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TITLE:

Determination of Tripartite Interaction between Two Monomers of a MADS-box Transcription Factor and a Calcium Sensor Protein by BiFC-FRET-FLIM Assay

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SUMMARY:

Here we present, a method to visualize ternary complex formation between three protein partners using fluorescent-tagged proteins by BiFC based FRET-FLIM assay. This method is valuable for studying protein-protein interaction complexes *in vivo*.

ABSTRACT:

Protein-protein interactions are an integral part of all biological processes in the cells as they play a crucial role in regulating, maintaining, and amending cellular functions. These interactions are involved in a wide range of phenomena such as signal transduction, pathogen response, cell-cell interactions, metabolic and developmental processes. In the case of transcription factors, these interactions may lead to oligomerization of subunits, sequestering in specific subcellular contexts such as the nucleus, cytoplasm, etc., which, in turn, might have a more profound effect on the expression of the downstream genes. Here, we demonstrate a methodology to visualize *in vivo* tripartite interaction using Bimolecular Fluorescence Complementation (BiFC) based Förster Resonance Energy Transfer (FRET) involving Fluorescence Lifetime Imaging (FLIM). Two of the proteins selected for this demonstration interact as BiFC partners, and their reconstituted fluorescence activity is used to assay FRET-FLIM with the third partner. Four to five-week-old growth-chamber-grown *Nicotiana benthamiana* plants have been used as the model plant system for this demonstration.

INTRODUCTION:

Protein-protein interactions (PPIs) form the basis of the proper functioning of the eukaryotic cells by regulating various metabolic and developmental processes. Some PPIs are stable, while others are transient in nature. The interactions may be categorized based on the number and type of members in the interaction such as dimeric, trimeric, tetrameric homomeric, and heteromeric[1]. The identification and characterization of protein interactions may lead to a better understanding of protein functions and regulatory networks.

Transcription factors are proteins that are involved in regulatory functions. They regulate the rate of transcription of their downstream genes by binding to the DNA. Sometimes oligomerization or formation of higher-order complexes by proteins is a prerequisite for carrying out their functions[2]. Plant MADS-box transcription factors are homeotic genes that regulate various processes such as floral transition, floral organ development, fertilization, seed development, senescence, and vegetative development. They are known to form higher-order complexes which bind to the DNA[3,4]. Studying PPI networks among transcription factors and their interactors provides insights into the complexity underlying transcriptional regulation.

Transient protein expression in *Nicotiana benthamiana* has been a popular approach to study protein localization or protein-protein interactions *in vivo*[5]. BiFC and FRET are methods for studying protein-protein interactions *in vivo* using fluorescent reporter systems[6]. A combination of these two techniques has been shown to reveal the interaction between three proteins[7]. FRET is measured using acceptor photobleaching, sensitized emission, and Fluorescence Lifetime Imaging (FLIM) technique. FLIM-based FRET has emerged as a tool that provides accurate quantification and spatiotemporal specificity to energy transfer measurements between two molecules based on their fluorescence lifetimes[8]. FLIM measures the time a fluorophore stays in an excited state before emitting a photon and is better than techniques that use intensity measurements alone[9,10]. Besides heterologous systems such as *Nicotiana benthamiana* and onion epidermal peels, more recent reports have demonstrated the use of *Arabidopsis* roots and young rice seedlings, etc., for *in vivo* analysis of protein-protein interactions under native conditions[11,12].

Other than a suitable expression system, the selection of interacting partners for BiFC and FRET assays is also crucial for the success of this experiment. The PPI among partners used in BiFC configuration should be validated using appropriate controls prior to their use as a conjugated partner in the FRET experiment[13]. BiFC utilizes the structural complementation of N- and C-terminal parts of the fluorescent protein. A common limitation in most if not all fluorescent proteins used in BiFC assays has been the self-assembly between the two derivative nonfluorescent fragments, contributing to false-positive fluorescence and decreases the signal-to-noise (S/N) ratio[14]. Recent developments, including point mutations or position of splitting the fluorescent protein, have given rise to BiFC pairs with increased intensity, higher specificity, high S/N ratio[15,16]. These fluorescent proteins can also be used for carrying out BiFC depending on the suitability of the experiment.

Traditionally, CFP and YFP have been used as the donor and acceptor pair in FRET experiments[17]. However, YFP or m-Citrine were found to be better FRET donors (when used with RFP as the acceptor) because of the high quantum yield (QY) during the native expression

of target proteins in the *Arabidopsis* root system. The selection of promoters (constitutive versus native/endogenous) and fluorophore also play a crucial role in designing a successful BiFC-FRET-FLIM experiment. It is essential to note that the efficiency of FRET donors and suitability of FRET pairs tend to change with the change in the promoter and the biological system being used for expression. The QY of the fluorophore, which relates to its brightness, depends on the pH, temperature, and the biological system in use. We suggest that these criteria be thoroughly considered before choosing the fluorophore pair for the FRET experiment. The biological system, promoters, and the proteins used for this protocol worked well with CFP-YFP fluorophores for the BiFC FRET-FLIM experiment.

In the present study, we incorporate the feature of FLIM to visualize the interaction between three protein molecules using BiFC based FRET. In this technique, two proteins are tagged with split YFP protein and the third protein with CFP. Since we were interested in studying the interaction of a MADS-box protein (M) homodimer with a Calcium sensor protein (C), these proteins were tagged with fluorescent proteins in pSITE-1CA and pSITE-3CA vectors[18]. Two of the interacting partners, in this assay, were tagged with N- and C-terminal parts of the YFP in pSPYNE-35S and pSPYCE-35S vectors[19], and their interaction results in the reconstitution of the functional YFP that acts as a FRET acceptor to the third interacting partner, which is tagged with CFP (acting as FRET donor) (**Figure 1**). In this particular case, the PPI between two M monomers and between M and C has been validated by performing BiFC in three different systems along with the yeast-two-hybrid system. These vectors were mobilized into *Agrobacterium tumifaciens* GV3101 strain by electroporation. The GV3101 strain has a disarmed Ti plasmid pMP90 (pTiC58DT-DNA) with gentamicin resistance[20]. A p19 *Agrobacterium* strain was added along with all infiltrations to prevent transgene silencing[21]. We recommend that the three proteins should also be used in opposite conformations to validate the tripartite interactions.

In this technique, we have employed FLIM, where first, the fluorescence lifetime of the donor (unquenched donor lifetime) is measured in the absence of an acceptor. After that, its lifetime is measured in the presence of the acceptor (quenched donor lifetime). This difference in donor fluorescence lifetimes is used to calculate FRET efficiency, which depends on the number of photons exhibiting a reduction in fluorescence lifetime. Mentioned below is a detailed protocol to determine the formation of a ternary complex between any three proteins by transiently expressing the fluorescent-tagged proteins in *Nicotiana benthamiana* and assaying their interaction by BiFC-FRET-FLIM.

PROTOCOL:

1. Cloning of genes in entry and destination vectors (Figure 2)

1.1. Amplify the coding sequence (CDS) of the genes of interest (M and C genes in our case) by PCR and clone them in appropriate entry vectors (e.g., pENTR/D-TOPO vector; *see* **Table 1** for vectors used in this experiment).

1.2. Grow the clones on plates containing antibiotics. Validate clones that are selected on the antibiotics by restriction digestion and DNA sequencing[22,23].

1.3. Mobilize the reconstituted CDSs from entry clones to the destination vectors (pSPYNE-35S, pSPYCE-35S, pSITE-1CA and pSITE-3CA) and confirm the transfer of sequences from the entry to the destination vectors by restriction enzyme digestion.

NOTE: All vectors used in this experiment are listed in **Table 1**.

1.4. Finally, transform *Agrobacterium* GV3101 (pMP90 ($Gent^R$)) cells with the destination vectors by electroporation (**Figure 3**)[24].

2. Growth conditions for *Nicotiana benthamiana* plants

NOTE: Grow *Nicotiana* plants till 4–6 leaf stage in control conditions.

2.1. To grow *Nicotiana* plants, prepare the soil mix by mixing commercially available soil mixes with cocopeat and compost in a ratio of 2:1:1.

