PREFACE

Although there is a copious supply of electron microscopy textbooks and monographs concerned with animal cells and tissues, there are few whose thrust is plant cell biology. Because of the unique characteristics of the higher plant cell, i.e., the cell wall and often a large central vacuole, the techniques employed for animal cells are often not directly applicable to plant cells. Because many of the available methods for the optical microscopy of plant cells/tissues are now quite dated, *Plant Electron Microscopy and Cytochemistry* includes chapters regarding light microscope cytochemistry, autoradiography, and immunocytochemistry. Recent developments in fluorescence, confocal, and dark-field microscopies are highlighted. Light microscopy is often employed in conjunction with electron microscopy as correlative microscopy. With regard to electron microscopy, recent advances in conventional transmission and scanning electron microscopies are presented together with highly contemporary ancillary techniques. The latter include: high-resolution radioautography, immunoelectron microscopy, x-ray microanalysis, and electron systems imaging, as well as atomic force and scanning tunneling microscopies. Prior to the summation, *Plant Electron Microscopy and Cytochemistry* concludes with a chapter centering about the uses of electron microscopy in molecular biology.

Although this manual is concerned with higher plants, some chapters present information relevant to lower plants. In this connection, the position of the fungi has been debated for years. Most taxonomists do not include fungi in the plant kingdom. However, this manual includes some fungal systems since they are involved in wood decay.

Finally, though Humana Press has published *Electron Microscopy Methods and Protocols* by M. A. Nasser Hajibagheri in 1999, *Plant Electron Microscopy and Cytochemistry* is dedicated to plant studies, and should be quite useful to professors, certain graduate and undergraduate students, and postdoctorals, as well as government and industrial scientists.

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2 Methods for the Cytochemical/ Histochemical Localization of Plant Cell/Tissue Chemicals

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Contents

Introduction
Purpose of the Chapter
Protocols
Conclusions
References

1. INTRODUCTION

1.1. Value of Cytochemistry and Histochemistry

Although many published volumes exist regarding the cytochemical/ histochemical localizations of cellular and tissue chemicals for animal systems *(1–10)*, there are only a few relatively recent monographs concerning plant cell/tissue cytochemistry and histochemistry *(11–15)*.

Perhaps the main reason for the rather numerous volumes centering about animal systems stems from the obvious importance of localizing cellular/tissue chemicals for clinical histopathology. For example, embryonic surface antigens appear during transformation of a healthy cell to a malignant one. Although there are plant cancers, e.g., crown galls, the development of cytochemical stains to reveal possible surface antigens in plant neoplasms has not been extensively explored. Nevertheless, plant cytochemistry has yielded a wealth of information regarding

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the distribution of cellular and tissue chemicals in diverse systems. Furthermore, cytochemistry/histochemistry has provided significant details about the organization of the vascular system in monocot and dicot roots and stems.

1.2. Continued Evolution of Cytochemistry

Plant cytochemistry/histochemistry continues to evolve as fluorescence microscopy *(16–19)*, confocal fluorescence microscopy *(20,21)*, and microspectrophotometry *(22)* expand our quantitative knowledge of the distributions of chemical constituents in plant cells and tissues. With regard to microspectrophotometry, this is possible for single cells, as the Arcturus Corporation (Mountain View, CA) has developed an instrument capable of isolating single cells.

2. PURPOSE OF THE CHAPTER

Since the classic plant histochemistry volume of Jensen *(23)*, a few volumes regarding the topic (see opening paragraphs of the introduction) have appeared over the last four decades. Certain of these volumes contain updated methods for fixation, dehydration, and embedding of plant cells and tissues for the light microscopic localization of certain of their chemical constituents. Much of the older botanical microtechnique volumes, e.g., Sass *(24)* abound with paraffin embedding and sectioning methods. These volumes remain very useful, as they contain highly relevant information regarding microtomy and affixing sections to slides.

