareness of laws regarding particular plant species in given areas is important because the collection of many may be limited given their rare status in a region. Necessary permits to collect on protected lands (e.g. provincial, national, state parks, or ecological reserves) may be required. Permission from landowners or government agencies may also be necessary.

B. Smith (✉) · C. C. Chinnappa
Department of Biological Sciences, University of Calgary, 2500 University Drive NW., Calgary, Alberta T2N 1N4, Canada
e-mail: smib@ucalgary.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_30

2. Collection of the sole specimen of a plant in a particular area is inadvisable. Specimen collection should occur only if the local population will not be adversely affected. Collection of 1 % or less of any given population is advised.

30.1.2 Materials

Jones and Luchsinger [1] suggest the following items be included on a collection expedition:

Plant press and newsprint, field notebook, plant identification book, pencils and marking materials, sealable plastic bags of various sizes, paper bags, tags for specimens not to be pressed flat (fruit, cones, etc.), maps of collection areas, handheld global positioning system (GPS), topographic and road maps, compass, spade, pocket knife, pruning shears, 10–40× magnifier, first aid kit (including insect repellent and sunscreen), lunch including water, pole pruners (if required), field press and cooler (if required), and drying cabinet.

Choice of clothing should be made according to the weather and terrain conditions. Apparel includes socks, boots (rubber, hiking, hip-waders), water repellent and wind-proof clothing, and insect-proof netting and gloves.

Contemporary technology can greatly aid a collection expedition. Cameras, phones, laptops, or iPads that contain stored descriptions and photographs of plant species can facilitate field identification and aid in navigation and mapping. Protect these items from weather and field conditions.

30.1.2.1 Plant Press

A plant press is designed so that plants can be dried quickly while being pressed flat. A plant press can either be purchased from a variety of on-line sources or constructed from simple materials. The basic plant press (17” × 12” dimension) is composed of two cross-slatted wooden frames or press ends, cardboards and blotting paper, and two straps with clips, for tightening the press once specimens have been placed in it for drying. Straps will have to be tightened periodically as the plant material dries and shrinks. The press is organized in the following order: the wooden form is placed on the bottom followed by cardboard, blotter, and the plant specimen in folded newsprint. The sequence is continued until the wooden form is placed on the top. In the case of thicker or damper specimens, the blotting sheet can be replaced, one or both, with foam of various thicknesses [4]. Although the press should not reach more than 3 ft when filled with plant specimens [1], a final height of 18” is preferred [4].

Each part of the plant press performs a necessary function. The plant press frames strengthen the press by allowing pressure to be spread uniformly over the plants being dried. The cardboards (ventilators) allow the air to diffuse through channels following corrugations across the cardboard. Channels should not run along the

length of the cardboard as this pattern is less effective in permitting the exchange of moist air from the interior of the press with the dry air from the outside. Blotters mould themselves around the stems and roots of the plant. This allows pressure to be spread over the surface of the plant. Blotters then absorb moisture which slowly diffuses into the cardboards. Blotters also cushion the cardboards against structures that otherwise might crush the corrugations in the ventilators. A good strap has a buckle which will cinch and bind the press tightly together [2].

Some botanists use metal corrugates instead of cardboards, and some use foam plastic instead of blotters. Metal corrugates, although heavier and more expensive than cardboard, are less easily deformed and do not become damp. Therefore, they are less susceptible to mildew. Foam plastic, although more bulky and a problem to store, moulds itself more tightly around plants then blotters. This allows more uniform pressure to be applied to the plant material in the press [2].

30.1.2.2 Field Press

A field press is thinner, lighter, and more easily transported during collection trips than a normal plant press. The field press contains only a few sets of cardboards and blotters enclosed by a rigid frame and straps. The press is intended for immediate field use for the collection of delicate or wilt-prone specimens [4]. The field press allows for quicker pressing of field specimens which produces higher quality plant specimens.

An example of the organization of a field press is as follows: bottom wooden form, cardboard, blotter, and ten sheets of torn newsprint (each sheet will be used for one specimen). The sequence is continued until ten groups of ten newsprints are present and the top wooden form is added. In another version of a field press, specimens may be wrapped and rolled in newsprint while in the field [1]. There are many types of field presses, many unique in design [4].

The length of time that plants can be kept in the field press without damage varies widely with the weather and the collection location. A few hours is the maximum time in hot dry conditions while in southern Canada up to 8 h is the maximum, and in the Arctic up to 8 days is possible [4].

30.1.2.3 Field Notebook

Collection information should be recorded while the collector is in the field. The information contained in the field notebook will be used later to create a label for the herbarium specimen. Add identification information to the field notebook once the specimen has been identified. Include the identification, authority, and keys or floras used to obtain the identification, name of the person making the determination and date of determination as well as the place where the specimen will be stored [5, 6]. The field notebook should contain pages of acid-free, long-lasting paper

written in permanent ink since they may contain notes and marginalia which do not appear on labels. Carry field notebooks in plastic bags, for their protection [7].

If collecting extensively (50 or more specimens per day) or on restricted time, the field notebook data may be abbreviated by referring to it in shortened form to other specimens collected from the same site such 'ditto' or 'same' indicating that the habitat of this specimen is the same as that of the previous specimen number. Field information may be written directly on the sheets in the field press. These sheets may be reused after the data have been entered in the field notebook. Data from the press sheets may be entered into the field notebook once field collecting is complete allowing the field notebook to not actually be taken to the field but left safely elsewhere [4].

A field notebook should contain space on the individual pages to allow the following data to be recorded.

1. Name of the plant—even if the exact identification is uncertain. The specimen may be described in general terms referring to an outstanding feature, such as 'tall grass' for *Phragmites* in the Poaceae, or 'hairy buttercup' for some species of *Ranunculus* in Ranunculaceae.
2. Name of collector and collection number (assigned either numerically, starting with #1, or according to the desired form of the collector). This number should be written on the main specimen sheet as well as any other pieces of the specimen kept separately, such as fruit, cones, or photographs [3]. Reference to any digital photographs which might have been taken should be included [6].
3. Date of collection.
4. Locality, including exact coordinates, if possible. The location of the specimen collected should be given using distances from the nearest permanent landmark, compass directions, Universal Transverse Mercator (UTM) grid coordinates, longitude/latitude, legal land descriptions, information from topographic maps or any other data which will fix the location as accurately as possible. Longitude and latitude or UTM are essential for georeferencing and can be obtained from topographic maps or GPS units (note the map sheet and date or the UTM system used) [3]. The field notebook might also contain more specific directions to the collection site for possible relocation [6]. Include country, province, state, or county in the description.
5. Habitat description, including associated species as well as site description such as community type, elevation, exposure, slope and aspect, approximate soil type and soil moisture, drainage conditions (wet, submerged, dry), and any disturbances to the habitat. Not all information will be readily apparent at all collection sites. Be as thorough as possible.
6. Remarks. When material is fresh, it is easier to determine the features necessary for species identification. For example, flower colour and scent are important to note since these may fade during drying. All morphological features such as annual/perennial habit, height (especially if incomplete plants must be collected), growth form (clumped, growing individually, or twining), presence of milky juice or other unusual characteristics. Any features which cannot be

ascertained by looking at a dried specimen should be noted including abundance and blossom-visiting insects [3, 4, 6].

Rather than a field notebook, a field collection label may be used. The field collection label may be blank or preprinted with basic headings for each type of information. Some collectors may tag specimens with their collection number in case specimens are separated from the newsprint [3].

30.1.3 Methods

30.1.3.1 Vascular Plant Specimen Preparation—Collecting Specimens

Plant specimens are essential for taxonomic and floristic research. Plant specimens should be collected to enable proper identification. The following guidelines should be considered when collecting plant specimens.

