Feulgen Staining of Intact Plant Tissues for Confocal Microscopy

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ABSTRACT. A method was developed to prepare plant structures for confocal laser scanning microscopy by combining Feulgen staining with pararosaniline and embedding in LR White™. This procedure preserves intact, delicate structures for three-dimensional imaging without loss from sectioning or squashing, and the slides can be viewed several times without serious photobleaching.

Key words: chromosomes, confocal microscopy, fertilization, Feulgen, pararosaniline, Schiff

Confocal laser scanning microscopy optically slices through biological structures and allows them to be viewed in three dimensions (Brakenhoff et al. 1989, Pawley 1990). Fluorescent dyes such as Lucifer yellow, fluorescein diacetate, and 5(6) carboxyfluorescein have been used extensively to study transport system of plants (Cole et al. 1991, Duckett et al. 1994, Oparka et al. 1994). Immunochemical approaches using fluorescently labeled antibodies have been favored by workers investigating structures within cells (Beven et al. 1991, Flanders et al. 1990, Stafford et al. 1992). Fluorophores for confocal microscopy of chromatin include acridine orange (Tsien and Waggoner 1990), chromomycin A3 (White et al. 1987), DAPI (Brakenhoff et al. 1989, Rawlins and Shaw 1988), ethidium bromide (Tsien and Waggoner 1990), Hoechst 33342 (Tsien and Waggoner 1990), mithramycin (Brakenhoff et al. 1985), and propidium iodide (Tsien and Waggoner 1990). Additional methods for nuclei have used DNA or RNA sequences labeled with fluorescein isothiocyanate (FITC) (Highett et al. 1993, Makowski and Ruzin 1994, Weterings et al. 1995). The fluorescence of these molecules diminishes rapidly when radiated with the laser (photobleaching), limiting the applicability of these approaches.

Cheng and Summers (1990) reported that as a fluorescent nuclear stain for confocal microscopy, pararosaniline (basic fuchsin) in the Feulgen procedure was more stable than chromomycin A3 and they used Feulgen staining for confocal studies of sea urchin development (Summers et al. 1993). Erlandsen and Rasch (1994) used Feulgen staining with confocal microscopy to visualize chromosomes and to quantify DNA content of the parasitic intestinal flagellate, *Giardia lamblia.*

During a study of interspecific hybrids of wild (*Solanum stoloniferum* ssp. *stoloniferum* Schlechtd. et Bche., *S. phureja* Juz. et Buk.) and domesticated potatoes (*Solanum tuberosum* L.), our analysis required that we prepare ovules for three dimensional imaging with confocal microscopy without distorting nuclear orientation within the egg apparatus. We developed a method combining Feulgen staining and embedding in LR White™ that allowed the nuclei of fixed, embedded, intact, and undistorted potato ovules to be viewed by confocal microscopy. This method may be applicable to confocal microscopy of a variety of plant tissues. Here we describe the method and present representative examples.

MATERIALS AND METHODS

Tissues

The following specimens were used in the present study: the apical 4–5 mm of adventitious roots from freshly germinated onion (*Allium cepa* L.) bulbs, first leaves of germinating seeds of *Luzula purpurea* Link., intact anthers of *Solanum phureja* on the first day of anthesis, and

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ovules from flowers of *S. stoloniferum* 3 days after pollination with pollen from *S. phureja.*

Fixation and Staining

The tissues were fixed overnight in 3:1 ethanol:glacial acetic acid at 4 C. Material may be stored in fixative for several weeks in the refrigerator. For longer storage, the fixative should be replaced with several changes of 70% ethanol and can be stored indefinitely in the freezer.

The tissues were rinsed three times with distilled water for 15 min each and hydrolyzed in 5 N HCl for 50–60 min at room temperature. After hydrolysis, the tissues were rinsed three times with distilled water for 5 min each and stained with Schiff's reagent prepared with pararosaniline for 2–3 hr at room temperature. We have used Schiff's reagent prepared commercially (Sigma, P.O. Box 14508, St. Louis, MO 63178, product number S 5133) or prepared by Lillie's (1951) method with Harleco basic fuchsin (C. I. No. 42510, Certification No. LF-54).