This chapter offers some select, recent developments regarding fixation, dehydration, and embedding. In addition, some tried-and-true procedures are described for the localizations of cellular and tissue chemicals in stems and roots of young *Zea mays* seedlings. Also provided are more recently developed fluorochromes for DNA and RNA localizations *(18,19)*.

Finally, the localizations of low-molecular-weight compounds requires special specimen preparation techniques, as these compounds are often diffusible, water- or organic-solvent soluble, and solubilized by conventional fixation and dehydration procedures. The reader is referred to ref. *(12)* for the processing of cells and tissues for the cytochemical and histochemical localization of these compounds.

3. PROTOCOLS

3.1. Preparation of Plant Cells and Tissues for Light Microscope Cytochemistry/Histochemistry

1. Cut tissue block, with at least one dimension a maximum of 5 mm, into 1.5% (w/v) formaldehyde, 2.5% (v/v) glutaraldehyde in 0.05 *M* phos-

Table 1
Fixatives Employed for Light Microscopy

Acetic acid 45%
Acetic acid—alcohol
Acetic acid—alcohol—chloroform
Chromium tetroxide
Chromium—formal
Ethanol 50-70% aqueous
Formalin—calcium
Formalin 10%
Formalin—alcohol—acetic acid (FAA)
Glutaraldehyde 20%[a]

[a] Harris et al. *(13)* suggest using a combination of glutaraldehyde and paraformaldehyde.

phate buffer, pH 7.0. Note that speed is important to prevent autolytic changes. *See* Table 1 for other fixatives employed for light microscopy.
2. Fix overnight at 4ºC (volume of fixative >10X volume of sample).
3. Wash twice in phosphate buffer, 30 min each time.
4. Dehydrate through a graded alcohol series (10%, 25%, 40%, 60%, 75%, 95%) with two changes at each step (15 min each change) and three 15–30 min changes in 200-proof alcohol.
5. Embed in 1:1 ethanol:polyethylene glycol 1000 overnight at 40ºC.
6. Infiltrate with polyethylene glycol for 48–72 h at 56ºC with changes to fresh PEG each morning and evening.
7. Place in prewarmed embedding molds with fresh polyethylene glycol and cool on ice at 4ºC.

3.1.1. Wax Embedding Procedure for Sectioning *(11,12)*

1. Wash with 2:1 ethanol: Histo-Clear for 2 h at room temperature.
2. Repeat with 1:1 and 2:1 ethanol: Histo-Clear and leave in Histo-Clear overnight.
3. Infiltrate with Histo-Clear: wax (or paraplast) at 1:1 for 8 h at 56ºC.
4. Infiltrate with wax (or paraplast) for 96 h at 56ºC with changes every 24 h.
5. Place in prewarmed embedding molds with fresh wax (or paraplast) and cool on ice at 4ºC. Adapted from ref. *(13)*. The reader is referred to Jensen *(23)* and Berlyn and Miksche *(11)* for older methods of clearing with xylene and subsequent progressive embedding in graded mixtures of paraplast and xylene or toluene with final embedding in pure paraplast. These methods have endured and are still widely used today. The reader is urged to examine the early papers of Rosen et al. *(25)* and Reynolds and Dashek *(26)* for celloidin-embedding procedures.

Table 2
Summary of the Specificity of Cytochemical Stains Available for the Detection of Various Classes of Cellular Chemicals [a,b]