1. Collect a typical specimen of the species noting in the field notebook if an aberrant form is collected. Do not collect the smallest specimen of a population merely because it will be easier to press and mount on the herbarium sheet [3].
2. The specimen should bear root, shoot, and leaf material along with flowers and fruit. If the plant is small, pull it up by the roots (do not uproot orchids). The roots as well as other floral parts may prove to be diagnostic [4].
3. Collect a few extra buds, flowers, and fruits so that they can be dissected without damaging the specimen during the identification process. Extra material may also be useful for future research purposes [3, 4].
4. If plants are to be sent away for identification, collect enough material for at least two sheets [4].
5. Collect specimens to fill the space available on a regular herbarium sheet. Leave room for the label in the lower right-hand corner and a small space for the herbarium accession stamp on the upper part of the sheet. Leave space for a fragment packet. Press the packet with the specimen [4].
6. If small herbaceous plants are collected, then several will be required to fill the available space on a herbarium sheet, which is the same size as the typical plant press cardboard. Collect six or eight plants if they are about 6-in. high. Collect a dozen or more if the plants are minute. Collect to show variability in the population [4].
7. Collection of a single large herbaceous plant may be satisfactory. Fold the specimen, if necessary, to ensure a good fit. Leave 1-in. margin all around. It may be necessary to trim off any excess shoots or leaves. Should the specimen be very large then cut off portions of the stem bearing the inflorescence as well as typical basal and mid-stem leaves. If the root or stem is very thick cut it longitudinally. Occasionally, two herbarium sheets may be required to provide proper representation of the specimen. Use the same label as the first specimen (1/2; 2/2) [4].
8. Plant specimens should not be collected unless at least 20 members of the species are present.

9. Collect each plant separately. When the specimen is placed in its newsprint or plastic bag include a slip of paper with the collection number written in pencil. Each specimen should have its own collection number if any collection factor is different [6].
10. If possible, do not collect in wet weather or heavy dew.
11. For best results, specimens ought to be pressed directly after collecting them in the field.
12. Collect critical material. For example, many groups, including Brassicaceae, Chenopodiaceae, Poaceae, Onagraceae, Apiaceae, Juncaceae, Salicaceae, and Cyperaceae, require well-formed mature fruit for precise identification. Some species of *Carex* that shed their fruits readily when fully ripe must be collected before shedding occurs. Species from Violaceae and Orchidaceae require flowers at maximum bloom when pollen is released [3, 4].
13. Collect both flowering and fruiting material whenever possible since most herbaria contain inadequate fruiting material of many plants. If flowering and fruiting does not occur simultaneously, such as the complex genus *Crataegus*, you should tag the tree from which flowers are taken and return later for the fruits [4].
14. Collect grasses after they have started to flower, when the anthers may be seen hanging from the florets. If grasses are taken fully ripe, the seed usually have dispersed and a poor specimen results [4].
15. Collect a portion of the underground structures of plants to show the annual, biennial, or perennial habit, which is diagnostic in many groups such as the Poaceae and Asteraceae [4].
16. Collect a portion of the stem to show the type of leaf arrangement and character of the winter buds, which is critical for the identification of some shrub and tree species [4].
17. Place specimens, folded to the correct length for a herbarium sheet, carefully into the plastic bag so that separate collections will not become tangled. Later, carefully remove the specimens by turning it upside down. Do not try to pull material out of the bag. This usually breaks up the specimens. Use large bags rather than small ones, since there will be less damage to the plants. Put large or heavy plants into separate bags [7].
18. Keep plastic bags partially filled with air as a cushion against crushing and keep collections as cool as possible (refrigerator or cooler). Leave specimens in plastic bags for no longer than a few hours before they are pressed. Specimens may be kept fresh during this interval period by placing a few moistened paper towels in the bag [3].
19. Remove as much of the soil as possible from roots to avoid disfiguring them. This allows the roots to be more easily cleaned before they are put in the plant press [4]. Shake all excess soil off the roots or wash them off carefully if possible [5]. A dense tangle of roots, particularly of rhizomatous grasses and sedges, presents a problem. Stand some clumps in water while working on others. Running water is particularly effective.
20. Collect healthy specimens, unless purposely collecting for fungal or insect damage as part of a research project [4].

21. Cut twigs off cleanly with a sharp knife or pruners. Do not break the twigs. This will strip the bark and ruin a specimen and likely cause damage to the living tree or shrub [5].
22. When collecting trees, shrubs or large herbs cut 2–3 dm. lengths from ends of stem or branches. These should include flowers and/or fruit where feasible. Also include a range in leaf sizes or types if these vary, and a small chip of bark from trees. Make notes on estimated height, trunk diameter at breast height (d.b.h.), habit, colour, and texture of the bark of old and young stems and note whether the collected material came from the same shrub or tree or from different individuals [3, 4, 6].
23. Some Arctic and alpine plants have several years' accumulation of dried basal leaves. Do not remove all of these leaves, because they are sometimes an aid in identification. Similarly, fibrous remains of old leaves (e.g. some *Carex* and Apiaceae) should not be cleaned off completely [4].

30.1.3.2 Vascular Plant Specimen Preparation—Specimen Pressing Tips

1. Lay the specimen out so that there is minimum overlap between parts. Starting near the folded edge of the newsprint hold the plant specimen in the desired position. Cover the specimen parts near the fold with newsprint and continue holding down each part of the specimen after the other and covering with newsprint until the entire specimen is covered and all the newsprint is folded down [5].
2. Ensure that no part of the specimen overhangs the newsprint. A mounting sheet is smaller than a sheet of newsprint so this is especially important [3].
3. Try to retain the natural posture of the living plant; if the stem is decumbent do not make it appear erect [3].
4. Arrange the plant materials loosely on the newsprint. Do not overcrowd [1].
5. When pressing a specimen write the name of the collector and collection number from the field book on the newsprint in pencil in the lower left corner. Adding the genus and species is also recommended [3, 6].
6. Vary the positioning of the thicker parts of plant specimens such as roots or the press will soon become lopsided [4].
7. Make a zigzag bend in the stems of tall plants to allow the specimen to fit in the press [5]. Fold tall plants to a length slightly less than the width of a newsprint [4]. Place a piece of paper with a slit in it over the elbow of the bend in grass or sedge (or other plants with narrow leaves) stems to hold all the leaves together [5]. Use pieces of paper roughly 2.5 × 1.5 in., with a slit about ¾ to 1 in. long cut or torn down the middle. Do not remove the papers until just before the specimens are mounted [4].
8. If the plant specimen has a great number of leaves, snip off some of the leaves but always leave part of the petiole so that it is evident that leaves have been removed [5]. Ensure some leaves from different areas of the stem are left since leaves may vary in form along the length of the stem. If possible, do not remove leaves but spread them out or bend petioles to prevent overlapping and crowding [4].

9. Bend twigs and branches to the correct orientation before the plant is arranged on the newsprint. Do not sever twigs or leaves [5].
10. Slice thick woody stems and roots lengthwise and lay them side by side for pressing. Split the lower part of a very thick stem or fleshy crown. Thick tuberous parts or large root crowns can be split with the pruning shears or knife [3]. Use foam, which moulds itself around the specimen to ensure even pressure, to press leaves along thick stems since, otherwise, they do not always get sufficiently pressed and may tend to wrinkle. Layers of paper may also be used instead of foam [4, 5].
11. Slice end of woody stems diagonally so that the colour of the wood and pith are displayed [5].
12. Use a piece of wet newsprint to hold wilted or folded petals or leaves so that they will lie flat for pressing [5].
13. The back of at least one leaf should be visible on a pressed specimen [5].
14. If there are several flowers on a specimen, then vary positioning of the flowers with some face up and others face down or sideways. Some should be pressed open and flat so that the inside is visible. To achieve this, use deliberate pressure with the thumb before the newsprint is folded shut [3, 5].
15. When pressing very small plants, place them into a paper packet. Place loose seeds and fruit into a small paper packet. Write the collector's name on the packet. Press the packet with the specimen [2, 5].
16. Pine cones, large dry fruits, bark, and other similar structures can be dried whole and filed separately from pressed specimens. Store objects in acid free boxes that cannot be satisfactorily mounted on sheet [8].
17. Place mosses, cones, fruits, and some cacti and succulents in small paper bags, number on the front, and dry unpressed. If stored in plastic bags, they rapidly mould, even after drying [7].
18. Some kinds of large fleshy fruits can be dried whole or sliced into sections and dried in a plant press. Change cardboards, blotters, and papers to remove excess moisture as required. Press small fruits with the vegetative and floral parts of the plant. Fleshy fruits may also be placed into jars of preservative fluids [2].
19. Plants with very large leaves such as palm trees can be pressed in pieces in several sheets of newsprint. Press a portion of the inflorescence and upper leaves, the stem and middle leaves, the lower leaves, and the main underground parts. Labels should be attached to each indicating the part pressed. Use the same collection number for all the sheets of the series [2, 3].

30.1.3.3 Vascular Plant Specimen Preparation—Specimen Drying Tips

1. A plant press is used to apply pressure to flatten plant specimens and dry them rapidly. A greater area of contact between plant, newsprint, blotter, and ventilator will result from greater pressure. The goal is to speed up the transfer of moisture from the plant to the ventilator where the passage of air evaporates it [3].

