**A cryohistological protocol for preparation of large plant tissue sections for screening intracellular fluorescent protein expression**

Elisabeth Knapp¹, Rosemary Flores¹, David Scheiblin²,³, Shannon Modla², Kirk Czymmek²,³, and Vidadi Yusibov¹

¹Fraunhofer USA Center for Molecular Biotechnology, Newark, DE, USA, ²Delaware Biotechnology Institute, University of Delaware, Newark, DE, USA, ³Department of Biological Sciences, University of Delaware, Newark, DE, USA


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In this study, we have developed a robust cryohistological method that allows imaging of virtually any type of plant cell or tissue while preserving fluorescent protein signals and maintaining excellent cellular and subcellular morphology. This method involves modified fixation of plant tissues (i.e., leaves, stems, and petioles), infiltration in a sucrose gradient, freezing, and collection of cryosections directly onto a cryoadhesive tape. Using this method followed by microscopic analysis, we demonstrated a localized accumulation of green fluorescent protein (GFP) in *Nicotiana benthamiana* plants agroinfiltrated with the movement-incompetent tobacco mosaic virus–based vector and systemic accumulation of GFP in plants infiltrated with the movement-competent vector. Overall, this simple cryohistological procedure reduced sample preparation time and allowed processing of tissue sections for high-resolution imaging of targeted fluorescent proteins in all plant tissues.

In recent years, the agricultural, medical, and pharmaceutical industries have been increasingly recognizing plants for their potential to produce large amounts of recombinant proteins (1,2). A variety of plant virus–based vector systems for expression of recombinant proteins in plants have been described (3–6). The development of recombinant protein expression platforms was greatly facilitated by the use of fluorescent marker proteins (7), which allowed live cell imaging of plant tissues to rapidly determine whether an expression strategy was feasible or not. However, live cell imaging of intact plants with handheld UV lamps or epifluorescence microscopes suffers from several limitations, namely low sensitivity and resolution, making it difficult or impossible to obtain details of protein expression beneath the epidermal cell layers. With very few exceptions, even with confocal or multiphoton microscopy, live cell imaging typically can only monitor fluorescent protein expression a few cell layers beneath the surface. Some further imaging improvements have been recently reported (8), demonstrating that better optical qualities of living plant leaves can be achieved by infiltration with perfluorodecalin, extending the imaging depth of confocal microscopy to about 50 µm. The optical limitations in plants are primarily due to such factors as absorbance, scattering, and severe spherical aberration resulting from refractive index changes associated with cell walls, cell contents, and air pockets. Ultimately, this makes visualization of expression patterns and protein localization in the vast majority of tissue beneath the plant cell surface problematic, especially with vascular and woody tissues and thick mesophyll layers with abundant chloroplasts.

To alleviate such optical effects, the sectioning or clearing of tissues is typically required. Clearing has been a tried and true method that facilitates deep imaging within plant tissues using both standard histological stains and contemporary probes. This procedure includes extraction of chlorophyll and other cell constituents/cell wall materials, staining of structures of interest, and finally, infiltration of tissue with compounds that ultimately render the sample transparent (9,10). The clearing technique is highly useful for studying plant cell/tissue architecture (11). However, due to the nature of clearing with harsh chemicals, only the most abundant and stable cell constituents are preserved. This severely limits the utility of the method for detecting host’s labile molecules and fluorescent proteins and application of various techniques for protein and nucleic acid localization.

For decades, traditional histochemical protocols have proven to be invaluable for imaging internal plant structures. The standard procedure consists of tissue fixation, dehydration, rehydration, and subsequent embedment of the plant material into paraffin or plastic polymers. After sectioning of the embedded tissue, the protein of interest is detected using antibodies labeled with a fluorescent molecule, an enzyme, or gold particles (12–15). Among the drawbacks of conventional histological procedures are the lengthy (up to 1 week) sample preparation and the need to work with relatively small (millimeter size) tissue samples to ensure good infiltration with chemical compounds. In addition, the most commonly used fixatives, such as glutar-
aldehyde and osmium tetroxide (OsO₄), preserve morphological details but may destroy or interfere with fluorescent signals and the integrity of target protein (16,17). Solvents required for dehydration and resin/wax infiltration can also quench fluorescent protein signals and modify or destroy protein’s native folding pattern.