The Schiff's reagent was decanted and specimens were rinsed three times for 10 min each with cold (4–5 C) distilled water. The last change of distilled water was replaced with 70% ethanol for 10 min; the ethanol turns pink. The specimens were dehydrated with one change of 95% ethanol and three changes of 100% ethanol for 10 min each. The ethanol may turn pink. The samples were stored overnight in the refrigerator. If the ethanol became pink, it was replaced with fresh 100% ethanol and changed continuously with 100% ethanol at hourly intervals until it remained colorless. For some material, this took several days. Material in fresh 100% ethanol may be stored in the freezer for several weeks until infiltration with LR White™.

Embedding

The samples in ethanol were brought to room temperature and LR White resin (soft) was added to make a 1:1 ethanol:LR White mixture. After mixing thoroughly, the samples were left for 1 hr with occasional stirring. The 1:1 ethanol:LR White mixture was replaced with pure LR White, mixed thoroughly, and allowed to stand for a minimum of 1 hr, with occasional stirring. The LR White was replaced with pure LR White and left overnight with occasional stirring.

The specimen was placed in fresh LR White on a standard glass microscope slide. If needed, the samples were dissected to expose structures

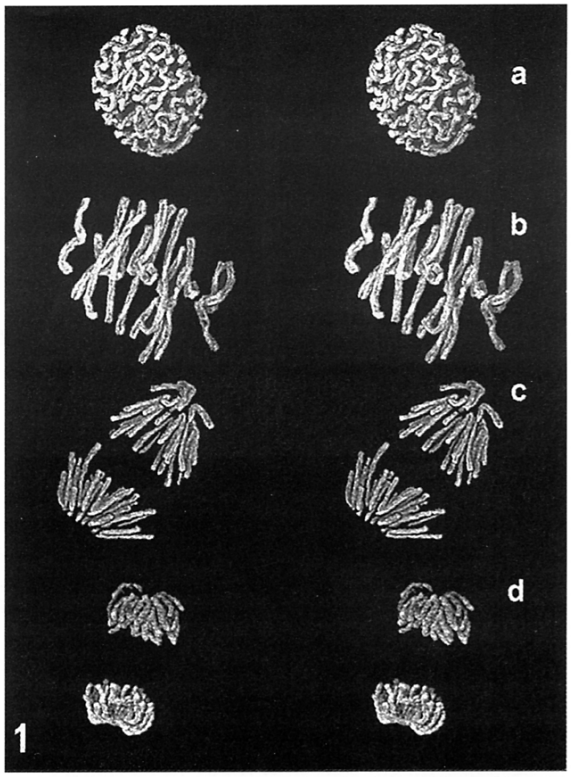

Fig. 1. Stereo pairs of mitotic stages in onion root tip. a) Prophase, b) metaphase, c) anaphase, and d) telophase. × 800.

that were to be examined by confocal microscopy. In the present study, root tips used for Fig. 1 were teased apart with dissecting needles into strands of five to ten cells thick. For Fig. 2, the intact first leaf that had emerged from a germinating seed was mounted whole in LR White. The pollen grain in Fig. 3 was released from an anther by teasing the anther apart with jeweler's tweezers. The fertilization image (Fig. 4) was obtained from an intact ovule that was scraped with a fine scalpel blade from the placenta of a potato ovary.

A coverglass (size 00) was gently lowered over the specimen in LR White taking care to avoid air bubbles in the resin. For delicate structures such as ovules, small pieces of broken coverglass were placed near the specimen to keep the coverglass from crushing the specimen.

The samples were incubated at 60 C overnight. The LR White shrinks and pulls away from the edge of the coverslip during polymerization. In addition, LR White in contact with oxygen will not polymerize completely; unpolymerized LR White can be removed from the slide

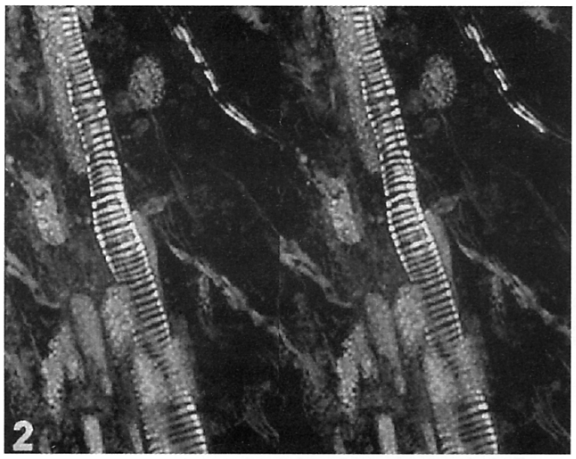

Fig. 2. Stereo pair of a portion of xylem element in young leaf of *Luzula purpurea.* × 800.

with 100% ethanol. An alternative is to incubate the slide in a vacuum oven to reduce the exposure of LR White to oxygen during polymerization.