2.2. Spread a 1-inch-thick layer of this soil mixture in a plastic tray to make the soil bed and saturate it with deionized water. Sprinkle about 200 seeds in this soil bed.

2.3. Transfer it to a bigger tray containing 1 cm of standing water. Cover this tray with plastic wrap to create a moisture chamber.

2.4. Transfer this set up to a growth chamber set at 23 ˚C with 16 h light and 8 h dark cycle with 150–170 µmol/m^2s light intensity.

2.5. After two weeks, transfer young seedlings to small, 3–4-inch pots containing water-saturated soil mix.

2.6. Place these pots in plastic trays and transfer them to the growth chamber for four more weeks.

3. Prepare bacterial strains for agro-infiltration

NOTE: For agro-infiltration, bacterial strains need to be freshly subcultured and mixed along with p19 strain of Agrobacterium in appropriate ratios.

3.1. Prepare 2xYT agar plates containing rifampicin (100 µg/mL), gentamicin (25 µg/mL) and kanamycin (50 µg/mL) for Agrobacterium harboring pSPYNE-35S and pSPYCE-35S vector. For pSITE vector containing strain, use rifampicin (100 µg/mL), gentamicin (25 µg/mL) and spectinomycin (50 µg/mL).

3.2. Streak the Agrobacterium strains containing the plasmids on these plates using sterile inoculation loops in a laminar flow hood.

3.3. Incubate these at 28 ˚C for 48 h in the dark.

3.4. Start this procedure by inoculating *Agrobacterium* GV3101 strain harboring BiFC and FRET constructs (prepared in pSPYNE-35S and pSPYCE-35S vectors) from streaked plates in 10 mL of 2xYT broth containing appropriate antibiotics (Rifampicin (100 µg/mL), gentamicin (25 µg/mL), kanamycin (50 µg/mL) or spectinomycin (50 µg/mL)).

3.5. Additionally, initiate a culture of p19 strain of Agrobacteria by inoculating 10 mL of 2xYT broth containing rifampicin (100 µg/mL) and kanamycin (50 µg/mL).

NOTE: p19 strain is added to prevent transgene silencing.

3.6. Cover the flask with an aluminum foil and keep them in the incubator shaker set at 28 ˚C and 170 rpm for 16 h in the dark.

3.7. After the overnight growth, transfer 1 mL of this culture to a disposable cuvette to measure the optical density (O.D.) of the cultures at 600 nm using a spectrophotometer.

3.8. Mix the cultures of appropriate BiFC and FRET partner containing strains so that the final O.D. of each culture is 0.5 and that of p19 is 0.3 in a total volume of 2 mL.

3.9. To achieve these ratios, use the formula mentioned below:

$$OD_{obtained} \times V_{culture} = OD_{final} \times V_{final}$$

$OD_{obtained}$ = O.D. of the culture measured at 600 nm
$V_{culture}$ = Volume of the culture required
OD_{final} = 0.5 for constructs and 0.3 for p19
V_{final} = Final volume for the infiltration, which is 2 mL

NOTE: The construct combinations used in this study are specified in **Table 2**.

3.10. Centrifuge the mixed Agrobacterium cultures at 3,000 x *g* for 5 min at room temperature and carefully discard the supernatant. Resuspend the pellet in 2 mL of freshly prepared infiltration buffer (10 mM MES, 100 µM of Acetosyringone, and 10 mM $MgCl_2$). Use a vortex mixer to make a homogenous cell suspension.

3.11. Incubate the tubes containing resuspended cells in the dark at room temperature for 3 h.

3.12. Meanwhile, label each plant pot with the construct mixture it is going to be infiltrated with. Use two plants for each infiltration mixture.

3.13. Fill a 1 mL needleless syringe with the agrobacterial mix. Gently but firmly press the syringe onto the abaxial side of the fully expanded leaf while supporting the leaf from the other side. Gently push the plunger till the solutions fill up in the leaf area equivalent to 2–3 times the syringe tip.

3.14. Infiltrate up to four spots on a leaf and 3–4 leaves per plant, as shown in **Figure 4**.

NOTE: Change gloves or wipe gloves with 70% alcohol between samples to prevent cross-contamination.

3.15. Transfer all the pots to a tray and incubate in a growth chamber under the same conditions as mentioned in step 2.

3.16. Check a small part of the agroinfiltrated leaf at different time points using a fluorescence microscope. When the fluorescence from both YFP and CFP are detectable in cells, proceed to the confocal microscope for BiFC-FRET FLIM assay. In this experiment, the analysis was carried out 3 days after agro-infiltration.

NOTE: Set the post-agroinfiltration period of incubation individually for every promoter and gene combination to avoid overexpression of chimeric proteins used in the BiFC-FRET FLIM assay. The overexpression of the partner proteins may lead to false-positive interactions.

4. Prepare slides for fluorescence visualization

4.1. When plants are ready for visualization, cut square leaf samples, 5–8 mm away from the infiltration wound, and mount them in distilled water on clean slides.

NOTE: To minimize background fluorescence, clean the slides with 80% ethanol followed by distilled water 3–4 times, air dry them, and keep them on an absorbent sheet.

4.2. Cover the leaf sample with a clean coverslip and seal using a nail enamel.

4.3. Visualize these samples under a confocal laser scanning microscope.

5. FRET-FLIM analysis using a confocal laser scanning microscope

NOTE: In this procedure, the basis of determining and quantifying interaction between two proteins is the reduction in fluorescence lifetime of the FRET-donor partner upon its interaction with the acceptor, which is used to calculate the efficiency of FRET. The complexity in the case of tripartite interaction increases further because the FRET-acceptor, in this case, is not a single molecule but a split YFP-BiFC pair, which should first get reconstituted *in vivo* to become a functional FRET-acceptor fluorophore. To carry out FRET-FLIM, one needs to determine the donor molecule's fluorescence lifetime—first alone and then in the presence of a FRET partner.

5.1. Open the FLIM application in the confocal laser scanning microscope, start the console and use pattern-recognition photon-counting to measure fluorescence lifetime. Select the standard ‘All photon counting’ measurement mode.

5.2. Analyze samples from two types of agro-infiltrated plants: one with the donor only (C-CFP) and the other with the donor and the acceptor (C-CFP, along with M-YFP) both.

NOTE: Because the interaction of M protein has already been validated with C protein using BiFC and Y2H, good FRET efficiency is expected with this interacting pair.

5.3. Next, scan the C-CFP agro-infiltrated leaf and focus on a cell showing good CFP fluorescence. Initiate the laser scanning mode and set the system for CFP visualization and FLIM measurements (λ_{ex} 440 nm pulse laser, λ_{em} 480–520 nm by hybrid detectors, Scan speed 512 x 512 pixels at 400 Hz).

5.4. Adjust the focus, zoom, and smart gain to focus on the area that needs to be captured.

5.5. Illuminate the sample at sufficient laser power to achieve the capture of approximately 0.1 photons per pulse. For samples with variable fluorescence intensity, capture 50 frames to collect adequate photons required for the lifetime measurement. CFP exhibits two fluorescence lifetimes due to its conformational adaptation; therefore, fit the data using the n-exponential reconvolution model while keeping the value of n equal to 2.

5.6. At these settings, the CFP shows two lifetimes of 1.0 and 3.2 ns. Here the higher, 3.2 ns, lifetime is used for all subsequent calculations[25,26].

5.7. To calculate FRET efficiency using FLIM, which is the measure of the degree of interaction between two proteins, take a leaf sample that has been co-infiltrated with C-CFP and M-YFP. Look for a cell that expresses both C-CFP and M-YFP and confirm their respective emission patterns by exciting them using λ_{ex} 440 nm pulse laser, λ_{em} 480–520 nm and λ_{ex} 514 nm white light laser with λ_{em} 526–550 nm. Sequentially scan and identify a cell that shows both CFP and YFP fluorescence.

5.8. After confirming the fluorescence from both the proteins, switch to the FLIM console to measure the lifetime of CFP using the same settings that we used earlier for measuring the lifetime of C-CFP (step 5.5).

