Chapter 9
Fluorescent Staining of Living Plant Cells

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9.1 Introduction

Fluorescence is the luminescence emitted when photons are absorbed and released by molecules known as fluorochromes (fluorophores); as a consequence of energy transference, the wavelengths of the fluorescent light are longer. Fluorescence imaging has many benefits, one of which being the high degree of sensitivity. Fluorochromes can be excited repeatedly, thus, a single molecule can absorb and release thousands of detectable photons. As a result of the amplified signal, fluorochromes allow for highly specific cellular and organellar differentiation with limited background. Additionally, fluorochrome detection is nonlethal and does not require chemical fixation, allowing for noninvasive in vivo and real-time imaging. This chapter highlights common fluorochromes, particularly fluorescent dyes, and how they can be applied to plant cell imaging.

9.1.1 Autofluorescence Imaging in Living Plant Tissues

When examining plant cells using fluorescence microscopy, one can excite fluorochromes already present or introduce them. The first option makes use of autofluorescence, wherein endogenous molecules absorb light in the UV and visible light spectra. The most abundant example of autofluorescence is chlorophyll fluorescence. While the majority of light energy absorbed by chlorophyll is allocated to photosynthesis, some of the absorbed energy can be dissipated as heat

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or luminescence (fluorescence). Even with only 1–2 % of absorbed light being diverted to chlorophyll fluorescence, the sensitive nature of fluorescence imaging allows for easy chlorophyll detection [1]. As chlorophyll molecules are embedded in the thylakoids of chloroplasts, red fluorescence emitted by chlorophyll (Table 9.1) allows for dye-free imaging of the entire plastid (Fig. 9.1). Other plant structures that can be imaged by autofluorescence include: cuticles of aerial organs (cutin [2]), pollen exine wall (sporopollenin [3]), secondary cell walls (lignin [4]), periderm (suberin [5]), and vacuoles (anthocyanin [6], flavonol [7]).

9.1.2 Exogenous Fluorochrome Application in Living Plant Tissues

If a cellular structure cannot be visualized through autofluorescence alone, exogenous fluorochromes are introduced. Synthetic organic dyes were the first fluorochromes applied to biological research. Over the past 100 years, derivatives of the original dyes have been further modified and novel probes developed. Fluorescent dyes are still commonly used for observing plant cells today, as they are convenient and easily applied to both living and chemically fixed tissues. The majority of fluorescent dyes are small (~0.5 nm) organic molecules [8] that can passively enter the cell or be taken up via active transport. Due to their small size, common fluorescent dyes such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) can also be conjugated to other biomolecules such as antibodies, without interfering in their biological functions. Cross-linking fluorescent dyes to protein-based probes allows for highly specific detection, however, exogenous protein application in living plant cells is problematic. Although semipermeable, plant primary cell walls only allow molecules and proteins up to 60–120 kDa in size to enter [9, 10], making them impermeable to antibodies exceeding this size limit. Thus, to increase the access of antibody, cell walls can be enzymatically removed and the released protoplasts studied [11].

For live-cell imaging, the expression of biological fluorochromes has become a valuable and powerful alternative to immunofluorescence. In 1994, green fluorescent protein (GFP) was cloned from jellyfish (*Aequorea victoria*) and used as a fluorescent reporter [12]. Since its early use, derivatives of the original GFP and many other fluorescent proteins have been developed for biological expression systems. Biological fluorochromes can be introduced to a large variety of species and expressed alone or fused to a protein of interest allowing protein function to be investigated [13]. Despite their numerous benefits, biological fluorochromes have limitations; most notably, stable transformation mediated by *Agrobacterium tumefaciens* can be time consuming and some plant species remain recalcitrant to this method. Additionally, expressing large amounts of light-emitting proteins can induce artificial responses or cell toxicity. Finally in protein fusion studies, the size of the fluorescent protein can also affect biological functions, ultimately altering the behavior of the protein [14].