The specimen in the center of the slide is embedded in LR White and can be cleaned without being crushed. The coverglass can be carefully removed from the polymerized LR White by sliding a clean razor blade under the coverglass; this does not affect the image through confocal microscopy when examined with oil immersion objectives. Slides may be stored at room temperature or in the freezer to prevent fading of pararosaniline.

The excitation λ for the laser was set at 488 nm and the detector at 535 nm and longer. Power of the laser, gain for the detector, and other parameters such as black level, may vary with specimen or the confocal instrument used and can be adjusted during observation to obtain the maximum image quality. All images shown in Figs. 1–4 were taken through a Nikon 60 × oil immersion objective.

RESULTS AND DISCUSSION

Figures 1–4 demonstrate the quality of images obtained with the Feulgen/LR White procedure. Nuclear structures fluoresce against a dark background revealing extremely fine detail including coiling of chromatids during mitosis (Fig. 1). Feulgen staining is not specific for DNA when viewed with confocal microscopy because xylem, pollen cell walls (Figs. 2 and 3), and some cytoplasmic components fluoresce.

The primary advantage of preparing plant material for confocal microscopy with the method described here is the ability to observe plant cellular structure and tissue organization in three dimensions with excellent resolution. Fixation and hydrolysis in the Feulgen/LR White method, however, does destroy some cellular components such as microtubules and RNA, thus preventing immunolocalization procedures with other fluorochromes. Confocal microscopy is less laborious than serial sectioning and allows preservation of delicate structures that may be destroyed during sectioning or squash preparations. Figure 4 in particular demonstrates the advantages of the Feulgen/LR White method. The orientation of polar nuclei, egg nucleus, and pollen germ units are maintained within an intact ovule, and their relative positions are clearly revealed in three dimensions. Specimens in Figs. 1–4 have been examined up to five times without significant photobleaching.

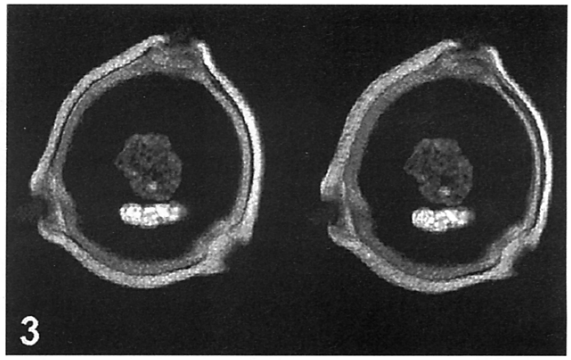

Fig. 3. Stereo pair of pollen grain of *Solanum phureja* with less dense vegetative (tube) nucleus and rod shaped, dense generative nucleus. Two layers of pollen walls also fluoresce revealing three pores. × 1500.

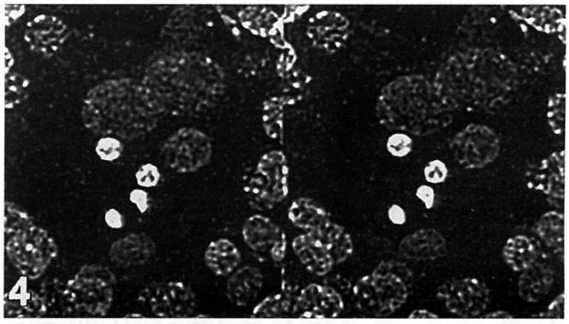

Fig. 4. Stereo pair of incipient double fertilization from an intact ovule in a cross between *Solanum stoloniferum* (ovulate parent) and *S. phureja,* with one sperm near the two polar nuclei and the other near the egg nucleus. The two smaller brightly fluorescent bodies are the x-bodies, remnants of the vegetative (tube) nucleus, and nucleus of the degenerated synergid. × 800.

The combination of staining with the Feulgen procedure and embedding in LR White has several advantages over mounting stained material in methyl salicylate (Cheng and Summers 1990). There is no overpowering aroma while viewing LR White embedded materials, there is less photobleaching of LR White embedded material than with methyl salicylate, there is no danger of crushing the material or distorting it by the weight of the coverglass, and slides of LR White embedded tissues may be stored and viewed repeatedly.

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