Chapter 5

Clearing Techniques for the Study of Vascular Plant Tissues in Whole Structures and Thick Sections

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Introduction

Transmission light microscopy as a matter of principle requires the passage of light through the object of study into the optical system of the microscope. For the study of plant and animal tissue, this requirement is traditionally fulfilled by slicing the tissue into sections sufficiently thin to permit light transmission. An alternative to sectioning methods, which are by nature quite arduous, is provided by a variety of clearing techniques. These techniques, whereby thick masses of tissue are made translucent through specific chemical treatment, fall into two categories.

In the first category, clearing is accomplished by removal from the cells much of the protoplasmic content which tends to make the tissue opaque. Study can be accorded only to the cleared portion that remains, and methods of this kind are, therefore, restrictive. In the second category by contrast, the tissue is structurally unaltered by the treatment process, and yet it becomes uniformly translucent. Although the mechanism operative is not presently understood, it is apparent that cellular organelles which ordinarily differ markedly in their refractive properties become more closely uniform in this regard. The effect of refractive uniformity in tissue examined with ordinary bright-field optics is a very low contrast among the structural components of the cells. However, with phase contrast or Nomarski interference optics, which intensify the small refractive differences among organelles, cellular structure can be examined throughout thick pieces of cleared tissue. Through selective staining of specific types of cells or cell organelles, techniques of this second category can be used effectively in conjunction with bright-field optics.

Of methods in the second category, the 4½ clearing technique has been rather broadly applied in the study of plant tissue. The following four exercises are designed to acquaint biology students with some of the underlying principles of this technique, with some of its specific uses, and with its limitations.

Materials

Items Required for Preparation of Plant Material in Advance

FPA50 (formalin, propionic acid, 50% ethanol; 5:5:90)
Carnoy's (Farmer's) fluid (absolute ethanol, acetic acid; 3:1)
Randolph's modified Navashin fluid (Solution A: chromic acid, 1 g; acetic acid, 7 cc; water, 92 cc. Solution B: formalin, 30 cc; water, 70 cc. Mix equal portions of A and B just before using.)
Ethanol: 70%, 95%, and absolute
4½ clearing fluid (lactic acid, chloral hydrate, phenol crystals, clove oil, xylene; 2:2:2:2:1, by weight)
Bordeaux Red 4½ clearing fluid (0.2 mg dye/g clearing fluid)
BB-4½ clearing fluid (4½ clearing fluid, benzyl benzoate; 9:1, by weight)
Lactic acid saturated with chloral hydrate
Radnoti planchets (Radnoti Glass Technology, Inc., 227 West Maple Ave., Monrovia, CA 91016)
Screw-cap vials and small jars

Items Required for the Exercises (Amount/number of students)

Dropping bottles:
- 70% ethanol (1/4; one for each four students)
- 4½ clearing fluid (1/4)
- BB-4½ clearing fluid (1/4)
- Lactic acid saturated with chloral hydrate (1/4)
- 1% phloroglucin in 95% ethanol (1/4)
- HCl (concentrated) (1/4)
- 0.05% aqueous toluidine blue 0 (1/4)
- Absolute ethanol (1/4)

Waste dish (1/4)
Lint-free cloth (1/4)
Wide mouth jar, 95% ethanol (for cleaning slides and coves) (1/4)
Standard forceps (1/2)
Standard dissecting needles (straight) (2/1)
Absorbent paper strips (10/1)
Standard microscopes slides and cover glasses (6/1)
Raj slides with slide thickness support mounts and cover glasses (1/1)
Raj slides with cover glass (#1) support mounts and cover glasses (1/1)
Minuten-pin dissecting needles (2/1)
Radnoti planchets (2/1)
Flat-bottom planchets (2/1)
Camel hair brush (#3) (1/1)
Pasteur pipets (1/1)
Elodea leaves (fixed and stored in 70% ethanol) (1/1)
Elodea leaves (fixed and stained with Bordeaux Red 4½, 0.2 mg/g) (1/1)
Selaginella apoda (strobilus), *Wisteria sinensis* (carpel), *Abelia grandiflora* (leaf), *Nymphaea odorata* (leaf fragment) (1/1 of each)
Chlorophytum, Setcreasea, Psilotum, Coleus root and stem sections, 240 µm thick (2/6 of each)

Coleus stem sections (360 µm thick) (1/1)
Cassia occidentalis and *Wisteria sinensis* carpels (1/2 of each)
Yucca filamentosa ovulary cross sections 60 µm thick (1/1)
Iphion uniflorum pistils (1/1)
Note: Above plant material fixed for 24 hours in FPA50 unless otherwise specified in the procedures.

Notes for the Instructor

Initially, all glassware used in these exercises must be clean and dry. Slides and cover glasses should be immersed in 95% ethanol and dried with a clean, lint-free cloth. Pipets and small dishes need not be cleaned between uses unless the second use is for a new fluid. Dissecting needles, forceps, and dropping bottles should be kept in an orderly arrangement for ready access. Whenever feasible, cleared preparations should be saved after the initial examination for review toward the end of the period. The quality of many clearings often improves with the passing of time.

Student Outline

Exercise 1:
Principles of Microscopy Important to the 4½ Clearing Technique

Procedure

Only two-cell-layers thick, fresh leaves of Elodea are highly suitable for the study of cell structure with bright-field optics. The cell organelles are readily visible, and the streaming cytoplasm carries the chloroplasts around the periphery of the cell so to verify the dynamic state of living protoplasm. The simplicity of cellular organization in these leaves permits an easy assessment of basic principles of microscopy that have an important bearing on the clearing of thicker and more complex plant tissues. These basic principles can be examined through the following procedures:

1. Place a fresh Elodea leaf with its lower side down in a drop of water on a slide and add a cover glass.

2. Examine the preparation with the low- and high-power objectives of a bright-field microscope and notice the marked differences in the depth of field for the two magnifications (about 25 µm for low and less than 1.0 µm for high magnification). As a consequence of this difference, both the large cells of the upper layer and small cells of the lower layer can be observed simultaneously, although not in sharp focus, with low magnification. With high magnification, the depth of field falls entirely within the upper layer of cells. Notice, however, that a lowering of the high-power objective to the focal plane of the lower layer does not entirely eliminate the image of the upper layer. The depth of field at high magnification is slightly thicker than a median optical section of the lower cell layer.

