Video Article

Bimolecular Fluorescence Complementation

Katy A. Wong[1], John P. O'Bryan[1]

[1]Department of Pharmacology, University of Illinois at Chicago

Correspondence to: John P. O'Bryan at obryanj@uic.edu

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Abstract

Defining the subcellular distribution of signaling complexes is imperative to understanding the output from that complex. Conventional methods such as immunoprecipitation do not provide information on the spatial localization of complexes. In contrast, BiFC monitors the interaction and subcellular compartmentalization of protein complexes. In this method, a fluororescent protein is split into amino- and carboxy-terminal non-fluorescent fragments which are then fused to two proteins of interest. Interaction of the proteins results in reconstitution of the fluorophore (Figure 1)[1,2]. A limitation of BiFC is that once the fragmented fluorophore is reconstituted the complex is irreversible[3]. This limitation is advantageous in detecting transient or weak interactions, but precludes a kinetic analysis of complex dynamics. An additional caveat is that the reconstituted flourophore requires 30min to mature and fluoresce, again precluding the observation of real time interactions[4]. BiFC is a specific example of the protein fragment complementation assay (PCA) which employs reporter proteins such as green fluorescent protein variants (BiFC), dihydrofolate reductase, b-lactamase, and luciferase to measure protein:protein interactions[5,6]. Alternative methods to study protein:protein interactions in cells include fluorescence co-localization and Förster resonance energy transfer (FRET)[7]. For co-localization, two proteins are individually tagged either directly with a fluorophore or by indirect immunofluorescence. However, this approach leads to high background of non-interacting proteins making it difficult to interpret co-localization data. In addition, due to the limits of resolution of confocal microscopy, two proteins may appear co-localized without necessarily interacting. With BiFC, fluorescence is only observed when the two proteins of interest interact. FRET is another excellent method for studying protein:protein interactions, but can be technically challenging. FRET experiments require the donor and acceptor to be of similar brightness and stoichiometry in the cell. In addition, one must account for bleed through of the donor into the acceptor channel and vice versa. Unlike FRET, BiFC has little background fluorescence, little post processing of image data, does not require high overexpression, and can detect weak or transient interactions. Bioluminescence resonance energy transfer (BRET) is a method similar to FRET except the donor is an enzyme (e.g. luciferase) that catalyzes a substrate to become bioluminescent thereby exciting an acceptor. BRET lacks the technical problems of bleed through and high background fluorescence but lacks the ability to provide spatial information due to the lack of substrate localization to specific compartments[8]. Overall, BiFC is an excellent method for visualizing subcellular localization of protein complexes to gain insight into compartmentalized signaling.

Video Link

The video component of this article can be found at https://www.jove.com/video/2643/

Protocol

A. BiFC Calibration

1. **Choose a fluorophore.** There are multiple fluorophores, such as YFP and Venus, that work well as BiFC fusion partners (Table 1). Amino- and carboxy-terminal ends of Venus are able to form a complex at 37°C, while the YFP BiFC fragments require a pre-incubation at 30°C in order to facilitate fluorophore formation[2]. This incubation at a low temperature may alter some cellular processes and should be taken into account when choosing fragments. Vectors for fusing Venus to the carboxy-terminus of proteins are available from Addgene (http://www.addgene.org/pgvec1; seepBiFC-VN173 and pBiFC-VC155) along with additional constructs for use as controls, e.g., pBiFC-bJunVN173 and pBiFC-bFosVC155[2]. Additional vectors, including amino-terminal Venus vectors (pFLAG-VN173 and pHA-VC155), are available at the following site: http://people.pnhs.purdue.edu/~hu1/.
2. **Tag the protein of interest.** The BiFC fragments are fused to the amino- or carboxy- terminal ends of the candidate proteins. Some proteins may not allow for tagging at either end due to disruption of protein function. For example, many members of the Ras superfamily of GTPases are lipid modified at the carboxy-terminus thus precluding attachment of the BiFC fragments at that end. Thus, it is important to have some idea of how attachment of the BiFC fragments may affect function of the proteins of interest. If it is unclear how tagging a protein will affect its function multiple combinations should be tested. In addition to the BiFC fragment, a peptide linker may be included to increase the flexibility between the fragmented fluorophore and the candidate proteins. While the multiple cloning sites (MCS) in the BiFC vectors encode short amino acid stretches that may provide sufficient flexibility, the RSIAT, KQKVMNH, and RPACKIPNDLKQKVMNH linkers have been successfully used in BiFC experiments[3,9]
3. **Determine transfection conditions.** Before testing multiple mutants, a few control experiments should be performed. The first two BiFC combinations that should be tried are two wild type proteins that are known to interact and one wild type and mutant that do not interact. Using these two combinations, different amounts of DNA and transfection times should be tested to determine optimum conditions for

