

Chapter 14

Fluorescent Probes and Live Imaging of Plant Cells



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1 Introduction

Fluorescent probes are used in almost all areas of plant research ranging from molecular biology to ecophysiology studies. Development of novel fluorochromes and fluorescent proteins in combination with advanced microscopy techniques allow us to analyze cells, tissues, organs and whole plants in great detail. Live fluorescence microscopy imaging of plants is of particular importance for ecophysiology studies where complex interactions of plants and their environment need to be understood at molecular, cellular and organismal level. Here we present an overview of fluorescent probes and live cell microscopy setup for plants and provide a detailed protocol for fluorescent live-dead viability assay using fluorescein diacetate and propidium iodide fluorescent dyes.

1.1 *Natural Fluorophores and Autofluorescence in Plants*

Naturally occurring fluorescence or intrinsic fluorescence is common to many molecules of living organisms, which in turn confer auto-fluorescence properties to the tissue or cell compartment they reside in. The excitation and emission wavelengths of the autofluorescence encountered in cells can be diverse, spanning the whole visible spectrum and beyond. Common examples of molecules with intrinsic fluorescence are the aromatic residues, phenylalanine, tyrosine and tryptophan of proteins, with

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fluorescence maxima at 282, 303 and 348 nm, respectively, where tryptophan accounts for about 90% of the fluorescence of the proteins. Certain cofactors such as NAD, FAD, FMN and porphyrins exhibit fluorescence, as well (Lakowicz 2007). There are several plant-specific molecules bearing intrinsic fluorescence. For example, plant cell walls rich in lignin fluoresce blue with emission peak around 360 nm (Chapman et al. 2005) and the chlorophyll of green plants emits red fluorescence between 650 and 750 nm range (Agati 1998). There are also several other plant-specific molecules, which fluoresce in blue and blue-green (certain alkaloids such as colchicine, terpene and flavonoids) or in yellow and orange (acridone, the polyacetylenes and isoquinoline) or in red (anthocyanins and azulenes) spectral ranges (Roshchina 2012).

During fluorescence imaging of plants, autofluorescence can be either advantageous or disadvantageous depending on the application. Red chlorophyll autofluorescence of plants, for example, can be used as an intrinsic probe to locate chloroplasts in green tissues during fluorescence microscopy imaging. Intrinsic chlorophyll fluorescence also allows assessing changes of photosynthetic apparatus, state of plant health, stress tolerance, disease onset and nutrient deficiency (Buschmann 2007). On the other hand, intense red chlorophyll fluorescence often interferes with red and far-red colored fluorescent probes in multicolor labeling experiments. In such cases, either a specific bandpass emission filter or spectral unmixing approach can be used to isolate the signal of interest (Berg 2004; Mylle et al. 2013). Similarly, the cyan and blue colored cell wall autofluorescence is suitable to mark cell and tissue borders. While being advantageous, cell wall autofluorescence may also cause interference in experiments involving blue and cyan colored exogenous fluorescent probes. Autofluorescence can also be induced by mechanical stress and wounding of plants, such as during sampling of plant parts for microscopy analysis. Fluorescent phenolic compounds are being formed when contents of burst vacuoles at the cut site mix with cytoplasmic enzymes. This may cause cut or injured sites of living plant samples to fluoresce. Although this property can be exploited in wounding response studies, often it creates unwanted background fluorescence during imaging. Therefore, mechanical stress and physical injury should be minimized during live analysis of plant samples.

Figure 14.1a, b show a setup for direct live analysis of a leaf of a potted maize plant analyzed with confocal fluorescence microscope without detaching the leaf. Using this setup, chlorophyll fluorescence is captured to locate chloroplasts of parenchyma tissue (Fig. 14.1c). Similarly, violet light-induced intrinsic fluorescence is used to capture guard cells of epidermis (Fig. 14.1d) and trichomes of the leaf midrib region (Fig. 14.1e).