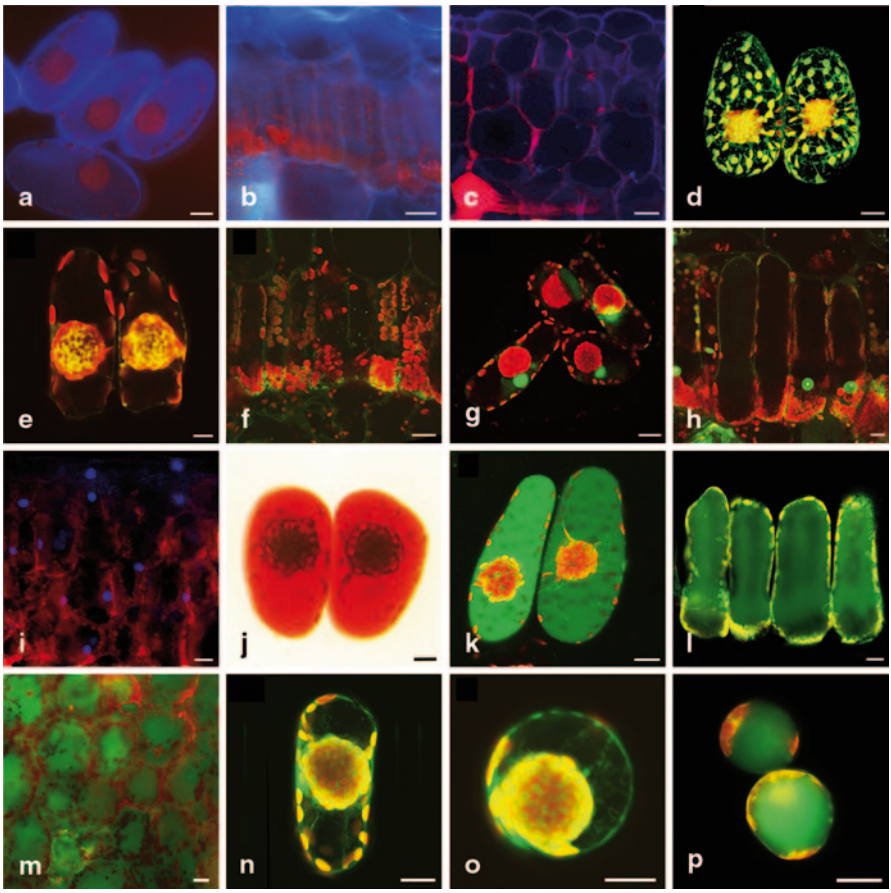

Fig. 9.1 Mesophyll cells and protoplasts of *Arabidopsis thaliana*, *Bienertia sinuspersici, Suaeda aralocaspica, Kalanchoe blossfeldiana*, and *Kalanchoe daigremontiana* stained with various fluorescent dyes to visualize cellular structures such as cell walls, ER, mitochondria, nuclei, and vacuoles. (**a**–**c**) Cell walls in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. daigremontiana* stained with fluorescent brightener 28 (*FB28*). **d** ER in *B. sinuspersici* mesophyll cells stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). (**e**, **f**) Mitochondria in mesophyll cells of *B. sinuspersici*, and *S. aralocaspica* stained with rhodamine 123 (*R123*). (**g**–**i**) Nuclei in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. daigremontiana* stained with acridine orange and 4′,6-diamidino-2-phenylindole (*DAPI*). (**j**–**m**) Vacuoles in mesophyll cells of *B. sinuspersici*, *S. aralocaspica,* and *K. blossfeldiana* stained with neutral red and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA). (**n**–**p**) Viability of mesophyll cells and protoplasts of *B. sinuspersici* and *A. thaliana* stained with FDA. Fluorescence is shown in false colors with green for acridine orange, R123, carboxy-DCFDA, $DiOC_6$ and FDA, blue for DAPI and FB28, and red for the autofluorescence of chloroplasts. Overlapping signals between green and red appear yellow. Panels **a**–**c**, **i**, **m**, and **p** represent epifluorescence micrographs. Panel **j** represents brightfield micrograph. Panels **d**–**h**, **k**, **l**, **n**, and **o** represent confocal micrographs. Scale bars = 25 μm