3. Under high magnification, observe one particular cell in the upper layer, preferably one showing cytoplasmic streaming (cyclosis). Add several drops of fixative (FPA50) to the left side of the cover glass and from the right side draw off the water from under the cover glass with a piece of absorbent paper. As the fixative replaces the water, dramatic changes will occur to indicate the fixation process. Cyclosis will cease, and the chloroplasts, nucleus, nucleolus, and vacuolar

boundaries will become very distinct. Fixation greatly increases the differences in light refraction among the cell organelles. Notice that the depth of field at high magnification appears little altered with fixation. The image in the median optical section of the lower layer includes the slightly unfocused outline of the cells in the upper layer.

4. Remove the cover glass and apply additional FPA50 to insure complete fixation of the leaf. After 3–5 minutes, replace the fixative with two changes of 70% ethanol allowing about 1 minute for each application. Tilt the slide over a waste dish and use absorbent paper to remove fluids. Replace the 70% ethanol with 4½ clearing fluid and place a clean cover glass over the leaf.

5. Place an Elodea leaf fixed in FPA50 and stored in 70% ethanol lower side down in a few drops of 70% ethanol on a slide. Observe the preparation with bright-field optics and notice the high resolution of cellular structure. Remove the cover glass, blot away the excess ethanol, apply 1 or 2 drops of 4½ clearing fluid, and add a cover glass.

6. Observe the preparation under low and high magnification and notice two marked effects of the clearing fluid: (1) reduction in definition and contrast of the organelles by diminishing the differences in their refractive properties; and (2) the apparent reduction of the depth of field so that both layers cannot be discerned simultaneously under low magnification. The focal plane of the median optical section of the lower cell layer does not include an image of cell outlines from the upper layer.

7. Observe the preparation with low- and high-power optics of a phase contrast or Nomarski interference microscope, both of which intensify refractive differences so to restore the definition and contrast of the organelles. At high magnification, the depth of field is very shallow, and the focal plane for each cell layer is distinct. Cell outlines of the upper layer are not discernible when the high-power objective is focused on the median optical plane of the lower layer.

8. Obtain an Elodea leaf previously cleared and stained for approximately 24 hours in Bordeaux Red 4½ clearing fluid.1 Place the leaf lower side down in several drops of fresh clearing fluid on a slide, add a cover glass, and observe under low and high magnification of a bright-field microscope. Although the stain is absorbed throughout the cell, it is sufficiently differential in its action to impart some definition and contrast to the organelles. The depth of field with low-power optics, however, extends through both cell layers and is comparable to that of fixed leaves not subjected to clearing fluid. Since the stain is somewhat general in its action, that is, not distinctly differential, it tends to restore an opaque quality to the tissue. Generalized, nondifferential staining is the antithesis of clearing. Were the stained Elodea leaf several cell layers thick, it would be too opaque for study with the transmission bright-field microscope. Only highly specific stains, those confined to specific organelles, can be regarded as useful in the study of cleared, multilayered tissue with transmission bright-field optics.

Summary

1. Acid stains (e.g., vital red, fast green, and aniline blue) in concentrations from 0.1 mg/g to 1.0 mg/g of clearing fluid are effective in the differential staining of nuclei and cytoplasm. Basic stains, readily soluble in clearing fluid, cannot be absorbed from the fluid by plant tissue.

The application of 4½ clearing fluid to plant tissue changes the refractive properties of cell organelles so to reduce their definition and contrast under bright-field optics. The minor differences in definition and contrast are sufficiently amplified by phase contrast and Nomarski interference optics to restore distinction among cell organelles and to permit the study of structure within cells of cleared tissue.

The clearing fluid, in effect if not in actuality, reduces the depth of field and thereby permits the examination of cells in a specific focal plane unobscured by cells in layers above that focal plane.

Highly specific stains may provide definition to certain organelles and so permit their examination with bright-field optics. Generalized stains, however, render cleared tissue opaque and thus unsuitable for study with transmission light microscopy.

Exercise 2:
The Clearing of Whole Structures for Study with Low Magnification, Bright-Field Microscopes

Procedure

The 4½ clearing technique can be applied to intact structures from flowering plants and from other vascular plants as well for the study of structural features with low magnification bright-field optics. It has been shown to be especially useful in studies of vascular distribution in leaves, and it has broader application to other structures and for other anatomical features as will be demonstrated here.

In some instances, and especially often with thick, bulky structures, direct application of clearing fluid does not produce adequate results. The type of fixative used when the structures were collected may be the cause, and with alternative fixatives good results can be obtained. Sometimes the fixed material requires a special pretreatment to remove substances that inhibit the clearing process. Lactic acid and lactic acid saturated with chloral hydrate are examples of pretreatment fluids which have been effectively applied. With the following structures, you can become acquainted with both the simple and the more complex clearing procedures.

Carpels (simple pistils) of *Wisteria sinensis*:

1. Place a carpel of *Wisteria sinensis*, fixed in FPA50 and stored in 70% ethanol, on a Raj slide with slide thickness support mounts. Cover it with BB-4½ clearing fluid and add a cover glass.
2. Immediately observe the specimen with high magnification of a dissecting microscope equipped to provide transmitted light. Adjust the mirror so that the light passes obliquely through the carpel and also observe the specimen with reflected light.

In 10–20 minutes, the ovary wall will become translucent so to reveal the locular space (the space the wall encloses) and a row of ovules, each one attached by its funiculus to the placental surface. At first the ovules will appear quite distinct because they will still be opaque after the ovary wall has become clear. Later the ovules too may become translucent and less distinct. As the young ovule clears, the central mass of tissue, the nucellus, and the two integuments that cover it become distinguishable. The mature ovule is not likely to clear sufficiently to reveal these features because of secondary wall thickening in the epidermis of the outer integument.

*Strobili of Selaginella apod*a:

1. Place a strobilus of *Selaginella apoda*, fixed in either FPA50 or Carnoy's (Farmer's) fluid and stored in 70% ethanol, in a flat-bottom planchet. Cover it with BB-4½ clearing fluid. After 10–30 minutes, transfer the strobilus to a Raj slide with slide thickness support mounts. Cover it with BB-4½ clearing fluid and add a cover glass.
2. Observe the specimen under the dissecting microscope with transmitted light and with low magnification of a standard bright-field microscope.

The strobilus of *Selaginella* is composed of four rows of specialized leaves, the microsporophylls and megasporophylls, each one of which bears respectively a microsporangium and a megasporangium. The megasporangia are more or less globose, and each one contains a single tetrad of megaspores. Smaller and somewhat ellipsoid, the microsporangium contains numerous tetrads of microspores. After the clearing process is complete, all four megaspores in the tetrahedral tetrad of each megasporangium can be discerned. The microsporangia, because of cell wall thickening in the sporangial wall, will appear less translucent, but that each one contains many microspores will be evident. Both sporophylls and the vegetative leaves below the strobilus contain a single central vascular trace extending from the base nearly to the apex of each leaf. On the upper surface of each sporophyll and vegetative leaf near the base is a tongue-like structure, the ligule, which appears darker than the leaves. The ligule appears very distinct in specimens cleared in fast Green 4½ fluid (see footnote 1) because they stain more intensely than does the surrounding leaf tissue.