Fig. 14.1 (continued) detection range. (e) Trichome autofluorescence is captured at the leaf midrib region using 405 nm laser excitation (emission: 425–525 nm). Multiple confocal optical sections are merged to obtain extended depth of focus image. (f) Timelapse imaging of tobacco BY2 suspension cells using differential interference contrast (DIC) technique. Arrow indicates translocation of a cytoplasmic cargo. Nuclei (N), nucleoli (n) and one of the vacuoles (V) are marked on the first image. (g) Fluorescein diacetate (FDA, green, live cells) and propidium iodide (PI, red, dead cells) live/dead staining of *Arabidopsis* (Col) suspension culture. (h) FDA/PI viability analysis of phosphinotricin (PPT) resistant transgenic maize suspension culture after treatment with PPT (15 mg L^{-1}) for 5 days

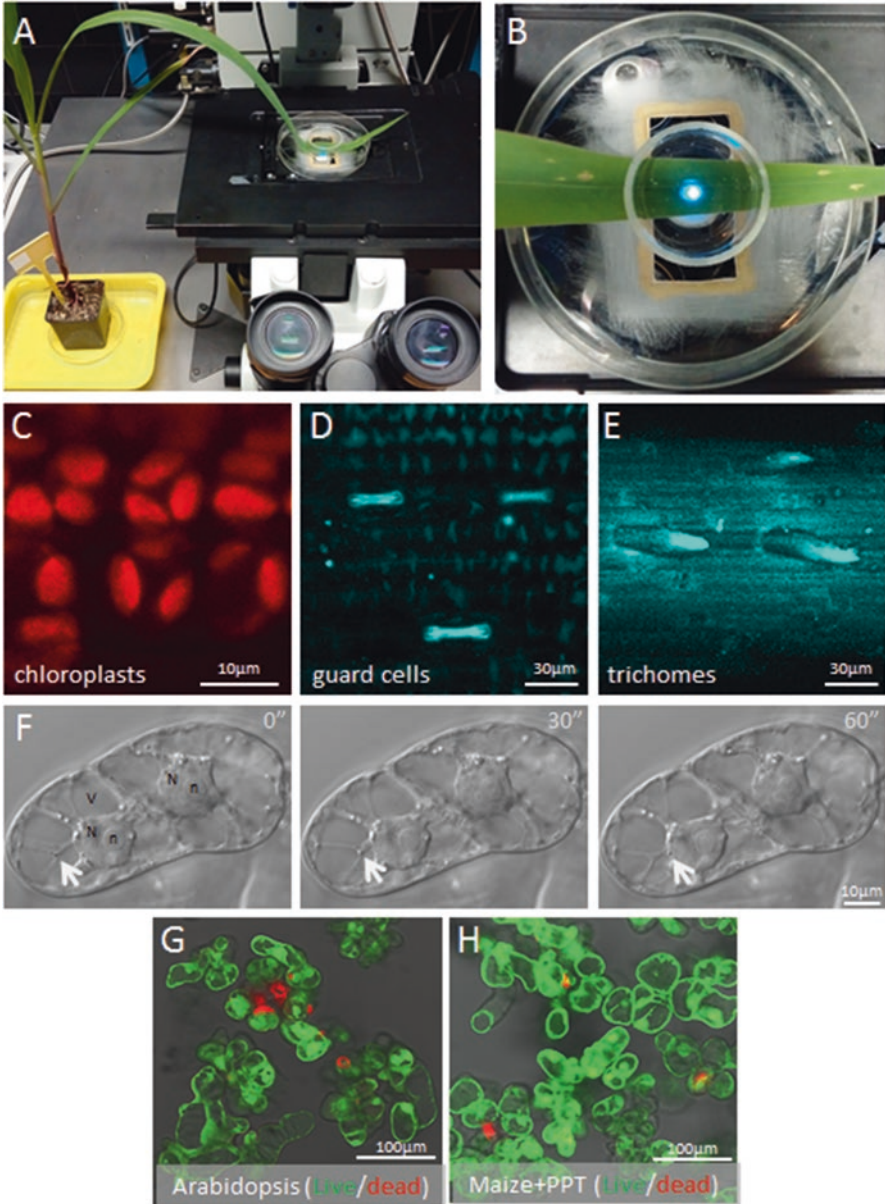


Fig. 14.1 Laser scanning confocal microscopy imaging of live plant samples. (a) A potted maize plant is placed next to the microscope stage and one of the leaves is layered onto a coverslip-bottom Petri dish (9 cm diameter) to image intrinsic leaf fluorescence using 40 \times oil immersion objective. (b) Closeup view of the same sample on the microscope stage. The leaf is immobilized on top of the larger Petri dish by using a smaller glass Petri dish (3 cm diameter). (c) Using the mounted leaf sample, maize leaf chloroplasts of parenchyma tissue are captured using chlorophyll fluorescence (emission: 650–750 nm) excited by 543 nm laser beam. (d) Guard cell autofluorescence of three stomata at the leaf surface is captured using 405 nm laser excitation and 425–525 nm emission

1.2 Organic Fluorescent Dyes and Reporter Molecules

During the last decades, a wide variety of synthetic fluorescent dyes and fluorescent reporter molecules with diverse spectral characteristics had been developed for various specific applications (Johnson and Spence 2010). According to the mode of attachment to the target to be studied, they can be divided into two broad categories, (i) probes that target the molecule of interest by *affinity binding*, (e.g. the nucleic acid dyes DAPI and propidium iodide) and (ii) fluorescent dyes that possess an active group through which the labeling is achieved in a *covalent* manner, either directly to the target molecule, or to an intermediate molecule – such as an antibody, that in turn will target the molecule of interest. Examples of this second category are the reactive fluoresceins, rhodamine dyes, acridines, fluorenes, naphthalimides, stilbenes, reactive AlexaFluor dyes and their derivatives with specific activities towards specific chemical groups.