2. When tightening the press equalize the pressure from side to side when tightening the straps. Stand or sit on the press while tightening the straps to increase the pressure [3]. Tuck in the loose ends of the straps. The press is now ready to be placed in the drier [4].
3. A press that will tighten down to about 15 in. deep is ideal. A press less than 12 in. deep will not be able to maintain adequate pressure. A press over 18 in. deep is very awkward to handle. If pressing only a few plants, then bring up the press to normal height with added blotting sheet and ventilators [4].
4. The heat in the plant press or drying cabinet must not exceed 80 °C (120 °F) or the plant specimen will become discoloured from damage to internal structure of leaves and flowers [8].
5. A simple solution for quicker drying is to place the press over two chairs with the cardboard flutes vertical over a low heat source (60–100 W desk lamp). Warm air will pass upward through the flutes and speed evaporation [3]. Instead of chairs this portable drier could be placed inside a box with a mounted, well-insulated light bulb. Do not allow any part of the apparatus to come into contact with the light bulb [2].
6. Changing the newsprint holding the plant specimen after the first 24 h enhances drying. The collector also can make adjustment to the specimen while it is still able to be manipulated. Folded leaves can be unfolded and flattened and flowers can be rearranged [5].
7. If a drying cabinet is available, leave the plant press in for a week to 2 weeks depending on the moisture content of the specimens [4].
8. Whether the specimens are in a drying cabinet or not, check them every 2–3 days, replacing newsprint, if the specimens are still moist. Remember to write the name of the collector and collection number on this newsprint [4].
9. Presses can be dried in the field by placing them in open, sunny, and breezy areas on rocks or on car roof racks, suitably tied down [4].
10. Do not expose the plant too closely to artificial heat source, or plants may be scorched [4].
11. Ensure that plants are thoroughly dry and free of pests before placing them in cabinets. Never use a microwave oven to dry or treat herbarium specimens for pests [8].

30.1.3.4 Troubleshooting for Successful Pressing and Drying of Difficult Plant Specimens

Succulents: Succulent or fleshy plants contain a large quantity of water, colloidally bound within the cells, as well as a thick, heavily cutinized epidermis which inhibits the loss of water. This construction makes them very difficult to dry properly in a plant press. It often takes a month or longer to properly dry some succulent plants and some can live for months inside a plant press under conditions that would rapidly dry out most other plants [2, 4]. *Opuntia* may produce etiolated shoots that grow out of the press and look nothing like the original specimen [4]. By the time

the plant is dry it may shrivel into a nearly unrecognizable remnant or it may get mouldy. *Portulaca* can be pressed in flower and removed in mature fruit with leaves shriveled and detached [2, 4]. Leaves of succulents fall when dried, unless previously immersed for a few seconds in boiling water but do not immerse flowers [3]. Drying succulents may involve killing the plant and removing the water. A chemical such as alcohol may be used to kill the plant. It may also be frozen or microwaved for a couple of minutes. To remove water, large fleshy plants may be sectioned and the fleshy tissue sliced away or a long period of drying may be undertaken involving the frequent removal of damp blotters and cardboards and substitution of dry replacements [2]. It is difficult to work around the needle-sharp barbed spines and care must be taken to ensure evenness of drying due to the differences in thickness between vegetative and floral parts [4]. Dry succulents should be separated from other specimens whenever possible. Flowers may be detached and dried separately or layers of newsprint may be used to bring the different parts of the plant to the same level.

Very spiny or thorny plants: These can be very troublesome to press. Delicate flower or fruits should be pressed separately from the coarse vegetative structures. Side branches can be clipped off and pressed separately. If the specimen is forced into the press, the plant and the cardboard ventilators will likely be damaged quite badly. The separately pressed parts of the plant can be recombined after the specimen is fully dry [2].

Glandular-viscid (sticky) plants: The removal of soil particles from plants which have viscid hairs is very difficult if not impossible. Sand grains may cause leaves to bruise and blacken. Because glandular-viscid beach plants such as *Glehnia littoralis* are robust but often found encrusted with sand, they may be washed in the sea or a pool of water without damage. Most of the sand may be removed by soaking the plants for a few minutes while wiping the submerged leaves. Sticky plants such as *Drosera* are so sticky that when they dry they stick to newsprint and even more firmly to wax paper. It is difficult to open the newsprint to remove them. When removing the plant from the paper pull the paper at an acute angle, rather than lifting it up. Ease the paper away from the plant. Use a sharp knife where necessary. Often the plant cannot be removed using this method and it must be dampened with a wet cloth and the paper peeled off as soon as it loosens. If the dampening is done quickly, the specimen remains almost perfectly dry and will not curl [4].

Conifers: These lose their needles during the drying process. Consider the following options to prevent needle loss: Once the material is dried, the needles can be shaken off and placed in a packet [5]; leaves of conifers and heaths can be immersed for a few seconds in boiling water (do not immerse flowers) [3]; and adhesive can be spread on the back of a fresh specimen which is then laid on a sheet of mounting paper, covered with wax paper, and pressed. The needles may separate from the stalk but are held in position on the sheet [4].

Submersed aquatic plants: If an aquatic plant is removed from water, the plant is limp, and droops and masses together since the stems and leaves are so weak they cannot support the plant out of water. The specimen will be very difficult to

arrange and press properly. Aquatic plants should be floated on a sheet of paper (smaller than the herbarium sheet) in a shallow tray or in the field [2]. In the field, loosen the roots from the mud and float the plant near the surface of the water. Insert a herbarium sheet (or part of one) into the water; keep it horizontal just below the surface; float the plant on top of the paper; and arrange the stem, leaves, and roots as desired. Gently move the sheet and plant specimen out of the water. Cover with a sheet of wax paper to prevent it from sticking to the newsprint. Place the wet sheet and specimen enclosed in newsprint into the plant press [3]. It is much easier to arrange the plant material while the stems and leaves are supported by water [2]. Alternatively, plants may be washed in the field then brought back in mass in the field press or water should be placed in a plastic bag with the specimen to prevent wilting while waiting for pressing [6].The specimen can then be floated out in a sink. The mucilaginous slime, often the product of microscopic algae covering many aquatic plants, serves to stick them to the mounting paper. Cover with wax paper, otherwise, the specimen may be ruined as it is apt to adhere more firmly to the newsprint and may be very difficult to remove successfully. Aquatic plants dry quickly. The delay caused by the use of wax paper is not significant [4].

30.2 Identification of Plant Specimens

It is very important to have the best possible specimen which displays the root, stem, leaf, and flower and/or fruit. Most classification and keys are based on characteristics of the flower. Where possible, bryophyte and lichen specimens should include capsules and fruiting bodies [5]. While fresh material is best for identification, preserved specimens are identified most successfully when they have been properly collected and pressed to show necessary characteristics of the species.

30.2.1 Materials

Equipment includes the following: reference books (flora of the region, field guide, illustrated botanical glossary or dictionary); hand lens, dissecting microscope, or both; fine forceps; mounted needles; sharp scalpel or razor blade [5]. Herbarium material may be softened for examination by boiling it in a 5 % liquid soap solution for a few seconds. This action makes the dried plant material more malleable and softer so that it can be examined [9].

For identification of lichens, the following chemicals are normally required: concentrated potassium hydroxide, calcium hyperchlorite (bleach), and phenylenediamine (carcinogenic). For identification of bryophytes, the following equipment is required: compound microscope, glass slides, cover slips, and a stain such as methylene blue [5].

30.2.2 Methods

Procedure for Identifying Flowering Plant Collections

1. Soften dried plant material for examination by covering the flower in a 5% liquid soap solution, bringing the mixture to a boil for a few seconds (over a Bunsen burner), cooling the mixture, pouring the contents into a watch glass, and examining it under a dissecting scope using mounted needles and fine forceps.
2. Examine the specimen, particularly the flower. Make drawings or notations regarding the composition of the flower for future reference when using the keys. See examples of typical shorthand floral formats (Fig. 30.1a floral formula, Fig. 30.1b floral diagram, Fig. 30.1c half flower illustration). These formulas and diagrams used together will aid in presenting a complete understanding of a flower.
3. Select a flora (reference books for the identification of plants) appropriate for the location of the plant collection. It is also useful to have an illustrated glossary of botanical terms or a botanical dictionary for reference should terms used in the keys require clarification.

