Chapter 9

Live Cell Imaging and Confocal Microscopy

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Abstract

The availability of more specific dyes for a subset of endomembrane compartments, combined with the development of genetically encoded probes and advanced microscopy technologies, makes live cell imaging an approach that goes beyond the microscopically observation of cell structure. Here we describe the latest improved techniques to investigate protein–protein interaction, protein topology, and protein dynamics.

Furthermore, we depict new technical approaches to identify mutants for chloroplast morphology and distribution through the tracking of chlorophyll fluorescence, as well as mutants for chloroplast movement.

Key words Plant cell, FRAP, iFRAP, BIFC, Protein interaction, Protein topology, Chloroplast movement, Photosynthesis, VAEM

1 Introduction

Cell biology has made huge progresses with the advent of confocal microscopy applied with genetically encoded fluorophores. The combination of these tools allows for in vivo studies at cellular level and in entire organisms. Also, the broad variety of the molecular dyes now available has made a strong impact. For example, dyes such as FM4-64 and DiOC6 have been used in several studies for membrane staining in live cells; nonetheless, the incorporation of these lipophilic dyes in prolonged time stains most the endomembranes making it very difficult to perform single organelles analyses. Advances in this field led to the development of new chemical dyes that allow the identification of new cellular structures. Furthermore, with the advent of genetically encoded fluorescent probes, studies at specific organelle level have become possible.

The use of molecular markers that target specific endomembrane compartment combined with the confocal microscopy allows for studying not only mechanisms for organelle targeting but also the identification of specific machinery components, protein–protein

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interactions, as well as the study of protein topology and protein intraorganelle and interorganelle dynamics. Confocal microscopy methodologies that enable this kind of analyses include fluorescence recovery after photobleaching (FRAP) (with all its variants like iFRAP and two-color FRAP) and bimolecular fluorescence complementation (BiFC).

Specific protein targeting to organelles in vivo is fundamental to investigate organelle function and biogenesis but also organelle interactions with other cellular compartments and components, underling that analyses in vivo using specific probes may be highly relevant to the understanding of the physiology of a specific cell type or entire organism.

Among all the cell types that best allow for high-resolution imaging of the secretory pathway are the leaf epidermal cells. This specific cell type has indeed the advantage not only to be large but also to have a large central vacuole that makes the cytoplasm confined to a cortical thin layer, which makes endomembrane imaging easier. Moreover in photosynthetic organisms, through the imaging of epidermal and mesophyll cells live cell confocal microscopy allows investigating chloroplasts morphology, biogenesis, and conduct chloroplast photo-relocation experiments by simply using the autofluorescence of chlorophyll without the need of introducing foreign fluorescence markers into the organism. Thanks to this kind of analysis, it is possible to have information also on the photosynthetic performance of plants. The newest methodologies that can be applied through live cell confocal allow also the study of important components of plant structures, an example is the total internal reflection fluorescence (TIRF) variant, also named variable epifluorescence microscope (VAEM). This technique can be applied to image cellulose microfibrils to study their orientation through the vital molecular dye Direct 23.

1.1 FRAP, iFRAP, and Two-Color FRAP

In order do study protein dynamics in vivo, it is possible to use techniques like FRAP (fluorescence recovery after photobleaching) and iFRAP (inverse FRAP). Photobleaching analysis allows monitoring the dynamics of proteins with a highly refined precision and in the range of subseconds. This method provides information such as protein retention at membrane level, interaction with other proteins and prediction of trafficking mechanisms. FRAP and iFRAP techniques can be performed to measure protein diffusion and the ratio of protein exchange between compartments. FRAP is performed by irreversibly photobleaching a fluorochrome in a specific area of the cell called region of interest (ROI) using high intensity illumination of a laser line that excites the fluorochrome; in contrast, for iFRAP a whole cell area or organelle is photobleached, except a selected small area of interest. By disrupting a fluorochrome integrity, the protocol enables monitoring only the pool of fluorochrome that has not been bleached. Only the