Covalently attached fluorophores are used for generation of *fluorescent reporter molecules*. Such reporter molecules available are numerous, since just about any molecule can be engineered as covalently-modified synthetic fluorophore. When such molecules are reintroduced into the cell, they can be monitored by their fluorescence and their localization and behavior could report on the endogenous molecules that they mimic.

Examples of fluorescent reporter molecule types are: polysaccharide reporters, which can be monitored during cellular internalization or interaction with various cellular molecules during trafficking; labeled metabolites; substrates for different enzymes; labeled fatty acids and lipids, which can be partitioned into cell membranes and can therefore be used to report on their environment. Also in this category and of special interest are the labelled immunoreagents such as fluorescently labelled primary and secondary antibodies. Such labelled antibodies are usually available with various functional groups and are available in various colors. They can be used to probe location or activity of diverse biological molecules or the molecular interactions they participate in. Labeling technologies were further advanced by the commercial efforts of several companies that developed novel fluorochromes (e.g. AlexaFluor, Bodipy, CyDyes, ATTO, Chromis, CAL fluor, Quasar and IR Dyes), which all possess several unique properties and advantages (e.g. photostability, high quantum yield and brightness) as compared to traditionally used fluorescein and rhodamine-based dyes.

Among the most common applications of fluorescent dyes is the visualization of cell structures and components. For almost all intracellular organelles diverse stable and bright dyes had been developed for visualization. Several of them can be used for live analysis in plants such as probes for mitochondria (mitotracker green, nonyl acridine orange), nucleus (DAPI, Hoechst 33242), intracellular membranes (DiOC₆), cell walls (calcofluor white), vacuoles (Carboxy DCFDA), lipid droplets (Nile Red, Ac-201) and plasma membrane (FM 4-64) (Johnson and Spence 2010; Kuntam et al. 2015; Lovy-Wheeler et al. 2007; Schoor et al. 2015)

Another common application of the probes is to monitor biological processes in cells. Several fluorescent labels were developed for signal transduction studies; for

tracking lipid metabolism, protein kinases, phosphatases and tracking nucleotide binding of proteins. Special probes are developed for studying ion channels and receptor binding, as well as endocytosis and exocytosis. A number of unique cellular functions can also be studied by specialized fluoroprobes, such as cell proliferation, cell cycle and apoptosis (Johnson and Spence 2010).

