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Topic Introduction

Live Cell Imaging of Plants

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INTRODUCTION

Live cell imaging is an essential approach for studying the structure, dynamics, and functions of cells in a living plant under normal or stressed growth conditions. The tiny flowering plant, *Arabidopsis thaliana*, provides an ideal system to apply various live microscopy techniques. Its small size allows fluorescent light to penetrate the tissues, and its plantlets contain different cell types with different ploidy levels and differentiation stages. Its 2C nucleus contains only five pairs of chromosomes in which heterochromatin domains are organized as chromocenters, and these domains are easily resolved under the microscope. In addition, the availability of powerful genetic tools facilitates the investigation of the molecular mechanisms underlying various cellular phenomena. In designing live imaging experiments, one must keep in mind that plants sense light, temperature, osmolarity, humidity, gravity, and nutrition. In addition, plants also have strong circadian rhythms of physiological behavior and gene expression. Moreover, plant tissues are normally thick (having multiple cell layers), and can have strong autofluorescence, especially in green leaves. Therefore, optimized culturing and imaging conditions are essential for successful live cell studies in plants. This article discusses the general experimental considerations and design for live plant imaging studies.

RELATED INFORMATION

Additional information on the properties and uses of various fluorescent proteins for live cell imaging is available in **Fluorescent Protein Tracking and Detection: Fluorescent Protein Structure and Color Variants** (Rizzo et al. 2009a) and **Fluorescent Protein Tracking and Detection: Applications Using Fluorescent Proteins in Living Cells** (Rizzo et al. 2009b). An overview of **Vectors and *Agrobacterium* Hosts for *Arabidopsis* Transformation** (Weigel and Glazebrook 2006a) is also available online, as are specific protocols for **Transformation of *Agrobacterium* Using Electroporation** (Weigel and Glazebrook 2006b) and **Transformation of *Agrobacterium* Using the Freeze-Thaw Method** (Weigel and Glazebrook 2006c).

GENERAL EXPERIMENTAL DESIGN AND CONSIDERATIONS FOR PLANT LIVE CELL IMAGING

The general procedure for plant live cell imaging is to fuse a protein of interest with an appropriate fluorescent protein (FP), express the fusion protein transiently or stably, and then visualize the expression, organization, and dynamics of the fusion protein in vivo using various live microscopy techniques. In addition, biosensor FPs have been used successfully in plants for reporting on a wide variety of metabolites, ions, and intracellular processes, such as glucose, GTPase, cAMP, Ca^{++}, pH, and phosphorylation (Berg and Beachy 2008).

Choosing a Fluorescent Protein

A series of different FPs with various excitation and emission spectra have been generated, including blue, cyan, green, yellow, orange, and red-shifted monomeric fluorescent variants, and a UV-excited green fluorescent protein (GFP) (see **Fluorescent Protein Tracking and Detection: Fluorescent Protein Structure and Color Variants** [Rizzo et al. 2009a]). More recently, Chapman et al. (2008)

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have engineered iLOV, a smaller (~10 kDa) flavin-based alternative to GFP (~27 kDa) derived from the light-, oxygen-, or voltage-sensing (LOV) domain of the plant blue-light receptor, phototropin. A number of factors should be considered when selecting the appropriate FPs suitable for a particular series of experiments (see, e.g., **Fluorescent Protein Tracking and Detection: Applications Using Fluorescent Proteins in Living Cells** [Rizzo et al. 2009b]). For example, enhanced yellow fluorescent protein (EYFP) and its variants (e.g., Venus) have increased brightness and are used widely in plant live cell imaging. However, although EYFP absorbs 50% more light energy per molecule than the enhanced green fluorescent protein (EGFP), the two have similar fluorescence quantum yields (Shaner et al. 2004). Consequently, EGFP is less sensitive to photobleaching and therefore more suitable for long-term time-lapse imaging. When red-shifted FPs or enhanced cyan fluorescent protein (ECFP) are exposed to light at their appropriate excitation wavelengths, the chlorophyll in green leaf tissues emits higher autofluorescence, making these FPs less suitable for use in plants. Also, the shorter wavelengths needed to excite ECFP samples are more energetic and potentially more damaging to the tissues. Nevertheless, because of their special fluorescence spectra, the red-shifted FPs and ECFP are useful for multicolor FP experiments. For colocalization analyses, double-labeling pairs include EGFP/far-red-shifted FP (such as mCherry or HcRed) and EYFP/ECFP, whereas triple labeling can be achieved with cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP)/mCherry. CFP is also useful for fluorescence resonance energy transfer (FRET) when paired with EYFP or GFP to investigate protein-protein interactions. Another simple method to study protein-protein interactions in vivo is bimolecular fluorescence complementation (BiFC), or so-called split FP (Ghosh et al. 2000; Hu et al. 2002), in which an FP is divided into amino-terminal and carboxy-terminal fragments, each of which is fused with one partner protein whose interactions are being probed. If the two proteins under study interact, the amino- and carboxy-terminal parts of the FP will be brought close enough together such that they fuse to form a functional FP. If the two proteins do not associate, no fluorescence is observed.