nonbleached fluorochromes, such as those of fluorescent proteins, can diffuse into the bleached area (FRAP) or outside (iFRAP) with a recovery rate that is specific for each protein fusion. From this kind of experiments, one obtains information about kinetic plots of the fluorescence changes in the ROI [1–4]. It is therefore possible to calculate protein mobile fraction and immobile fraction. The mobile fraction represents the fraction of fluorescent probes that can exchange between the bleached region of interest and nonbleached area, while the immobile fraction is the fraction that cannot exchange between these two regions. The evaluation of mobile fraction values can be important to understand protein–protein interactions or protein–membrane domain association.

FRAP can also reveal the existence of intercompartmental communication and rates of movement of molecules within or between compartments. This kind of information generally is useful to capture a broad picture of the functional organization of the cell. For example, in plant cells FRAP is useful to examine protein exchange between compartments that could be functionally or physically connected. Indeed, using a fluorescent protein targeted to the stroma of plastid this approach has been used to test whether chloroplasts could exchange protein molecules through stromules, which are narrow chloroplast–chloroplast connecting structures [5].

Another useful variant of FRAP is the two-color FRAP. Two-color FRAP is used to investigate mutual mobility of two proteins which belongs or form a complex. The principle of technique is similar to the single channel FRAP; the only difference is that we can use two proteins, fused to different fluorophore (which possesses different spectral properties to avoid signal overlapping). Simultaneous FRAP of both fluorophores can be performed using two channels with specific excitation and emission wavelength ranges. This allows for measuring the protein turnover of the two molecules at the same time.

1.2 Use of Chlorophyll Autofluorescence to Identify Possible Photosynthetic Mutant and Chloroplast Fractal Analysis

The use of confocal microscopy is convenient for studying photosynthetic organelles like chloroplast in plants or algae. By taking advantage of chlorophyll autofluorescence, it is possible to visualize these organelles without tagging them with foreign fluorophores. With this approach, it is possible to identify chloroplast mutants with defects in the morphology or distribution of these photosynthetic organelles [6]. Furthermore, the availability of fast scan Z-drive available in the newest microscope systems now gives the possibility to acquire Z-stacks in a very short time without, thus limiting the potential artifacts owing to movement of chloroplasts during the image acquisition. The images acquired and reconstructed in the Z-direction provide abundant information, including number and size of each chloroplast, shape, and spatial arrangement. The latter can be analyzed in detail using fractal

analysis from which we can obtain fractal dimensions (D) and lacunarity parameter (Λ) for each acquired image. The obtained values of D allow establishing the geometric complexity of the distribution of chloroplasts for comparison across different experimental conditions or mutants. Instead, the heterogeneity (e.g., the gaps) in the distribution of chloroplasts is defined by Λ (Fig. 1).

Another useful analysis is the photo relocation of chloroplasts, which can be performed to calculate the velocities of this organelle in different conditions or mutants. Chlorophylls can be excited with laser line of 514 nm and light emitted can be detected at 650–700 nm.

1.3 Bimolecular Fluorescence Complementation (BIFC)

BIFC is a useful technique to study protein topology, protein–protein interaction or to screen a prey genomic library tagged with half moiety fluorescent protein for possible interactors against bait fused with the other half fluorescent protein [7]. Unfortunately, if not properly executed, this technique can provide false positive results, so specific precautions and improvements have been developed in the recent years.