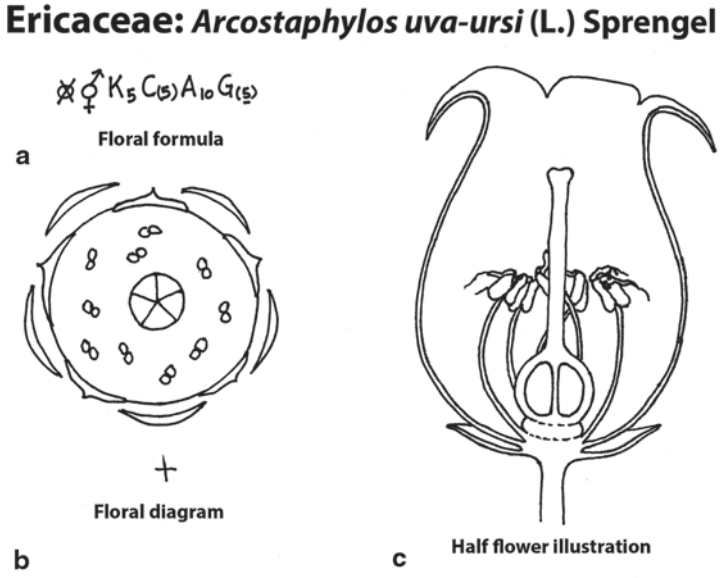

Fig. 30.1 Ericaceae: *Arctostaphylos uva-ursi* (L.) Sprengel (Illus. by L. Lassiter). **a** Floral formula, **b** Floral diagram, **c** Half flower illustration

4. Using keys in the flora, determine the family, genus, and species of the specimen. Most keys used today are dichotomous. A dichotomous key presents two contrasting choices at each step. Each pair of choices is called a couplet. The key is designed so that one part of the couplet will be accepted and the other rejected. The first contrasting characters in each couplet are referred to as the primary key characters, which are usually the best contrasting characters. Characters following the lead are secondary key characters [1]. See Table 30.1 [10].
5. Do not expect to use a key successfully without significant practice.
6. Compare the specimen to a plant of the same species using a herbarium collection or on-line resources. Read family, genus, and species descriptions found in floras for complete species information. If the plant appears different from the reference specimen and its associated description, repeat step 4 [9].

Table 30.1 Examples of taxonomic keys

Family key [10]		
Key to ferns and fern allies (Pteridophyta)		
1	Leaves small, usually scale-like	2
	Leaves broad, never scale-like	4
2	Stems jointed and hollow	Equisetaceae
	Stems not jointed	3
3	Stems less than 6-mm wide; plants less than 3-cm tall	Selaginellaceae
	Stems more than 7-mm wide; plants taller than 4 cm	Lycopodiaceae
4	Sporangia (spore sacs) produced on leaf underside	Polypodiaceae
	Sporangia produced in terminal clusters on specialized stalk	Ophioglossaceae
Genus key [10]		
Key to ferns		
1	Sporangia (spore-sac) located along leaf-margin	*Pellaea*
	Sporangia not located along leaf margin	2
2	Fronds once-pinnate	3
	Fronds 2 to 3-pinnate	5
3	Fronds evergreen	*Polystichum*
	Fronds dying in autumn	4
4	Sporangia 4–8 per pinna; frond stalk (stipe) reddish brown	*Asplenium*
	Sporangia many per pinna; frond stalk (stipe) yellow, green or brown	*Woodsia*
5	Ferns growing in moist, wooded areas	*Athyrium*
	Ferns growing on rocky ledges, crevices or slopes	6
6	Veins reaching edge of pinna	*Cystopteris*
	Veins not reaching edge of pinna	*Woodsia*
Species key [10]		
Key to Pellaea		
1	Fronds of 1 type; stipes brown	*P. glabella*
	Fronds of 2 types; stipes purplish black	*P. atropurpurea*

30.3 Herbarium Procedures

The herbarium is a collection of pressed and dried plant specimens generally organized either in a systematic or alphabetical classification sequence. Each herbarium will usually include many other diverse materials depending on the interests and resources of a particular herbarium including, but not limited to, photographs, drawings, books, reprints, and cone and fruit collections. Herbaria also preserve type specimens, historically significant collections, and they serve as a depository for experimental vouchers. Contemporary herbaria are complex and multifaceted: as Radford [11] observes, "The herbarium of today is a research, training and service institution that serves as a reference center, documentation facility, and data storehouse."

The effectiveness and value of a collection "depends on the care with which the specimens were preserved and mounted, the accuracy and completeness of identification and labelling and the satisfactory arrangement and maintenance of the collections so that they do not deteriorate and can be readily referenced when required" [5]. Herbarium collections are used in teaching, graduate and undergraduate students, as a resource for identification, a centre for biodiversity exhibited by its many collections, and a source for research materials. Plants collections and other herbarium materials are fundamental references for basic and applied research in botany, biology, agriculture, medicine, pharmacy, and genetics [11].

Herbarium collections arranged in sequence by plant family, either alphabetically or, more typically, by one of the systems of classification. "Each herbarium has an alphabetical index to the families which indicates the cabinet number in which the family is housed. Each cabinet has a shelf list which lists the contents of the cabinet by family and then genus." [4]. Specimens may be separated geographically through the use of folders of different colours. Specimen folders are stored in a cabinet designed to preserve the collection by decreasing exposure to light, insects, moisture and controlling the temperature [6].

Some special collections, such as type specimens, historically significant or rare specimens, may be housed in separate cabinets to offer extra protection. A reference collection may also be present to reduce the use of the main collections for identification checks or study purposes. Specimens within these collections will often be kept in special folders. The special collections will be arranged in the same order as the main collections.

30.3.1 Specimen Preparation

30.3.1.1 Arrangement tips

1. Following identification, the plants may be laid out for mounting. Before beginning, write in pencil the collector and collection number on fragment packets and in the lower right-hand corner of the mounting sheet, even if the label is available

and will be applied right away. If the specimen gets separated from its label, then valuable information could be lost. Paper-clip any tags, field notes, or other identifiers to the sheet as well until the finished label is applied [3].

2. Specimens should be laid on the sheet in an attractive, space-filling way [4] (Fig. 30.2). All plant parts such as the following should be clearly displayed: leaves (both surfaces), roots, fruits, flowers (both faces and side views) [3]. Leave space in the lower right-hand corner for the herbarium label. Fill the sheet as full as possible without crowding the sheet; therefore, the specimen should not occupy more than about three quarters of the sheet. The specimen should be placed on the sheet, so that it does not come within an inch of the edge at any point [4]. When the arrangement is completed, the specimen may be attached to the sheet [5].
3. An annotation label may be attached to the sheet following examination by a specialist (Fig. 30.3). These labels are generally attached by one edge only and they should not overlap the specimen [4].
4. If plant material is removed from the specimen, the material should be put in a packet attached to the sheet, so that the next examiner will not have to disturb the specimen further [4] (Fig. 30.2).
5. Do not pack a large number of small specimens on a sheet. Display enough specimens to show the variation in size and other characters and to provide ample flowers and fruit [4].
6. Duplicates of specimens should be laid on sheets of newsprint or other light paper. As a check against the mixing of labels, write the collection number on each sheet [4]. Do not mount duplicate material. Leave the material in the original flimsy with a duplicate label [3].
7. Do not mount solitary small specimens or packet containing specimens in the center of the sheet, since if we collect several specimen in one folder, there will be a bulge at this point. Practice a rotation in mounting such specimens [4].
8. Although it is generally better to have the base of the plant toward the bottom of the sheet, the position should be varied in plants with very thick bases so that the sheets stack evenly and occupy the minimum of shelf space [4].

30.3.1.2 Attachment of Plant Specimen to Herbarium Sheet

Vascular plant specimens may be attached to herbarium sheets with glue, plastic strapping, or linen tape. All materials for attaching plant specimens to herbarium sheets have their advantages and disadvantages and many herbaria have their own preferred method of mounting specimens.

Glue which dries quickly, transparently, and contains no ingredients likely to harm or alter the specimen is most effective (Fig. 30.2). Do not use rubber cement. Glues which have been used successfully include Weldbond and Elmer's Glue-all. However, pasting does not allow easy removal of parts for examination. Glue may not have the strength of tape or strapping to hold the specimen firmly in place.