NOTE: This cell is also expressing M-YFP that can potentially interact with C-CFP and cause a reduction in the lifetime of C-CFP.

5.9. Fit the graph obtained using the n-exponential reconvolution model, with n = 2. A decrease in the CFP lifetime from 3.2 to 2.6 ns was observed, indicating Förster resonance energy transfer between CFP and YFP (**Figure 5A**).

5.10. Now start the FRET console in the software and calculate the FRET efficiency by manually entering unquenched donor lifetime in the equation provided in the software. And the observed FRET efficiency is: 56%.

5.11. Tripartite interaction

5.11.1. Finally, to visualize the interactions between three partners, take the leaf sample from a plant that was co-infiltrated with C-CFP, M-YFPn, and M-YFPc.

5.11.2. Scan the leaf explant for a cell that shows both CFP and reconstituted YFP fluorescence emanating from BiFC interaction between two M proteins. Use the same laser and emission wavelengths as used earlier.

5.11.3. Subsequently, switch off the 514 nm laser and move to the FLIM console.

NOTE: If the M-YFP dimer interacts with C-CFP, one should see a reduction in the lifetime of C-CFP as observed during its interaction with M-YFP. However, if the C-CFP fails to interact with the M-YFP dimer, its fluorescence lifetime should stay at 3.2 ns.

5.11.4. Using similar settings as mentioned above, measure the CFP lifetime in the presence of reconstituted YFP. Fit the graph obtained using the n-exponential reconvolution model, with n = 2, and move to the FRET console.

NOTE: There is a decline in the CFP lifetime from 3.2 to 2.3 ns. Calculate the FRET efficiency as described above. The calculated FRET efficiency is 55%. The reduction in donor lifetime and good FRET efficiency of 55% confirms tripartite interaction between two M proteins and the C protein *in vivo* (see **Figure 5B**).

REPRESENTATIVE RESULTS:

This protocol represents an optimized method to study *in vivo* tripartite protein-protein interactions in plants. The basic principle of the protocol is to combine two fluorescence-tagged protein-interaction techniques, i.e., BiFC and FRET, to create an assay to measure ternary complex formation between three protein partners. Here, we have used FLIM to measure the fluorescence lifetime of the FRET donor partner in the presence and absence of the FRET acceptor. A reduction in the fluorescence lifetime of the donor was expected if there were a positive interaction between the two proteins. We started with measuring the fluorescence lifetime of the donor, i.e., the C-CFP protein. We have used the 'All photons counting' measurement mode, which creates a fluorescence decay curve with all the photons in the region of interest (ROI) and gives a measured lifetime with a smaller error than the traditional time-correlated single-photon counting. The high-speed FLIM filter ensures the usage of single-photon events between the pulses. A suitable mathematical model was then chosen for pixel-by-pixel calculations and fitting of the decay curve[27].

The CFP variant used here (and is also the most commonly used) has conformational adaptation, which results in two fluorescence lifetimes. Therefore, we chose to fit the fluorescence decay curve using a model for the multi-exponential donor. This results in the separation of the two lifetimes of CFP, which in our case were observed as 1.0 ns and 3.3 ns. For calculating the FRET efficiency, we used the higher CFP lifetime, i.e., 3.3 ns (average fluorescence lifetime, n = 10).

In the case of positive control (C-CFP and M-YFP), we observe a reduction in the fluorescence lifetime of the donor (C-CFP) from 3.3 ns to 2.5 ns (**Figure 6**). And the FRET efficiency using the following equation was 56%.

$$E = 1 - \frac{\tau_{AvAmp}}{\tau_D}$$

E = FRET Efficiency

τ_D = Unquenched donor Lifetime (donor only sample, C-CFP)

τ_{AvAmp} = Mean Decay time – Amplitude weighted average lifetime

A similar exercise with reconstituted YFP resulting from the interaction between two M monomers and C protein also resulted in a positive interaction leading to the reduction of fluorescence lifetime of the acceptor (C-CFP) to 2.6 ns. The calculated FRET efficiency was about 55% in this case (**Figure 6**).

If a CFP variant or any other donor fluorophore has a single fluorescence lifetime, the decay curve will need fitting using the mono-exponential donor model. In that case, the efficiency of FRET can be calculated using the formula:

$$E = 1 - \frac{\tau_{Quench}}{\tau} = 1 - \frac{\tau_{donor,FRET}}{\tau_{donor,No\ FRET}}$$

τ is the fluorescence lifetime of the sample containing donor only

τ_{quench} is measured in presence of the acceptor (while FRET is taking place)

The raw fluorescence lifetime readings from at least 10 cells for each experimental setup have been collated as a scatter plot using the PlotsOfData online tool (https://huygens.science.uva.nl/PlotsOfData/, **Figure 6C**)[28]. The reduction in the fluorescence lifetime of the FRET donor provides strong evidence for a tripartite interaction between these three proteins.

FIGURE LEGENDS:

Figure 1: Schematic representation of the principle of BiFC and BiFC FRET-FLIM method. Interaction between two M proteins results in reconstitution of YFP, and this pair can then act as an acceptor molecule. The third protein C-CFP acts as a FRET donor here. Thus, a positive FRET signal proves tripartite interaction among two M proteins and one C protein.

Figure 2: Cloning of M and C genes in final destination vectors. The coding sequences of the genes are amplified and first cloned in pENTR/D-TOPO vector followed by mobilization of the constructs in final destination vectors pSITE-1CA, pSITE-3CA, pSPYNE-35S, and pSPYCE-35S by LR Clonase II recombination reaction.

Figure 3: Using electroporation, the desired vector combinations are delivered in GV3101 strain of *Agrobacterium tumefaciens.*

Figure 4: Diagrammatic illustration of agro-infiltration of Nicotiana plants and the construct combinations used for the experiment. (**A**) C-CFP containing agrobacterial culture acts as donor only sample (**B**) C-CFP and M-YFP serve as positive FRET control, (**C**) C-CFP and M protein in BiFC conformation; M-YFPn and M-YFPc are used for studying tripartite interaction.

Figure 5: Representation of fluorescence lifetimes of the samples in the absence and presence of the acceptor. (**A**) The lifetime of C-CFP in the presence of M-YFP is reduced to 2.67 ns as shown by the bar graph. Similarly (**B**) shows the reduction in lifetime of C-CFP to 2.3 ns in case of tripartite interaction.

Figure 6: Tripartite interaction between two M monomers and a C protein analyzed using BiFC based FRET-FLIM assay. (**A**) A cell showing either only CFP (i) or both CFP and YFP (ii and iii) emanating from donor and acceptor proteins using respective excitation and emission wavelengths. (**B**) i, ii, iii represents FLIM image showing fluorescence lifetime of CFP in the cell. Color scale bar represents the pseudo-color corresponding to the lifetime. Scale bar = 50 µm. (**C**) Scatter Plot showing fluorescence lifetime of CFP when present alone or with the interacting partners where n = 10. The plot is generated using the online tool PlotsOfdata (https://huygens.science.uva.nl/PlotsOfData/); mean fluorescence lifetime is represented as the line between the sample dots.

Supplementary Figure 1: Negative control experiment for the validation of interaction between proteins M and C by BiFC. Interaction between the mutated form of M Protein (MΔCBD), which lacks all C protein binding regions, and the C protein-YFPc fusion is attempted to serve as a negative control for BiFC analysis. Upper panels show transient expression of MΔCBD-GFP in onion peel cells, and the results of attempted interaction between MΔCBD-YFPn and C-YFPc are shown in the lower panels. The absence of fluorescence from reconstituted YFP is indicative of the lack of interaction between MΔCBD and C protein. N, nucleus; C, cytoplasm; BF, bright field; FL, fluorescence; OL, digital overlay, the cytoplasmic region is shown using white arrows. Scale bar = 10 µm.

Table 1: Vectors used in the study.

Table 2: Construct combinations used in the study.

