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

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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
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-
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 1

<table>
<thead>
<tr>
<th>Fixatives Employed for Light Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid 45%</td>
</tr>
<tr>
<td>Acetic acid—alcohol</td>
</tr>
<tr>
<td>Acetic acid—alcohol—chloroform</td>
</tr>
<tr>
<td>Chromium tetroxide</td>
</tr>
<tr>
<td>Chromium—formal</td>
</tr>
<tr>
<td>Ethanol 50-70% aqueous</td>
</tr>
<tr>
<td>Formalin—calcium</td>
</tr>
<tr>
<td>Formalin 10%</td>
</tr>
<tr>
<td>Formalin—alcohol—acetic acid (FAA)</td>
</tr>
<tr>
<td>Glutaraldehyde 20%*</td>
</tr>
</tbody>
</table>

* Harris et al. (13) suggest using a combination of glutaraldehyde and paraformaldehyde.
Table 2
Summary of the Specificity of Cytochemical Stains Available for the Detection of Various Classes of Cellular Chemicals

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

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)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Plant material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesive such as Haupt’s</td>
<td>Zea mays seedlings</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Prepare in advance</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Plant material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Excise stem or roots</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>(see Introductory Material)</td>
</tr>
<tr>
<td>Fast green</td>
<td></td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Analytical balance and weighing paper</td>
</tr>
<tr>
<td>Paraplast</td>
<td>Greenhouse or hood light banks (Grolux)</td>
</tr>
<tr>
<td>Periodic acid</td>
<td>Incubator</td>
</tr>
<tr>
<td>Permound or Polymount</td>
<td>Light microscope with or without camera</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>Microtome with blade</td>
</tr>
<tr>
<td>Polyethylene Glycol 1000</td>
<td>Ocular micrometer</td>
</tr>
<tr>
<td>Safranin</td>
<td>Slide warmer</td>
</tr>
<tr>
<td>Schiff’s reagent</td>
<td>Tissuetek or Paraplast dispenser</td>
</tr>
<tr>
<td>Sudan stains</td>
<td>(optional—embedding can be accomplished without them—see text)</td>
</tr>
<tr>
<td>Vermiculite or perlite</td>
<td></td>
</tr>
<tr>
<td>Xylene (histological grade)</td>
<td>Top-loading balance and weighing boats</td>
</tr>
<tr>
<td>or Histo-Clear, a recent commercially available clearing agent</td>
<td>Water bath</td>
</tr>
</tbody>
</table>

**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
- Permound
- 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

Deparaffinize with two xylene changes, 5 min each

Use freeze-dried or freeze-substituted tissue; can also use chemically fixed

(continued)
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 dH₂O 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 H₂O; 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 dH₂O stirring; cool solution to 50°C and filter, add 30 mL NHCl and then 3 g K₂S₂O₅

Keep in dark for 25 h in a well-stopped 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 SO₂H₂O, each 10 min (INHCl, 5 mL K₂S₂O₅, 5 mL dH₂O, 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

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

Nucleic acids—azure 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 37°C, 20–24 h

Rinse in two changes of absolute ethanol

Rinse in dH₂O

Immerse in Schiff’s reagent (see PAS method) for 10–30 min

Rinse in dH₂O

Place in 2% sodium bisulfite for 1–2 min

Wash in running tap water 10–20 min.

Dehydrate through an increasing alcohol series
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### 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

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4 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 H₂O₂ Investigations**

<table>
<thead>
<tr>
<th>Tests employed</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-3-Diaminobenzidine; horseradish peroxidase, and ABTS or o-dielsidine; titanium reagent</td>
<td>Forney et al. (27); Highley and Murmanis (28); Illman and Highley (30); Micales and Highley (31)</td>
</tr>
</tbody>
</table>

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### 3.3. Cytochemical/Histochemical Localizations of Low-Molecular-Weight Compounds—H₂O₂

Some of the most comprehensive investigations of H₂O₂ localizations in plant tissues have been those of Highley and his co-workers (Table 3). These investigators were concerned with localizing H₂O₂ in decaying wood and wood decay fungi as H₂O₂ 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 H₂O₂. With modification for systems differences, the tests cited in Table 3 should be applicable to a wide variety of plant systems.

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### 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

Chapter 2 / Localization of Plant Cell/Tissue Chemicals

Methods in Plant Electron Microscopy and Cytochemistry
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