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Visualization of G protein-coupled receptor (GPCR) Interactions in Living Cells Using Bimolecular Fluorescence Complementation (BiFC)

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Abstract

Members of the G protein-coupled receptor (GPCR) superfamily have been shown to homo- and hetero-oligomerize both *in vitro* and *in vivo*. Although the functional and pharmacological significance of GPCR oligomerization is far from being completely understood, evidence suggests that, depending on the receptor, oligomerization may influence ligand binding, G protein coupling, and receptor targeting. Bimolecular fluorescence complementation (BiFC) is a technique based on the complementation of fragments from fluorescent proteins which allows the measurement and visualization of protein interactions in living cells. It can be extended to the simultaneous detection of distinct protein-protein interactions using a multicolor setup. This unit describes the application of BiFC and multicolor BiFC to the visualization of GPCR oligomerization in a neuronal cell model. Oligomerization of GPCR fusions to BiFC tags is visualized and measured using fluorescence microscopy and fluorometry. The effect of ligands on the relative formation of distinct oligomeric species is monitored with a ratiometric multicolor BiFC approach.

Keywords

G protein-coupled receptor; oligomerization; bimolecular fluorescence complementation; multiple protein-protein interactions; neuronal cells; ligand effects

Protein complexes mediate many cellular functions, from the integration of extracellular signals to the regulation of gene expression. The functional characteristics of many proteins are influenced by their interacting partners and the spatial context in which the interactions take place. The identification and localization of protein-protein interactions is therefore critical for the understanding of biological functions. G protein-coupled receptors (GPCRs), in addition to interacting with ligands and effector molecules such as G proteins, have been shown to homo- and hetero-oligomerize. GPCR oligomerization, at least in some instance, is necessary for proper receptor targeting (Pin et al., 2007). GPCRs localize to the plasma membrane, as well as to different intracellular compartments including the ER, Golgi, vesicles, and the nuclear envelope. The subcellular context of GPCR oligomers may influence their signaling. Moreover, oligomerization is increasingly recognized as a mechanism to modulate the pharmacological and functional characteristics of the receptors (Maggio et al., 2005; Fuxe et al., 2007).

This unit describes the use of bimolecular fluorescence complementation (BiFC, Hu et al., 2002) for the visualization of GPCR interactions in living cells. The approach relies on the

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complementation between two nonfluorescent protein fragments brought together by the interaction of their fusion partners (Fig. 1). When detected with microscopy, BiFC allows direct visualization of the subcellular locations of GPCR dimers in living cells (see Basic Protocol). A number of receptors have been shown to homo-oligomerize and to hetero-oligomerize with several other GPCRs. The multicolor BiFC assay (Hu and Kerppola, 2003), based on the complementation of fluorescent protein fragments with distinct spectral characteristics, enables parallel visualization and detection of multiple GPCR dimers in the same cell (see Alternate Protocol 1). Although microscopic analysis of BiFC signals provides invaluable insight on the localization of the GPCR interaction, fluorometric BiFC measurements on large cell populations provide rapid, unbiased, and quantitative results that are amenable to high-content experiments (see Alternate Protocol 2).

BASIC PROTOCOL

MICROSCOPIC DETECTION OF GPCR INTERACTIONS IN LIVING CELLS USING BIMOLECULAR FLUORESCENCE COMPLEMENTATION (BiFC)

Protein interactions can be directly visualized in living cells with the BiFC assay which is based on complementation between two nonfluorescent fragments of fluorescent proteins (FPs). This protocol illustrates the application of BiFC to visualize GPCR oligomers.

Materials—Plasmid vectors containing the BiFC fragment sequences of Venus (e.g. pBiFC vectors (Shyu et al., 2006), Fig. 2) pBiFC vectors and their sequences may be requested from Dr. C.-D. Hu (email: cdhu@pharmacy.purdue.edu).