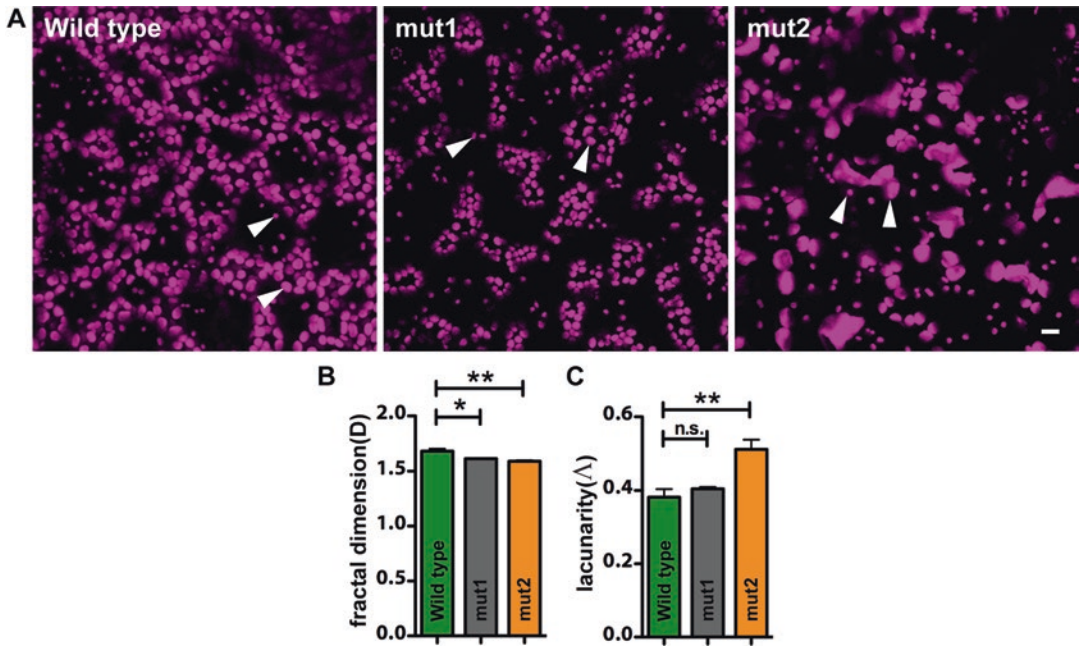

Fig. 1 (**a**) Confocal images of the chloroplasts from wild type, mut1 (mutant 1), and mut2 (mutant 2) plant leaves. Arrowheads indicate chloroplasts. Each image in panel A is the result of a Z-stack obtained from 25 confocal planes acquired starting from the abaxial surface of the leaf. Scale bar = 10 μm. (**b**) Fractal dimensions (D) of the wild type and mutant plants calculated from 10 images to compare the chloroplast distribution and geometry. (**c**) Lacunarity parameters (∧) calculated from 10 images. Error bars indicate SEM. **0.001 > P < 0.01; *0.01 > P < 0.05; n.s., not significant

1.3.1 BiFC for Topology

The classical BIFC experiments have been performed by splitting the YFP in a first half containing amino acids 1–154 (N terminal YFP) and the second half containing amino acids 155–239 (C terminal YFP). These two halves are generally fused to the proteins for which an interaction is to be tested. In this classical setup the nonspecific reconstitution of the entire YFP protein is recurrent. However, such feature has proven advantageous to establish protein topology. Specifically, this method can be used to study protein orientation with respect to a membrane in most organelle with the premises that a reference protein for each side of the compartment under investigation is available. Once a reference protein with an established subcellular is selected (preferable a soluble protein) then it should be fused to either the N or C terminal half of YFP while the complementary half should be fused to the protein to be investigated. It is worth to mention that for this approach YFP is commonly used; however, any fluorescent variant can be used [8]. Detection of a fluorescence signal will inform us if the two proteins are facing the same compartment with respect to a membrane or to the organelle investigated, and as a result it is possible to determine the protein orientation (Fig. 2) (*see* **Note 1**).

1.3.2 BIFC for Protein Interaction

As stated before the commonly used BiFC approaches are generally prone to false results, showing fluorescent protein reconstitution even when the two proteins are not true interactors [9–11]. A more reliable system has been optimized to test and quantify the level of interaction at the same time. This is the ratiometric BIFC (rBIFC) approach [12] that consists of a single binary vector that has multiple expression cassettes. The system named "2 in 1" has the half N-terminal YFP (1–155) in one cassette and the other C-terminal YFP (156–239) in another cassette. This system possesses also a soluble monomeric red fluorescent protein (mRFP) that allows the ratiomentric analysis because it works as internal control of expression. This system can be used in different organism (*see* **Note 2**).