Leaves of *Abelia grandiflora*:

1. Place fixed *Abelia* leaves, stored in 70% ethanol, in lactic acid in a closed container and keep them at room temperature for about 3 weeks.
2. Replace the lactic acid with lactic acid saturated with chloral hydrate and keep them in a closed container at room temperature for 3–6 days. The leaves may be subjected to 60°C for 2–6 hours during this period.
3. Mount the cleared leaves, lower side up, in a few drops of fresh lactic acid saturated with chloral hydrate on a slide and add a cover glass. Leaves may be transferred from the lactic acid-chloral hydrate directly to 70% ethanol for indefinite storage. So stored, they may be dehydrated to absolute ethanol and returned to lactic acid-chloral hydrate for observation.
4. Examine the preparation with low magnification of a bright-field microscope and notice the distribution pattern of the veins. Also focus on the surface of the lower epidermis with high magnification. Focus down with the fine adjustment and observe the spongy mesophyll, veins, palisade mesophyll, and the upper epidermis in a series of paradermal optical sections.

Abelia leaves exhibit netted venation as is typical for dicotyledonous plants. The leaf margin is dentate, and each tooth is actually a hydathode, a secretory structure which exudes water (guttation) during a brief time in the early morning when the water in the xylem is under pressure. In each hydathode, a large cluster of vessel elements end blindly against a mass of thin-walled, loosely arranged parenchyma (epithem). At the tip of the tooth is a small pore flanked by two cells, much like guard cells of a stoma but usually without the mechanism for opening and closing the pore. The cells of the lower and upper epidermis and those of the mesophyll as well are nearly devoid of protoplasm because of the rigorous clearing process used. Removal of the protoplasm provides the translucent quality that permits examination of the cellular organization through a series of

paradermal optical sections. Definition of the cell walls throughout the leaf can be effectively increased by a brief application of 0.05% toluidine blue 0 (aqueous).

There are numerous techniques for clearing leaves, and at least several of these are sufficient to permit the observations described here.

Leaf fragments of *Nymphaea odorata*:

1. Place a rectangular leaf fragment of Nymphaea odorata, fixed in Carnoy's fluid and stored in 70% ethanol, on a slide with its upper (slightly concave) surface against the slide.
2. Cover it with BB-4½ clearing fluid and carefully balance a cover glass over the lower surface of the thick fragment.
3. Fill in the space under the cover glass with clearing fluid and observe the specimen with low magnification of a bright-field microscope.

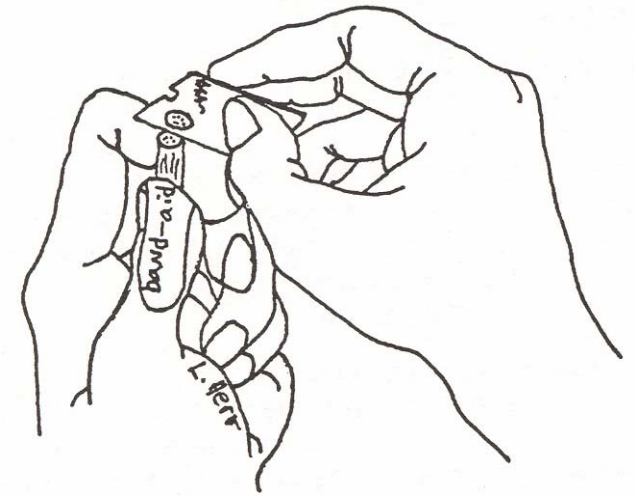
Thick water lily leaves clear more rapidly than might be expected because much of the thickness is accounted for by the presence of large air chambers. The blades are permeated with specialized, highly branched sclerenchyma cells referred to as astrosclereids. In typical thin cross sections of the leaf, the astrosclereids appear as isolated fragments of lignified cells widely separated from one another in the leaf mesophyll. From a cleared wholemount preparation, however, the abundance and arrangement of these cells pointedly indicates their function of support in providing a degree of rigidity to the blade.

Exercise 3:
The Clearing and Differential Staining of Thick Sections for Study with Bright-Field Optics

Procedure

Sections of fresh, herbaceous roots and stems one-tenth to one-half of a millimeter in thickness can be readily cut with a hand-held razor blade and more precisely so with specialized microtomes. These sections can be fixed in FPA50 or in other plant fixatives, stored in 70% ethanol, and later cleared and differentially stained for anatomical study.

To prepare hand sections of an object, place a drop of water on the surface of a thin razor blade near its cutting edge. Dip the object to be sectioned in water and grasp it between the thumb and forefinger of the left hand (if one is right-handed), and hold the razor blade in the right hand. The thumb of the left hand should be covered with a band-aid (see figure on the next page). Steady the razor blade by resting it against the left forefinger and slice through the object toward the thumb. As each section is cut, it should float in the drop of water on the razor blade from which it can then be transferred to a collecting dish of water. With only a little practice, useful sections may be obtained, particularly if the following precautions are observed: (1) Use a thin, sharp razor blade. (2) Try to make a slice that tapers in thickness. (3) Do not attempt to make a complete section of a very large object; try to obtain tapering sections of a small portion. (4) Make many sections and select the best ones for study. Separate the thinner ones which can be stained following fixation and storage, from those thicker ones that must be cleared prior to the application of stains.



Prepare sections about 240 µm thick from *Chlorophytum* and *Setcreasea* roots and from *Psilotum* and *Coleus* stems; fix in FPA50 and store in 70% ethanol. Further prepare sections by the following procedure:

1. Use a camel hair brush to transfer two sections from storage 70% ethanol to a Radnoti (round-bottom) planchet. Cover the sections with 95% ethanol for 5 minutes and then with absolute ethanol for 5 minutes.
2. Replace the ethanol with lactic acid saturated with chloral hydrate and place the planchet in a small petri dish for 12–24 hours at room temperature.
3. Replace the lactic acid-chloral hydrate with three changes of 70% ethanol allowing at least 5 minutes for each change. If the sections are to be cleared later, store them in a screw-cap vial in the third change of 70% ethanol.
4. Return the stored sections to a Radnoti planchet. Cover the sections with 1% phloroglucin in 95% ethanol and add a few drops of concentrated hydrochloric acid. Allow about 1 minute for the mixture to react with lignified cells.
5. Replace the acid-phloroglucin with absolute ethanol. After about 1 minute, remove one section with a camel hair brush, mount it in lactic acid saturated with chloral hydrate on a slide, and add a cover glass.
6. Replace the absolute ethanol in the planchet with a second application of absolute ethanol. After about 1 minute, mount the second section in BB-4½ clearing fluid on a slide and add a cover glass.
7. With low and high magnifications of a bright-field microscope, examine the sections for the distribution of lignified cells. It is very important that the iris diaphragm is closed to the specific optimal diameter for each objective used. Otherwise, the major distinction between the two preparations will not be evident.