DNA encoding the receptor coding sequences

Cell system in which to express the BiFC constructs (e.g., HEK293, COS-1, HeLa, or the neuronal CAD cell line; also see Critical Parameters)

Appropriate growth medium

Lipofectamine2000 (Invitrogen) or other transfection reagent

Cell culture vessels appropriate for microscopy (e.g., chambered coverglass (Nunc) or cluster plates with round glass coverslips added at the bottom of the wells)

Phosphate buffered saline (PBS)

Culture medium lacking pH indicator

Inverted fluorescence microscope (e.g. Nikon TE2000-U) equipped with:

- 20x and 60x objectives
- Excitation source (e.g., mercury lamp)
- Filter sets for
  - Venus/YFP (excitation at 500/20 nm; emission at 535/30 nm)
  - Cerulean/CFP (excitation at 430/25 nm; emission at 470/30 nm)
  - mCherry/DsRed (excitation at 572/23 nm; emission at 640/50 nm)

A sensitive digital monochrome CCD camera (e.g., Photometrics CoolSNAP-ES) Image acquisition software (e.g., Metamorph, Molecular Devices)

Reagents and equipment for protein immunodetection and radioreceptor binding assays

Construct GPCR-BiFC fusion vectors—1. Insert the receptor coding sequence in the pBiFC vectors (Fig. 2), in the same reading frame as the N-terminal epitope tags and the Venus yellow FP fragments (VN and VC).

> Use cDNAs of the receptors of interest as PCR templates. Oligonucleotides should contain the appropriate restriction sites and should omit the stop codon of the GPCR coding sequence. Fragments from the Cerulean cyan FP may also be used (see Table 1). Verify the vector sequences by DNA sequencing.

If the interaction interface is known, construct plasmids with mutations in the interaction interface as controls. Alternatively, prepare BiFC fusions to related receptors that do not interact with the receptor(s) under investigation to serve as additional controls (see Critical Parameters).

Verify expression and functionality of the BiFC-tagged receptors—2. Use receptor binding experiments (UNIT 1.4) or other appropriate approaches to determine if the addition of a C-terminal Venus fragment alters the ligand-binding characteristics of the receptor fusion protein. In addition, determine whether functionality of the receptor is retained by measuring downstream signaling (e.g., cAMP accumulation (UNIT 7.12) or ERK phosphorylation).

Transfect cells—3. Seed cells at the appropriate density that will allow them to grow to sub-confluency over 24 hours, incell culture vessels appropriate for microscopy.

> Commonly used mammalian cell lines, including HEK293, COS-1, and HeLa may be used for the BiFC assay. The authors have also used Cath.A differentiated (CAD) neuronal cells for the expression of BiFC fusions (Vidi *et al.*, 2008a; Vidi *et al.*, 2008b).
>
> Cluster plates may be used when multiple samples need to be analyzed, however, cells should be grown on glass coverslips (e.g., chambered coverglass) for high-resolution imaging with short-working-distance objectives.

4. Transfect the cells the following day, when cells have reached 60–80% confluency, with the vectors encoding the BiFC fusions (step 1).

> Prior optimization of the transfection conditions (e.g., using receptor fusions with the full-length Venus) will determine minimum amounts of plasmid DNA and Lipofectamine2000 transfection reagent to achieve optimal expression of the receptor fusion proteins of interest. The transfection procedure is as recommended by the manufacturer of the transfection reagent. BiFC fusion vectors may be co-transfected with markers for subcellular compartments, provided that the latter have a distinct chromophore (e.g., Cerulean or mCherry in combination with -VN/-VC fusions).

Detect fluorescent signals—5. Twenty-four hours post-transfection, delicately rinse the cells with warm PBS and replace the growth media with warm, colorless media lacking a pH indicator, or with warm PBS. This step will remove cell debris, which often have intrinsic fluorescence.

6. Image fluorescent signals using an inverted fluorescence microscope. Use YFP excitation and emission filters (500/20 and 535/30, respectively) to detect complemented Venus signals. A 60x immersion objective with high numerical aperture allows for high-resolution

imaging and determination of sub-cellular localization of fluorescent signals (see Support Protocol 1).

Quantify BiFC fusion expression—7. Determine the density or expression levels of the BiFC receptor-fusions under the conditions used for microscopy.

Radioreceptor binding experiments provide an estimate of receptor densities and should be performed on total cellular membrane extracts, so that plasma membrane as well as intracellular receptor pools are measured. Immunodetection (e.g., immunoblotting or dot blots) may be used as a complementary technique for the measurement of BiFC fusion expression or may be required if radioligands are not available for the receptor of study. The pBiFC vectors (Fig. 2) encode HA, MYC, or FLAG epitope tags that may be used for immunodetection.

Assess results—8. It is assumed that the interaction between receptors A and B is studied and signals from A-VN + B-VC, as well as A-VN + B′-VC transfections are measured, with B′ a mutated form of B or a non-interacting receptor serving as negative control. Compare BiFC fluorescent signals in cells expressing A-VN + B-VC and in cells expressing A-VN + B′-VC. Determine the expression level of A-VN in both transfections, as well as B-VC and B′-VC (as described in step 7).