Recently, after different attempts of splitting the fluorescent proteins in different positions or introducing point mutations to eliminate nonspecific self-assembling of the complementary halves, a novel modified approach has been proposed, which, as stated by the authors, should have "zero background" BIFC [13]. In this new system a binary vector is composed of two expression cassettes driven by the same promoter and contains an additional fluorescent marker (mTurquoise2) to discriminate between transformed and non transformed cells (internal control of successful transformation). In one cassette, the vector contains the half N-terminal YFP (1–210) and in the other cassette the C-terminal YFP (211–239). This split position reduces the nonspecific signal frequently obtained with the approaches described above to a zero background signal [13].

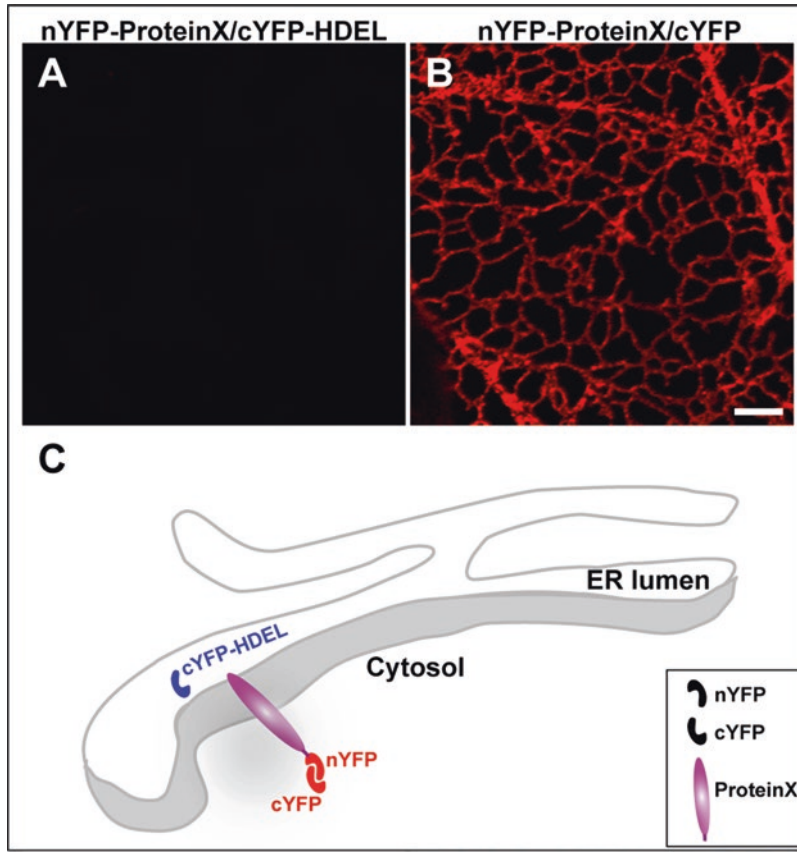

Fig. 2 Example of BIFC to investigate membrane protein topology. To map the N-terminal region of a ProteinX fused with half YFP, coespression analysis is performed in presence of the other half YFP expressed in the cytosol or ER lumen. (**a**) Confocal image of *N. tabacum* epidermal cells expressing ProteinX fused with half YFP and half YFP fused to an ER luminal protein. (**b**) Confocal image of *N. tabacum* epidermal cells expressing ProteinX fused with half YFP and half YFP fused to a cytosolic protein. Fluorescence detection in this case indicates that the protein face the cytosol with its N-terminal region. (**c**) Cartoon depicting the approach used to map the N-terminal of an ER ProteinX

1.4 Variable-Angle Epifluorescence Microscopy (VAEM)

VAEM technique derives from total internal reflection fluorescence microscopy (TIRFM). TIRFM is a useful technique to observe fluorescent probes on the plasma membrane edge of animal cells. In plant cells, the thick cell walls surrounding the plasma membrane form a barrier, limiting the use of the TIRF. However, VAEM represents a useful variant which allows for imaging plasma membrane molecules and study their behavior [14]. Here we describe how to use VAEM technique to investigate the behavior and orientation of cellulose microfibrills in *A. thaliana* seedlings stained using a well-characterized fluorescent probes known as Pontamine Fast Scarlet 4B or Direct RED 23 (Fig. 3) [15].