9.1.3 Fluorescent Staining of Plant Structures and Organelles

When selecting a fluorescent dye, the spectral properties of the fluorochrome should be closely examined. One major concern is ensuring that a fluorescent dye's emission spectrum does not overlap with that of another. Maximum wavelengths of absorption and emission are typical terms used to refer to a fluorochrome, however emission spectra can be very broad [8]. To avoid fluorochrome "bleed-through," it is important to ensure that the microscope is equipped with appropriate optical filters or light sources and the dyes in use exhibit distinct emission spectra. Special care needs to be taken in photosynthetic tissues as the strong chlorophyll fluorescence often masks photon emissions from other fluorochromes [15]. As chlorophyll fluoresces at >600 nm, dyes emitting red light are particularly affected, but fluorescence of all color spectra can be reduced as a result of autofluorescence. To increase fluorescence signals, ethanol treatment can be used to lower chlorophyll levels, however such treatment impairs cellular metabolism and causes cell damage. As an alternative to pigment removal, non-photosynthetic cells can be studied and/or only dyes with strong fluorescence utilized. Here we describe several common fluorescent stains that are routinely used to facilitate microscopic observation of plant structures.

9.1.3.1 Cell Wall

Primary cell walls are mainly composed of cellulose, hemicellulose, and pectin. Unlike the heavily lignified secondary walls of wood tissues which produce endogenous fluorescence, primary cell walls do not typically autofluoresce. For live-cell imaging of primary walls, *calcofluor white* (Fluorescent Brightener 28) is one of the most widely used fluorescent dyes. Calcofluor white binds to β-linked cellulose [16] where it exhibits a broad excitation and emission spectra; calcofluor white has an excitation maximum of 410 nm (Table 9.1) but any filter set with UV or violet/blue excitation wavelengths will generate strong blue fluorescence (Fig. 9.1a, b, and c). Due to the dye's emission spectrum, calcofluor white pairs nicely with other fluorescent dyes that emit 500 nm and above. Finally, as calcofluor white binds to the outer cell wall, membrane permeability is not an issue and the dye is readily taken up by tissues and individual cells (Table 9.2).

9.1.3.2 Endoplasmic Reticulum (ER)

In 1984, 40 years after the endoplasmic reticulum was first discovered by transmission electron microscopy, fluorescence imaging of the membrane network was achieved using *3,3'-dihexyloxacarbocyanine iodide (DiOC$_6$*; [17]). DiOC$_6$ had been previously characterized as a mitochondrial dye [17], but higher concentrations of the fluorochrome resulted in ER localization [18]. Unlike earlier attempts using

Table 9.1 Fluorescent dyes commonly used to visualize plant organelles and structures

Cellular structure	Fluorescent dye	Excitation (nm)	Emission (nm)
Cell wall	Calcofluor white	410	455
Chloroplast	Chlorophyll autofluorescence	488	685(740)
ER	3,3′Dihexyloxacarbocyanine iodide ($DiOC_6$)	482	504
Mitochondria	$DiOC_6$	482	504
	Rhodamine 123	511	534
Nucleus	Acridine orange (dsDNA)	502	525
	Acridine orange (ssDNA, RNA)	460	650
	4′, 6-diamidino-2-phenylindole (DAPI)	350	470
Vacuole	Acridine orange	475	590
	5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA)	504	529
	Neutral red	495	540
Viability	Fluorescein diacetate (FDA)	494	518

ER endoplasmic reticulum

immunofluorescence, which required treatments with organic solvents and detergents to increase antibody permeability, $DiOC_6$ stained both living and glutaraldehyde-fixed cells without damaging the ER membrane (Fig. 9.1d; [18]). Although immunofluorescence and fluorescent proteins have since been further developed, $DiOC_6$ is still commonly used. $DiOC_6$ is a potential membrane-sensitive, cationic fluorescent dye. It is the dye's hydrophilic groups that allow it to strongly accumulate in intracellular membranes where it absorbs blue light and emits a strong green fluorescence signal (Table 9.1). $DiOC_6$ only works with living cells but they can quickly become damaged from photodynamic toxicity; to prevent cell death, it is recommended to limit excitation times. Finally, $DiOC_6$ is a strong dye but it is not very photo-stable thus anti-fade reagents may be added to the mounting medium.