The following conclusions may be drawn:

Specific interaction supported: When i) fluorescence is detected in the cells expressing A-VN + B-VC, ii) signal is eliminated or significantly reduced in A-VN + B′-VC transfections, and iii) expression levels of the tagged receptors are similar.

Nonspecific interaction: When both transfections (A-VN + B-VC and A-VN + B′-VC) lead to similar BiFC signals. If this is the case, the BiFC assay may not be an appropriate assay to study the receptors of interest.

Non-conclusive result: The lack of fluorescence complementation does not prove the absence of interaction (Also see Critical Parameters).

SUPPORT PROTOCOL 1

QUANTIFICATION OF MICROSCOPIC FLUORESCENT SIGNALS

In transient transfections, as well as in stable cell lines, individual cells may express different levels of recombinant proteins. It is therefore essential to measure BiFC signals over large cell populations to prevent variations in cell-to-cell expression levels from influencing the results. Moreover, to compensate for differences in transfection efficiencies across conditions, a FP with distinct spectral properties is co-expressed with the BiFC fusions and used to normalize BiFC signals. Provided that imaging conditions are identical (i.e., exposure time, gain, binning), quantitative data reflecting the relative efficiencies of BiFC can be obtained.

Additional Materials *(also see Basic Protocol)*—Plasmid encoding Cerulean or mCherry to be used as normalizing FP (assuming -VN/-VC was used in the Basic Protocol)

ImageJ image processing and analysis software (http://rsbweb.nih.gov/ij/) or equivalent

Express of BiFC fusion receptors in cells—1. Follow steps 1 to 5 of the Basic Protocol, but add 20–50 ng of plasmid encoding the normalizing FP to each transfection mix.

If possible, Cerulean or mCherry normalizers should be expressed in the same subcellular compartment as the BiFC-tagged receptors. Membrane targeting of Cerulean or mCherry can be achieved by expressing the FPs as fusions to the N-terminal portion of neuromodulin/GAP43 ('Mem' vectors, Clontech). These membrane-targeted markers are useful for receptors predominantly expressed at the plasma membrane. Other fluorescently tagged markers may be useful for receptors that are localized intracellularly. The authors have used markers for the endoplasmic reticulum (FP fused to the ER targeting sequence of calreticulin), transmedial Golgi (FP fused to residues 1-81 of the 1,4-galactosyltransferase), and endosomes (FP fused to RhoB)(Vidi *et al.*, 2008a).

Image collection and analysis—2. For each microscopic field, image sequentially the normalizer and BiFC signals. To avoid bias, select the fields to be imaged based on the normalizer signal and image at least 100 cells. Convert the two images into an image stack for subsequent analysis.

Verify in preliminary experiments that the emission and excitation filters efficiently separate Venus and Cerulean (or Venus and mCherry) fluorescent signals.

3. Quantify the normalizing FP (i.e. Cerulean or mCherry) and BiFC signals on a cell-to-cell basis. Fluorescent signals may be measured over the entire area of each cell. Alternatively, if high-resolution imaging is desired, plasma membrane and/or intracellular signals may be quantified independently (see Fig. 3). For each filter setting and in each microscopic field, determine background fluorescence by measuring signal intensities in regions devoid of cells. Subtract the respective background values from both the normalizer and BiFC signals.

The authors use ImageJ software, which is in the pubic domain. This software is particularly convenient for this type of analysis, since multiple pixel intensity measurements can be performed on image stacks. The reader is referred to the ImageJ website (http://rsbweb.nih.gov/ij/) or to resources from the McMaster Biophotonics Facility(http://www.macbiophotonics.ca/imagej/index.htm) for tutorials and documentation.

4. Calculate the ratio of BiFC fluorescence over normalizer fluorescence for each cell.

This represents the relative complementation efficiency for the combination of receptors expressed as BiFC fusions.

5. Calculate the median of the BiFC/normalizer fluorescence ratio. Average this median value from at least 3 independent experiments.

Median values are calculated because fluorescence intensities usually follow a non-Gaussian distribution (Hu *et al.*, 2002).

6. Assess results.

If the BiFC/normalizer ratio is significantly different in the A-VN + B-VC compared to the A-VN + B′-VC transfections, and the expression levels and localization of the proteins are comparable, the observed A-VN + B-VC fluorescence is likely to represent a specific interaction between A and B. If the ratios are similar, the fluorescence signals may represent nonspecific complementation. Alternatively, an interaction may also occur between A and B′.