Compound	*Stain*
Carbohydrates	Periodic Acid—Schiff's
Callose	Aniline blue fluorescence
Cellulose	Zinc chlor-iodide
Pectin	Hydroxylamine—ferric chloride
	Ruthenium red
Nucleic acids	Calcofluro white M2R fluorescence
DNA/RNA	Methyl green—pyronin
	Azure B
DNA	Feulgen
	Acridine orange as a fluorochrome
	Ethidium bromide as a fluorochrome
	4', 6'- diamido-2-phenylindole as a fluorochrome
Lignin	Acidic phloroglucinol
Lipids	Nile blue
	Sudan black B
	Sudan IV
Phospholipids	Acid haematin
	Bromine—Sudan black
	Bromine—Acetone—Sudan black
Protein (total)	Fast green pH2
	Ninhydrin—Alloxan Schiff's
	Mercuric-bromphenol blue
Proteins	
Containing tyrosine	Million's diazotization
Containing arginine	Sakaguchi reaction
Containing tryptophan	N-(1-Naphthyl)-ethylenediamine
Containing sulfhydrils or disulfide	Rosindole
Tannins	Tetrazolium
	Mercaptide formation
	Ferric chloride—HC1

[a] Adapted from refs. *(23)*, *(11)*, *(12)*, *(18)*.
[b] *See* ref. *26a*.

3.2. Protocol

3.2.1. Cytochemical/Histochemical Localizations of Chemicals in Stems and Roots of Zea mays Seedlings (*see* Table 2)

Chemicals	*Plant material*
Adhesive such as Haupt's	*Zea mays* seedlings
dH_2O	Prepare in advance

Chemicals	*Plant material*
Ethanol	Excise stem or roots
Glutaraldehyde	(*see* Introductory Material)
Fast green	*Equipment*
Paraformaldehyde	Analytical balance and weighing paper
Paraplast	Greenhouse or hood light banks (Grolux)
Periodic acid	Incubator
Permount or Polymount	Light microscope with or without camera
Phosphate buffer	Microtome with blade
Polyethylene Glycol 1000	Ocular micrometer
Safranin	Slide warmer
Schiff's reagent	Tissuetek or Paraplast dispenser
Sudan stains	(optional—embedding can be
Vermiculite or perlite	accomplished without them—*see* text)
Xylene (histological grade)	Top-loading balance and weighing boats
or Histo-Clear, a recent	Water bath
commercially available	
clearing agent	
Supplies	
Aluminum foil	
Camel's hair brush	
Coplin jars or staining dishes	
Coverslips 22 × 50 mm	
Embedding molds	
Embedding rings	
Flats for growing corn seedlings	
Graduated cylinder	
Ice bucket	
Kimwipes	
Microscope slides—frosted end	
Pasteur pipets	
Permount	
Pipets 1,5, and 10 mL	
Probes	
Pro-pipets	
Pyrex bottles	
Single-edge razor blades	
Vials for fixation	

3.2.2. Use Coplin Jars or a Rack of Staining Dishes

Carbohydrates—periodic acid—Schiff (use ref. 12 controls)	*Nucleic acids—azure blue*[a]
Deparaffinize with two xylene changes, 5 min each	Use freeze-dried or freeze-substituted tissue; can also use chemically fixed

(continued)

Carbohydrates—periodic acid—Schiff (use ref. 12 controls)

Hydrate
100% ethanol, 5 min
95% ethanol, 5 min
70% ethanol, 5 min
Staining
Place sections in 0.4 g periodic acid, 35 mL; absolute ethanol, 5 mL 0.2 *M*; sodium acetate, 10 mL dH_2O for 10 min
Rinse the sections in 70% aqueous ethanol
Transfer the section to reducing bath for 3 min
Reducing bath = 1 g potassium iodide and 1 g sodium thiosulfate in 30 mL absolute ethanol and 20 mL distilled H_2O; add 0.5 mL 2NHCl (make reducing bath fresh daily)
Rinse the section in 70% aqueous ethanol
Stain the section in Schiff's reagent for 20 min
Schiff's—dissolve 1 g basic fuchsin in 200 mL boiling $dH_2O\bar{c}$ stirring; cool solution to 50ºC and filter, add 30 mL NHCl and then 3 g $K_2S_2O_5$
Keep in dark for 25 h in a well-stoppered bottle; add 0.5 g charcoal and shake for 1 min; filter and store filtrate in dark in tightly stoppered bottle
Wash the sections in three changes of freshly prepared SO_2H_2O, each 10 min (INHCl, 5 mL $K_2S_2O_5$, 5 mL dH_2O, 100 ml)
Dehydrate the sections through a graded ethanol series
Mount in Permount, Polymount, or Euparol
Aldehyde groups stain pink