DISCUSSION:

The present protocol demonstrates the use of BiFC-based FRET-FLIM assay to ascertain the formation of a ternary complex between two monomers of a MADS-box protein and a calcium sensor protein. The protocol is adapted from a report by Y. John Shyu et al. where they have developed a BiFC-based FRET method to visualize ternary complex formed between Fos-Jun heterodimers and NFAT or p65 using the sensitized emission method[7]. Earlier, a three-fluorophore FRET system was developed by Galperin and co-workers in the year 2004[29], but it requires six filters containing a sophisticated microscope setup. Also, rapid movement of organelles, such as endosomes, during image acquisition imposed experimental difficulties. On the other hand, BiFC based FRET-FLIM uses filters for only two fluorophores and has a simplified microscope setup. This setup allows direct visualization of the ternary complex, unlike the 3-FRET system. This also has an advantage over the BiFC-FRET system in being more robust and quantitative than the FRET measurements involving sensitized emission[7]. In this assay, fluorescence lifetimes of individual photons are measured for calculating the FRET efficiencies, rather than overall changes in the relative fluorescence intensities of donor and acceptor molecules. Another advantage of the FLIM-based method over the BiFC-FRET method is that only the donor's lifetime has to be measured; the qualities of fluorescence emission of the acceptor do not affect the outcome of the FRET measurements. The advantages and simplicity of this method make BiFC-based FRET-FLIM an easier, more straightforward, and more quantitative method for studying tripartite interactions. However, one needs to have access to a confocal setup with pulse lasers, fast detectors capable of single photon counting and a compatible software.

For the success of this experiment, it is important that the interaction between the two protein partners used in BiFC conformation is validated by using multiple methods and appropriate controls prior to using them as FRET acceptor. It is advisable to use negative controls such as a mutated form of interacting partner or an unrelated colocalizing protein as a negative interacting partner[16]. The negative controls for validating the BiFC interactions between M and C proteins used in this protocol included mutations in the putative binding domain of these proteins, which completely abolished the BiFC signal (**Supplementary Figure 1**). Moreover, this interaction was also validated by Y2H and FRET methods in different systems.

Also, temporal optimization of the expression of proteins in *Nicotiana* is recommended so that the adverse effects of overexpression of proteins, which may lead to false-positive results, can be minimized. It is also necessary to include the p19 strain along with the agrobacterial strains containing constructs to prevent post-transcriptional gene silencing. p19 is a suppressor of gene silencing from tomato bushy stunt virus and enhances the transformation efficiency up to 90%[30]. Recently, better versions of the fluorophores, namely, m-turquoise-SYFP2, SYFP2-mRFP, and SCFP3a-SYFP2 have been developed. These can be used as FRET pairs depending on their suitability to the target system and cell type[11]. The FRET-FLIM measurements should be performed in a minimum of ten cells to obtain statistically significant data. Wherever possible, adding a greater number of samples in the measurements will further improve the quality and reliability of the data. Depending upon the donor fluorophore, the mathematical model to fit the data with the fluorescence lifetime decay curve should be chosen.

The protocol described here is a powerful tool to determine the tripartite interactions in living cells. It helps determine the intracellular location of the interacting complex, which is important in deciphering the function and physiological significance of any protein complex. It can also resolve the interacting partner's relative concentrations based on the FRET-FLIM data, that cannot be elucidated by intensity measurement methods alone[10]. Conclusively, BiFC based FRET-FLIM assay provides an opportunity to study and characterize important aspects of tripartite protein interactions, which can be utilized in animal as well as plant systems.

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DISCLOSURE:

The authors declare no conflicts of interest.

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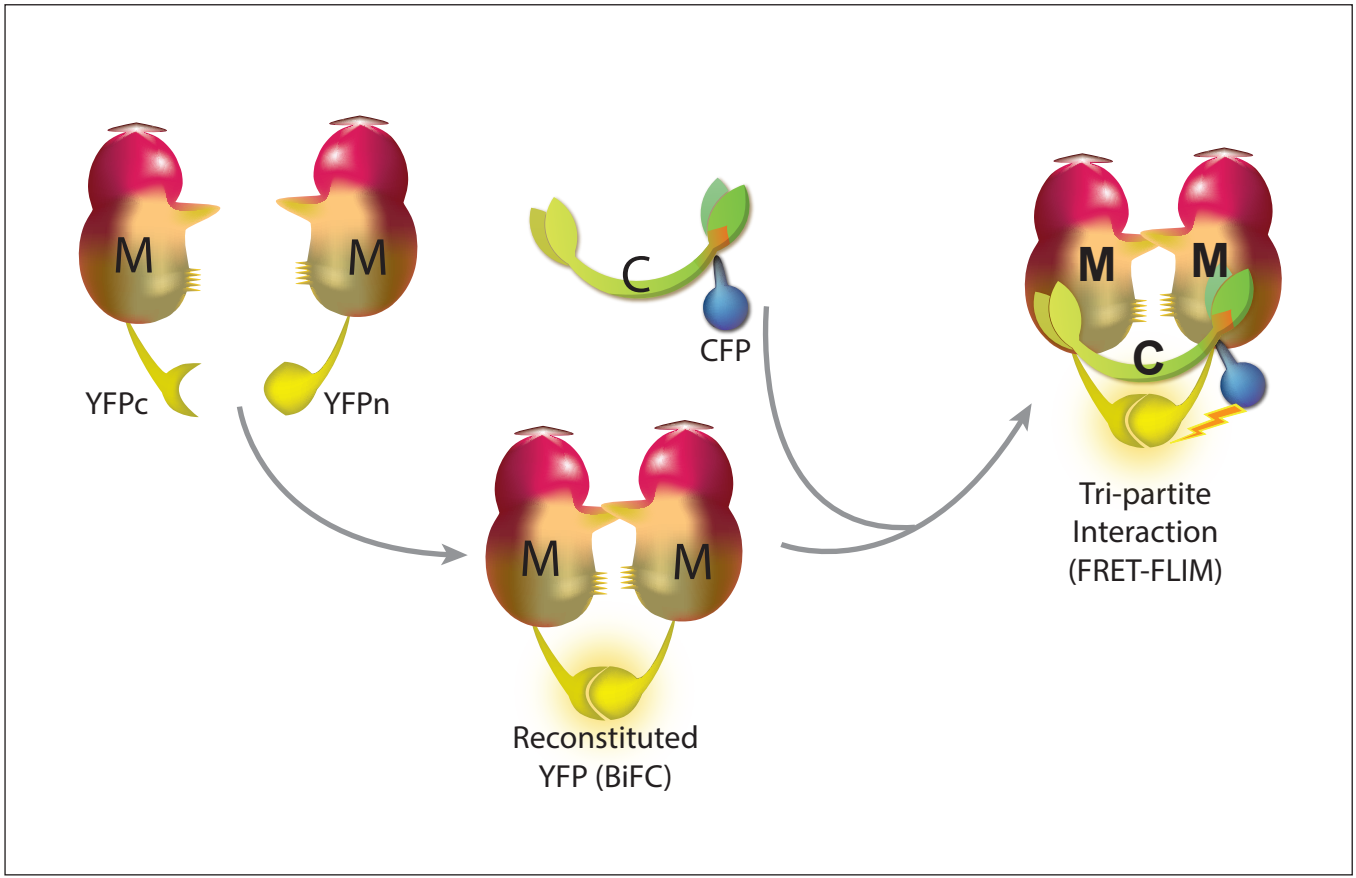
582