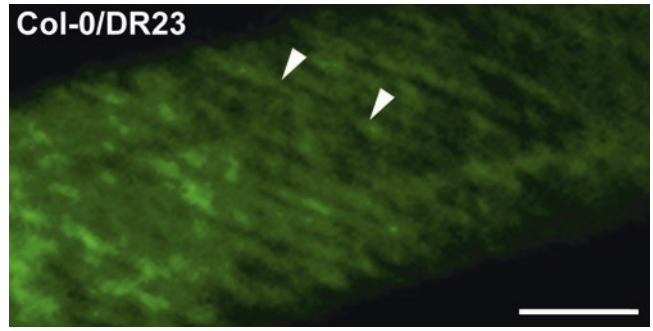

Fig. 3 Direct RED 23 (DR23) fluorescence acquisition by VAEM of *A. thaliana* hypocotyl epidermal cells. Arrowheads indicate cellulose microfibril. Scale bar = 5 μm

2 Material

2.1 FRAP

1. Plant plasmids: The gene of interest can be fused to any fluorescent protein variant in two ways: (1) Subcloning the gene in plant binary vectors already containing the XFP. (2) Fusing the CDNA of the gene of interest with the XFP by overlapping PCR [4, 16] and then cloning the obtained product in a binary vector without the XFP sequence.
2. Agrobacterium and Media:
   (a) Strain of agrobacterium: GV3101::mp90 containing the binary vector created as described above.
   (b) Antibiotics to select agrobacteria and plasmid: for GV3101::mp90 selection Gentamycin 25 μg/mL and rifampicin 50 μg/mL, plasmid selection varies depending on the specific resistance of the plasmid utilized, for the binary vector pVKH18En6 the antibiotic added to the media for selection in addition to gentamicin and rifampicin, will be kanamycin 100 μg/mL.
   (c) LB media for bacteria growth: tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L) pH 7.0.
   (d) Infiltration Buffer: 0.5% D-glucose, 50 mM MES, 2 mM Na_3PO_4* 12 H_2O, 0.2 mM acetosyringone.
3. Three-four weeks old *Nicotiana tabacum*, grown in growth chambers at 23 °C for 18 h light and 18 °C for 6 h dark.
4. Stable lines of *Arabidopsis thaliana* obtained accordingly to the protocol of Clough and Bent [17] and expressing the gene of interest can be used at different growth stages.
5. 25 mM latrunculin B solution.
6. Confocal microscope equipped with 10×, 40× and 60× objectives.

2.2 Chlorophyll Autofluorescence Imaging and Chloroplast Fractal Analysis

1. *Arabidopsis thaliana* EMS mutagenized or tDNA insertion lines grown in chambers with controlled temperature conditions 20 °C and 125 μmol m^{-2} s^{-1} white light with a photoperiod of 16 h of light followed by 8 h of dark.
2. Growth substrate:
   (a) Substrate for stratification: 0.1% Phytoblend.
   (b) Soil: sterilized mixture of Bacto High Porosity Professional Planting Mix, vermiculite and perlite with ratio 1:1:1
   (c) Sterile substrate for in vitro growth: Linsmaier and Skoog (LS) medium containing 0.5% Phytoblend, 2% sucrose.
3. Sterilization solutions:
   (a) 70% ethanol containing 0.5% Triton X-100.
   (b) 95% ethanol.
   (c) Sterilized filter paper.
4. Confocal microscope equipped with 10× and 40× objectives.