Thus, we modified the conventional protocol for fixation and cryosectioning of plant material to achieve the goals of reliable cryosectioning and excellent morphology. In order to minimize tissue disruption and leaching/loss of fluorescent proteins and other cell constituents due to ice growth and segregation artifacts, we chose to cryoprotect sucrose gradient-infiltrated tissues and used a commercially available cryocompatible tape to facilitate retention and maintain the integrity of the plant tissue. Our straightforward procedure reduced sample preparation time to 3 days, allowed the handling of tissue samples of at least 1 cm² in size, preserved cellular and subcellular morphology details, and permitted detection of fluorescence signals from the green fluorescent protein (GFP) tag expressed in plant cells using the tobacco mosaic virus (TMV)–based expression vector. These observations further suggest that detection of immunofluorescence signals also should be also fundamentally possible, as previously described for cryosections of Medicago truncatula plant root nodules treated with a fluorescently labeled anti-syntaxin (SYP132) antibody (18) and for cryosections of tobacco leaf tissue treated with a fluorescently labeled anti-calseotide antibody (19), with the caveat that as for any technique/probe/tissue, specific optimization would be required.

All plant tissues (leaves, petioles, and stems) were screened expeditiously for TMV movement protein (MP) expression and localization using epifluorescence microscopy. The results obtained using cross-sections were fully compatible with those of more detailed subcellular fluorescent protein localization studies using confocal microscopy and ultimately allowed us to determine the extent of TMV MP expression patterns in different plant tissues.

Due to the inherent difficulties associated with imaging large (and even small) internal plant structures, we expect that the technical protocol described herein will be highly valuable to plant researchers.

**Materials and methods**

**Plasmid DNA**

All constructs were built using standard recombinant DNA techniques (20). The binary plasmid pCASS4Rz has been described previously (21). All TMV derivatives were made from the infectious wild-type TMV cDNA clone (22), ∆Cla151, a TMV defective RNA (23), was PCR-amplified to attach a Stul site to the 5’ untranslated region (UTR) and a Kpn1 site to the 3’ UTR. The amplified fragment was digested with Stul and Kpn1 and inserted into the similarly digested pCASS4Rz plasmid to create pCASS∆Cla151 (E. Knapp, unpublished data), which was used for further cloning. pTMVD4∆MP, which lacks nucleotides (nts) 4920–5180 within the MP gene (D.J. Lewandowski, Ohio State University), and pTMVD4GFP, which contains GFP in place of the coat protein (CP) gene, (3,5) were digested with Xmal and Kpn1, and the resulting fragments were cloned into the similarly digested pCASS∆Cla151 plasmid.

**Agroinfiltration**

The binary pCASS4 plasmids harboring the TMV derivatives were electroporated into Agrobacterium tumefaciens strain GV3101 in 100 µL following the Bio-Rad MicroPulser manual (Bio-Rad Laboratories, Hercules, CA, USA). Transformed A. tumefaciens were suspended in 1 mL lysogeny broth (LB) medium and grown for 2 h at 28°C under rotation at 220 rpm in a shaker. Fifty to one hundred microliters A. tumefaciens solution were plated onto LB plates containing 50 µg kanamycin/mL medium. Multiple colonies were inoculated into 3 mL LB plates and grown overnight. Aliquots of the overnight cultures were inoculated into minimal AB medium (24) and grown overnight to an OD600 of 1.0. Nicotiana benthamiana plants at the six-leaf stage were submerged into the agrobacteria solution and infiltrated under vacuum for 1 min at 28 psi.