9.1.3.3 Mitochondria

Detection of mitochondria exploits the electron transport system and their accumulation of positively charged redox dyes inside the inner membrane. Similar to $DiOC_6$, *Rhodamine 123 (R123)* is another potential membrane-sensitive, cationic green fluorescent dye (Table 9.1) that is readily sequestered by active mitochondria (Fig. 9.1e, f; [19]). As a result of electric potential across the mitochondrial

membranes, R123 selectively accumulates in the mitochondria. Moreover, under recommended concentrations and incubation times, stained cells often exhibit no accumulation of the dye in other subcellular compartments. However, it has been reported that exposure of plant cells to R123 over long periods of time results in vacuolar accumulation [20]. Although R123 has no cytotoxic effect, the dye and hence fluorescence intensity are easily lost once the mitochondria experience a loss of membrane potential and thus the dye requires living cells with functional mitochondria [20].

9.1.3.4 Nucleus

The porous nuclear envelope readily allows fluorescent dyes to passively enter the nucleus. Fluorochromes for visualizing nuclei bind to and sometimes alter nucleic acids, making long-term exposure toxic to living tissues. One commonly used nuclear dye is *acridine orange*, a cationic dye that binds to both DNA and RNA via electrostatic attractions [21]. When bound to double-stranded DNA, acridine orange has an excitation and emission maxima of 502 and 525 nm (green), respectively (Fig. 9.1g, h), but it has an excitation of 460 nm and emission of 650 nm (red) when bound to the phosphate groups of single-stranded DNA or RNA [21]. Acridine orange is readily taken up by both living and fixed cells (Table 9.2), and its detection of both RNA and DNA makes it ideal for cell cycle studies [21]. A potential drawback to using acridine orange is signal overlap with other fluorochromes with similar emission spectrum.

4',6-diamidino-2-phenylindole (DAPI) is a classic nuclear stain for fluorescence microscopy in both living and fixed cells [22]. DAPI strongly binds to the minor groove of double-stranded DNA, particularly adenine- and thymine-rich regions, and absorbs light in the UV spectrum (Table 9.1); despite excitation maximum being in the UV spectrum, DAPI will readily absorb violet light. Once excited, DAPI emits blue or cyan fluorescence making it ideal for pairing with other fluorochromes that emit longer wavelengths (Fig. 9.1i). Additionally, when bound to DNA, fluorescence emitted from DAPI is 20 times greater than that of unbound, greatly reducing background signals [23]. Thus, its selectivity for DNA and high cell permeability allow for efficient staining of nuclei with limited cytosolic fluorescence.

9.1.3.5 Vacuole

Accumulation of pigments in the vacuole often provides some autofluorescent activity [6, 7], however the levels of these metabolites are dependent on the cell type and the stage of development. An alternative method for vacuole detection exploits the organelle's acidity and subsequent accumulation of basic dye such as *acridine*

Table 9.2 Working concentrations and incubation times of fluorescent dyes for living plant cells

Fluorescent dye	Organelle	Solvent	Final (μg/mL)	Incubation (min)	Treatment pH
Acridine orange	Nucleus	DMSO	10	30	7
	Vacuole		1		7.4–8.5
Calcofluor white	Cell wall	Water	1	5	7
DAPI	Nucleus	Water	10	15	7
Carboxy-DCFDA	Vacuole	DMSO	1.25	20	5.5
$DiOC_6$	ER	Ethanol	5	5	7.4
	Mitochon-dria		0.25		
FDA	Nucleus/ vacuole	Acetone	10	5	7.6
Neutral red	Vacuole	Water	1–10	15	7.2–8.0
Rhodamine 123	Mitochon-dria	Methanol	10	30	7