Figure



Figure

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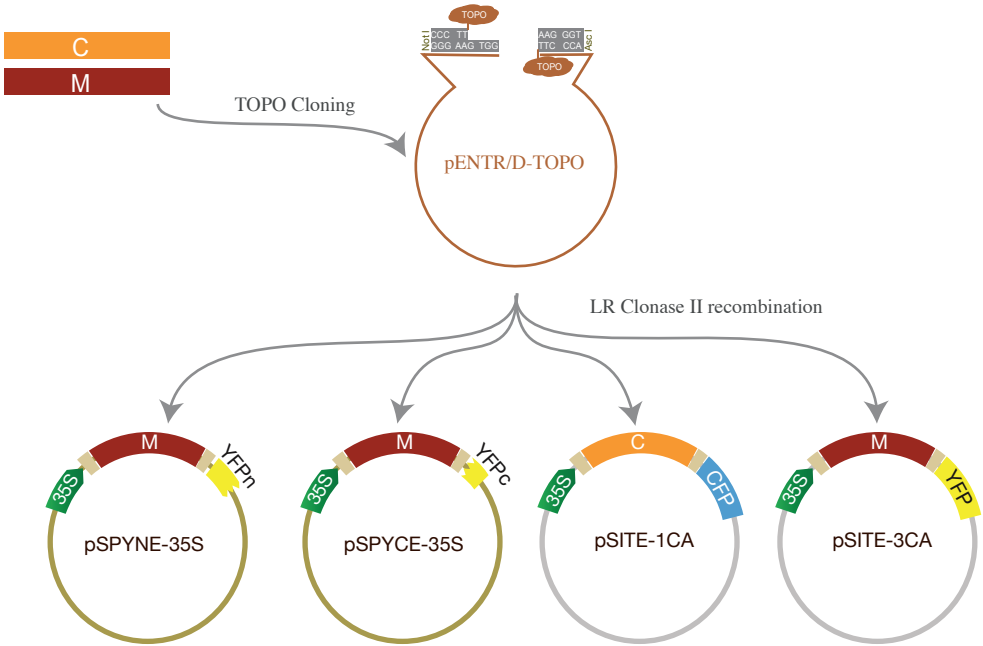

Figure

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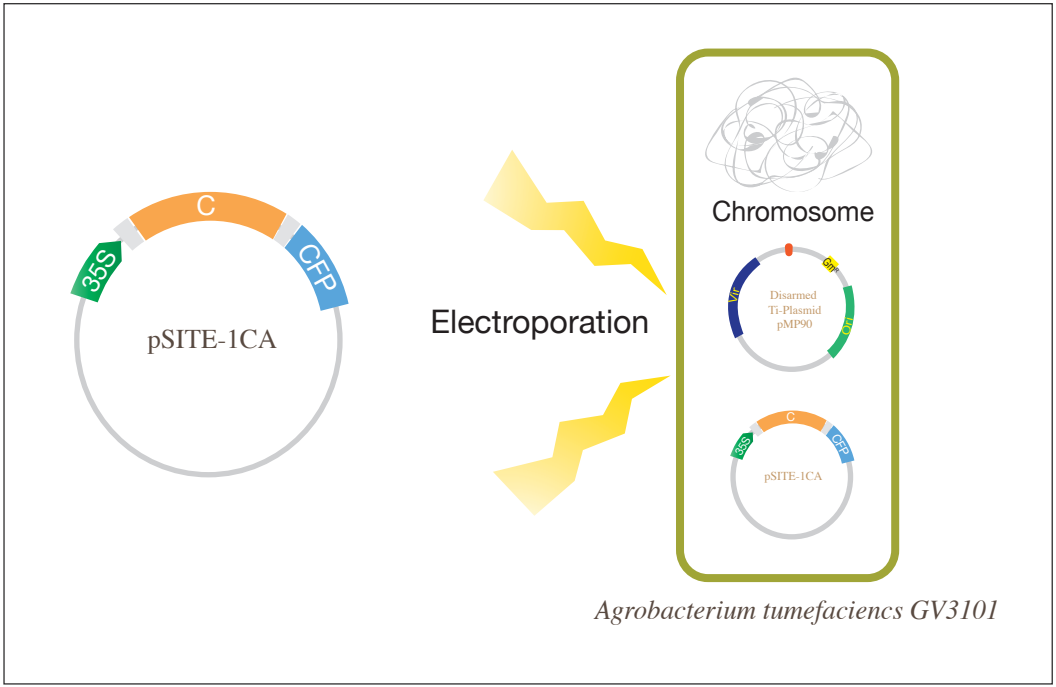


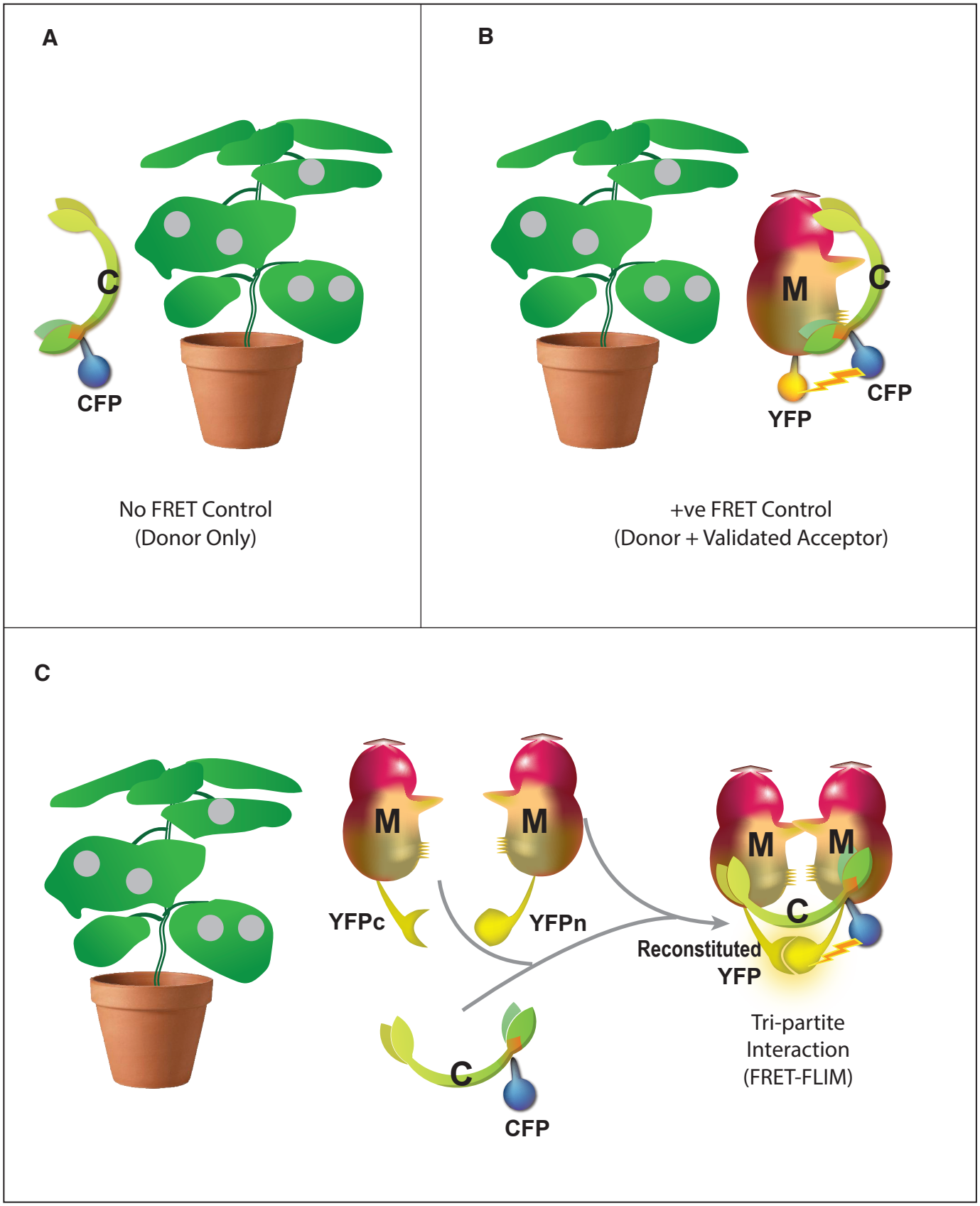
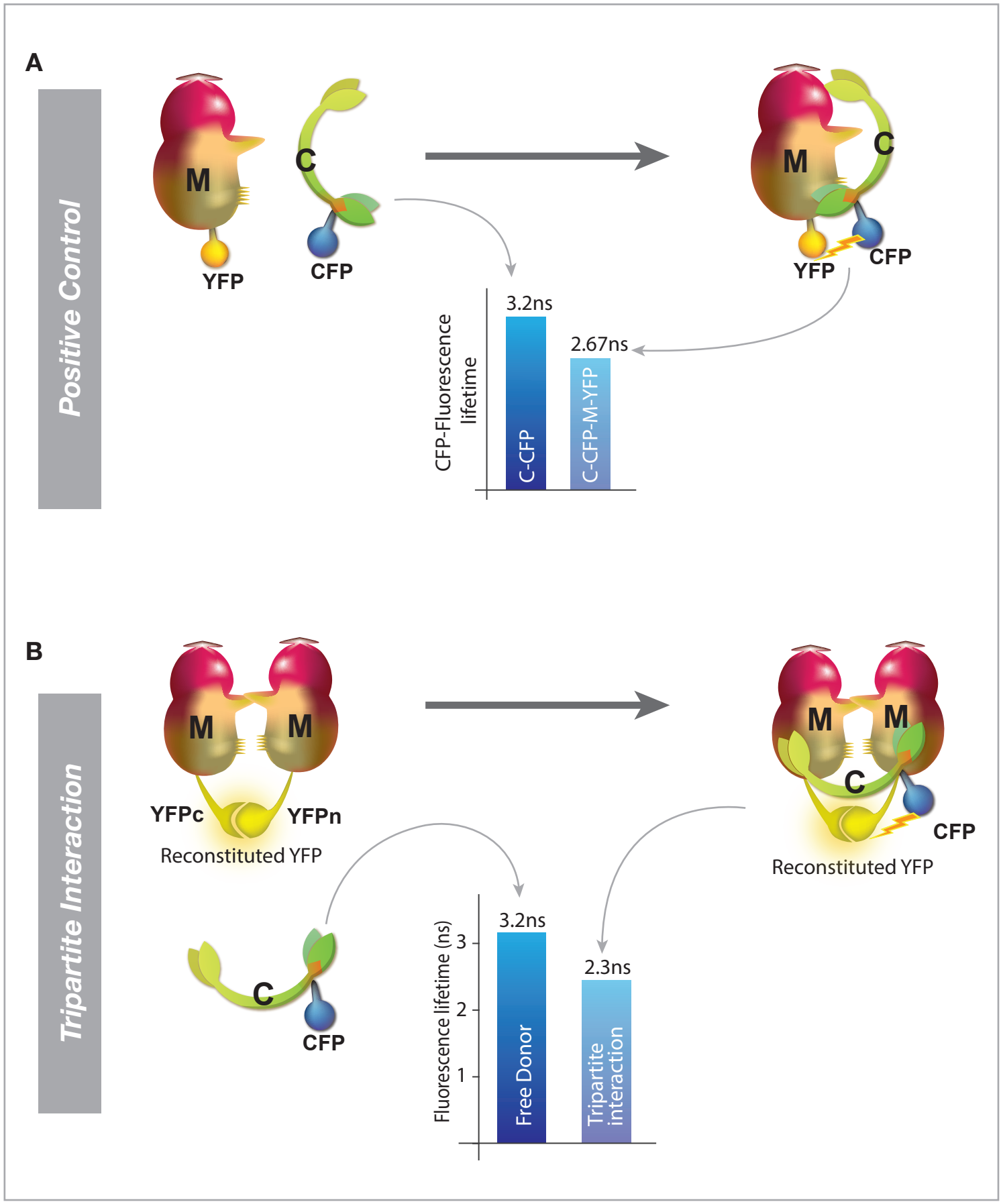