detecting a BiFC signal for the candidate proteins. We suggest testing 0.25ug, 0.5ug, 1.0ug of each BiFC construct for a single well of a 6 well dish. The day after transfection monitor cells by fluorescence microscopy to determine optimum time for signal development. The pBiFC-bJunVN173and pBiFC-bFosVC155[2]constructs are useful positive controls for BiFC and are available from Addgene (see above). In addition, Western blot analysis should be performed to confirm equal expression of the constructs. Conditions should be chosen such that a fluorescent signal is observed between the two wild type proteins but little to no signal is observed between the wild type and mutant proteins. Finally, it is best to keep protein expression as low as possible to prevent any non-specific interactions.

4. **Determine if the addition of the BiFC fragments alters the localization of the proteins of interest.** Each BiFC construct contains either an HA or FLAG epitope tag. Immunostain transefected cells for both the HA or FLAG epitope tag as well as well the endogenous protein (if possible) to determine if the BiFC tag affects localization of the proteins of interest.

B. Plating and Transfection of Cells

1. COS cells ($1.3x10^5$) are plated each onto one glass bottom Matek plate and two wells of a 6-well plate per sample. Allow cells to settle overnight at 37°C. Alternative cell types that are more relevant to the candidate proteins of interest may also be used.
2. Prepare DNAs for transfection. We typically utilize Lipofectamine (Invitrogen) for COS transfections. However, other reagents may be more appropriate for the cell line of interest. Since the transfection mixture will be split between one glass bottom dish and two wells of a 6-well plate, use the appropriate amount of DNA to account for this division. Dilute DNA in 250uL of serum free (SF) DMEM. Add CFP at 1/5 the amount of total BiFC DNAs as a transfection control. For BiFC quantification, only cells which are positive for CFP will be analyzed for the presence of a BiFC signal. Note that the CFP spectra will overlap with some of the BiFC pairs in Table 1; therefore an alternate transfection control maybe needed. Dilute Lipofectamine in 250uL SF DMEM (10uL Lipofectamine/1ug of DNA). Mix the DNA and Lipofectamine dilutions. Incubate at room temperature for 20min.
   Note: The Lipofectamine:DNA ratio can vary depending on cell type. Use the appropriate transfection method for the cell line of interest.
3. Rinse cells 2x with warm SF DMEM. Add 2mL of SF DMEM to each glass bottom plate or well of a 6-well dish.
4. Split each transfection mixture evenly between one glass bottom dish and two wells of a 6-well dish.
5. Incubate cells at 37°C for 5hrs.
6. Remove transfection media and replace with complete media (DMEM+10%FBS).
7. Incubate cells overnight at 37°C. The length of incubation following transfection will vary depending on the expression levels of the proteins of interest. Prolonged incubation may result in non-specific interaction so this step will need to be determined empirically.

C. Preparation of Cells for Imaging

1. Cells are initially examined under an epifluorescent microscope to ensure that the positive control is fluorescent. If not, it may be necessary to allow cells additional time at 37°C until signal is observed.
2. Rinse cells 3x with PBS (pH 7.4). To the cells in the glass bottom dish add 2%paraformaldehyde (pH 7.4). Fix cells for 10min on ice. Rinse cells 3x with PBS (pH 7.4). Store cells at 4°C covered with 1mL PBS (pH 7.4). Cells do not have to be fixed for imaging, but once the BiFC fragments reform an intact fluorophore it is irreversible thus preventing analysis of dynamic interactions[3]. Also, keep in mind that unfixed cells will continue to develop signal. Lyse the cells in the 6-well dish and prepare lysates for Western blot analysis. It is important that all cells are prepared at the same time so that the lysed cells are representative of the imaged cells.

D. Imaging Cells

1. The fluorescence intensity will be calculated per cell. Be sure to image individual cells.
2. CFP was included as a tranfection control and only CFP positive cells are selected for analysis of BiFC signals. We make the assumption that if the cell is transfected with CFP, it is also transfected with the BiFC constructs. This approach ensures that imaged cells which lack a BiFC signal are negative due to a lack of an interaction between the proteins of interest and not due to absence of one or both BiFC expression constructs in that cell.
3. We use a Zeiss LSM 510 confocal microscope for cell imaging. When using this microscope it is important to keep the zoom, pinhole, detector gain, amplifier offset, frame size, scan speed, scan average, and laser power consistent. When using any imaging system it is important to keep settings constant so that the fluorescence is comparable between samples. Also, when quantifying fluorescence it is important that pixels are not saturated.