Lipids and fatty acids—Sudan III

Unfixed or fixed frozen sections
Take sections to 50% aqueous ethanol

Nucleic acids—azure blue[a]

tissues if chromic acid or other heavy metals are absent
Deparaffinize with two changes of xylene
Hydrate through a decreasing alcohol series
Immerse slides in an 0.25-mg mL^{-1} solution of azure B in pH 4.0 citrate buffer at pH 4.0 for 2 h at 50ºC
Wash in dH_2O
Place in tertiary butyl alcohol (TBA) for 30 min
Take through two changes in TBA for 30 min each time
If additional differentiation is required, can allow sections to remain overnight in TBA
Xylene two changes, 5 min each
Mount in Permount, Polymount, or Euparol
DNA = blue green
RNA = purple or dark blue

Total protein—ninhydrin—alloxan—Schiff's reaction (see Jensen, 1952 for Deamination and Acetylation Controls)

Unfixed, freeze dried, freeze substituted, or chemically fixed
Chemically fixed, use 15–25 μm sections
Deparaffinize with two changes of xylene
Place sections in 0.5% ninhydrin or in 1.0% alloxan in absolute alcohol at 37ºC, 20–24 h
Rinse in two changes of absolute ethanol
Rinse in dH_2O
Immerse in Schiff's reagent (see PAS method) for 10–30 min
Rinse in dH_2O
Place in 2% sodium bisulfite for 1–2 min
Wash in running tap water 10–20 min.
Dehydrate through an increasing alcohol series

Lipids and fatty acids—Sudan III
Stain in Sudan III in 70% ethanol for 30 min
Rinse sections in 50% aqueous ethanol
Mount in glycerine
Avoid the use of absolute ethanol, as lipids will be soluble
Mount in Permount, Polymount, or Euparol xylene two times, 5 min each time
Neutral fats and fatty acids stain red

[a] The specificity of azure B for DNA and RNA must be verified in each system by DNase and RNase treatments as well as other cytochemical tests, The Feulgen reaction for DNA and acridine orange (DNA and RNA) coupled with fluorescence microscopy are particularly useful. Similarly, the specificity of fast green at pH2 for total protein must be verified by treating sections with proteases.

Table 3
Summary of Wood-Decay Fungal H_2O_2 Investigations

Tests employed	*References*
3' 3-Diaminobenzidine; horseradish peroxidase, and ABTS or o-diansidine; titanium reagent	Forney et al. *(27)*; Highley and Murmanis *(28)*; Illman and Highley (30); Micales and Highley *(31)*

3.3. Cytochemical/Histochemical Localizations of Low-Molecular-Weight Compounds—H_2O_2

Some of the most comprehensive investigations of H_2O_2 localizations in plant tissues have been those of Highley and his co-workers (Table 3). These investigators were concerned with localizing H_2O_2 in decaying wood and wood decay fungi as H_2O_2 is thought to function in proposed Fenton chemistry-mediated wood decay (*see* Chapter 12 and refs. *32*). Highley and his co-workers both present and cite methods for localizing H_2O_2. With modification for systems differences, the tests cited in Table 3 should be applicable to a wide variety of plant systems.

4. CONCLUSIONS

The future of cytochemistry resides in its usefulness as an adjunct to biochemistry. As mentioned, fluorescence *(33–35)* and confocal *(36–40)* microscopies have provided new dimensions to cytochemistry.