2.3 BIFC

1. Plant plasmids—the gene of interest can be fused to the half N or C terminal portion of YFP in two ways: (1) Subcloning the gene in plant binary vectors already containing the N terminal or C terminal half portion of YFP. (2) Fusing the CDNA of the gene of interest with the half YFP by overlapping PCR [4, 16] and then cloning the obtained product in a binary vector without the YFP sequence.
2. Agrobacterium and Media:
   (a) Strain of agrobacterium: GV3101::mp90 containing the binary vector created as described above.
   (b) Antibiotics to select agrobacteria and plasmid.
   (c) LB media for bacteria growth: Tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L) pH 7.0.
   (d) Infiltration Buffer: 0.5% D-glucose, 50 mM MES, 2 mM Na_3PO_4*$12H_2O$, 0.2 mM acetosyringone.
3. Plants: 3–4 weeks old Nicotiana tabacum, grown in growth chambers at 23 °C for 18 h light and 18 °C for 6 h dark.
4. Confocal microscope equipped with 10×, 40× and 60× objectives.

2.4 Variable-Angle Epifluorescence Microscopy (VAEM)

1. 0.01% (w/v) Direct RED 23 in liquid 0.5× LS or MS medium.
2. LS or MS medium.
3. Confocal laser scanning microscopy (CLSM) with VAEM mode (100× objective)

3 Methods

3.1 FRAP

FRAP and iFRAP techniques can be applied to endomembrane compartments or structures that are non-cytoskeleton driven or endomembranes/organelles that are guided by actin or microtubules. In this case the use of depolymerizing agents prior to imaging is generally necessary to reduce organelle movement significantly. For example, latrunculin B (actin depolymerizing agent) is generally used to stop Golgi movement [18, 19] and microtubule depolymerizing agents (oryzalin, nocodazole) can be used for organelles whose motility is driven by this cytoskeletal component.

FRAP analysis with the newest confocal systems has been very easy to perform thanks to the user-friendly software preinstalled in most of the systems available on the market. Here we describe a FRAP setup for Golgi bodies in a stable transgenic line of *A. thaliana* expressing a Golgi-specific fluorescent marker.

1. Use a cotyledon of young seedlings (i.e., 9 days after germination (DAG) (*see* **Note 3**).
2. Start the experiment by treating the sample to be investigated with an actin-depolymerizing agent in order to stop the movement of the Golgi stacks. To do so, submerge the tissue into 25 mM latrunculin B solution for at least 30 min (*see* **Note 4**).
3. Mount the tissue on the slide with the same solution using coverslips with the same thickness for all the experiments (*see* **Note 5**).
4. Open the microscope software and set up excitation and emission wavelengths for the fluorochrome of choice. Keep in mind that they should provide maximum signal yield with minimal laser intensity power and avoid pixel imaging oversaturation or undersaturation.
5. Acquire one image and select the following areas:
   (a) Draw a control region of interest (background ROI), where the fluorescent signal is visible. This ROI will allow checking for nonspecific bleaching during the process and will define the levels of fluorescence that should be reached upon the bleaching.
   (b) Select the number of preferred bleached regions (typically we use 2–3 ROIs), away from the background ROI. These regions should all be of similar area.
   (c) During FRAP analysis the background fluorescence curve will be analyzed against the bleached curve to eventually correct for any nonspecific bleaching.
6. Set up the confocal microscope software to acquire 10 frames before the bleaching period (prebleach time), and then acquires

images until the recovery of the fluorescence reaches a plateau in the bleached area (The acquisition time for postbleach depends on the protein investigated.).

7. Acquire at least 20 scans containing ROI background and bleached ROIs. Perform all the experiments using the same confocal settings, which include laser intensity, pinhole, objective, and zoom [20].