E. Quantifying Fluorescence

1. Fluorescence may be quantified using any imaging software. We utilize ImageJ which is freely available from the NIH (http://rsb.info.nih.gov/ij/). Open image files in ImageJ. Go to AnalyzeSet Measurements. Check the boxes for Area and Mean Gray Value in the Measurements box.
2. Using the 'free hand selection' tool, draw an outline around the edge of the entire cell in the CFP channel.
3. Leaving this outline in place, shift to the YFP channel. Go to AnalyzeMeasure.
   The Mean Gray Value is the sum of the grey values of all the pixels in the selection divided by the number of pixel (i.e., the average fluorescence intensity per area of the cell).
4. For each image draw a circle in the YFP channel in an area that does not contain a cell. Take a measurement for this area as background. Subtract the background from each image.
5. Average the Mean Gray Value minus the background for all the cells imaged in one sample. This will be the average fluorescence intensity for a population of cells. We would suggest about 60 cells over three experiments be quantified.

F. Representative Results:

Our lab focuses on the multi-domain scaffolding protein, intersectin (ITSN) which interacts with numerous proteins to regulate multiple biochemical and signaling pathways[10,11,12,13,14]. ITSN contains two Eps15 homology (EH) domains, a coiled-coiled region, and five Src homology 3 (SH3) domains. The longer isoform of ITSN also contains Dbl homology (DH) and pleckstrin homology (PH) domains that act in concert as a guanine nucleotide exchange factor for Cdc42[15]. This modular structure promotes protein:protein interactions and makes ITSN an ideal candidate for BiFC experiments. The subcellular localization of ITSN can alter its binding partners and therefore alter the pathways regulated by ITSN (unpublished data). Recently, our lab has demonstrated that ITSN regulates neuronal survival through regulation of a novel class II PI3K, PI3K-C2β [11]. The amino-terminal Pro-rich domain of PI3K-C2β contains two binding sites for ITSN's SH3 domains. Using co-immunoprecipitation with ITSN and PI3K-C2β truncation mutants we demonstrated that ITSN's SH3A and SH3C domains interact with the amino-terminal region of PI3K-C2β. Next, we used BiFC to visualize the subcellular localization of this complex. ITSN was fused to the amino-terminus of Venus (pFLAG-VN173) and PI3K-C2β constructs fused to the carboxy-terminus of Venus (pHA-VC155). As another negative control, a non-specific peptide was fused to pHA-VC155. VN-ITSN and VC-PI3K-C2β formed a BiFC complex with a punctuate distribution (Figure 2A, upper panels). Mutations in the Pro-rich domain of PI3K-C2β that disrupt co-precipitation of ITSN and PI3K-C2β decreased the BiFC signal (Figure 2A, lower panels)[11]. This difference in BiFC signal between ITSN and the two PI3K-C2β proteins was not due to differences in protein expression (Figure 2B).

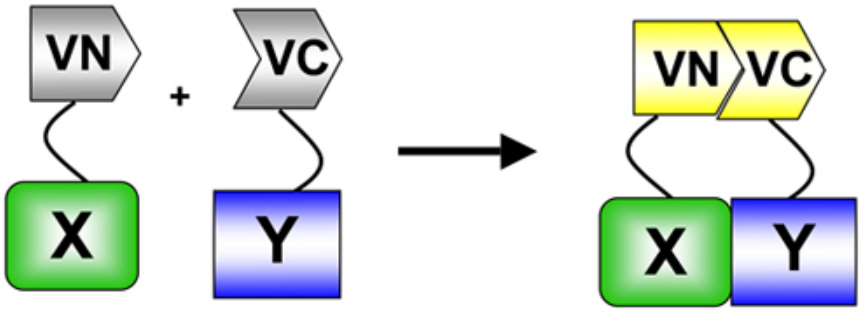

Figure 1. In BiFC, a fluorophore (in this case Venus) is split into amino(VN)- and carboxy(VC)-terminal ends. These ends are fused to two proteins of interest. When the two proteins interact, the VN and VC fragments re-associate resulting in reconstitution of the fluorophore and fluorescence at the sites of interaction. BiFC is a specific example of the protein fragment complementation assay (PCA) used to measure protein:protein interactions[5].