Making FP Fusions: Cloning Strategies

When FPs are fused with a protein of interest, several questions need to be considered. Should the FP be fused to the amino terminus, the carboxyl terminus, or the middle of the protein? Is a linker necessary between the FP and the protein of interest? Is the FP fused to genomic DNA or a cDNA? Is an endogenous or exogenous promoter being used to drive the expression of the fusion? Is the exogenous promoter constitutive, inducible, tissue-specific, or tissue-specifically inducible? Does the expression vector backbone contain suitable cloning sites and bacteria and plant selection markers?

Generally, the FP can be placed on either the carboxyl or amino terminus of the protein. However, if one end of the protein is close to a functional domain, then the other end should be chosen for initial experiments. In cases where neither an amino- nor a carboxy-terminal fusion is appropriate, the FP should be placed internally (Tian et al. 2004). For cloning convenience, as well as to increase the structural flexibility at the junction, a linker rich in glycine and alanine can be added between the FP and the protein of interest. Successful linkers can contain up to 20 amino acid residues. Although a linker normally is not harmful, its impact can be difficult to evaluate.

Use of the endogenous promoter to drive expression of the fusion protein can reduce overexpression-related and ectopic-expression-related artifacts. In addition, the use of genomic DNA-containing introns and exons provides a better chance of expressing the fusion at appropriate levels. A convenient way to make a genomic DNA fusion is to amplify the genomic DNA including the coding region containing all introns and exons, the 5′-untranslated region, and the upstream regulatory sequence. For constitutive expression, the actin, ubiquitin, and cauliflower mosaic virus 35S promoters are commonly used. Sometimes, a tissue-, cell-type-, or developmental-stage-specific promoter is necessary to reduce issues related to constitutive expression. A relatively low and controllable level of gene expression can be achieved using inducible promoters, such as those responsive to dexamethasone, ethanol, estradiol, tetracycline, copper, benzothiadiazole (an inducer of pathogen-related proteins), herbicide safeners, the insecticide methoxyfenozide, and temperature (Padidam 2003). Also, tissue-specific inducible expression can be achieved by combining these two approaches (Deveaux et al. 2003).

FP fusion sequences are usually inserted into *Agrobacterium* binary plasmids between the left and right borders, where a plant selection marker gene is normally included. The binary vectors, which also contain replicons for both *Escherichia coli* and *Agrobacterium tumefaciens*, can be amplified in these bacterial strains. Several families of binary vectors, such as the pBin19, pBI121 (Brasileiro et al. 1991), pPZP (Hajdukiewicz et al. 1994), pGreen (Hellens et al. 2000), and pCAMBIA

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(http://www.cambia.org.au/) series, include vectors conferring different bacterial selection markers, such as kanamycin, streptomycin or spectinomycin, gentamycin, and tetracycline, as well as plant-resistance genes for agents, such as the antibiotics kanamycin and hygromycin or the herbicides phosphinothricin and glufosinate ammonium. These different bacterial selection markers and plant-resistance genes provide compatible vector/*Agrobacterium* combinations for the generation of transgenic plants coexpressing two or three genes. Most *Agrobacterium* strains are resistant to rifampicin; GV3101 (pMP90) is resistant to gentamycin; and GV3101 (pMP90RK) is resistant to gentamycin and kanamycin. Restriction enzymes, tritemplate polymerase chain reaction (TT-PCR) (Tian et al. 2004), and Gateway recombination cloning technology (Invitrogen) are commonly used to generate FP fusions and insert them into binary vectors. To facilitate subcloning of FP fusions into plant binary vectors, a set of binary vectors supporting amino- or carboxy-terminal fusions to a set of FPs have been developed (Fang and Spector 2007).