Fig. 30.2 Herbarium specimen of *Gentiana platypetala* Griseb

Plastic strapping is transparent and strong but the ingredients for plastic strapping are highly carcinogenic (toluene), and in the presence of moth balls the plastic may become tacky [5]. For neatness, strength, and speed of application, plastic reinforcement is better than linen tape. Because plastic reinforcement is fully transparent it does not conceal details such as leaf teeth. If a specimen reinforced with this material is put under strain, the paper tears before the plastic breaks. With a little practice

Fig. 30.3 Herbarium specimen of *Carex nigricans* C. A. Mey

it can be applied very quickly and neatly. Apply a strip of plastic across the stem or other part and anchor it to the sheet on each side. If working with large leaves, put a drop here and there, half over the edge. A drop squirted into a mass of fibrous roots will anchor them more firmly and much more neatly than a long linen strip [4]

Linen tape (Fig. 30.3) is strong but may cover some areas of the plant and be considered unsightly. The linen tape method is time-consuming but it is still the most desirable for several reasons. Linen tape and plastic strapping may be removed without damaging the plant specimen. Very little of the specimen is in contact with the glue backing and thereby not contaminated by it. There remains enough flexibility that the plant can slide within its bindings somewhat, thus preventing some wear and tear during handling. Since no drying time is required, the specimen processing is complete once the tap is applied [3]. Never use cellulose tape or surgical tape for reinforcement. The adhesives in these materials spread out at the edges of the strip, picking up dirt and disfiguring the specimens. Cellulose tape also becomes yellow and brittle with age [4].

30.3.1.3 Mounting Supplies

Materials required for mounting specimens are very dependent upon the type of method used for attaching the specimens to the herbarium sheets. Mounting paper (16.5" (420 mm) × 11.5" (297 mm)) is available in various callipers. One calliper should be chosen and used consistently. Basic supplies required for most methods include the following: mounting board, adhesive (gummed linen tape, glue, or plastic strapping), forceps, fragment packets, dissecting needles, herbarium labels, kimwipes, wax paper, corrugated cardboard, and weights. If sewing is involved, then a needle (crewel embroidery type) and linen thread will be required. All paper products should be acid-free and pH neutral 100% cotton rag paper, so that they will not turn yellow or crack with age. Water-soluble cellulose glue such as Elmer's should be used, and trays or jars for glue should be available. Paintbrushes of varying sizes may be used to spread glue. Cardboard or plywood with wooden pegs may be used to separate specimens and assist in drying [3, 4, 6, 12]. The plastic strapping contains ethyl cellulose (ethocel) standard 7 eps (250 gm), Dow resin 276 V-2 (75 gm), methanol (200 cc) and toluene (800 cc) [4].

The following materials will also be required in the cellulose glue method: pieces of foam, plate glass (sheet 12" × 18") or an enamel tray for herbarium specimens, and a smaller piece of plate glass (ca. 5" × 5"), optional, for putting glue on labels, fragment folders [3].

30.3.2 Methods

30.3.2.1 Vascular Plants

1. Determine the best fit for the specimen on the herbarium sheet (mounting paper). Leave sufficient space for a label (lower right-hand corner), an accession stamp (upper left-hand side of the sheet, upper left corner preferably) and a fragment packet (vary placement on the sheet). Also vary placement of bulky portions of

the plant specimen from sheet to sheet. This will result in an even stack of plant specimens. Mount a few leaves with the lower surface facing up. The specimen should be arranged on the sheet to be as aesthetically pleasing as possible [12].

2. Apply white glue to the specimen either by evenly spraying the back of the plant with the glue while it is on the newsprint in which it came or by dabbing glue along the underside of the plant. Mask some flowers and fruits with small pieces of paper or lightweight cardboard or leave some free of glue for easy access for future study. Blot the glue if necessary [12].
3. Carefully place the plant on the mounting paper, glue side down in the position determined in step 1.
4. Cover the specimen with a sheet of wax paper. Use weights to hold the specimen down. Weight down 'ends' of specimen so that the specimens do not curl up while drying. Plastic strapping glue or gummed linen tape may be used to secure strategic areas such as stems, ends of stems (especially woody ones), peduncles, pedicels, scapes, petioles, and the tips of large leaves. Leave some leaves or flowers free. Plastic strapping should be as thin as possible with one inch being a safe maximum length. Longer straps may break or cause the sheet to curl upwards. Sewing the plant to the sheet using linen thread is preferable to punching holes to provide contact points for plastic straps [12].
5. Glue on the label and annotation labels, if present. "To attach label, spread glue over the upper half of the label back. Turn label right side up and press upper half firmly to lower half of label beyond edge of sheet. Then, slowly slide label up until even with lower edge of sheet. This distributes glue evenly over back of label, and prevents wrinkling or loose edges. Cover mounted label with wax paper then weights. Place annotation label just above the label on the right side. To attach annotation labels, use the same basic procedure. Spread glue on back of left side of label and slide label to the left" (Fig. 30.3) [12].
6. Select a suitable size of fragment packet and glue it onto the sheet by placing a strip of glue along the centre of the back of the packet. Place on sheet and cover with wax paper then weights. Fold fragment packets to the required size using pH neutral or rag paper. Fragment packets should not overlap the specimen [12] and should be labelled with your name and collection number [3].
7. If several plants are to be mounted, a stack of drying specimens may be created by covering the first specimen with a sheet of wax paper, a felt then the next specimen, wax paper, felt, etc. until the final specimen which will be covered with wax paper, felt, a heavy cardboard, and weights [12].
8. Specimens should be left to dry under the weight for several hours or preferably overnight [4]. Then, remove weights.
9. When dry remove the specimens from between the wax paper and felts and reinforce them using gummed linen tape or plastic strapping if this has not yet been done in step 4. Reinforce the stem every 3 in. or so, preferably in the middle of the internodes. Reinforce large leaves near the tip and on the sides and reinforce large inflorescences at several points. If tape is used do not cover the actual tip of the leaf [12]. Do not cover all examples of a given characteristic. Important features should remain visible [3].

10. Weldbond glue—Weight down the plant around the glue to prevent warping and to ensure a tight bond to the paper. The glue should make a continuous thin strip across the specimen and make contact with the paper at both ends. Weights are not required with linen tape since the gummed adhesive will bond in place within a few seconds [3].
11. Linen tape is cut into strips of various widths and lengths in advance. Manipulate the strips using curved forceps. Moisten and gummed backing using a moistened sponge. If too much water is applied, the glue will take too long to bind to the paper or may wash the glue off the tape completely. Use the strips to tack down the main stem of the specimen. Use only as many strips as are required to immobilize the specimen. Tape may be required on parts too close to the edge or where parts curl up [3].
12. The strip of linen tape should be extended about half an inch beyond the part to be covered (Fig. 30.3). Moisten the tape and hold it down firmly with bent forceps. Avoid long tapes as they give almost no support, especially when covering broad masses of grass leaves and stems [4].
13. If a specimen consists of several small plants (less than 5" and/or delicate), then the specimen may be mounted in a large packet which is then attached in varying position to the sheet. One or two specimens can be mounted directly on the sheet as an example if desired [12].
14. Sewing. Large or bulky portions of plants may be sewn to the sheet if a plastic strip would be too long or too thick. After the glue has dried, use heavy linen thread to sew woody stems, cones, large roots, large fruits, and broad leaves. "Cut a length of thread appropriate for the structure to be sewn. Place the sheet at the edge of the mounting label so that the area to be sewn overhangs the table. Wet the thread and bring the threaded needle up through the sheet from beneath" [12]. Pass it twice or more over the specimen and knot it on the back of the sheet [4]. Tie each stitch off separately on the underside of the herbarium sheet. "When sewing flat structures such as leaves, the needle should come straight up through the paper. When sewing bulky structures, the needle should come through at an angle slanted toward the structure to be sewn. Leave the end of the thread dangling below the sheet and bring the thread (straight or angled depending on the structure) over the structure and down through the paper. Knot the two loose ends firmly" [12]. Use a square knot with a double overlap at the beginning, followed by a single overlap. Trim the thread. Cut a piece of linen tape about 2 in. long and secure the knot. Use a separate piece of tape for each knot, unless they are very close together. Do not use long strips of tape [12].
15. With some objects, such as large pieces of bark or *Opuntia* pads, pairs of small holes may be drilled through which the thread can be passed. Glue another sheet of herbarium paper underneath or cover the stitches with a strip of linen tape or gummed paper to cover the threads on the back of the sheet and to prevent damage to the specimen immediately below [4].