**Plant tissue fixation and sucrose gradient**

Leaves, petioles, and stems were excised from agroinfiltrated N. benthamiana at 4–12 days postinfiltration (dpi). Samples that were submerged in a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.01% Fluorescent Brightener 28 (cat # F3543; Sigma-Aldrich, St. Louis, MO, USA) were vacuum-infiltrated at 28 psi for 1 min and incubated for 2 h. Following five 10-min washes in phosphate-buffered saline (PBS; pH 7.0), samples were fixed overnight at 4°C in the above buffer devoid of Fluorescent Brightener 28. Leaves were washed for 15 min in PBS, cut into squares (1 cm²), and
subjects to 2.3 M sucrose gradient infiltration. The gradient consisted of 25%, 33%, 50%, 66%, 75%, and 100% 2.3 M sucrose, respectively, in 0.1 M Sorenson's buffer. Infiltration steps with 25%, 33%, 50%, 66%, and 75% 2.3 M sucrose were performed for 1 h each at room temperature, whereas the final 100% 2.3 M sucrose was infiltrated overnight at 4°C.

**Cryosectioning**

Leaf tissue (1 cm²), petioles, and stems (1 cm length each) were embedded into optimum cutting temperature (OCT) compound (cat #62550-01; Electron Microscopy Sciences, Hatfield, PA, USA) and frozen at -30°C in a CM3050S Cryostat (Leica, Buffalo Grove, IL, USA). Samples could also be frozen at -80°C or in liquid nitrogen if desired, although we found that liquid nitrogen–frozen samples in large OCT blocks were prone to cracking. The frozen block with the sample was trimmed, and thick sections were taken and sectioned until the region of interest was reached. Sections (10, 25, or 50 μm) containing the intact plant material were cut and placed onto the adhesive side of a cryocompatible clear adhesive tape (cat #62800-72S; Electron Microscopy Sciences), which was then placed onto a standard glass slide with the section facing upward. While we evaluated a small range of section thicknesses for this work using conventional light microscopy, 25 μm were optimal for containing sufficient cellular material for clear identification of cell types, morphology, and contrast. Confocal microscopy worked well with 25-μm and thicker sections (25). The sections were mounted in SlowFade Gold (cat # S36936; Invitrogen, Eugene, OR, USA), covered with 20 mm × 50 mm no. 1.5 coverslips, and sealed with nail polish. The slides were stored in the dark at 4°C until imaged. Frozen OCT-embedded plant sections and tissue blocks were stored at -80°C in sealed bags for up to 1 week and 2 months, respectively.

**Screening of plant tissue for target protein localization**

**Light microscopy**

Slides were warmed to room temperature prior to viewing using light microscopy. Micrographs were recorded with a digital Zeiss AxiosCam HR camera mounted onto a Zeiss Axioskop2 MOT microscope (both from Carl Zeiss, Oberkochen, Germany) equipped with a 100 W mercury arc lamp. Both camera and microscope were controlled by the Zeiss AxiosCam HR software (version 4.2; Carl Zeiss). Filter sets for fluorescence imaging were from Chroma Technology Corp. (Rockingham, VT, USA). The following filter sets were used: for GFP, a D480/30× excitation (Ex) filter and a D535/40M emission (Em) filter; for calcofluor (Fluorescence Brightener 28) or 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain, a D350/50× Ex filter and D460/50× M Em filter; and for chloroplast autofluorescence, a HQ620/60× Ex filter and HQ700/75×M Em filter. Zeiss 10× Plan-Neofluar (numerical aperture 0.3), 20× Plan-Neofluor (numerical aperture 0.5), and the 40× C-Apochromat [numerical aperture (NA) 1.2] objective water lenses were used (all from Carl Zeiss).

**Confocal microscopy**

Samples imaged using confocal microscopy were acquired with a Zeiss LSM 710 attached to an Axio Examiner (Carl Zeiss) using a C-Apochromat 40× NA 1.2 water immersion objective lens. The 405 nm and 488 nm diode lasers were used with a 405/488/561 nm main beam splitter, and the Quasar detector with 422–470 nm, 494–592 nm, and 648–700 nm detection windows for Fluorescent Brightener 28, GFP, and chloroplasts, respectively. Ten-micrometer-thick z-stacks were acquired at a 0.5-μm z-interval.

Each target protein localization experiment was repeated at least 10 times with 2–4 sections being examined each time.