3.2 Chlorophyll Autofluorescence Imaging and Chloroplast Fractal Analysis

1. Use a rosette leaf from an Arabidopsis plant grown for 3 weeks under standard condition (22 °C temperature, 50% humidity with 16 h light and 8 h dark cycle).
2. Place the leaf sample (around 25 mm^2) (*see* **Note 6**) on a glass slide and place the abaxial or adaxial side facing the objective.
3. Make sure to use the fast scan Z-drive in order to minimize the imaging time and avoid long exposure of the sample to the laser.
4. Use a 20× or 40× objective. Here we used a 40×/1.30 objective mounted on an inverted confocal laser-scanning microscope.
5. Excite chlorophylls using at 514 nm using argon laser and collect the fluorescence between 650 and 700 nm [21, 22].
6. To compare the different samples, acquire the images using the same fixed laser intensity, pinhole, gain, and zoom.
7. Acquire for each Z stack between 25 and 50 sections of 1 μm (make sure to collect the same amount of section between samples) from the abaxial or adaxial surface scanning up to the region of the spongy mesophyll cells.
8. Collect at least ten scans for each sample or mutant to be analyzed.
9. Using the software provided with the confocal or ImageJ plugin extrapolate the maximum intensity projections from each Z-stack acquired.
10. Calculate the fluorescence values for each image using the software provided with the microscope and plot the values.
11. Calculate fractal dimensions and lacunarity parameters using the same maximum intensity projections by fraclac, an ImageJ plugin [23].

3.2.1 Photorelocation of Chloroplasts

1. Use a rosette leaf from an Arabidopsis plant grown for 3 weeks under 35 μmol m-2 s-1 white light (22 °C temperature, 50% humidity with 16 h light and 8 h dark cycle).
2. Place the leaf sample (around 25 mm^2) (*see* **Note 6**) on a glass slide and place the adaxial side facing the objective.
3. Use a 20× or 40× objective.

4. Set the confocal software to perform chloroplast irradiation (bleaching set-up on the software) by repeatedly laser pulse the region of interest for 2–3 s with a 488 nm laser at 2–5% of maximum power throughout the photorelocation experiment.
5. Acquire images of chlorophyll fluorescence between irradiations of the region of interest, using a 633 nm laser and collect fluorescence from 650 to 700 nm [21, 22].
6. The movement of the chloroplasts located in the cortical region of the spongy mesophyll was acquired.
7. Calculate chloroplast velocities using the Multi Kymograph plugin from ImageJ.
8. Analyze at least 25 and 40 kymographs, from ten independent cells to obtain a good statistics.

3.3 BIFC

3.3.1 Binary Vector

Use any of the binary vectors described above that is helpful for your experiment based on the requirements of the background levels. Here we used and describe the methodology for pVKH18En6 with the N- or C-terminal half of YFP fused to a protein of interest, but the same approach can be used for other vectors. The YFP in this vector is split at amino acid 155. The half YFP portion can be fused at the N- or C-terminus of our protein of interest; this choice depends on the properties of the protein investigated in order to preserve its functionality without affecting distribution and function.

3.3.2 Tobacco Infiltration

BIFC experiments lead generally to a stable protein–protein interaction. Therefore, transient expression should be preferred to stable transformation where reconstitution of the YFP bearing two irreversibly interacting proteins may cause developmental problems. Here we describe a BiFC protocol for transient transformation in tobacco leaf epidermal cells:

1. Prepare the infiltration buffer using 50 mg of D-glucose, 1 mL from a stock solution of 500 mM MES, 1 mL from a stock solution of 20 mM Na_3PO_4 $12H_2O$ (*see* **Note 7**), and 10 μL from a stock 200 mM of acetosyringone.
2. Perform Agrobacterium infiltration in tobacco leaf epidermis using 1 mL syringe containing the resuspended agrobacteria solution that will be pushed through the abaxial side of the leaf, balancing the pressure with your finger on the adaxial corresponding region.
3. Use the lowest bacterial optical density (OD) at 600 nm to limit protein overproduction, which can lead to false positives. To avoid nonspecific results, successful experiments have been performed using an OD with range between 0.0025 and 0.005 [11].
4. Observe the samples transformed, 48–72 h after bacterial infiltration.