Probes called fluorescent tracers and indicators (or sensors) are also used in imaging studies. Various types of tracers had been developed (Kumar and Gilula 1996; Nashmi et al. 2002; Sukhorukov et al. 1995; Vercelli et al. 2000) to trace cell morphology and cell lineage for example. Such probes are usually dextran conjugates or peptide and protein conjugates, or fluorescent microspheres and they are tracers for either lipophilic membranes or polar, cell-injectable cytoplasmic tracers. Requirement for tracers is to be biologically inert and non-toxic for the host, while allowing prolonged tracking.

The general procedure for using organic dyes in plant live cell imaging involves preparing the fluorescent probe in a live cell compatible physiological solution and its delivery to plant cells. In plant cells, one particular limitation for dye delivery is the plant cell wall. Cell wall impermeable but plasma membrane permeable probes can be delivered to plant cells by preparing protoplasts. In case of plasma membrane impermeable probes, electroporation, microinjection, ester loading or low pH loading techniques can be used as an alternative (Fricker et al. 2001).

1.3 *Fluorescent Proteins in Plant Cell Imaging*

The cloning of the green fluorescent protein (GFP) of *Aequorea victoria* in 1992 (Prasher et al. 1992) and its first use for genetically tagging cellular proteins (Chalfie et al. 1994) represented a major turning point for fluorescence imaging of biological samples and initiated an exponential increase of its applications for biological studies. The possibility of genetically tagging proteins of living cells with fluorescent proteins has opened the gates for studies of cellular proteins in their natural environment by fluorescence microscopy. Ever since, great efforts are being made to search for other varieties of either naturally occurring fluorescent proteins, or improving and diversifying the existing ones by evolving them through mutations. Hence, their application possibilities are being considerably widened (Chudakov et al. 2010).

Structurally, GFP-like proteins possess β -barrel structures with a short helix tethered inside in the middle of the barrel, harboring the posttranslationally acquired chromophore. The diversity of the structures of the chromophores and of the nearby amino acid residues in these proteins leads to a range of emission colors and varied spectral properties for these proteins. Exploiting these, numerous efforts had been devoted to generate mutants with new, refined or enhanced spectral properties (Rizzo et al. 2009; Shu et al. 2006), and at present, a wide range of fluorescent proteins are available. Their color palette covers the entire spectral range from deep-blue to far-red region, allowing not only for single but also for multicolor labeling of different targets simultaneously in a living cell, hence, opening doors to versatile applications.

By using fluorescent proteins to tag different endogenous proteins, not only structural organizations can be monitored but also molecular interactions and dynamic processes within the cell, by using live cell imaging (Shaw and Ehrhardt 2013).

The basic procedure for using fluorescent proteins in plant cell imaging involves construction of the gene of interest in fusion with a fluorescent protein gene in a suitable vector, its delivery into plant cells (e.g. using *Agrobacterium* mediated delivery or microprojectile bombardment) to express the protein either transiently or stably and visualization of the expressed protein using live imaging microscopy setup (Cui et al. 2016; Groover and Jackson 2007). In green plant tissues, the possible interference of red chlorophyll fluorescence should be taken into consideration when choosing fluorescent proteins for tagging and imaging. For green/red dual labelling and colocalization experiments, enhanced green fluorescent protein (EGFP) and mCherry combination can be used with appropriate filter sets to prevent interference of chlorophyll fluorescence. For triple labelling, enhanced cyan fluorescent protein (ECFP), enhanced yellow fluorescent protein (EYFP) (or its brighter derivative Venus) and mCherry can be used with appropriate filters. Beside protein localization studies, fluorescent protein fusions are also successfully used in plants to assess protein-protein interactions by using fluorescence resonance energy transfer (FRET) technique. ECFP and EYFP fused proteins are often used as FRET pairs, however, several novel alternative FRET pairs are being developed with better spectral properties (Müller et al. 2013). Another *in vivo* technique for protein interaction studies is the so called bimolecular fluorescence complementation (BiFC) where a fluorescent protein is split into carboxy-terminal (C-terminal) and amino-terminal (N-terminal) fragments, each of which is fused with a partner protein whose interactions are being assessed (Walter et al. 2004). In BiFC method, if the two proteins of interest interact with each other, the C-terminal and N-terminal parts of the split fluorescent protein will be brought close together such that they form a functional fluorescent protein. If the proteins do not interact, no fluorescence is observed. However, it is essential to include appropriate internal controls especially in BiFC type protein-protein interaction experiments, as the fluorescent protein halves are prone to self-assembly independently of protein-protein interactions (Horstman et al. 2014).