By design, plants of relatively close (*Chlorophytum* and *Setcreasea*) and distant (*Psilotum* and *Coleus*) relationship and respectively similar and dissimilar structure were chosen for this study. The selection demonstrates that in comparative studies the technique can be useful in the discernment of minor as well as major structural differences.

Chlorophytum and *Setcreasea*: These genera are monocots, and their roots exhibit a polyarch xylem composed of orderly files of vessels in a ring around a central pith region. Patches of phloem, unstained in this exercise, alternate with the files of xylem vessels. A layer of cells, the pericycle, surrounds the xylem and phloem and lies just inside of the innermost layer of the cortex, the endodermis. The endodermal cells in *Setcrease*a are equipped with casparian strips, partially lignified bands that for each cell form a belt around the radial and transverse cell walls. In cross sections, the portion of these strips in the radial walls can be observed. The endodermal cells of *Chlorophytum* by marked contrast have thick, secondary cell walls.

Psilotum: A primitive, rootless vascular plant, Psilotum is composed of a system of underground and aerial stems, the vascular system of which is highly variable in its organization within a single plant. Cross sections of the stem just above ground level, as provided here, show the xylem in several arms radiating from a central pith region composed of sclerenchyma fibers. In acid-phloroglucin treated sections examined in 4½-type clearing fluids, the red color of the sclerenchyma is slightly different in shade than that displayed by the xylem tracheids. The phloem, surrounding the xylem in a continuous band is devoid of lignified cells and therefore is unstained. Prominent casparian strips can be observed on the radial walls of the endodermal cells, and sclerenchyma is often abundant in the outer portion of the cortex.

Coleus: As is very common for members of the phylogenetically-advanced mint family, the stems of *Coleus* are quadrangular. Stem cross-sections show that a major vascular bundle is associated with each of the four corners. Adjacent to the four sides, midway between the corners, are four minor vascular bundles. In older stems, the vascular cambium differentiates around the entire circumference. This lateral, secondary meristem produces secondary xylem and phloem within the original eight bundles and mainly sclerenchyma in the regions between the bundles. The lignified cells of the primary xylem in the eight bundles and the band of sclerenchyma around the circumference (in older stems) are distinctly revealed in cleared, acid-phloroglucin preparations.

Uniformly in the root and stem sections investigated above, definition and contrast of unstained cells are so severely reduced by BB-4½ clearing fluid that they are almost invisible under bright-field optics. In lactic acid saturated with chloral hydrate, however, the contrast and definition of the unstained cells are greatly improved, and the size, shape, and arrangement of these cells can be determined.

The following procedures are designed to increase further the definition and contrast of cells throughout cleared, thick sections. These procedures rely on a partial retention of the translucent quality of the section after it has been removed from the clearing fluid to a medium where it can be differentially stained.

1. Remove a *Coleus* stem section 360 μm thick from storage in 70% ethanol to a vial a quarter full of lactic acid saturated with chloral hydrate, tightly cap the vial, and place it in an oven at 40–60°C for 12–24 hours.
2. Replace the lactic acid-chloral hydrate with 4½ clearing fluid for 12–24 hours at room temperature.

3. Replace the clearing fluid with two changes of absolute ethanol allowing 10 minutes for each application.
4. Gradually hydrate the sections through 95%, 70%, 50%, and 30% ethanol allowing about 5 minutes for each application. To accomplish this hydration, place the sections and absolute ethanol in a small petri dish. Remove the fluids by tilting the dish.
5. Replace the 30% ethanol with two changes of water applied one immediately after the other. Be sure that the sections sink to the bottom of the dish in the second change of water before proceeding to step 6.
6. Transfer a section to a slide and cover it with 0.05% aqueous toluidine blue 0 stain for 20–40 seconds.
7. Blot away the stain with absorbent paper and cover the section with water. Replace the water with several drops of fresh water and add a cover glass.
8. Examine the preparation under low and high magnifications of a bright-field microscope. Study the cellular organization from the periphery at one of the corners along a line to the center of the section.

From the periphery at a corner along a line to the center of the section, the following cellular organization occurs: (1) an epidermis which produces numerous uniseriate, multicellular trichomes; (2) three to five layers of angular collenchyma cells (unevenly thick walls, thickest in the corners of the cells); (3) three to four layers of cortical parenchyma (thin-walled cells); (4) phloem with small patches of sieve tube members and companion cells surrounded by larger phloem parenchyma cells; (5) vascular cambial zone of three to five layers of cells in distinct periclinal files (like stacked bricks); and (6) files of large diameter metaxylem vessels leading to small-diameter protoxylem vessels next to the very large parenchyma cells of the pith.

Exercise 4:
The Clearing of Ovules for Study with Phase Contrast and Nomarski Interference Optics

Procedure

The 4½ clearing technique was originally developed for the study of ovule and female gametophyte development in flowering plants. For some plants, the technique is quite simple and requires only the treatment of fixed ovules with 4½ clearing fluid. Satisfactory results for other plants, however, require modification of the original technique to include a variety of pretreatment procedures and the use of alternative clearing fluids as well.

In this exercise carpels of *Cassia occidentalis* and *Wisteria* sinensis are fixed in Randolph's modified Navashin fluid and stored in 70% ethanol.

Wisteria: The Clearing of Fixed Ovules in BB-4½ Clearing Fluid

1. Transfer a fixed carpel from storage in 70% ethanol to a Radnoti planchet.
2. Add BB-4½ clearing fluid to cover the carpel.
3. Place the planchet on the stage of a dissecting microscope and use minuten-pin dissecting needles to remove ovules from the carpel.
4. With a Pasteur pipet, transfer the ovules in a drop of clearing fluid to a Raj slide with cover glass (0 to 2) support mounts.
5. Place a cover glass over the support mounts and add enough clearing fluid to the edge to fill the covered space.
6. Observe the preparation with low and high magnification of a bright-field microscope with the iris diaphragm closed to a diameter less than that of the objective lens used.
7. Examine the ovules in optical median sagittal section and identify the features of structure and development that can be readily recognized. Examine this preparation or a similar one with either a phase contrast or a Nomarski interference microscope.