16. Mount tree and shrub branches, conifer cones, or large cactus on heavier weight herbarium sheets or if not available glue a second sheet to the back of the original [3, 4].
17. Fumigate (if out of the cabinet for more than one hour), accession, and file.

30.3.2.2 Cellulose Glue Method

The University of Alberta Herbarium [3] describes the procedure as follows:

1. Spread glue of a good consistency out on a sheet of plate glass or in an enamel tray using a paintbrush. The specimen must be able to slide off the glass relatively easily. Brush away any plant debris that accumulates during the mounting process. Adjust the consistency of the glue by adding more water or glue.
2. Place the label (face up) on the glue-topped glass. Remove and place it on the sheet of herbarium paper. Smooth out bubbles and mop up any excess glue with a tissue or paper towel. Determine the arrangement of the plant specimen. Place the plant specimen down gently in the glue. Carefully tap down on parts that are not in contact with the glass spreading the glue on all parts of the specimen.
3. Slide the specimen off the glass at an angle of roughly 45°. For particular specimens such as woody stems paint the glue directly onto the specimen. Place the plant specimen onto the herbarium sheet. Blot up any excess glue. Glue the fragment packet using the same process as the label.
4. Cover the herbarium specimen with a sheet of waxed paper, polyurethane foam, and a stiff piece of cardboard and weigh it down with weights. The polyurethane foam will cushion bulky or fragile specimens. The foam is not required for flat specimens. Remove weights and stack additional specimens in a similar fashion, then replace the weights. The glue should be left to dry overnight. Gummed linen tape may also be used to reinforce bulky or stiff material.

30.3.2.3 Mending and Remounting Specimens

When specimens require mending they should be put aside in a cabinet. When mending a specimen, use linen tape, sewing or glue just as when mounting new specimens. If desired, a packet may be mounted on each sheet whether or not one is presently needed. In extreme cases, the entire specimen may require remounting on a new sheet of herbarium paper or other substantial repair is required [12]

To remount, remove the specimen carefully from the paper (tape or plastic strapping) or cut the paper around it (glue). Mount the specimen as described in the above procedure if the specimen has been removed from the original herbarium sheet. If the paper was cut around the specimen, glue the paper directly to a new sheet of herbarium paper. Cut the label off and glue label in the lower right corner of the new sheet. Stamp the new sheet with the herbarium stamp. Set the automatic numbering machine to the accession number on the old sheet and stamp the new sheet with this number [12]

30.3.2.4 Mounting Nonvascular Plant Specimens

Tips—Bryophytes and Lichens

1. Once bryophyte and lichen specimens are dry (not pressed but air-dried in small bags or packets), they may be stored permanently in herbarium packets. These should be of good quality, 100 % rag paper or acid-free paper. If the packets are folded around a 10–15-cm index card, they will be stiffer and of uniform size [5].
2. The advantage of these packets is that they can be opened flat for examination of the specimen [5]. These packets may be stored upright or glued onto 13 cm × 20 cm sheets of herbarium paper [12]. They may be stored in shoe boxes or other suitable boxes [5].
3. Write information directly on the front of the envelope or on a herbarium label pasted onto it.
4. Ensure that the specimen does not become separated from its label. Write the name and number of the collector on the bag or packet which contains the specimen [12].
5. A thin sheet of foam may be placed in the packet to help keep the specimen from sliding down to the bottom of the packet. Since lichen and bryophyte specimens are stored vertically, this is a necessary precaution [12].
6. Bryophyte and lichen specimens may be accessioned by stamping with an automatic numbering machine on the left side of the 13 cm × 20 cm sheet so that the oval of the stamp has its long axis vertically on the card [12].
7. File the specimen [12].

Tips—Algae

1. Algae are usually mounted by the collector in the field. Float the algae in a flat pan with water from its habitat. Slide the specimen onto herbarium paper (full size). Cover the specimen with wax paper to prevent sticking, then place the specimen in a regular plant press for drying. When dry, alga has enough mucilage to glue itself to the paper [12].
2. If specimens are received on full size herbarium paper, then glue on label, accession, and file. Otherwise if the specimen is received on paper smaller than herbarium sheet size then glue the smaller sheet to a full size sheet of herbarium paper. Attach label, accession, and file [12].

30.3.2.5 Labels

A herbarium label is a permanent record of the collection data (Figs. 30.2, 30.3). Most of the data are drawn from the field notebook. A properly prepared herbarium specimen can last for hundreds of years [2]. A typical label requires the organization of data into the proper format. The label information must include [3]:

Heading: Name of the Herbarium/Company or the project/thesis
Title: "Plants of ……." (region, province and country)
Name: Genus (capitalized) species (lower case), underline or italicized, Authority
Family: (optional), Common Name (optional)
Location and district, Lat. & Long., Elevation
Habitat and Abundance
Notes: Description of plant (flower colour, habit, height, etc.), if applicable
Name of Collector, Collection #, and date
Det.: Name of person who identified the specimen (i.e. yourself, initials may be used if the same name as the Collector, or the full name of another expert) and the date

The information on the label can be broken into three categories: (1) Information that remains the same on all labels. This may be preprinted on the label form, and often includes the heading and sometimes the collector and other data. (2) Information that remains the same for all plans collected together at the same location and the same time. This includes the collector, date, locality, elevation, and general summary of the habitat. (3) Information that changes from plant to plant. This includes the scientific name, the family, the relative abundance and specific habitat, flower colour, and collection number [2].

Tips

1. To avoid confusion or misinterpretation, write out dates in full. For example, "June 12, 1981" is preferred over "6/12/81" or "12/6/81."
2. Use waterproof ink which will not run if it becomes wet or smear when touched [3].
3. The label must be printed on plain, pH neutral, unlined white 100% rag bond paper (not index cards).
4. Herbarium sheet labels are typically 8 cm × 13 cm or 8 cm × 10 cm [3]. The size of the label can vary but most are not more than about 10 cm × 13 cm, and they are often smaller.
5. Attach the label to the lower right corner of the sheet. Either attach the label by a narrow strip along the top edge, or if there is insufficient space for the label, attach it by the right-hand edge and allow it to overlap the specimen. Do not paste down the whole label since this might make the corner of the sheet curl [4].
6. Most herbaria now keep specimen records in a database and have programs which create labels automatically. Before collecting and donating specimens to a herbarium, determine the database requirements so that information can be provided in an acceptable format [5]
7. The following additional information may be placed on labels when appropriate: (1) associated plants, (2) flower colour, (3) pollinators, (4) bark, (5) abundance, (6) height or diameter at breast height (DBH), (7) life form, (8) economic uses, and (9) folklore [1].
8. Determine the number of duplicates and print labels for the main sheet and duplicates at the same time.

30.3.2.6 Accession Numbers

Accession numbers are used for keeping track of specimens used in research and teaching [6]. Accession any new plants added to the herbarium through exchanges, donations, or staff collections. Specimens are normally accessioned in sets so all the specimens in that set have consecutive numbers. If sets (exchange specimens or specimens from collection trips) are too large, this may not be possible. Specimens should be accessioned using the following procedure [12].

1. Record the material that is being accessioned in the accession book by stamping the accession number for each sheet in the accession book and writing the genus and species next to each number. The herbarium logbook or accession book is used to keep track of the specimens accessioned into the collection [6].
2. All sheets should be previously stamped with the herbarium stamp in the upper left-hand corner of the mounted specimen [12], whenever possible, or on the upper part of the sheet (Fig. 30.2) [4].The herbarium stamp typically has a space left for the accession stamp.
3. Number sheets consecutively, usually with a consecutive numbering machine. Check the accession book to find out what the first number should be for the set of plants to be accessioned [12].
4. Stamp each herbarium sheet with its own accession number. Turn the dial on the front of the numbering machine to 'consecutive' and stamp the first sheet by placing the number stamp in the centre of the herbarium stamp (upper left corner of the herbarium specimen). Stamp this same number as the beginning number in the first space in the accession book. Do not stamp over any part of the plant, envelope, or label [12].
5. Do not continue stamping with fading numbers. Apply ink to the stamp pad as required. Let the ink soak in, and reapply several times. Stamp the number on scrap paper several times until the image is clear [12].
6. Number all specimens in the set except the last one.
7. Turn the dial on the numbering machine to 'repeat'. Number the last sheet and also stamp this number as the ending number in the second space in the accession book [12].
8. If the accessioned specimens have been out of the cabinet for more than a few hours, place them in the fumigator or freezer. Once treated, place in the 'to be filed' cabinet [12].