ALTERNATE PROTOCOL 1

MICROSCOPIC DETECTION OF MULTIPLE GPCR INTERACTIONS IN LIVING CELLS

Proteins often have several different interacting partners. In particular, a large number of GPCRs are known to homo-oligomerize as well as to hetero-oligomerize with other receptors. Two different GPCR-GPCR interactions can be visualized simultaneously in living cells using multicolor BiFC (see Fig. 1 and Table 1). Most residues determining the color of GFP-derived FPs such as Venus and Cerulean are located upstream from residue 155. N-terminal BiFC fragments from different FPs, upon complementation with a common C-terminal BiFC fragment, therefore reconstitute FPs with distinct spectral properties. For example, Cerulean C terminal fragments (-CC) complement with both Cerulean and Venus N terminal fragments (-CN and -VN). The two complemented FP populations (-CN/-CC, Cerulean and -VN/-CC, Venus) are consistent with two different species of receptor dimers.

Note: The excitation and emission spectra of complemented -VN/-CC are green-shifted relative to Venus (Shyu *et al.*, 2006, and Table 1). These modest shifts are due to the absence of the T203Y mutation (characteristic of Venus and other yellow-shifted GFP variants) in the C-terminal of Cerulean. However, signals from -VN/-CC complementation are efficiently detected and quantified using YFP filter sets and are referred to as Venus fluorescence in the text below.

Additional Materials *(also see Basic Protocol)*—Plasmid vectors containing the following BiFC fragments: -VN (Venus N-terminal); -CN (Cerulean N-terminal); -CC (Cerulean C-terminal). pBiFC vectors may be used (Shyu et al., 2006).

Construction of multicolor BiFC vectors—1. Using pBiFC vectors (or starting from Venus or Cerulean-containing vectors), construct the A-VN, B-CC, and C-CN BiFC fusions, with A, B, and C the GPCRs of interest (Fig 1). Note that if receptor homo-versus hetero-oligomerization is studied, the [A-VN/B-CC/B-CN] or [A-VN/A-CC/C-CN] combinations may be used to transfect cells.

Transfect and image cells—2. Follow steps 2–7 of the Basic Protocol. Use YFP excitation and emission filters (500/20 and 535/30, respectively) to detect -VN/-CC complementation signals. Use CFP filters (excitation at 430/25 nm; emission at 470/30 nm) to detect Cerulean complementation signals.

3. Analyze fluorescent signals with high-resolution fluorescence microscopy. Information can be gained on the localization of the A-B and B-C interactions. In particular, the relative intracellular distribution of A-B and B-C signals can be determined by comparing the Venus and Cerulean images. The localization of the interactions relative to defined cellular structures can also be established when appropriate localization markers have been included in the transfection reaction.

SUPPORT PROTOCOL 2

RATIOMETRIC ANALYSIS OF MULTIPLE GPCR INTERACTIONS USING FLUORESCENCE MICROSCOPY

Information on the relative formation of multiple protein complexes can be obtained by a quantitative analysis of the multicolor BiFC experiments detailed in Alternate Protocol 1. Specifically, the effect of ligand or drug treatment on the formation of GPCR oligomers can be determined by examining multicolor BiFC signals from cells expressing the BiFC-tagged receptors (Vidi et al., 2008a).

1. Follow Alternate Protocol 1 (see also the Support Protocol 1). Add 20–50 ng of plasmid encoding a red fluorescent marker of the plasma membrane (e.g., mCherry-Mem) to each of the transfection mixes.

If ligand or drug effects are to be studied, it is important to optimize the temporal relationship between transfection of the cells and the treatment. The authors have treated cells with receptor ligands 4 hours post transfection and measured fluorescence 24 hours post transfection. Both the duration of the drug treatment and the time at which BiFC signals are measured should be optimized for the cell system used and the receptor under study. Drugs are typically prepared as 100x stocks and are added to the cell growth media. Vehicle control samples are treated with the diluent only. If Lipofectamine2000 is used for transfection, the growth media (including DNA and Lipofectamine) may be changed 4 hours post transfection, prior to drug treatment.

2. Collect Venus (-VN/-CC), Cerulean (-CN/-CC), and mCherry fluorescent signals using YFP, CFP, and Texas Red filter sets. To avoid bias, select the fields to be imaged based on the mCherry signals and image at least 100 cells for each condition/drug treatment. Convert the three images into an image stack for subsequent analysis.