Finally, photomicrography is the culmination of the preparation of specimens for optical microscopy. This is a very technical area requiring proper illumination *(41–43)*, focusing, choice of films, as well as exposure and appropriate film development. This critical area of microscopy should see continued technological innovations as much of photomicrography is being computerized (44, 45). This effort is witnessing the concomitant

improvement of basic measuring techniques for light microscopy and image analysis *(46–48)*.

REFERENCES

1. Galigher AE, Kosloff EN. *Essentials of Practical Microtechnique*, Lea and Febiger, Philadelphia, PA, 1964.
2. Hayat MA. *Stains and Cytochemical Methods*, Plenum Press, New York, 1993.
3. Horubin RW. *Understanding Histochemistry: Selection, Evaluation and Design of Biological Stains*, Hopwood, Chichester, UK, 1988.
4. Kiernan JA. *Histological and Histochemical Methods: Theory and Practice*, Pergamon Press, Oxford, UK, 1990.
5. Lillie RD, Fuller HM. *Histopathology Techniques and Practical Histochemistry*, McGraw-Hill, New York, 1976.
6. Pearse AGE. *Histochemistry Theoretical and Applied*, Little, Brown, Boston, MA, 1964.
7. Sannes PL. *The Histochemical and Cytochemical Localization of Proteases*, Deerfield BEA, Stuttgart, Germany, 1988.
8. Sheehan DC. *Histotechnology—Theory and Practice*, The CV. Mosby Co., St. Louis, MO, 1980.
9. Horobin RW. Histochemistry and the light microscope, in *Light Microscopy in Biology* (Lacey AJ, ed.), IRL Press, Oxford, UK, 1989, pp. 137–162.
10. Sumner BEH. *Basic Histochemistry*, Wiley, Chichester, New York, 1988.
11. Berlyn GP, Miksche JP. *Botanical Microtechnique and Cytochemistry*, Iowa State University Press, Ames, IA, 1976.
12. Gahan PB. *Plant Histochemistry and Cytochemistry: An Introduction*, Academic Press, London, UK, 1984.
13. Harris N, Spence J, Oparka KJ. General and enzyme histochemistry, in *Plant Cell Biology* (Harris N, Oparka KJ, eds.), IRL Press, Oxford University Press, Oxford, UK, 1994, pp. 51–68.
14. Vaughn KC. *Handbook of Plant Cytochemistry*, CRC Press, Boca Raton, FL, 1987.
15. Vigil EL, Hawes CR. *Cytochemical and Immunological Approaches to Plant Cell Biology*, Academic Press, London, UK, 1989.
16. Rost FWD. *Quantitative Fluorescence Microscopy*, Cambridge University Press, New York, 1991.
17. Rost FWD. *Fluorescence Microscopy*, Vols. I and II, Cambridge University Press, New York, 1992.
18. Oparka KJ, Read ND. The use of fluorescent probes for studies of living plant cells, in *Plant Cell Biology* (Harris N, Oparka KJ, eds.), IRL Press, Oxford University Press, Oxford, UK, 1994, pp. 27–50.
19. Oparka KJ, Roberts AG, Santa Cruz S, Boevnik P, Prior D, Smallcombe A. Using GFP to study virus invasion and spread in plant tissues. *Nature* 1997; 388: 401–402.
20. Wilson T. *Confocal Microscopy*, Academic Press, New York, 1990.
21. Shuming N, Chiu DT. Probing molecules with confocal fluorescence microscopy. *Science* 1994; 266: 1018.
22. Cherry RJ. *New Techniques of Optical Microscopy and Microspectrophotometry*, CRC Press, Boca Raton, FL, 1990.
23. Jensen WA. *Botanical Histochemistry*, Freeman, San Francisco, CA, 1952.
24. Sass JE. *Botanical Microtechnique*, 3rd ed., Iowa State College Press, Ames, IA, 1958.
25. Rosen WG, Gawlik SR, Dashek WV, Siegesmund KA. Fine structure and cytochemistry of *Lilium* pollen tubes. *Am J Bot* 1964; 51: 61–67.