Cassia: The Clearing of Fixed and Pretreated Ovules in BB-4½ Clearing Fluid

1. Transfer a fixed carpel from storage in 70% ethanol to a Radnoti planchet.
2. Cover the carpel with lactic acid saturated with chloral hydrate for 6–12 hours.
3. Replace the lactic acid-chloral hydrate with three changes of 70% ethanol allowing at least 10 minutes for each change. If the ovules are to be cleared later, store the carpel in the third change of 70% ethanol.
4. Return the stored carpel to a Radnoti planchet and replace the 70% ethanol with BB-4½ clearing fluid.
5. Place the planchet on the stage of a dissecting microscope and use minuten-pin dissecting needles to remove ovules from the carpel
6. Use a Pasteur pipet to transfer the ovules in a drop of clearing fluid to a Raj slide with cover glass (0 to 2) support mounts.
7. Place a cover glass over the support mounts and add enough clearing fluid to the edge to fill the covered space.
8. Observe the preparation with low and high magnification of a bright-field microscope with the iris diaphragm closed to a diameter less that of the objective lens used.
9. Examine the ovules in optical median sagittal section and identify the features of structure and development that can be readily recognized. Examine this preparation or a similar one with either a phase contrast or a Nomarski interference microscope.

The young ovule of *Wisteria sinensis* and *Cassia occidentalis* is anatropous; that is, bent against the funiculus which attaches it to the carpel wall. In later development, the mature ovules of both species become campylotropous. The young ovule consists of a central mass of tissue, the nucellus, surrounded by two envelops of tissue, the inner and outer integuments. Because of the anatropous form, the apex of the nucellus is pointed downward toward the placenta and is exposed to the locular space by a zig-zag opening in the integuments, the micropyle. A very large cell, the megasporocyte, develops in the center of the nucellus several cell layers below the apex and undergoes meiosis I and II to produce a linear or T-shaped tetrad of megaspores. Three of the megaspores closest to the micropyle degenerate, and the remaining one produces a female gametophyte which in form and development is typical for most flowering plants. As the functional megaspore enlarges, its nucleus divides to produce a two-nucleate female gametophyte which in succession produces the four-nucleate and eight-nucleate stages. Differentiation establishes the mature stage composed of a central egg cell flanked by two synergids at the micropylar end of the female gametophyte, three antipodal cells at the opposite end, and two free nuclei, the polar nuclei, in the center.

Optical sagittal sections of the ovules will likely reveal one of the following developmental stages: (1) a megaspore tetrad with the megaspore furthest from the micropyle distinctly larger than the other three; (2) a megaspore tetrad with three degenerating micropylar megaspores; (3) a two-nucleate female gametophyte beneath the degenerated, defunct megaspores; and (4) a four-nucleate female gametophyte with two nuclei at the apex (micropylar end) separated by a large vacuole from a pair of nuclei at the base.

The gynoecium of *Yucca filamentosa* is a syncarp composed of three carpels. In the fusion process, the margins of each carpel contribute to a central column of tissue in the basal portion (ovary or ovulary) to which six rows of anatropous, bitegmic ovules are attached (axile placentation). Each carpel has a large median bundle located in the wall of the syncarp and two marginal bundles located in the central column. Ovular traces from the marginal bundles extend into the funiculi of the ovules.

Prior to processing sections of *Yucca* ovularies 60 µm thick prepared with a Hooker fresh-section microtome are fixed in Randolph's modified Navashin fluid for 24 hours and stored in 70% ethanol. To process a section do the following:

1. Use a camel hair brush to transfer a section of the *Yucca* ovulary from storage 70% ethanol to a slide.
2. Cover the section with BB-4½ clearing fluid and add a cover glass.
3. Examine the preparation with a dissecting microscope using transmitted light to observe the vascular distribution and the position of the ovules.
4. Examine this preparation or a similar one with either a phase contrast or a Nomarski interference microscope.

In the sections for this exercise, the bitegmic ovules should contain mature female gametophytes, each one situated beneath one layer of cells at the apex of the nucleus. The micropylar portion of the gametophyte is quite broad whereas the chalazal portion (opposite pole) is very narrow. As is typical for many flowering plants, the vacuole of the egg cell is located in the micropylar portion of the cell while that of each synergid is situated in the chalazal portion. The polar nuclei fuse early, prior to the entrance of the pollen tube, to form a very large secondary nucleus located toward the chalazal portion of the gametophyte. The antipodals are ephemeral but may still be evident in some preparations in the very narrow chalazal pole of the female gametophyte. A broad funiculus attaches each ovule to the axile placenta. In most preparations,

the integuments are likely to be nearly equal in length with the inner one tightly closed around the apex of the nucellus.

A member of Amaryllidaceae, *Iphion uniflorum* (Spring Starflower), bears erect funnel-form flowers each typically with six perianth segments, six stamens, and three carpels. In large populations, atypical flowers with 8-8-4 and 10-10-5 arrangements are common. Placentation is axile, and each loculi of the ovulary contains two rows of hemianatropous, bitegmic ovules. The ovules, nearly mature before anthesis, contain a female gametophyte in the apical portion of the pseudocrassinucellate nucellus. Of typical form, the gametophyte contains an egg, two synergids, two polar nuclei or a single very large secondary nucleus, and three antipodal cells. Occasional periclinal divisions in the apical portion of the nucellar epidermis indicate the pseudocrassinucellate condition. The inner integument extends beyond the outer one so that the micropyle is formed by the endostome (opening in the inner integument) alone.

1. Transfer a pistil (syncarp) from storage ethanol to a Radnoti planchet and cover it with BB-4½ clearing fluid.
2. Place the planchet on the stage of a dissecting microscope and observe the syncarp with transmitted light. The rows of ovules will become clearly visible through the ovulary wall.
3. Use minuten-pin dissecting needles to remove the ovules and with a Pasteur pipet transfer them in clearing fluid to a Raj slide with cover glass support mounts. Place a cover glass across the mounts.
4. Observe the preparation with a bright-field microscope with the iris diaphragm closed to a diameter less than that of the objective lens used.
5. Examine the ovules in optical median sagittal section and identify the features of structure and development that can be readily recognized. Examine a preparation with either a phase contrast or a Nomarski interference microscope.