Image Analysis—3. After subtracting background fluorescence from each image, use the fluorescent marker (e.g., mCherry) to select the cells for analysis. Measure signal intensities at the membrane, as well as within the intracellular area (see Fig. 3). Determine the Venus/Cerulean fluorescence ratios on a cell-to-cell basis and calculate the median of these ratios. Average this value from at least three independent experiments.

An approximation of plasma membrane signal intensities can be obtained by tracing lines perpendicular to the cell surface and retrieving the maximal pixel intensity along this line (see “1” in Fig 3). As an estimate of the intracellular signals (presumably reflecting receptor internalization and/or retention in the endoplasmic reticulum or Golgi apparatus), regions of interest (ROIs) are drawn that cover the cell area (see “2” in Fig 3), but exclude the cell surface. The average pixel intensities of the ROIs are calculated.

4. To obtain a quantitative evaluation of the cellular distribution of the BiFC signals (membrane-bound versus intracellular signals), calculate the ratio of surface to intracellular signals for each cell. Determine the median value of this ratio for each treatment. Average this value from at least three independent experiments.

5. Assess results. Compare the BiFC fluorescence ratios (Venus/Cerulean), as well as the signal distribution ratio (surface/intracellular) are compared across the different treatments. Determine significant differences by statistical analysis.

Differences in BiFC fluorescence ratio can be interpreted as changes in the propensity of the receptor to oligomerize, i.e., changes in the oligomerization preferences (see Commentary). Drug-induced changes in BiFC may also reflect alterations in the expression level of the BiFC-tagged receptors that can be examined using receptor binding (UNIT 1.4) or immunodetection (see step 7 of the Basic Protocol and Commentary).

ALTERNATE PROTOCOL 2

BiFC and MULTICOLOR BiFC MEASUREMENTS USING FLUOROMETRY

Microscopic analysis of BiFC signals (see above) has the tremendous advantage of providing spatial information on the receptor interactions. However, quantification of

microscopic BiFC signals is time-consuming and prone to experimental bias: only a limited number of cells can be imaged and analyzed for each condition and these are typically chosen by the experimenter. Fluorometry is a complementary approach for the measurement and quantification of fluorescence complementation signals. Measurements are performed over large cell populations and the technique allows multiple treatment conditions to be analyzed in parallel. Both BiFC and multicolor BiFC signals can be quantified by fluorometry. However, one disadvantage of fluorometric analysis as opposed to microscopic analysis of BiFC signals is the measurement of whole cell fluorescence does not allow for spatial resolution of the signal in the cell (i.e., this method does not allow for the evaluation of intracellular versus membrane-localized signals of the BiFC complexes). Moreover, cells that may aberrantly overexpress BiFC fusion proteins and hence display very strong fluorescence cannot be excluded.

Additional Materials *(also see Basic Protocol)*—Twelve-well cluster plates

Reagents for protein concentration determination (e.g., BCA from Pierce)

Black 96-well plates

Multi-well plate reader that efficiently separates Venus and Cerulean fluorescent signals (Fusion, Packard-Perkin Elmer)

Express BiFC fusion receptors in mammalian cells—1. Seed cells at the appropriate density in 12-well cluster plates that will allow them to grow to sub- confluency over 24 hours.

2. On the following day, when cells have reached 60–80% confluency, transfect the cells with the vectors encoding the BiFC fusions (see Fig. 2). For BiFC experiments, transfect A-VN and B-VC BiFC constructs together with a full-length Cerulean construct as a normalizer (see Support Protocol 1). For ratiometric multicolor BiFC experiments, transfect A-VN, B-CC, and C-CN receptor-BiFC constructs (see Alternate Protocol 1). The experimental layout should include each transfection condition (or treatment) in triplicate. Mock-transfected cells (no DNA added to the transfection mix) should also be included.

Prepare cell suspensions—3. Twenty-four hours after transfection, use fluorescence microscopy to provide a crude assessment of the transfection by the observation of the fluorescent signals. Following the microscopic examination, delicately rinse the cells with 500μL of warm PBS, resuspend the cells in 330μl of PBS, and transfer the cell suspensions into a microcentrifuge tube. Use 10μl of the cell suspensions in triplicate to determine protein concentrations. During that time, maintain the remaining 300μl of cell suspensions at 37°C.

> The BCA protein assay (Pierce) can be performed in a 96-well plate format to expedite the measurements.

4. Equalize protein concentrations for all samples by diluting with PBS. The authors typically dilute to 200 ng/μl.

Measure fluorescence intensities—5. Transfer 200–400 μl of each cell suspension into a black 96-well plate.