30.4 Preservation of Specimens

30.4.1 Protection from Insects

Common herbarium pests include the following: silverfish, book lice (*Psocids*), dermestids (cigarette or tobacco beetle (*Lasioderma*), black carpet beetle, and the

drugstore beetle (*Stegobium panicuem*) [1, 8]. All of the above can cause extensive damage to specimens in cabinets; however, the most destructive pest is the cigarette beetle that multiples very quickly. Its life cycle is completed in 45–50 days with three to six generations per year [1]. Proper protection against insect damage may require not only suitable cabinets but also fumigation of an entire building in which the cabinets are stored.

Moth balls (naphthalene or paradichlorobenzene) are no longer used to repel insects because of their toxicity [8]. Herbaria in tropical climates are more vulnerable to insect infestations, and may require careful use of chemicals. All compounds used as repellents or fumigants are hazardous to some degree, so much care must be taken in their use.

The following precautions will minimize damage.

1. The whole herbarium should be in insect-proof storage units. All specimens should be returned to these cases at night. Keep specimens in the herbarium as much as possible. Keep cabinets tightly closed. Close the door immediately after removing specimens and leave the door closed until the specimens are returned [12]. Regularly inspect cabinets to ensure that gaskets between doors and frame provide an effective seal [8].
2. All incoming specimens must be opened promptly on arrival, checked for dryness, and put in the drier if necessary. Fumigate all specimens coming into the herbarium even if they were removed for only a few hours since fumigation kills insects which may be hiding on the dried plant specimens [12]. Incoming specimens may be treated by heating at 60 °C for a period of 6 h. This temperature will effectively kill all stages of the dermestids [1]. Fumigation, isolation, and freezing of specimens are all useful tools in preventing insect damage.
3. In the freezing of newly collected plants, the specimens must be completely dry. Transfer the press into a no-frost freezer and leave for 1 week or for at least 7–10 days at −20 °C [8]. All dried or loaned specimens need to be frozen before they are filed in the herbarium collection. This process kills pests such as the herbarium beetle (*Stegobium paniceum*) or the tobacco beetle (*Lasioderma sericorne*) [6] as well as any eggs or fungal spores. Another method is to freeze plants for 5 days; remove for a day or two; freeze an additional 5 days. This process is considered the best way to ensure that all possible insect pests are eliminated [13].
4. Keep herbarium, preparation rooms, and supply rooms uncluttered, so that thorough cleaning can be conducted. Any litter may contain insects and the greater the population the smaller the probability of a perfect kill in fumigation. There should be no baseboards or crevices between wall and floor. If necessary, clean cabinets before new specimens are placed in them [4].
5. The temperature of the herbarium should be below 21 °C and the humidity should be at 30–40 %. Low humidity as well as proper drying will protect the specimens from mildew [1]. There should be sufficient lighting.

30.4.2 Handling of Specimens

Herbarium specimens are valuable and irreplaceable scientific material. Because they are brittle and easily damaged, they must be handled with extreme care. The following rules should be observed:

1. Store the material safely in herbarium cabinets. Always return specimens to the cabinet immediately after use unless staff instruct otherwise. Do not leave specimens on the bench overnight. Material must be studied in the herbarium, rather than in offices or other locations [3].
2. Do not overfill shelves or storage space lest the material be damaged during removal and return [3]. The weight of the top specimens should be limited to prevent damage to the lower specimens [8].
3. Do not place books or heavy objects on herbarium specimens. Do not lean on stacks of specimens.
4. Do not squeeze the cover of specimen folders since such pressure can severely damage dried plants. Do not slide specimens from between other specimens in the folder. Lift the specimens that cover the desired sheet and set them down elsewhere. Remove the desired sheet [12].
5. Use two hands to support the stack from both sides when removing folders from cabinets. Transport the stack on a sheet of cardboard to prevent bending of the sheets. Keep specimens mounted on sheets flat. Move the stack to a bench or table for examination [3]. Keep specimens aligned in folder when returning them to the cabinet [6].
6. Pick each specimen sheet up directly off the stack with both hands so that it does not slide across the sheet below [3]. Do not turn specimens upside down. Replace specimens in the same order as they were in when removed from the cabinet [8].
7. Do not flip through a stack like the pages of a book [3] and never hold folders vertically [6].
8. Always keep sheets in their flimsies (folded clean newsprint or white paper). This prevents the loss or mixing of any fragments. Make fragment packets and attach to sheets as necessary [3].
9. Microscopes and tools are available. Do not dissect or remove samples of the specimens unless specific written permission for removal of material has been received from staff in advance. Each individual specimen must receive individual permission. Permission is never granted in the case of type specimens [3]. Examine prepared specimens on the flat surface [8].
10. Annotations or notes should be made on standard annotation labels with permanent black ink. The label information should include the new determination and/or other notes, the name of the examiner, his or her affiliation, and the date. Use paper clips to attach the labels to the sheets; they will be permanently attached to the sheet by staff. Use an annotation label for each specimen; do not use one label for an entire folder [3].

11. Inform staff if you notice insects, insect damage, and/or evidence of the presence of insects such as shed exoskeletons or a powdery residue around damaged flowers or leaves [3].
12. If specimen labels or folder labels are becoming unglued please inform the staff. The labels should be attached with paper clips to the appropriate sheet if they have not become separated. Tape should not be used. If bits of plants are broken or damaged during use please inform the staff so that the pieces may be collected and placed in packets. Do not dispose of any plant material from the specimens [8].
13. Personal specimens brought into the herbarium must be pressed and dried. They must then be frozen at −20 °C for a minimum of 24 h (or −10 °C for 4 days), before they can be brought into the room [3]. If the specimens are removed from the herbarium they should be treated as incoming material before being refilled [6]. Fresh field specimens are not allowed in the herbarium since they could introduce contamination or insects [3]
14. Unmounted dried plant specimens in newsprint are more fragile than mounted plants. Support newsprint folders from beneath and do not flip them over [12].

30.4.3 Destructive Sampling for Molecular Research

Scientists may use material from herbarium sheets to obtain material for DNA research. A balance must be achieved between the need for scientific research and the preservation of herbarium specimens. When requesting a loan or examining specimens at a herbarium, written permission must be received in advance before such sampling may occur. The University of Alberta Herbarium [3] recommends the following guidelines:

1. Remove the absolute minimum leaf material per sheet. When possible, take material from packets. A 5-mm diameter circle (paper punch size) or less is the goal, especially for small plants.
2. Annotate specimens to the effect that a sample was taken, the purpose, name of researcher, institution, and data.
3. The annotation label should state the DNA accession details for successful sampling, i.e. DNA accession number and storage location. Also, note if no successful results were obtained.
4. Any publication using the data should note the herbarium accession number and a copy of the publication should be sent to the herbarium.

30.5 Protocol for Filing Specimens [12]

Specimens in the herbarium are filed by family. Families are arranged phylogenetically or alphabetically. Within a family, genera are arranged alphabetically. Within a genus, species are arranged alphabetically. Within a species, subspecies and/or varieties

are arranged alphabetically. At the end of each genus is an A–Z folder, in which species represented by fewer than three specimens are filed. After the A–Z folder there may be an 'undetermined' folder for specimens identified only to genus [12].

1. Specimens to be filed are stored in a separate cabinet.
2. File each specimen according to the name on the label. If annotated, file according to the most recent annotation. Carefully determine the family to which each plant belongs.
3. Arrange sheets in order of the families. Refer to the alphabetical list of families, with their associated cabinet numbers, posted in the herbarium. Locate the family and cabinet number for the specimen.
4. Refer to the cabinet shelf list to locate the proper genus and species folder. If the folders are arranged geographically, make sure to insert the specimen in the proper folder. Otherwise, place the specimen in any of the species folders available. If no folder exists for the species or the folders are full, make new folders as necessary. Label folder '*Rosa woodsia*' I, '*Rosa woodsia*' II, etc. [6, 12].
5. If there is no species folder for the specimen to be refiled, check in the A–Z folder before making a new folder. Insert the specimen sheet alphabetically into the A–Z folder. Make a new folder for the species if, with the addition of the new sheet, there would now be a total of three sheets.
6. Likewise, if there are more than two sheets of a subspecies or variety, place that subspecies or variety in its own folder.
7. Each folder should have two labels. On the lower left corner, write Genus or Genus and species; on the lower right corner, write Family. Styles for labelling of folders vary from herbarium to herbarium.
8. Lichens and bryophytes are filed alphabetically and, typically, vertically in packets by species within a genus. Genera and species are separated by index cards. Write the generic and/or species name on the index card.