26. Reynolds JD, Dashek WV. Cytochemical analysis of callose distribution in *Lilium longiflorum* pollen tubes. *Ann Bot* 1976; 40: 409–416.
26a. Prasad B. *Staining Techniques in Botany*, State Mutual Book and Periodical Services, New York, 1986.
27. Forney LJ, Reddy CA, Pankvatz HS. Ultrastructural localization of hydrogen peroxide production in ligninolytic *Phanerochaete chrysosporium* cells. *Appl Environ Microbiol* 1982; 44: 732–736.
28. Highley T, Murmanis LL. Determination of hydrogen peroxide production in *Coriolus versicolor* and *Poria placenta* during wood degradation. *Mater Org* 1985; 29: 241–252.
29. Highley T. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium. *FEMS Microbiol Lett* 1987; 48: 373–378.
30. Illman B, Highley TL. Hydrogen peroxide formation by wood decay fungi in liquid medium. *Phytopathology* 1988; 78: 1590.
31. Micales JA, Highley TL. *In vitro* production of hydrogen peroxide by degradative and non-degradative isolates of brown-rot wood decay fungi. *Phytopathology* 1989; 77: 988.
32. Highley TL, Dashek WV. Biotechnology in the study of white-rot and brown-rot decay, in *Forest Products Biotechnology* (Bruce A, Palfreyman JW, eds.), Taylor and Francis, London, UK. 1998, pp. 15–36.
33. Slavik J. *Fluorescence Microscopy and Fluorescent Probes*, *Proceedings of a Conference Held in Digre, Czech Republic*, Plenum Press, New York, 1996.
34. Tarke HJ. *Fluorescence Microscopy*, Bios Scientific UK, Cornett Books, Oxford, UK, 1996.
35. Wang XF, Herman B. *Fluorescence Imaging Spectroscopy and Microscopy*, Wiley, New York, 1996.
36. Van der Voort HTM, Valkenburg JAC, Van Spronsen EA, Woldringh CL, Brakenhoff GJ. Confocal microscopy in comparison with electron and conventional microscopy, in *Correlative Microscopy in Biology. Instrumentation and Methods* (Hayat MA, ed.), Academic Press, Orlando, FL, 1987, pp. 60–81.
37. Pauley B. *Handbook of Biological Confocal Microscopy*, Plenum Press, New York, 1990.
38. Corle TR, Kino GS. *Confocal Scanning Optical Microscopy and Related Imaging Systems*, Academic Press, New York, 1996.
39. Sheppard C, Shottan, D. *Confocal Laser Scanning Microscopy*, Springer-Verlag, Berlin, Germany, 1997.
40. Paddock SW. *Confocal Microscopy Methods and Protocols*, Humana Press, Totowa, NJ, 1998.
41. Smith RF. *Microscopy and Photomicrography. A Working Manual*, 2nd ed., CRC Press, Boca Raton, FL, 1994.
42. Thompson DJ, Bradbury S. *An Introduction to Photomicrography* (Book 13), Oxford University-Royal Microscopical Society, Oxford, UK, 1991.
43. Weiss DG, Marle W, Wick RA. Video microscopy, in *Light Microscopy in Biology: A Practical Approach* (Lacey AJ, ed.), IRL Press, Oxford, UK, 1989, pp. 221–278.
44. Bradbury S. *Basic Measurement Techniques for Light Microscopy*, Oxford University- Royal Microscopical Society, Oxford, UK, 1991.
45. Swatland HJ. *Computer Operation for Microscopy Photometry*, CRC Press, Boca Raton, FL, 1997.
46. Hader DP. *Image Analysis in Biology*, CRC Press, Boca Raton, FL, 1992.
47. Russ JC. *The Image Processing Handbook*, CRC Press, Boca Raton, FL, 1995.
48. Jahne B. *Practical Handbook on Image Processing for Scientific Applications*, CRC Press, Boca Raton, FL, 1997.