3.3.3 Microscope Observations

1. Place the sample (around 25 mm^2 of leaf section) on a glass slide with the abaxial side towards the coverslip and scan with a confocal microscope.
2. Use a low magnification objective, for example, a 10×.
3. Check first the negative control for nonspecific results; this sample should not be fluorescent. On the contrary if it is fluorescent do not proceed to the next step, but repeat the whole experiment reducing the OD of bacteria.
4. Observe the sample containing the protein of interest to test occurrence of an interaction.
5. If an interaction takes place, acquire images with a 40× or 60× objective to obtain more information about the localization of the interactors.

3.4 Variable-Angle Epifluorescence Microscopy (VAEM)

1. Use young seedlings (3–12 DAG).
2. Incubate a seedling with 0.01% (w/v) Direct RED 23 in liquid 0.5× LS or MS medium for 30–45 min (*see* **Note 8**). The seedlings can be incubated in a 1 mL eppendorf tube.
3. Wash the seedling by transferring them into a new tube supplemented with fresh medium without the probe. Wash the sample at least three times.
4. Place the seedling on a glass slide and observe the sample with confocal laser scanning microscopy (CLSM) to confirm that the fluorescent probes stained your tissue.
5. Switch from CLSM to VAEM mode and set-up the system to image the sample of interest. Below the procedure for Nikon A1RSi TIRF system equipped with ANDOR camera (iXon Ultra):
   (a) Turn on all the components of the microscope.
   (b) Install the TIRF stage.
   (c) Select the 100× (NA 1.49) TIRF objective.
   (d) Open the NIS Elements software using ANDOR.
   (e) Open the following windows available through the software: TiPad, DU-897 settings, TIRF/SR-active, LUT.
6. Place the sample mounted on glass slide on the stage and cover with the TIRF stage coverplate.
7. Focus on the sample.
8. Perform a coarse alignment of TIRF as follow:
   (a) Select from top toolbar the TIRF_UP optical alignment configuration specific for the laser being used.
   (b) Turn the laser ON using the icon AOTF located in the TIRF/SR-active window.

(c) Center the laser using the knob on TIRF arm (*see* **Note 9**).

(d) With the apposite slider in unlock position focus the laser beam until the beam is as tight as possible.

(e) Turn the laser OFF using the same icon as in **step b**.

9. Perform a fine alignment of the TIRF angle as follow:

(a) Select the TIRF_ optical configuration specific for the laser being used.

(b) Using the DU-897 settings window select NO Binning, 1 frame exposure time, EM Gain 17 MHz at 16 bit, EM gain ~300.

(c) Turn the laser ON using the same icon as in **step 8b**.

(d) Select PLAY to turn the camera ON to acquire images. (*see* **Note 10**)

10. Use TiPad window to tune the TIRF angle in live mode. (*see* **Note11**)

11. Record movies for further analysis.

4 Notes

1. In the specific case of the localization of the protein in compartment that might affect fluorescent protein stability compartment the YFP can be replaced with more resistant for of fluorescent proteins like RFP, or mCherry.
2. The 2in1 system can be used in different cell or expression systems cloning using the enzymes SpeI and PsiI [12].
3. Different age seedling can be used, as well as leaf form mature plants.
4. Incubation with Latrunculin B should be performed for a time no longer than 90 min in order to reduce excessive cellular stress that may confound the results.
5. Coverslip thickness influences the results of the experiment.
6. On a glass slide and place the adaxial side facing the objective.
7. MES and Na3PO4 12H2O Stock should be sterilized by filtration and stored at 4 °C. Acetosyringone stock solution is prepared in DMSO and aliquoted in an Eppendorf tube and stored at −20 °C until use.
8. Incubation time with the fluorescent probe depends on the age of the seedlings.
9. Wear laser safety goggles.

10. If you do not see image, increase the laser power until fluorescence is detected.
11. Keep in mind that a value of TIRF equal to 4000 is close to the critical angle.

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