Exercise 5:
New Uses for Calcium Chloride as a Clearing and Mounting Medium

Throughout the first half of the 19th century, microscopists were in diligent search for mounting media which would preserve whole mount and hand sectioned specimens on microscope slides. The various substances employed were actually referred to as preservatives rather than as mountants. In 1841, Professor P. Harting of Utrecht introduced calcium chloride solution as a superior preservative and, according to Rooseboom (1956), published his account in 1843. Moleschott's (1844) account of Harting's method, along with an addendum to his comments by von Mohl (1844), served to popularize the use of this medium. Von Mohl considered solutions of calcium chloride as superior to aqueous sugar solutions or sugar and acetic acid mountants introduced by Oschatz (1843). The most detailed accounts of the calcium chloride technique were offered by Schleiden (1849) and Schacht (1853). The hygroscopic nature of calcium chloride negates the need for hermetically sealing the preparations, and slides prepared in 1848 by Hermann Schacht and Ernst Hallier, students of Schleiden, are still in good condition in the Museum for the History of Science in Leiden (The Netherlands).

Glycerine was introduced as a mountant by Warington (1849) and glycerine jelly by Deane (1852). These media, and especially the improved formula for glycerine jelly offered by Lawrence (1859), gradually replaced calcium chloride solutions. However, calcium chloride as a mountant continued to appear in treatises on microtechnique. Balfour (1855) mentions his preference for a solution of one dram of calcium chloride in a half ounce of water (approximately 11%). Griffith

and Henfrey (1556) report that solutions of calcium chloride (no specific concentrations cited) are highly refractive and frequently render the specimen, "so transparent that it is almost or completely invisible." They state further that the medium apparently dissolves some cell components. Much later, however, Poulsen (1884) recommends aqueous solutions of calcium chloride in concentrations of two to three parts water to one of the salt as sometimes useful as a mounting medium for permanent preparations. He states further that although glycerine by that time had largely replaced it, Treub (1876) had used calcium chloride combined with glycerine for clearing tissues. By the close of the 19th century, use of calcium chloride as a mountant was for the most part abandoned, and most texts on microtechnique excluded any mention of the procedure.

In the study reported here, calcium chloride solution is shown to be useful as a clearing agent, as a medium for specifically staining lignified tissue with acid-phloroglucin or with toluidine blue 0, and as a mountant for sections and whole mounts of plant specimens.

Procedure

The procedures for staining lignified walls either red with acid-phloroglucin or blue with toluidine blue 0 may be applied to the whole structures (e.g., *Nymphaea* leaf fragments) and to the 240 µm thick sections (e.g., *Psilotum* stems) studied by techniques already presented.

Leaf fragments of *Nymphaea odorata*:

1. Place a leaf fragment of Nymphaea odorata, fixed in FPA50 or Cornoy's (Farmer's) fluid and stored in 70% ethanol, in a vial containing lactic acid.
2. Plug the vial with cotton and autoclave it for 15 minutes at 15 psi.
3. Alternatively, clear the fragments in lactic acid or lactic acid saturated with chloral hydrate at room temperature for several weeks, or at 60°C for 24 hours, or at 35°C for 48 hours.
4. Replace the clearing agent with three changes of 95% ethanol allowing at least 5 minutes for each change.
5. Place the leaf fragment in 95% ethanol in a flat-bottom planchet. Gradually hydrate the fragment through 70%, 50%, and 30% ethanol and two changes of water.
6. Replace the water with an acid-phloroglucin-calcium chloride solution (1% phloroglucin [250 mg] in 25 ml 20% calcium chloride solution plus 4 ml concentrated hydrochloric acid). Allow at least 5 minutes for the fluid to penetrate the fragment.
7. Transfer the fragment to a standard slide or preferably to a Raj slide with slide thickness support mounts. The upper surface of the leaf fragment should rest against the slide surface.
8. Cover the fragment either with acid-phloroglucin-calcium chloride solution or with a fresh calcium chloride solution (20%, without HCl and phloroglucin). Place a cover glass over the fragment.
9. Observe the specimen with low magnification of a bright-field microscope.

The astrosclereids will appear richly defined by the bright red color of lignin in their secondary cell walls. Lignin in the astrosclereids can be specifically stained bright blue through the following procedures:

1. Process the leaf fragments through step 5 above.
2. Replace the water with aqueous 0.05% toluidine blue 0 for 5 minutes or longer.
3. Replace the stain with 20% calcium chloride solution which in approximately 10 minutes will destain all portions of the specimen except lignified cell walls.
4. Mount the fragment on a standard slide or Raj slide, upper side down, in 20% calcium chloride solution, add a cover glass, and observe the bright blue astrosclereids under a dissecting microscope with transmitted light and under low magnification of a bright-field microscope.

Stem sections of *Psilotum nudum*:

1. Process thick hand sections or microtome sections 240 μm thick or thicker as previously described for thick sections.
2. Transfer thin sections (120 μm) or processed thick ones in 70% ethanol and downgrade them to water (50%, 30% ethanol, and water).
3. Use a camel hair brush to transfer a section to a slide and blot away the excess water.
4. Place a few drops of acid-phloroglucin-calcium chloride (1% phloroglucin [250 mg] in 25 ml 20% calcium chloride plus 4 ml concentrated hydrochloric acid) and add a cover glass.
5. Observe the preparation under low and high magnification of a bright-field microscope.

The treatment with acid-phloroglucin-calcium chloride brings about an almost instantaneous reaction to produce a deep-red color in the lignified walls of the peripheral tracheids and a lighter orange-red color in the walls of the fibers in the center. Sclerenchyma in the outer cortex of older stems likewise shows the orange-red color. The broad casparian strips in the endodermis take on the deep-red coloration that characterizes the tracheids. Lignin in *Psilotum* stems can also be differentially stained with toluidine blue 0 by the following procedures:

1. Process the sections as described in steps 1–3 above.
2. Place a drop of aqueous 0.05% toluidine blue 0 on the section for 20–30 seconds.
3. Blot away the strain and replace it in turn with water and then 20% calcium chloride solution for 2–5 minutes.
4. Replace the initial calcium chloride with fresh solution and add a cover glass.
5. Observe the preparation with low and high magnification of a bright-field microscope.

Although a mechanism for the effect is not presently known, calcium chloride solutions remove toluidine blue from all cellular components except lignified cell walls. Toluidine blue applied as described here thus becomes a reliable test for lignin comparable to the acid-phloroglucin test. Tracheids stain a dull, dark blue in contrast with the bright, light blue of the central fibers of the stele and the peripheral ones in the cortex. Lignin in the casparian strips shows the dark blue color that characterizes the tracheids.