DAPI 4′, 6-diamidino-2-phenylindole, *FDA* fluorescein diacetate, *DMSO* dimethyl sulfoxide, *ER* endoplasmic reticulum

orange and *neutral red* [24]. Proton motive force, which drives the transport of protons from the cytosol to the vacuole, regulates the cytoplasmic pH and facilitates the movement of solutes into and out of the vacuole. As a consequence of protonation, the vacuole becomes acidic (pH 5–5.5) and acts as an ion trap for membrane-permeable bases. Therefore, vacuolar dyes are often used as pH indicators. As discussed in Sect. 1.3.4, acridine orange is a nuclear stain; however, when applied under basic conditions (Table 9.2), the dye also accumulates in the vacuole [25] and emits red fluorescence (Table 9.1). Neutral red is used for both its strong red fluorescence (Table 9.1; [26]) and its distinctive red color that can be observed under bright-field microscope (Fig. 9.1j). Since neutral red requires active transport into the vacuole, only living cells may accumulate the dye. However, the working concentrations of neutral red vary depending on the tissue types and plant species under investigation. For example, low concentrations are used to stain *Arabidopsis* cell suspensions, 0.1 mg/L [27, 28], *Brassica* protoplasts, 1 mg/L [29], and *Arabidopsis* roots, 1 mg/L [30], whereas high concentrations are required for onion parenchyma cells, 4 mg/L [31] and whole carrots seedlings and embryos, 20 mg/L [25].

5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (*carboxy-DCFDA*) is another group of vacuolar stains that rely on intracellular esterases to release negatively charged carboxyfluorescein fluorochromes. Carboxy-DCFDA is nonfluorescent until the acetate groups of the fluorochrome are released by intracellular esterases [32]. Once cleaved, carboxy-DCFDA becomes 5-(and-6)-carboxy-2′-dichlorofluorescein, a fluorochrome that absorbs blue light and emits green fluorescence (Table 9.1, Fig. 9.1k, l, and m). Carboxy-DCFDA is membrane-permeable and the fluorescein derivatives are strongly sequestered in the vacuole using anionic transporters (Table 9.2).

9.1.4 Fluorescent Staining to Assess Cell Viability

Viability, or the ability of an organism or a cell to maintain itself, can be an important factor in experimental procedures. Fluorescent dye such as R123 requires mitochondrial activity [19] and can be used as indicators of cell viability; however, *fluorescein diacetate* (FDA) is the most routinely used for assessing cell viability. FDA is a cell-permeant dye that exhibits no fluorescence until intracellular esterases release fluorescein [33]. The strong green fluorescence of fluorescein (Table 9.1) is used as an indicator of both enzymatic activity and membrane permeability, as intact membranes allow fluorochrome accumulation. Once cleaved, fluorescein localizes to the cytosol, nucleus, and vacuole of the living cell (Fig. 9.1n, o, and p).

9.2 Materials

9.2.1 Equipment

Major equipment: confocal microscope (optional) or compound microscope equipped with light source capable of delivering excitation wavelengths near UV and blue range, pH meter, and centrifuge with swing-bucket rotor (optional).

9.2.2 Supplies for Tissue Preparation

Laboratory supplies: double-edge stainless steel razor blade (Electron Microscopy Sciences 72000), mortar and pestle, Pasteur pipettes, microcentrifuge tube (1.5 mL), nylon mesh (40 μm; Spectrum Laboratories Inc. 146502), staining dishes, fine-tipped forceps, glass slides and cover slips, depression slides, and number 1 Whatman filter paper.

9.2.3 Chemical Reagents

1. Solvents (acetone, dimethyl sulfoxide (DMSO), methanol, ethanol, or deionized water).
2. Chemicals for buffer preparations: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma H4034); 2-(*N*-morpholino) ethanesulfonic acid (MES; BioShop MES503); 1,4-piperazinediethanesulfonic acid (PIPES; Sigma P6757); dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$; BioShop SPD579); monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$; BioShop SPM400); sodium chloride (NaCl; BioShop SOD002); ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic

acid (EGTA; BioShop EGT202); magnesium sulfate (MgSO4; Fisher 964443); D-mannitol (Fisher 937002); and potassium hydroxide (KOH; BioShop PHY202).
3. Fluorescent dyes: acridine orange (Sigma A6014), carboxy-DCFDA (Invitrogen C369), DAPI (Sigma D9564), $DiOC_6$ (Sigma 318426), FDA (Sigma F7378), Fluorescent brightener 28 (Sigma F3543), Neutral red (Sigma 4638), and R123 (Sigma R8004).