1.4 Live Cell Imaging of Plants

Basic live cell imaging of plant cells can be performed even with a simple light microscope equipped with a detector. Keeping the cells alive and healthy is the first and foremost concern during live cell imaging, since living cells are responsive to minute changes in environmental conditions. Therefore, it is vital to keep cells in an environment close to their natural or *in vitro* growth conditions during the entire duration of imaging. Parameters such as temperature, light, pH, humidity, osmolarity, nutrient and oxygen supply are among the most important factors that need to be regulated.

For long term observations, special environmental chamber inserts or whole-microscope enclosures are available from several manufacturers that allow for keep-

ing cells, seedlings or plants under constant temperature and humidity with options to precisely regulate oxygen and carbon dioxide content of the chamber, as well. For short term observations, simpler observation chambers and imaging setups can be used. For *in vitro* grown suspension cultures, standard plastic Petri dishes provide the simplest solution for low magnification imaging. On the other hand, majority of the high magnification, high numerical aperture objectives are of oil-immersion type and require very short working distances. These objectives are often designed to be used with 0.17 mm thick, No 1.5 size coverslips. Therefore, for high resolution imaging, coverslip-bottom containers and invert microscopes should be preferred. For simultaneous handling of multiple samples, coverslip-bottom 4-compartment Petri dishes or 8-compartment chambered coverglass systems can be used. For high-throughput imaging, there are also 96 well, 384 well or even 1536 well coverslip-bottom plates available. However, all of these coverslip-bottom containers designed specifically for high resolution imaging applications, are significantly more expensive than standard plastic-bottom ones. As a low cost alternative, it is also possible to prepare imaging chambers by cutting out part of the bottom of regular plastic containers and attaching a coverslip at the bottom using a non-toxic adhesive such as pure silicon rubber, which is also used for building aquariums. (For sterilization: After complete curing of adhesive, dip the container and lid in 70% ethanol for 10 min, rinse with absolute ethanol and let it dry face down on a sterile tissue paper in laminar hood).

Figure 14.1b shows an example of a custom-made coverslip-bottom live cell imaging chamber for observation of plant cells using high resolution oil immersion objectives. For imaging of deeper layers, water immersion objectives should be preferred to better match the refractive index of the living plant tissue. In addition to bottom, inserting a coverslip to the lid of the container allows the use of a contrast enhancement technique called differential interference contrast (DIC), which benefits from all-glass light pathway. As opposed to standard bright field imaging, DIC imaging offers higher contrast to better resolve cellular and intracellular details in case of transparent, unstained samples such as the one shown in Fig. 14.1f. Note that nuclei (N), nucleoli (n), vacuoles (V) and cytoplasmic strands are clearly visible in the cultured tobacco BY-2 cells shown in Fig. 14.1f, where time-lapse live DIC imaging shows translocation of a cytoplasmic cargo to the vicinity of nucleus (arrows). In addition to DIC, there are several other contrast enhancement techniques such as phase contrast, Hoffmann modulation contrast, darkfield illumination or polarized illumination (Dawe et al. 2006). Depending on the nature of the sample, these non-intrusive techniques can provide significantly enhanced image for unstained samples during live observation of plant cells. All of these methods, however, require special microscope accessories attached to the basic light microscope. This is also the case for fluorescence imaging, which requires additional hardware to excite and detect fluorescent signals. Unlike transmitted light imaging modes, fluorescence imaging techniques do not require transparent samples. Majority of modern fluorescence microscopes work in reflection mode, hence non-transparent samples can also be conveniently observed.