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APPENDIX A
A Clearing Technique for the Study of Ovule and Female Gametophyte Development in Angiosperms

The clearing technique described here was devised specifically to extend investigation beyond the limits imposed by traditional section methods. Known originally as the 4½ clearing technique, a name derived from the ratio of ingredients in the clearing fluid, the procedure has undergone several modifications to broaden its use. The basic principle of the method remains unchanged and involves the use of phase contrast or Nomarski interference optics for the examination of cell structure in optical sections of whole ovules.

Standard fixation and storage procedures used for ovule and female gametophyte development should be followed. Best results are achieved if pistils or ovularies excised from flower in all stages of anthesis are fixed for 24 hours in FPA50 (formalin, propionic acid, 50% ethanol, 5:5:90), Allen's modification of Bouin's fluid, or Randolph's modified Navashin fluid. Other fixatives may prove useful, but some, for example, Carnoy's and Farmer's fluids, have limited value. Fixed material may be stored indefinitely in 70% ethanol.

The whole ovulary or ovules dissected out in 70% ethanol should be placed in standard 4½ clearing fluid composed of lactic acid (85%), chloral hydrate, phenol (dry crystals), clove oil, and xylene (2:2:2:2:1, by weight) for 2–24 hours. A small concave dish, such as a Radnoti planchet (Radnoti Glass Technology, Inc., 227 West Maple Ave., Monrovia, CA 91016), is recommended for the clearing process. Cleared ovularies and ovules appear nearly transparent under the dissection microscope equipped with transmitted light. By means of a Pasteur pipet, the ovules should be transferred with a drop of the fluid to a Raj slide for microscopic examination. A Raj slide is an ordinary microscope slide with two cover glasses (No. 0–2, 12 or 18 mm2) affixed with permount or a similar mountant 1–1.5 cm apart. Freshly prepared slides must be placed on a warming table at 50°C for 3 days to insure adequate hardening of the mountant. The drop of clearing fluid with several ovules is placed in the center of the 1-1.5 cm space, and a cover glass is then placed over the preparation so to rest on the two permanently mounted cover glasses. These support cover glasses must be of sufficient thickness to eliminate any pressure of the top cover of the ovules.

The preparation should be examined with either phase contrast or Nomarski interference optics. Ovules in all stages of anatropous or campylotropous development tend to lie sideways; that is, with the long axis of the nucellus parallel to the slide. Downward focusing, therefore, reveals ovule structure through a series of optical sagittal sections. By slight shifting of the cover glass on the support mounts, small ovules can be reoriented for optical sections in the frontal or transverse planes.

After brief exposure to the clearing fluid, the ovules become quite fragile. If, therefore, the cover glass is lightly and repeatedly pressed with a dissecting needle midway between the support mounts, the cells of the ovule gradually become spread apart. This squash procedure, performed under observation with a dissecting microscope, does not disrupt the structural integrity of the individual cells. Furthermore, it provides a method for extending the observations to detailed features of specific structures such as megasporocytes, megaspores, and developing female gametophytes previously identified within ovules.

The clearing process in some cases may be greatly enhanced if the stored ovularies or dissected ovules are dehydrated and/or pretreated prior to application of the clearing fluid. Modifications of the standard 4½ clearing fluid too may produce an improved clearing image. Often and perhaps in the majority of cases, dehydration of the materials to absolute ethanol (70%, 95%, 100% ethanol) is necessary for best results. For some species, a pretreatment procedure is required for successful clearing. Where these procedures are applied, the ovules should be passed through a graded series of absolute ethanol and the pretreatment fluid. The most widely tested pretreatment fluids to date are: lactic acid (applied 24 hours at 60°C), lactic acid-phenol-benzyl benzoate, 1:1:1, by weight (applied for 24 hours at 60°C). The latter two fluids should be applied in closed containers. Pretreated ovules may be transferred directly or by way of an ethanol series to clearing fluid.

Of several modifications of the standard 4½ clearing fluid the two mixtures that have been most widely applied are IKI-4½ and BB-4½. To make IKI-4½, add 100 mg iodine and 500 mg potassium iodide to 9 g of the standard fluid. IKI-4½ enhances the phase contrast image and permits detection of accumulated starch. BB-4½ is concocted by the addition of one part benzyl benzoate by weight to the standard fluid. In many

cases, use of BB-4½ markedly increases the definition and contrast of cell contents. With the passing of time from a few minutes to several days, the contrast of the cell contents begins to diminish. The effect is progressive such that gradually the protoplasm in all cells throughout the ovule becomes homogeneous, and cytological features (nuclei, vacuoles, etc.) are not distinguishable. Concomitantly, the cell outlines at all focal planes becomes sharply defined. The effect is discernible with phase contrast and to a greater advantage with Nomarski interference optics. The surface topography of cell layers at any focal plane can be perceived, and the examination of the surface aspects of internal layers reveals several manifestations of growth and development which are not easily detected with phase contrast microscopy. In short, the effect is invaluable for interpreting the tectonic or architectural aspects of ovule structure.

Recently, the utility of these techniques has been enhanced by methods for making preparations permanent, and two of these procedures will be described here. Cleared ovules can be permanently mounted in piccolyte or permount by replacing the clearing fluid with absolute ethanol and upgrading the ovules in mixtures of ethanol and xylene (3:1, 2:2, 1:3, and xylene). The ovules are mounted on Raj slides in either mountant and covered. Optical sagittal sections through the ovules have a marked similarity to microtome sections. The protoplasts are shrunken sufficiently to distort the outlines of cell walls. This image, so characteristic of permanent preparations, has become an acceptable artifact variably assessed as to cause. Another method for permanently mounting cleared ovules leaves the cleared image unaltered and indicates xylene as the actual cause of the artifact. The mountant used contains the ingredients of Spurr low-viscosity embedding medium. Vinylcyclohexene dioxide (10 drops) is combined with diglycidyl ether of polypropyleneglycol (6 drops) and nonenyl succinic anhydride (26 drops). BB-4½ is mixed with part of this epoxy medium in a graded series of 3:1, 2:2, and 1:3. Ovules treated for 24 hours in BB-4½ are passed through this series with 15 minutes exposure to each mixture. The 1:3 mixture is replaced with 20 drops of pure epoxy medium for 15 minutes. One drop of dimethylaminoethanol, the cure accelerator, is then added and the ovules are mounted and covered immediately on a Raj slide. The preparation is cured in an oven at 60°C for 24 hours or at room temperature for several days and then observed with phase contrast or Nomarski interference optics.