9.2.4 Solution Preparation

1. 0.1 M HEPES buffer, pH 7–8.2: For 100 mL, dissolve 2.38 g of HEPES in 90 mL of deionized water, adjust the pH to the desired value with 1 M KOH and then adjust the volume to 100 mL with deionized water.
2. 0.1 M MES buffer, pH 5.5: For 100 mL, dissolve 2.13 g of MES in 90 mL of deionized water, adjust the pH to 5.5 with 1 M KOH and then add deionized water to 100 mL.
3. 0.1 M PIPES buffer, pH 7–7.5: For 100 mL, dissolve 3.02 g of PIPES in 90 mL of deionized water, add 1 M KOH until the desired pH is obtained and then adjust the volume to 100 mL with deionized water.
4. 0.1 M sodium phosphate buffer, pH 7–7.8: (a) Prepare the 0.1 M solution of $NaH_2PO_4 \cdot H_2O$ (monobasic) by dissolving 1.38 g in 100 mL of deionized water. (b) Prepare the 0.1 M solution of $Na_2HPO_4 \cdot 7H_2O$ (dibasic) by dissolving 2.68 g in 100 mL of deionized water. For 100 mL, the desired pH can be obtained by mixing the monobasic and dibasic solutions as follow:

pH	Monobasic (mL)	Dibasic (mL)
7.0	39	61
7.1	33	67
7.2	28	72
7.3	23	77
7.4	19	81
7.5	16	84
7.6	13	87
7.7	10.5	89.5
7.8	8.5	91.5

5. Stabilization buffer: For 100 mL, dissolve 0.877 g NaCl (final 150 mM), 0.38 g EGTA (final 10 mM), 0.06 g $MgSO_4$ (final 5 mM), 5.47 g D-mannitol (final 300 mM) in 0.1 M HEPES, pH 7. Store solution at 4 °C to reduce potential contamination.
6. Staining solution: The fluorescent dye solutions are prepared as 1000-fold stock solutions; for example, dissolve 100 mg of DAPI in 10 mL deionized water for a

concentration of 10 mg/mL (please refer to Table 9.2 for appropriate concentrations and solvents). To remove precipitate, filter stock solution with Whatman paper (No. 1) and store in the dark, ≤4 °C. To obtain working concentration of dye, mix 1 µL of 1000-fold stock solution with 999 µL buffer (Table 9.2).

9.3 Methods

9.3.1 Fluorescent Staining of B. sinuspersici Chlorenchyma Cells

1. Excise four mature leaves (>2 cm) and gently crush in 2 mL of stabilization buffer (*see Note 1*) using a mortar and pestle.
2. With a Pasteur pipette, transfer the cell homogenate onto a 40-µm nylon mesh filter. Discard broken cells of the flow-through and collect intact cells in 1.5-mL centrifuge tube by washing the mesh with 1 mL stabilization buffer.
3. Add appropriate amount of fluorescent dye to cells (Table 9.2). Gently mix by inverting the tube twice.
4. Incubate cells in the dark for 5–30 min at room temperature.
5. After cells have settled to the bottom of the tube, discard the supernatant and wash with 1 mL of fresh stabilization buffer. Gently mix and allow cells to settle for 10 min (*see Note 2*).
6. Repeat step 5.
7. After washing cells twice in stabilization buffer (*see Note 3*), resuspend cells in 50 µl of stabilization buffer.
8. Using a Pasteur pipette, transfer the stained cells in stabilization buffer to a depression slide (*see Note 4*) and place a glass cover slip over the sample.
9. Image cells using compound or confocal microscope immediately after staining (*see Note 5*).