Figure 5



Figure

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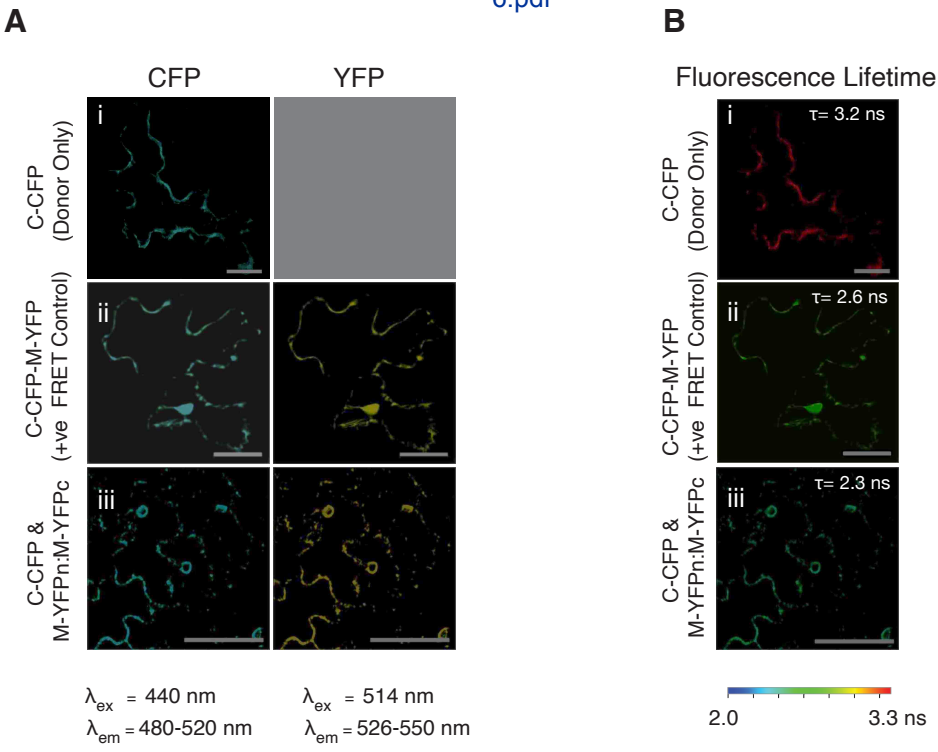

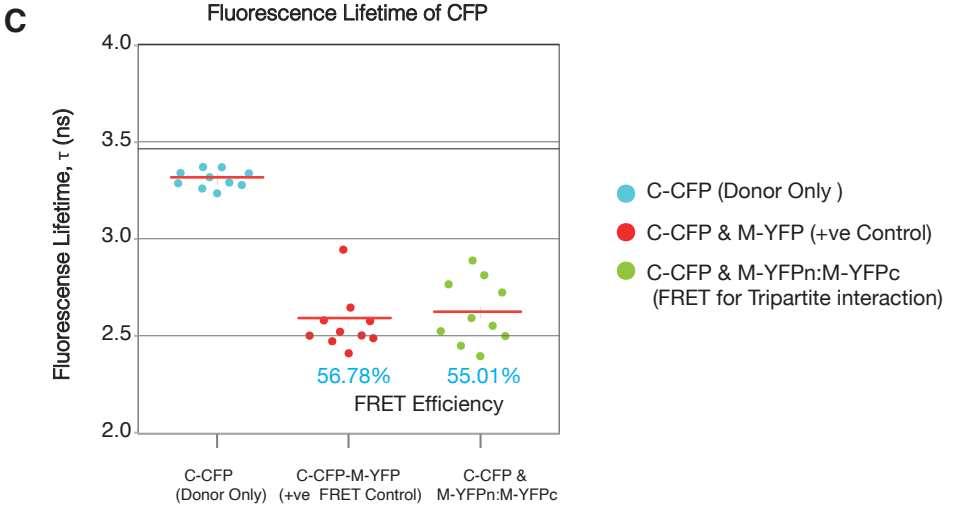

Table 1: Vectors used in the study

S.No.	Vector	Antibiotic selection
Entry Vector		
1	pENTR/D-TOPO	Kanamycin
Destination Vectors		
2	pSPYNE-35S	Kanamycin
3	pSPYCE-35S	Kanamycin
4	pSITE-1CA	Spectinomycin
5	pSITE-3CA	Spectinomycin

Table 2: Combinations of constructs used in the study

S.No.	Interaction	Combination of constructs
1	Tripartite Interaction	C-CFP + M-YFPn:M-YFPc + p19
2	Positive control	C-CFP + M-YFP + p19
3	Negative Control	C-CFP + Empty-YFP + p19
Optional (opposite conformations)		
4	Tripartite Interaction	M-CFP + M-YFPn:C-YFPc + p19
5	Positive control	M-CFP + C-YFP + p19
6	Negative Control 1	M-CFP + Empty-YFP + p19
7	Negative Control 2	Empty -CFP + C-YFP + p19
8	Negative Control 3	Empty -CFP + M-YFP + p19

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Table of Materials

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Dear Dr. Bajaj,

We like to thank the editorial team and the reviewers for their comments, suggestions and patience.