Fluorescence microscopy, however, requires fluorescent molecules such as endogenously produced lignin or chlorophyll, or externally delivered fluorescent

dyes or transgene-expressed fluorescent proteins. Detection of the reflected fluorescence emission allows to image thick leaves, stems or roots alive under fluorescence microscope, however, the quality of the image provided by conventional wide-field fluorescence microscopy deteriorates significantly when focused deep into the sample, due to a blur originating from the out-of-focus fluorescence emission.

The technique called confocal imaging greatly enhances the image quality by eliminating fluorescence emission originating from out-of-focus regions. This filtering out in confocal imaging is achieved by using a pinhole or slit-based system in front of the detector, so that it allows only the emission arising from focal point to enter the detector. These systems require sensitive detectors and also high illumination intensities such as those provided by lasers, high-power light-emitting diodes or high performance arc-discharge lamps. Using these powerful light sources during live analysis requires extra care as high intensity illumination can cause phototoxicity and photodamage especially for chlorophyll containing green tissues. A laser scanning confocal fluorescence microscope imaging setup for live observation of a leaf of potted maize plant is shown on Fig. 14.1a, b. In this configuration, a maize leaf is layered on a custom-made coverslip-bottom Petri dish and it is observed from below using an inverted microscope and an oil-immersion objective. A similar setup can be used for live observation of roots of a potted plant by using a pot with a coverslip/coverglass window at the bottom. Live, high resolution cellular imaging of intact plants is particularly important for ecophysiology studies where minimal disturbance of plant growth and metabolism is essential. To assess the health and viability of plant cells and tissues, below we present an example protocol based on using dual color fluorescence and fluorescence microscopy imaging that enables determination of live-dead cell ratio for the specimens.

2 Protocol: Live-Dead Viability Assay in Plant Cells Using Propidium Iodide and Fluorescein Diacetate

Determination of plant cell viability is essential to optimize growth conditions and to assess cell health following various experimental procedures and treatments (e.g: protoplastation, stress treatment, genetic modification). Fluorescence-based viability assays employ fluorescent stains and/or dye-conjugated substrates. One of the widely used approaches is dual staining with propidium iodide and fluorescein diacetate. Propidium iodide (PI) is a nucleic acid intercalating dye, which has very low plasma membrane permeability hence unable to penetrate living cells. It penetrates to plasma membrane compromised, dead cells and stains nuclear DNA as well as cytoplasmic and nuclear RNA. When excited by green light (e.g: 543 nm HeNe laser or mercury lamp with Texas red/Rhodamine filter set), propidium iodide fluoresces red and marks dead cells. Contrary to PI, the live cell staining agent fluorescein diacetate (FDA) is cell permeable, but nonfluorescent. However, when the acetyl groups of FDA molecules are cleaved by intracellular esterases, fluorescein molecules are being released inside the living cells. When excited by blue light (e.g: using 488 nm

laser or mercury lamp with fluorescein filter set), the bright green fluorescence of the fluorescein molecule serves as a marker of the cellular viability. Hence co-incubation of cells with FDA/PI can be used as a convenient dual-color viability assay. As an example protocol, below we present detailed steps for viability assay of a non-transgenic *Arabidopsis* culture (Fig. 14.1g) and of a herbicide phosphinotricin (PPT)-resistant transgenic maize culture following treatment with PPT (Fig. 14.1h).