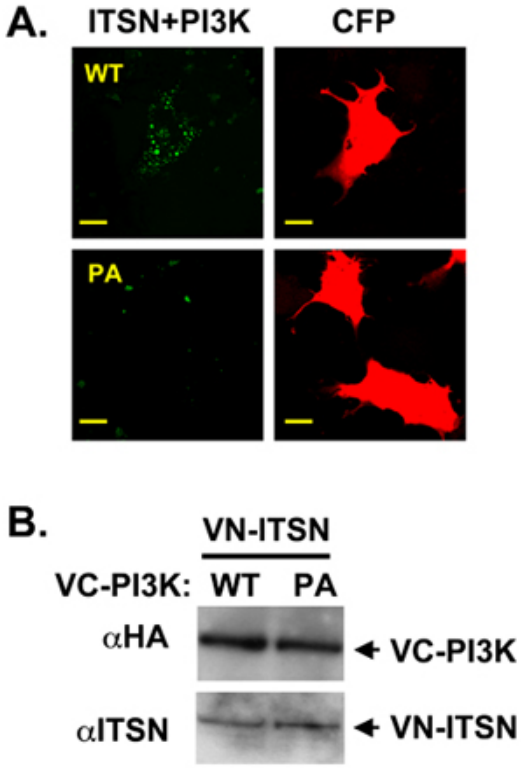

Figure 2. ITSN and PI3K-C2β form a BiFC complex. A. VN-tagged ITSN was co-tranfected with VC-tagged PI3K-C3β WT or a proline-rich domain mutant (PI3K-C2β-PA). ITSN and WT PI3K-C2β form a complex (green). CFP (red) was used as a transfection control B. A Western blot was performed to demonstrate equal expression of the constructs. The VC-tagged constructs are HA tagged.

BiFC Fragments	Excitation	Emission
YN155/YC155	515 nm	527 nm
YN173/YC173	515 nm	527 nm
YN155/CC155	503 nm	515 nm
YN173/CC155	503 nm	515 nm
GN173/YC173	513 nm	521 nm
GN173/CC155	488 nm	512 nm
CN173/YC173	466 nm	497 nm
CN155/CC155	452 nm	478 nm
CN173/CC155	452 nm	478 nm
VN155/VC155	515 nm	528 nm
VN173/VC173	515 nm	528 nm
VN173/VC155	516 nm	529 nm
CitN155/CitC155	515 nm	528 nm
CitN173/CitC173	516 nm	529 nm
CitN173/CitC155	516 nm	529 nm
CerN173/VC155	456 nm	478 nm
CerN173/CC155	439 nm	479 nm
VN173/CC155	504 nm	513 nm
CitN173/YC155	517 nm	527 nm
CitN173/VC155	516 nm	528 nm

Table 1. There are multiple vectors that are compatible with BiFC. YN155: 1-155aa of YFP; YC155: 155-238aa of YFP; YN173: 1-172aa of YFP; YC173, 173-238aa of YFP; VN155: 1-154aa of Venus; VC155: 155-238 of Venus; VN173: 1-172aa of Venus; VC173: 173-238aa of Venus; CN155 1-154aa of CFP; CC155 155-238aa of CFP, GN173: 1-172aa of GFP, CitN155: 1-155aa of Citrine, CitC155: 155-238aa of Citrine, CitN173: 1-172aa of Citrine, CitC173: 173-238aa of Citrine, CerN173: 1-172aa of Cerulean [2,3,9].

Discussion

BiFC is an excellent method for visualizing protein:protein interactions in whole cells and determining the subcellular localization of these complexes. The advantages of BiFC are that only interacting proteins are fluorescent, transient interactions are stabilized, and post-processing of the imaging data is minimal. Two disadvantages of this method are the maturation time for the fluorophore and the irreversibility of fluorophore complex. Under some applications this irreversibility can be used as an advantage. For example, one may use an enzyme and an activator of that enzyme in BiFC complex, resulting in constitutive activation. One could then determine what proteins are recruited to this complex. Furthermore, the irreversibility of the BiFC complex allows for the identification of weak or transient interactions. Proper controls are important for interpretation of BiFC experiments. Empty BiFC vectors should not be used as controls as these reagents result in high background fluorescence. Proper controls include using a mutation in one of the proteins of interest that is known to block the interaction of the two proteins. An unrelated protein that should not complex with the protein of interest may also be used. It is also important that Western blot analysis is performed to ensure that expression of the different constructs is equal and that differences in fluorescent signal are not due to varying levels of protein expression. High expression can also lead to non-specific interactions and fluorescence; therefore expression levels should be kept relatively low.

BiFC is a practical method that is readily combined with other fluorescent techniques. For example, BiFC may be combined with standard immunofluorescent co-localization to examine trimolecular complexes although this approach is still subject to the resolution limits of the confocal microscope. Recent studies have combined BiFC with FRET to allow for examination of trimolecular complexes of transcription factors[16]. For this application, a CFP tagged protein is used as the donor and the BiFC complex is used as the acceptor (i.e. YFP, Venus). This approach allows for the temporal monitoring of a protein complex and a third interacting protein of interest. These combinations of techniques allow for great versatility in the application of BiFC technology to determine protein:protein interactions. Defining the localization of protein complexes in the cell is essential to deciphering physiological processes. Thus, BiFC represents an excellent tool in aiding this understanding.

Disclosures

No conflicts of interest declared.

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