We have tried our best to modify the manuscript and the video in accordance with the suggestions and comments.

Following are our answers to the comments, suggestions and queries made by the production team and the reviewers in a pointwise manner.

We now hope that the modified version of the manuscript and the video will be acceptable in its present form.

Editorial and production comments:
Text:
1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Response: We have retained the style and format.

2. Please address all the specific comments marked in the manuscript.

Response: We have replied to all the comments mentioned in the document.

3. Please make the protocol text more homogenous to the video narration. However, please remember that there can be steps in the protocol text which are not presented/described in the video.

Response: We have tried to make the text more homogenous to the video narration.

4. Please address all the reviewers comments as well.

Response: We have responded to all the comments and suggestions of the reviewers below.

Video:
1. Please reduce the duration of the video below 15 mins by cutting unnecessary content or video clips. It seems the video has a lot of interview statements which can remove and only important interview statements can be used.

Response: We have reduced the duration to around 14:31 minutes.

2. Please change the representative result and data analysis title to Representative result only.

Response: The title has been changed to Representative result.

Please do not include interviews in this section. This section should show all the representative results figures.

Response: The interview videos have been removed from representative results and rest have been reduced considerably.

3. All interview statements should be confined to the introduction and conclusion section only.

Response: We have kept the interview videos in introduction and conclusion mainly but a small part is there in protocol also.

4. Please change the discussion and conclusion section title to Conclusion only.

Response: We have made the changes.

5. Title Cards:
• 12:20 Please maintain the same style of the chapter title card.

Response: We have changed the style to match the rest of the video.

6. Audio Editing and Pacing:
• Audio levels are a little high. Please decrease the audio level and ensure audio level peaks average around -9 dB.

Response: The MacOS iMovie's software doesn't allow us to set average decibel levels. It has a 0-100 scale for audio clips. In the earlier version, the audio level was at 60, which we have now reduced to 45. In case this is also higher please arrange for an online meeting and let us know how can we take care of this problem.

7. Video Editing Content:
• 02:24 Please use the Cross dissolve properly. There seem to be a glitch between three shots

Response: We have made this change.

• 05:46 Please remove the animation on the title card.

Response: The animation has been removed.

• Please use cross dissolve on hard cuts to make the video look smooth.

Response: We have used cross dissolve in all video transitions now.

Once done please ensure that the video is no more than 15 min in length. Please upload the video at: https://www.dropbox.com/request/c1L7FPz2p9nLA43YsQ34?oref=e

Reviewers' comments:
Reviewer #1:
Concerns:

1. The authors have responded to most of my concerns, except for two. First, the obvious overexpression of their proteins (Points 7 and 8 in the responses).
   Fig 6A is still the image showing extreme overexpression and mislocalization (e.g. the ER) of the proteins. The authors have now added a timeframe for their experiment, which is a 24-hr induction of protein expression, 3 days post infiltration. In my experience, this long timeframe, especially the 24 hrs induction for a transcription factor, will indeed lead to severe overexpression, as is visible in the image.
   The authors address my criticism, that such overexpression will ALWAYS lead to positive interactions in a BiFC-assay, by reiterating that they have confirmed the interaction via yeast-two-hybrid assay, and that the positive interaction is therefore not an artifact caused by overexpression. This may well be true, and since the authors do not reveal the identity of their proteins, there is no way to confirm this. But this is beside the point I was trying to make: This protocol is supposed to show users how the experiment is properly performed. And as it is right now, it is teaching potential users to massively overexpress their proteins. And especially for a BiFC assay, this is a big problem. So even is the interactions shown here is indeed unaffected by the severe overexpression, it is still sending the wrong message to potential users.

   Response: We have removed the 24 hrs time frame and replaced it with a more flexible ("at different time points") expression. We have also added a cautionary note (see below and section 3.15 in the revised manuscript; modified regions are highlighted).

   However, in our experience, the three-day incubation window works the best because at earlier time points there is a lot of autofluorescence (probably because of cell damage), and fluorescence from both CFP and YFP can be visualized from a very few numbers of cells. A similar incubation timeframe has also been suggested for tobacco and Arabidopsis agroinfiltration protocols by Goto-Yamada et al. (2018; DOI: 10.1007/978-1-4939-7871-7_16) and Zhang et al. (2020; DOI: 10.1016/j.xplc.2020.100028), respectively. In both these papers (like in many others) a 2-5 day post-agroinfiltration incubation time period has been recommended.

3.15 Check a small part of the agro-infiltrated leaf at different time points using a fluorescence microscope. When the fluorescence from both YFP and CFP are detectable in cells, proceed to the confocal microscope for BiFC-FRET FLIM assay. In our case, this analysis was carried out three days after agro-infiltration.

NOTE: Set the post-agroinfiltration period of incubation individually for every promoter and gene combination to avoid overexpression of chimeric proteins used in the BiFC-FRET FLIM assay. The overexpression of the partner proteins may lead to false positive interactions.

2. The second point concerns the negative control for BiFC. A good negative control is essential for a BiFC experiment, which I have criticized as lacking (Point 9). The authors have now included a proper negative control, but the data is only in an image for me (image 1B for reviewer), while the text says 'Data not shown' (line 350). Given how essential this negative control is for BiFC, I strongly believe that this should be part of the protocol.

   Response: The image 1B (for reviewers) has now been added to the manuscript as Supplementary Figure 1. Please see line # 427-435 for Figure legend. The 'Data not shown' (Line #471) has been changed to 'Supplementary Figure 1.'

3. appreciate the use of a scatter plot.

   Response: We thank the reviewers for this suggestion. It is a helpful tool, and we have started to use it in other studies also.

4. Minor point: Reference 20 (Voinnet et al. 2003) is a retracted paper. I see that this change was requested by another reviewer, but a retracted paper must not be cited. An alternative would be http://dx.doi.org/10.1046/j.1365-313X.2003.01803.x which is one of the earliest uses of p19 as silencing suppressor.

   We thank the reviewer for his suggestion. In fact we were also hesitating in citing the retracted paper. Reference 20 has been replaced with the suggested paper by Van Der Hoorn et al. (2003)

Reviewer #3:

Major Concerns:

1. The helper plasmid for the Agrobacterium strain is missing. GV3101 is the chromosomal markers, but you still need a helper plasmid. This is a crucial error that should be addressed. If you have not been selecting for the helper plasmid then you might have been lucky as they are not thrown out soo fast, but it still happens.

Response: We are sorry to have missed out on this correction although we mentioned it in our response. The following has now been added at line #115 "The GV3101 strain has a disarmed Ti plasmid pMP90 (pTiC58DT-DNA) with gentamicin resistance."

2. YFP "halves" is incorrect - the YFP is split non-symmetrically.

   Response: YFP "halves" on pages 2 and 3 have now been changed to "parts". See Line # 83 & 109.

Minor Concerns:
Here are my comments/notes on the PDF:

3. Page: 3: Author: reviewer Subject: Highlight "halves" Date: 13-Aug-21 18:24:18. parts- the section in non-symmetrical !!!

   Response: YFP "halves" on pages 2 and 3 have now been changed to "parts". See Line # 83 & 109.

4. Page: 4: Author: reviewer Subject: Highlight Date: 13-Aug-21 18:25:07
   small q, (QY) can be used, if needed later.
   Author: reviewer Subject: Highlight Date: 15-Aug-21 08:38:33
   Author: reviewer Subject: Highlight Date: 15-Aug-21 08:42:28
   Helper plasmid is missing...
   pMP90 (GentR)? pMP90RK (GentR & KanR)? other.....?
   the binaries are not mobilized without a helper plasmid ..