9.3.2 Fluorescent Staining of Free-Hand Leaf Sections of K. blossfeldiana, K. daigremontiana, or S. aralocaspica

1. Cut freshly excised leaf (4–5 cm long) into 0.1–0.2 mm thick sections using a double-edged razor blade (*see Note 6*).
2. Place leaf sections into staining dish containing the buffer with an appropriate pH (Table 9.2) and incubate for 5 min (*see Note 7*).
3. Move sections to a well containing fluorescent dye diluted in appropriate buffer (Table 9.2). Incubate in the dark for 5–30 min at room temperature.

4. Wash leaf sections twice in fresh buffer without the dye for 10 min each (*see Note 3*).
5. Mount leaf sections on a depression slide, cover samples with fresh buffer, and place a glass cover slip on top.
6. Observe leaf sections using either a compound or confocal microscope, image sections within an hour of preparation (*see Note 8*).

9.4 Notes

1. HEPES can be replaced with PIPES, sodium phosphate, or MES buffer but consult Table 9.2 for the appropriate pH. Some plant species may also prefer sucrose as an osmoticum in place of mannitol.
2. As an alternative to allowing cells to naturally settle to the bottom of the tube, cells may be pelleted at 100 g for 2 min using a swing-bucket rotor. The use of fixed-angle rotor generally results in higher number of damaged cells.
3. Fluorochrome detection can be obscured by high background fluorescence if unbound dye is not sufficiently removed. By replacing staining solution, excess stain is diluted and background fluorescence is greatly reduced. If background is still too high, additional wash steps can be performed or the concentration of dye used can be lowered.
4. When working with non-fixed cells, one need to be careful not to crush cells by applying cover slip on a conventional glass slide. A depression slide is recommended because it provides the minimum space of 0.1 mm needed between the slide and the cover slip.
5. Observation and imaging should be done as soon as possible to ensure fluorescent dye is retained and cell death is minimized. Since fluorescent dyes are prone to photobleaching, minimize time of exposure of stained cells to the excitation wavelength.
6. Ideally, tissue sections should be 2–3 cell layers thick so that the dye has proper access and that the cellular detail can be revealed. For plant tissues that are not strong enough for free-hand sectioning, other support material such as Styrofoam or carrot root can be used. Alternatively, a vibratome may be used if the desired thickness is difficult to obtain using free-hand sectioning. To avoid crushed and damaged cells, leaf sections must be cut with a fresh razor blade.
7. Typically buffer alone is sufficient for staining of leaf sections. If an osmoticum is required, then 0.3 M mannitol or sucrose can be added to buffer.
8. If the uptake of dye is low, infiltration can be improved by: increasing dye concentration, adding 0.01 % (v/v) DMSO or 0.1 % (v/v) Triton X-100 to fluorescent dye incubation buffer and/or vacuum-infiltrate leaf sections with fluorescent dye for 5–15 min. If membrane permeability is required for fluorescent dye uptake, one must include controls with just the DMSO or detergent alone to ensure the signal observed is not an artifact due to cell damage.

9.5 General Comments

This chapter is intended to serve as a general guideline for staining of living plant cells or tissue sections and that there may be some variation with the overall results for other plant species. Thus, in order to obtain reliable and reproducible results, parameters such as osmolarity, buffer pH, dye concentration, incubation time, and wash procedure need to be empirically determined and optimized. For example, the optimum time of staining varies for different plant tissues and should be tested individually. Too long staining time will lead to over-staining and nonspecific staining whereas too short will result in under-staining. Another factor affecting the staining specificity of cellular structures is the pH of the buffer. In particular, pH sensitive dyes such as carboxy-DCFDA which has a low pKa and can be loaded into cells under acidic condition. It is extremely useful for monitoring pH changes in acidic organelles such as vacuoles. When optimizing a fluorescent staining protocol, it is of great importance to include appropriate controls. The preparation and observation of unstained samples alongside that of your stained specimen can be crucial in differentiating true signal from that of background autofluorescence or artifacts.

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