Figures 5.1 to 5.6 illustrate some of the major features of clearing technology discussed here

Figure 5.1. An early two-nucleate female gametophyte of *Cassia occidentalis* observed with Nomarski interference optics beneath approximately 12 layers of cells. The cleared ovule from which this optical median sagittal section was photographed is permanently mounted in the epoxy mountant related to Spurr low-viscosity embedding medium.

Figure 5.2. Two microspores of *Michelia fuscata* with nuclear division in progress to establish the generative and tube cells, photographed from a hand section of an anther cleared in BB-4½ clearing fluid. The nuclei are at metaphase, a polar view on the left and a side view on the right.

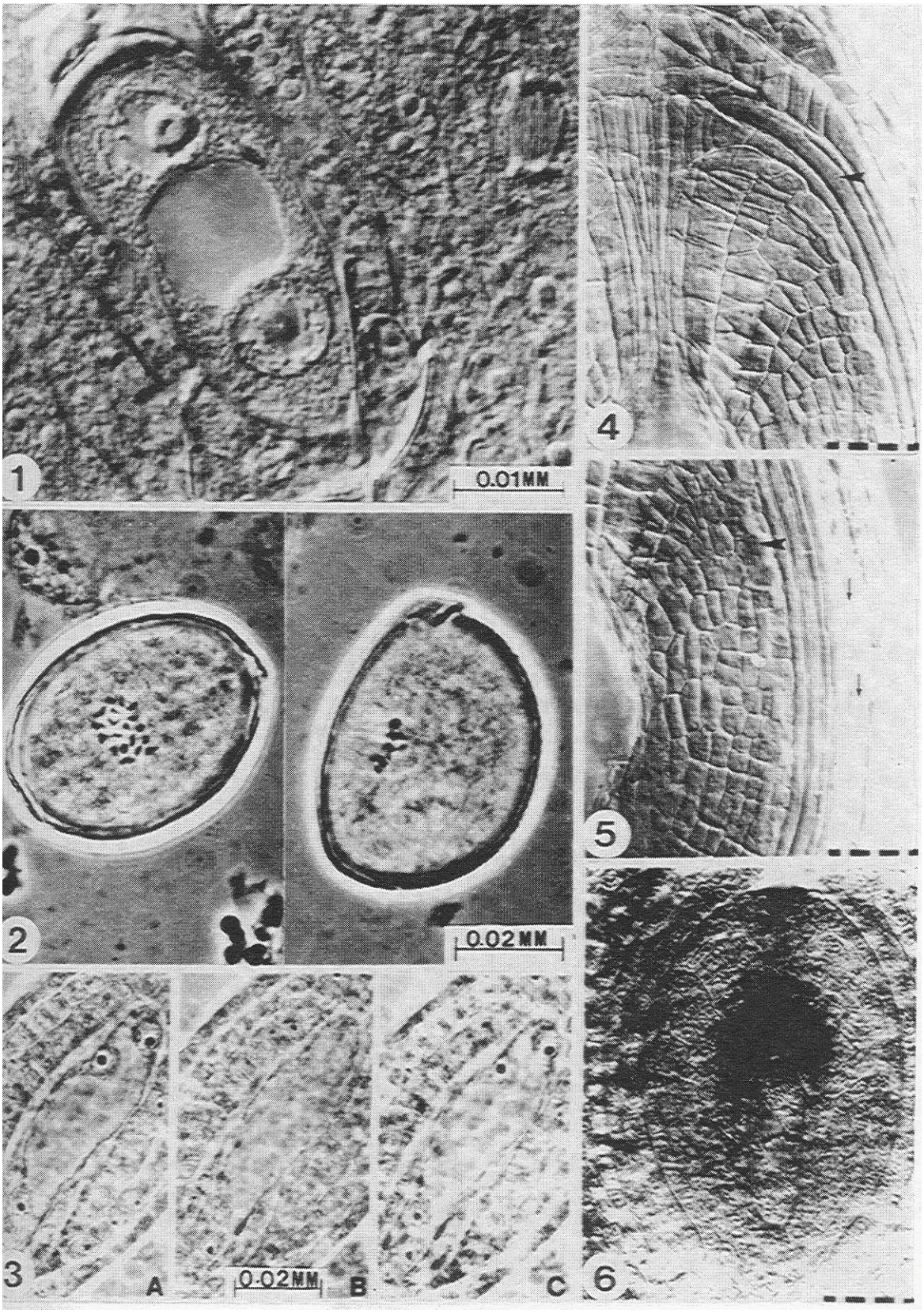
Figure 5.3. A four-nucleate female gametophyte of *Oxalis corniculata* in a median sagittal section of an ovule cleared in IKI-4½ clearing fluid. (A) One chalazal and two micropylar nuclei are visible. (B) A second chalazal nucleus is revealed. (C) A composite photograph of (A) and (B). The photographic combination of two focal planes (A and B) allows for an accurate assessment of the position of the nuclei in the four-nucleate female gametophyte (C).

Figure 5.4. Apical nucellus and inner integument in an ovule of *Ludwigia uruguayensis* at the two-nucleate stage. The cytological features of the cell contents have disappeared under the influence of several days exposure to BB-4½ clearing fluid thus to reveal surface aspects of the cells in this optical median sagittal section observed with Nomarski interference optics. The periclinal files of cells in the nucellus adjacent to a central column of elongated cells produce sufficient pressure to distort the cells of the inner epidermis of the inner integument (arrow). The anticlinal walls of these pressured and stretched cells are not visible.

Figure 5.5. A lateral view of the nucellus and inner and outer integuments of the ovule photographed in Figure 5.4. In this optical median sagittal section, the outer portion of the nucellus appears buckled (broad arrow) from the pressure caused by the proliferating lateral nucellus. This pressure is apparently completely absorbed by the inner integument. Note that the anticlinal walls in the outer integument are distinct (two narrow arrows) indicating that the layers are not under stress.

Figure 5.6. Nucellus in an ovule of *Ludwigia uruguayensis* in an optical median sagittal section under Normarski interference optics. The ovule was fixed in FPA50, pretreated in lactic acid at 60°C for 24 hours and cleared in IKI-4½ clearing fluid. Starch is amassed in a crescent-shaped zone in the nucellus above the megasporocyte at metaphase I. The optical section is approximately 14 cell-layers deep.

In **Figures 5.4, 5.5**, and **5.6**, the magnification is indicated by three white bars in the lower right corner of each photograph. The space between the bars represents 0.01 mm.


1
0.01MM
2
0.02MM
3
A
0.02MM
B
C
4
5
6

APPENDIX B
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