   Response: 'Quantum yield' in line # 93 has been changed to 'quantum yield (QY)'

   Step 1.4: Line # 147: (pMP90 (Gent^{R})) has been added

5. Page: 5: Author: reviewer Subject: Highlight Date: 15-Aug-21 08:41:10
   write out - what is RO water?

   Response (Step 2.2; Line # 158): 'RO' has been replaced with 'deionized', as it is essentially tap water filtered by using a combination of sedimentation and reverse osmosis (RO) filters.

6. Page 5: Author: reviewer Subject: Highlight Date: 15-Aug-21 08:43:13
   line 3.8 is not clear. correct.

   Response: Step 3.8 has been changed to "3.8. Mix the cultures of appropriate BiFC and FRET partner containing strains so that the final O.D. of each culture is 0.5 and that of p19 is 0.3 in a total volume of 2 mL."

7. Page: 6: Author: reviewer Subject: Highlight Date: 15-Aug-21 08:45:04
specify the exact model and make here too, not just in the video - line 187: Make of specific microscope needs to be in title of step 5.

Response: JOVE guidelines do not allow the use of any commercial term. Therefore, make of the microscope etc. have not been added. However, these details are listed in "Table of materials".

8. Page: 8
Author: reviewer Subject: Highlight Date: 15-Aug-21 08:53:18
extra space after URL

Response (Line # 389): The extra space after the URL has been removed. The line now reads as: "(https://huygens.science.uva.nl/PlotsOfData/; **Figure 6C**)[27]"

9. Page: 10
Author: reviewer Subject: Highlight Date: 15-Aug-21 08:53:58
remember: helper plasmid is missing.

Response: The name and a diagrammatic representation of the helper plasmid has been added in the figure.

10. Page: 11
Author: reviewer Subject: Highlight Date: 15-Aug-21 08:55:15
line 350: uppercase not needed in (Data not shown)

Response: (Data not shown) has been changed to (Supplementary Figure 1). And the data is shown therein.

11. Page: 15
Author: reviewer Subject: Sticky Note Date: 15-Aug-21 09:00:32
keep in mind that the YFPc is the last 4 b-strands of 13 aa each, and the YFPn is the rest of the protein.
I also kept saying halves for a long time...but that is inaccurate...the YFPn is a larger tag!

Response (Figure 2): As we could not see the original 'sticky notes' on the PDF, we assume that these comments were for Figure 1 or 2. In Figure 1, the YFPn is already shown as slightly larger than YFPc. In Figure 2 (Lower panel), YFPc now has been made smaller than the YFPn.

12. Page: 17
Author: reviewer Subject: Sticky Note Date: 15-Aug-21 09:01:54
Helper plasmid is missing.
What is going to mobilize your T-DNA into the plant cell? The majority of those are on the helper plasmid in GV3101.

Response: A representation of the helper plasmid pMP90 has been added in Figure 3.

13. Page: 18
    Author: reviewer Subject: Sticky Note Date: 15-Aug-21 09:02:46
    you could add an arrow or zappy thing that shows that energy moves from the CFP to the YFP...
    or a glow maybe.... as an idea.

    Response: Figure 4. Necessary changes have been made in the figure 1 and figure 4
    Thank you for the comments.

14. Page: 19
    Author: reviewer Subject: Sticky Note Date: 15-Aug-21 09:05:56
    less confusing is the M on your blobby protein and the YFP next to the YFP... same thing for the CFP.
    also the graph should be blue, it is a reduction in the lifetime of the CFP and not the YFP (as well as the brightness of CFP goes down and YFP up - which you could also use even if that is not measured here).
    Author: reviewer Subject: Sticky Note Date: 15-Aug-21 09:06:41
    see comments about colors on the previous side....
    CFP is reduced - should be blue..and then fainter blue.

    Response: Thank you for these comments. The figures have been suitably modified.

Supplemental File 1

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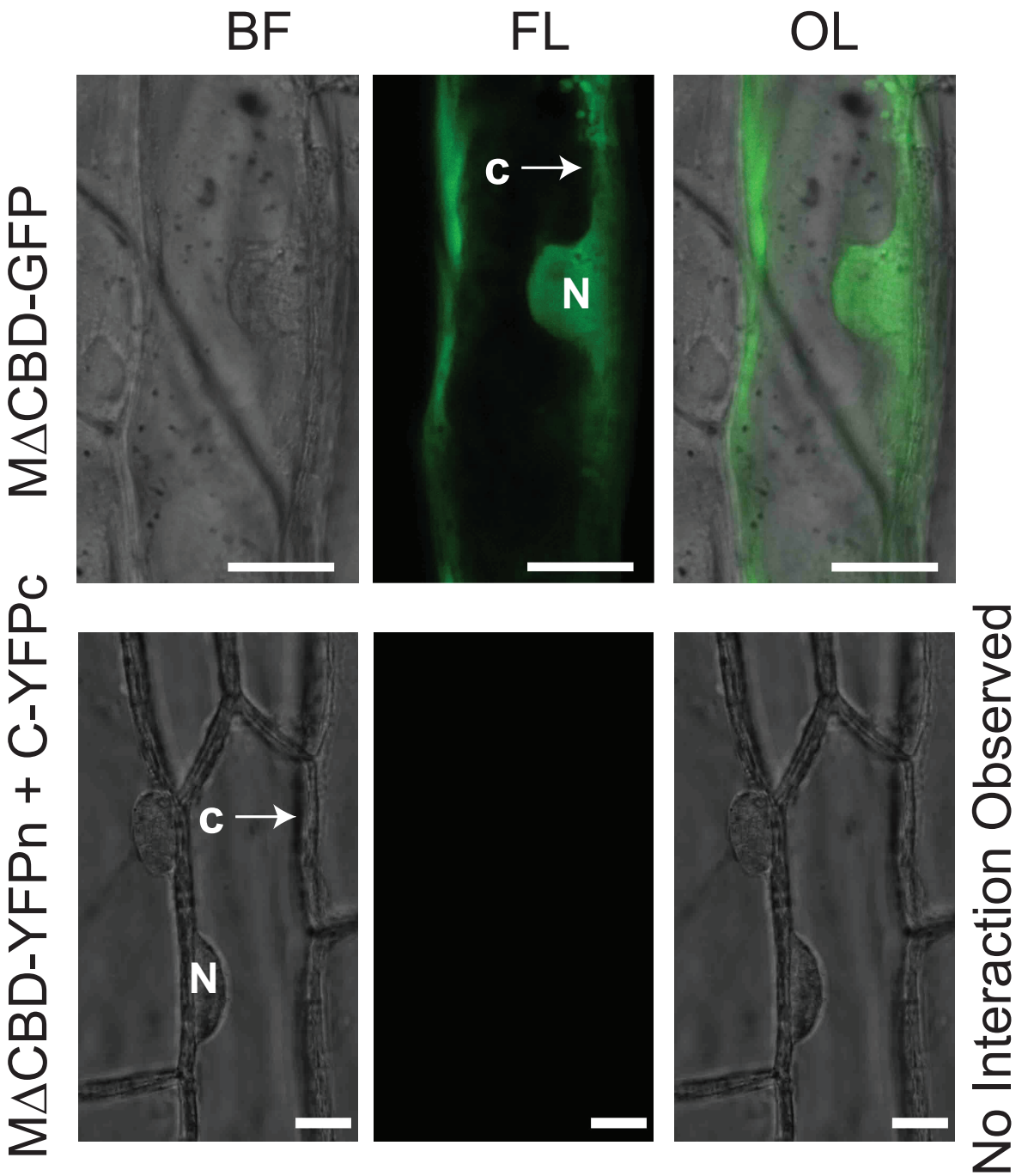
Supplementary figure 1



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Author(s):	Neelima Boora*, Vibha Verma*, Ridhi Khurana, Gautam Gawande, Sanchi Bhimrajka, Komal Chaprana, Meenu Kapoor and Sanjay Kapoor**

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