Transferring the Binary Vector with the FP Fusion into *A. tumefaciens*

A variety of disarmed *Agrobacterium* strains are available for plant transformation (Zupan and Zambryski 1995). The most commonly used strains include LBA4404, GV3101, AGLO, AGL1, A281, EHA101, and EHA105. The species of plant to be transformed, the transformation method, and the competence of the bacterial selection marker will affect the choice of an appropriate *Agrobacterium* strain (see, e.g., **Vectors and *Agrobacterium* Hosts for *Arabidopsis* Transformation** [Weigel and Glazebrook 2006a]). Binary vectors containing FP fusions normally are transformed into *A. tumefaciens* by electroporation (**Transformation of *Agrobacterium* Using Electroporation** [Weigel and Glazebrook 2006b]), the freeze-thaw technique (**Transformation of *Agrobacterium* Using the Freeze-Thaw Method** [Weigel and Glazebrook 2006c]), or triparental mating methods (see Wise et al. [2006] for details).

Validating the FP Fusion Protein by Rapid, Transient Expression

Transient expression by bombardment or agroinfiltration to plant cells provides a fast method to evaluate the fusion construct and assay the localization of the fusion protein. However, expression levels in transient studies are normally high, and therefore caution should be taken when analyzing the results because a transient dominant negative phenotype and/or ectopic localization of the gene product can occur.

Generating Transgenic Plant Lines Expressing FP Fusions

It normally is necessary to study the organization and dynamics of the FP fusions in transgenic plants. Bombardment and *Agrobacterium*-mediated transformation are the most commonly used methods to generate transgenic plants. If the FP fusion can rescue the mutant in plants, this indicates that the fusion protein is functional. Alternatively, rescue of a yeast mutant with an FP fusion could indicate if the plant protein is an authentic homolog of the yeast gene.

Preparing Plant Samples for Live Cell Imaging

To prepare live plant samples for imaging, optimal growth conditions should be preserved as much as possible, including light, temperature, osmolarity, humidity, gravity, and nutrition.

Microscopes, Data Collection, and Analysis

The most commonly used microscopy systems for plant live cell imaging include confocal laser-scanning microscopy, deconvolution microscopy, and spinning-disk confocal microscopy. Plant samples are normally thick, so confocal microscopy is suitable for plant live cell imaging because the laser beams can penetrate thick tissue. A wide-field microscope equipped with a motorized stage and deconvolution software allows collection of time-lapse image stacks along the *z*-axis with the efficient removal of out-of-focus light. A water objective lens is suggested, because plant tissues contain a large amount of water, and such a lens will match the refractive index of the live sample. It is essential to reduce photo-oxidative damage during imaging, and approaches for achieving this include the use of lower excitation light intensities, fewer image sections, and reducing the frequency of imaging in a time-course experiment. Cells in green tissues, pollen grains, and stressed or photodamaged cells normally have a higher degree of autofluorescence. Sometimes, photodamaged cells have cytoplasm

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that appears more granular and highly vacuolated with the nucleus being pushed to the side of the cell.

The fluorescence and degradation of FPs are pH-dependent. This property has been used to probe intracellular pH in plants (Gao et al. 2004). Rapid light- and pH-dependent degradation of GFP in the vacuole by cysteine proteases has been reported (Tamura et al. 2003). To some degree, the fluorescence of YFP has been reported to be Cl^--dependent (Griesbeck et al. 2001).

Software programs that are part of the imaging system normally allow for the processing of three-dimensional image stacks and time-lapse image stacks (i.e., four-dimensional data), such as deconvolution, projection, volume rendering, modeling, measuring signal intensity, and distances between particles, as well as the ability to save a data set as a movie or another format. Specialized software may be required for processing tasks, such as image registration, alignment, and particle tracking in two or three dimensions. Free public software such as ImageJ (http://rsbweb.nih.gov/ij/) from the National Institutes of Health with plug-ins for various functions is also very useful.

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