**Results and discussion**

**Detection of GFP-tagged proteins in plant tissues**

In order to assess the suitability of our cryohistological protocol for identification of individual GFP-expressing cells and to determine the optimum conditions of treatment, we used N. benthamiana leaves infiltrated with A. tumefaciens harboring the TMV expression vector D4ΔMP within the binary plasmid pCASS4 (pCASS4ΔD4ΔMP) [21]. Because D4ΔMP has a portion of its MP gene deleted, it is unable to spread from initially infected cells to adjacent cells. Additionally, N. benthamiana plants were agroinfiltrated with the TMV expression vector D4GFP, which has an intact MP gene and can move which has an intact MP gene and can move within the binary plasmid pCASS4 [26]. We determined the optimum conditions of fixation by examining the morphology and cell integrity of infiltrated plants. Following infiltration with sucrose minimized cell freezing process. Furthermore, slow infiltration with sucrose minimized cell cytoplasmic shrinkage away from the cell walls.

Initially, we used the standard Cryo-Jane Tape transfer method utilized widely with mammalian cell/tissue systems to mount sections ranging in thickness from 10 to 100 μm onto slides. For this, sections were collected on the adhesive side of a Cryo-Jane transfer tape that was placed onto Cryo-Jane slides and gently rolled with a soft roller while still frozen to ensure the tissue was in good contact with the polymer-coated slide. The sections were then exposed to UV light (360 nm for 8 ms) to cross-link the sections with the pretreated polymer surface of the slides. However, none of the tissue sections remained intact upon removal of the tape, indicating that tobacco plant tissues, in particular the leaves, were too fragile to withstand the required roller-induced compression needed to attach sections to the slides. To avoid this significant technical problem, we modified the transfer method by simply collecting the sections on the adhesive side of a cryocompatible clear adhesive tape and then placing them facing upward directly onto a standard glass slide. The advantage...
of this method was that it maintained a large area of the plant tissue intact, without distortion or compression, and prevented section folding.

Several mounting media conditions were also tested for using in this procedure, including distilled water, Gel Mount (Electron Microscopy Sciences), and SlowFade Gold. SlowFade Gold proved to be the most suitable mounting media. SlowFade Gold is a reagent that aids in the resistance to photobleaching and does not cure over time, allowing for it to be used for immediate imaging of the sample. Distilled water dissolved OCT, quenched the fluorescence, and limited the long-term section storage capabilities. Gel Mount also did not preserve the fluorescence during either short-term or long-term section storage as compared with SlowFade Gold. The best results were obtained when the sections were viewed within 1 week of processing. Sections stored and monitored beyond the first week tended to develop an increased background autofluorescence, presumably due to leaching of chlorophyll and/or fluorescent proteins. Tissues embedded in blocks of OCT were storable for an excess of 2 months at -80°C without adverse effects on the quality of cryosections and imaging.

The major steps in the modified cryohistological protocol designed to screen large plant tissue sections for intracellular fluorescent protein localization are summarized in Figure 1.

**Monitoring of TMV MP-GFP expression in infiltrated plants**

GFP expression in leaves infiltrated with either D4ΔMP or D4GFP was visible to the naked eye by 4 dpi at the latest, but the patterns of GFP accumulation resulting from the movement-competent and movement-incompetent vectors were different. The non-moving D4ΔMP vector induced a non-uniform distribution of GFP in infiltrated leaves. The percentage of GFP-expressing cells ranged from 0% to 90%, depending on the areas within the leaf that were viewed. As shown in Figure 2A, D4ΔMP was delivered into epidermal, palisade, and spongy mesophyll cells, as well as into bundle sheath cells of class V veins. These observations demonstrated a localized accumulation of GFP in *N. benthamiana* plants infiltrated with the movement-incompetent TMV vector. In addition, the analysis of GFP expression at the cellular and subcellular levels indicated that the infection of plant cells via agroinfiltration varied within different areas of the leaf.