2.1 Equipment and Reagents

1. *Arabidopsis thaliana* cell line (ecotype Columbia) in Murashige and Skoog (MS) medium with 0.24 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.014 mg L⁻¹ Kinetin (Murashige and Skoog 1962).
2. Transgenic maize cell line (*Zea mays* 'cv. H1233') with resistance to herbicide phosphinotricin (PPT) (Tiricz et al. 2018) in N6M medium with 0.5 mg L⁻¹ 2,4-D and 15 mg/l PPT (Morocz et al. 1990).
3. Phosphinotricin (Duchefa Biochemie BV, The Netherlands) for herbicide treatment. 10 mg mL⁻¹ in water. Sterile filtered, kept frozen in aliquots.
4. Propidium iodide (PI) 1000× stock solution: Prepare 2 mg mL⁻¹ PI solution in water. Caution: PI is a nucleic acid intercalating dye; hence it should be handled with extreme care due to health hazards.
5. Fluorescein diacetate (FDA) 1000× stock solution: Prepare 1 mg mL⁻¹ FDA stock solution in dimethyl sulfoxide (DMSO)
6. Consumables: sterile 1.5 mL microfuge tubes, pipettes, sterile pipette tips, microscope slides and coverslips.
7. Equipment: laminar flow hood, desktop centrifuge with swing-out rotor, conventional or confocal fluorescence microscope with fluorescein and Texas Red (or tetramethylrhodamine, TRITC) filter sets.

2.2 Method

1. Under a laminar flow hood, sample 0.5 mL from plant cultures (*Arabidopsis* and maize) into a 1.5 mL microfuge tube and let the cells to settle. (For PPT treatment, maize cells were incubated for 5 days prior imaging in 15 mg L⁻¹ PPT containing growth medium). Alternatively, cells can be centrifuged briefly (3 min, 100 g, swing-out rotor) to aid settling. Care must be taken not to mechanically stress the cultures. Using wide bore pipettes or cutting the tip of plastic pipettes with a sterile scalpel can help reduce mechanical stress during handling of cells.
2. Replace culture supernatant with 1.5 mL fresh culture medium and resettle the cells. This washing step helps minimize residual esterase activity present in old culture medium. On the other hand, performing FDA/PI labeling in unwashed culture medium may help identify possible bacterial, yeast or fungi contamination present in the culture.

3. Freshly prepare 0.5 mL solution of 1 mg mL⁻¹ FDA and 2 mg mL⁻¹ PI in plant growth medium or in phosphate buffered saline (PBS). If fresh culture medium contains components with esterase activity, preparation of labeling reagents in PBS (or in MS macro salts) is preferred to prevent premature cleavage of FDA, which results in increased background fluorescence due to free fluorescein. When working with protoplasts, proper osmotic adjustment (e.g. using 0.3–0.5 M Sorbitol, depending on cell type) is essential to prevent bursting of cells during assay.
4. Incubate 0.5 mL of labeling reagent with settled cells for 2–5 min by gently inverting the tube.
5. Take 50–100 µL from stained samples onto a microscope slide, coverslip or coverslip-bottom Petri dish and observe cells using a fluorescence microscope configured for imaging fluorescein (green fluorescent living cells) and propidium iodide (red fluorescent dead cells).
6. Count at least 500 cells in 3 replicates to assess the ratio of green and red fluorescent cells.

Acknowledgements We thank Dr. Sándor Mórocz (Cereal Research Non-Profit Ltd., Szeged, Hungary) for providing non-transgenic Zea mays (H1233) cultures, Prof. Dénes Dudits (BRC, Szeged, Hungary) for providing *Arabidopsis thaliana* (Col) cultures; Béatrice Satiat-Jeunemaitre (CNRS, Gif sur Yvette, France) for providing *Nicotiana tabacum* (BY-2) cultures; Katalin Török and Ildikó Váلكony for maintaining maize plants and suspension cultures. This work was supported by the National Research, Development and Innovation Office, NKFIH (Grant Number K116318) and by GINOP-2.3.2-15-2016-00001 grant.

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