30.6 Protocol for Loans and Exchanges

30.6.1 Loans

1. Loans are usually made to an institution, not to an individual. When a loan is requested by another institution, pull and send the loan [12]. Borrowers must adhere to all provisions of the loan [1].
2. Researchers requesting to borrow material should have a letter sent from their curator. Loans are normally for a period of 1 year [3]. Large loans and loans made to distant herbaria are given for longer lengths of time. Requests can be made for longer loan periods, and loans can be renewed [12].
3. Researchers requesting the loan have the responsibility of annotating the specimens and revising identification. Dissections are made with care and all

fragments placed in fragment folders. If material is removed, special permission must be obtained in advance. The borrower must annotate the specimens before returning the loan. Do not make annotations directly on the collection labels or on the sheets. Use annotation labels. The annotation labels should be glued above the herbarium label. Each annotation label should have the complete scientific name of the plant including authority as well as the full name of the person making the annotation, and the year in which the annotation was made. A convenient way to indicate agreement with the identification is an exclamation point (!), meaning 'I have seen it and agree with the identification' [1].

30.6.2 Shipping Loans [12]

1. Pack specimens as carefully as possible to avoid damage. Pack ten specimens in each folder. Create a packing folder by taping two genus covers together.
2. Mounted specimens. Protect each mounted sheet with a single sheet of newsprint on top of it. Stack ten specimens with the sheets of newsprint in the folder, and seal the folder with a long strip of brown gummed tape. Label the folder (e.g. 10 *Arnica*). Record date and destination.
3. If sending unmounted specimens, pack ten unmounted specimens with labels (in newsprint) in a folder. Seal the folder and stack five folders of specimens together between sturdy cardboard. Tie the folders together with string. Keep the stack as flat as possible by reversing the order of the folders.
4. Pack the folders in a box, without undue pressure on the specimens, but as tightly as possible using packing materials.
5. Fill out mailing labels. Place one inside the box.
6. Tape the box closed, wrap in brown wrapping paper, and seal the box with the gummed paper tape.
7. Use two strips of nylon filament tape, encircling the box, to reinforce the package. Do not use string.
8. Attach the second mailing label to the outside of the package.
9. If sending type specimens, then enclose each type specimen, along with its protective single sheet of newsprint, in its own folder. Clearly label the folder as a type specimen (holotype, isotype, etc.) of a particular genus and species. Pack type specimens separately in bundles of type folders.
10. Mark folders or shelves in the main herbarium and associated collections where specimens have been removed.
11. Complete loan forms (triplicate). Original—returned to the loaning herbarium when the study is complete; second copy—returned to the loaning herbarium when the shipment is received; third copy—placed in the files of the loaning institution. The third copy is discarded when the second copy is returned. This process is now undertaken by e-mail.
12. Fill out particular loan details (number and names of specimens, loan date, loan period, and herbarium) in the herbarium loan records.

30.6.3 Re-filing Returned Loans [12]

1. Check the loan specimens for damage. Put any loose fragments from a sheet in the packet on that sheet. Verify the packing list.
2. Check in the specimens against the loan forms and confirm that they have all been returned, then sign the loan form and return this form to the borrowing institution.
3. Cancel the loan and enter this information with the date of cancellation in the loan book.
4. Fumigate, then sort the specimens into groups according to the original labels, or annotation labels, if these have been appended to the specimens. Make any new folders which may be required.
5. Remove the loan markers from the main herbarium and associated collections.

30.6.4 Receiving and Returning Loans from Other Herbaria [12]

1. The person who requested the loan must receive and return loans from other herbaria. Space will be provided in herbarium cabinets for these specimens and for specimens sent for examination.
2. Unpack and count specimens carefully. Record each species using a temporary loan number.
3. Fumigate specimens.
4. Place in cabinet space allotted by herbarium. Keep the specimens in the cabinet, securely closed, except for examination and study.
5. When returning the loan, count and verify the number of specimen. Double check against the loan forms from the institution.
6. Pack specimens and fill out mailing labels.
7. Fill out, photocopy and return any forms required by the lending herbarium. Attach the photocopy to the records of the loan.

30.6.5 Exchanges

Selection, Sorting, and Shipping Exchange Specimens

1. Keep credit or debit records for each exchange institution. Keep records in a chronological sequence with a master total, either credit or debit under columns Date, Balance, Specimens Sent, and Specimens Received [4, 12].
2. Shipments will probably be in duplicate sets. Sort the specimens into exchange sets. One specimen from each duplicate set goes into an exchange set [12].

3. Number the exchange sets. The originating herbarium gets exchange set number one. Because this herbarium has a set of these specimens, start with exchange set number two (2). Temporarily store exchange sets in herbarium cabinets [12].
4. After the lots of specimens are sorted, they are arranged alphabetically, and the name, accession number, and host are entered for each institution. This practice accomplishes the following: it prevents the inadvertent sending of duplicates or near duplicates, and allows for tracing of duplicates so that name changes may be sent when necessary [4].
5. Do not overload exchange sheets lest the recipient discard the specimens in excess of the sheet capacity. Instead, make extra duplicates [4].
6. Exchange specimens are sent to particular herbaria based on many factors including the following: the known interests of the particular researchers at a herbarium; the need for wide distribution of rare or critical collections; the balance of the exchange account; or the requirement to distribute specimens such as isotypes based on published information [4].
7. Mark the destination on each packet lightly in pencil as soon as the distribution is decided [4].
8. Pack specimens tightly in firm boxes that will not be crushed in shipment. Use crumpled paper or other elastic packing to keep the specimens from moving during transit. Very large shipments are put in two or more boxes [4].
9. When all the specimens are in the box, a shipping label and packing are added to the box which is then closed and sealed. Wrap the box in heavy brown paper and tie with well-knotted cord. Send the specimens with exchange forms [12].
10. Address the parcel and attach necessary customs forms. The contents are declared as 'Dried Botanical Specimens—No Commercial Value' [4].

30.6.6 Receiving Exchange Specimens [12]

1. Upon receipt of a shipment of exchange specimens, unpack immediately, then count the specimens. Fill out a shelf marker which will remain with the specimens until they are counted and accessioned.
2. Sign the form sent with the specimens and return it to the sending herbarium. Record exchange shipment in the exchange book, changing the running balance. File correspondence relating to the shipment in the exchange file for the sending herbarium.
3. Fumigate.
4. After fumigation, place specimens with the shelf marker in the assigned herbarium cabinet. Mount and accession the specimens. Re-fumigate, and place the specimens in the 'to be filed' cabinet.

Acknowledgments We would like to thank Lynne Perras for proofreading the manuscript. As well, we would like to thank Lynne Lassiter for providing illustrations of *Arctostaphylos uva-ursi* (L.) Spreng.

References

1. Jones SB Jr, Luchsinger SE (1986) Plant systematics, 2nd edn. McGraw-Hill, New York
2. Walters DR, Keil DJ (1988) Vascular plant taxonomy, 3rd edn. Kendall/Hunt, Dubuque
3. University of Alberta, Department of Biological Sciences herbaria (nd) Herbarium handbook. University of Alberta, Edmonton
4. Savile DBO (1962) Collection and care of botanical specimens. Publ. 1113. Queen's, Ottawa
5. Bowles JM (nd) Guide to plant collection & identification. Queen's University, Kingston
6. Central Michigan Herbarium—http://cmcherbarium.bio.cmich.edu/collection_collection.html
7. Missouri Botanical Garden—http://mobot.org/MOBOT/Research/Library/liesner/intro.html.
8. National Park Service (2009) Preparing and storing herbarium specimens. Conserve O Gram Number 11/12
9. Chinnappa CC (1980) Botany 441; taxonomy of flowering plants lab manual. University of Calgary, Calgary
10. Hallworth B, Chinnappa CC (1997) Plants of Kananaskis country. University of Calgary and University of Alberta Press, Calgary
11. Radford SE (1986) Fundamentals of plant systematics. Harper & Row, New York
12. Washington State University (1976) Manual of procedures for Marion Ownbey Herbarium. Washington State Universiity, Pullman
13. Mitrow G (2014) Personal communication Department of Agriculture Herbarium (DAO), Ottawa

Index

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols*,
DOI 10.1007/978-3-319-19944-3