GFP expression from the movement-competent D4GFP vector was initially observed in foci of infected cells (mainly non-vascular) that were adjacent to non-infected cells (data not shown). By 6 dpi, D4GFP had spread into all non-vascular cells and moved into some major and minor veins. In class V veins, for instance, GFP was observed in bundle sheath cells and phloem parenchyma cells (data not shown). By 8 dpi, GFP was detectable in all cells of the epidermis, cortex, and inner and outer phloem of the major vein, but not in the xylem (X) tissues. (D) Expression of GFP from the D4GFP vector in a cross-section of a lateral root. GFP distribution (green) was irregular and observed in the cortex (C) and the epidermis (E), but also in peripheral regions of the vascular tissue (V). Scale bar: (A) 10 µm; (B) 20 µm; (C) 60 µm; and (D) 100 µm. Leaf tissues were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde, infiltrated with sucrose, embedded in OCT compound, and cryostat-sectioned at the 25-µm thickness.

At 12 dpi, we assessed intracellular GFP localization by confocal microscopy. Cross-sectioning of a leaf provided a longitudinal view through the minor vein and revealed details of GFP localization in all specialized vascular cells, with the exception of the xylem, throughout the entire thickness of the leaf (Figure 3, A–F). At this time point, GFP was already observed in the adjoining lower stem portion (data not shown), demonstrating spreading of D4GFP through the petioles of the infiltrated leaf (data not shown) and systemic accumulation of the protein. These findings indicate that the uniform infection of leaves requires the ability of vectors to move effectively from cell to cell and to enter the phloem of the vasculature.

We have developed a simple and robust cryohistological procedure that reduced sample preparation time to 3 days and allowed for handling of large tissue samples (at least 1 cm² in size) of all plant cell/tissue types. This protocol preserved cellular and subcellular morphology, as well as fluorescent signals, which allowed for performing high-resolution imaging of the subcellular protein localization using confocal microscopy.

This approach is morphologically consistent with conventional methods for plant tissue fixation and sectioning, based on the use of paraffin (27–29), epoxy resin (13,14), methacrylate (30), and cryopreservation (18), but is much more favorable...
than the use of methacrylate and paraffin due to the reduced processing time and the preservation of fluorescent protein signal. It is worth noting that in some high-expression animal systems GFP has been shown to survive paraffin (31) and LR White (32) processing, and it is likely that plants could be prepared similarly. Furthermore, GFP-MP and anti-callose antibody labeling have been previously reported for tobacco leaf tissue cryosections (19). However, in our hands, although preparing standard cryosections with tobacco tissues was technically possible (without tape transfer), obtaining targeted, reliable, and intact sections, especially of fragile leaf tissue and large tissue areas, was highly problematic, hence the development of the tape method described herein provided considerable methodological benefits.

Furthermore, the presented method is compatible with a cell wall counterstain and visualization of chloroplast autofluorescence, suggesting its suitability for other fluorescent affinity probes. The addition of sucrose for cryoprotection prevented the formation of ice crystals that can severely damage/alter cellular structure and light fixation–minimized GFP mobility without significant loss of fluorescent signal, which is a common problem with non-cryoprotected and/or unfixed cryosections. Indeed, our approach is a modification of the classical transmission electron microscopy (TEM)–based method by Tokuyasu (33) using sucrose-infiltrated, resinless, ultrathin cryosections to perform immunogold localization, which has been shown to be compatible with high-resolution fluorescent protein localization in plants (34). An ultrathin cryosectioning approach would not be suitable for rapid screening of large areas of tissue or three-dimensional confocal microscopy of entire cells. However, a modification of the described protocol with our tape transfer method was compatible with a novel fluorescent protein fusion, PDLPS5-GFP, confirming its localization to plasmodesmata of Arabidopsis hypocotyl cells via correlative confocal microscopy and TEM, as well as demonstrating its suitability for other tissue types and plant species (25). Other recent advances in high-resolution imaging approaches, such as serial block-face imaging (35,36), also hold great promise for plant cell biology studies despite the need for the specialized equipment and low-throughput. However, we believe that the cryohistological protocol described herein will complement these sophisticated approaches and be highly valuable to plant researchers who investigate the localization of fluorescent probes or fluorescently labeled recombinant proteins in planta.

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Competing interests

The authors declare no competing interests.

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