6. Measure Venus and Cerulean fluorescent signals using a multi-well plate reader. Use emission and excitation filters specific for YFP and CFP (Table 1).

Pilot experiments should be performed to ensure minimal bleed-through between Venus and Cerulean signals. Bleed-through coefficients for Cerulean and Venus channels are calculated with cells expressing either Venus or Cerulean (or corresponding BiFC pairs). The measured Venus and Cerulean fluorescence intensities are noted V and C, respectively. The C/V fluorescence ratio (termed the " x coefficient") is determined in cells expressing only Venus. The V/C fluorescence ratio ("y coefficient") is determined in cells expressing only Cerulean. Corrected Venus (V_{cor}) and Cerulean (C_{cor}) signals are calculated in cells co-expressing Venus and Cerulean (or BiFC constructs) with the following equations: $V_{cor}=(V - yC)/1 - xy$ and $C_{cor}=(C - xV)/1 - xy$.

As an alternative to a multi-well plate reader equipped with bandpass filters, a spectrofluorometer may be used for fluorescence measurements.

Assess results—7. Assess results. Average signal intensity values from mock-transfected cells. These represent background fluorescence that should be subtracted from each fluorescent signal.

8. Correct the Venus and Cerulean signals using bleed-through coefficients (x and y) determined with cells expressing only Venus or Cerulean FPs (see step 6).

9. Calculate the Venus/Cerulean fluorescence ratios. Average theses values for the three replicates.

For BiFC experiments, the fluorescence ratio correspond to the relative complementation efficiency for the combination of receptors. In multicolor BiFC experiments, the ratios are compared across conditions (see Support Protocol 2 and Commentary).

COMMENTARY

Background Information

Most proteins are part of multi-protein complexes that function in a defined cellular context. The determination of the composition and the localization of protein complexes are essential for understanding the biological function of a protein. Multiple protein interactions are involved in GPCR signaling cascades and are central to signal transduction. Ligand binding induces a conformational change that propagates from the receptor to interacting heterotrimeric G proteins, promoting GDP to GTP nucleotide exchange at the G protein α subunit. The GTP-bound Gα subunits dissociate from (or change conformation relative to) the receptor and the β/γ subunits (Lambert, 2008). As a result of the modified interaction interfaces, Gα as well as Gβ/γ subunits engage with effector proteins. Increasing evidence suggests that pharmacological characteristics of GPCRs as well as their coupling to G proteins is modulated by GPCR interactions and oligomerization (Fuxe et al., 2007; Milligan, 2007; Pin et al., 2007).

Protein fragment complementation assays such as BiFC are among the most powerful approaches for the detection of protein-protein interactions in living mammalian cells (Remy and Michnick, 2007). BiFC is based on the complementation of fluorescent proteins (Hu et al., 2002): two non-fluorescent FP fragments, when brought in close contact by fusion partners, assemble and reconstitute functional chromophores. The technique allows not only the detection of protein-protein interactions, but also has the ability to determine the sub-cellular localization of the protein-protein interaction. The use of BiFC is rapidly growing (see http://sitemaker.umich.edu/kerppola.lab/kerppola.bifc/bifc_results), reflecting the usefulness of the technique. A major limitation of BiFC, however, is its irreversible nature

(Hu et al., 2002). As a consequence, dynamic complex formation and dissociation cannot be evaluated with BiFC assays but may be analyzed using fluorescence resonance energy transfer (FRET; Vogel et al., 2006), another very popular method for the visualization of protein interactions in live cells. FRET and BiFC have distinct advantages and limitations, which make the two methods complementary. FRET usually requires rather high expression levels of fusion proteins and depends on the very close proximity (<30 nm) and the orientation of the chromophores. These criteria are not always easily met, especially with large proteins or when prior structural knowledge is lacking. In contrast, BiFC may stabilize weak protein interactions, allowing their detection. FRET requires complex fluorescence measurements (fluorescence lifetime or precise intensity measurements), whereas BiFC signals can easily be detected with a simple fluorescence microscope.

Critical Parameters

Choice of fluorescent protein fragments and construction of BiFC fusions—

The BiFC method was originally described with GFP (Ghosh et al., 2000) and has been extended to YFP and CFP variants (Hu et al., 2002; Hu and Kerppola, 2003). In YFP-based BiFC assays, the complementation signal can be localized relative to CFP-tagged subcellular markers. Moreover, YFP complementation signals can be normalized with co-expressed CFP in quantitative BiFC assays. Site-directed mutagenesis has yielded brighter versions of YFP and CFP: Venus (Nagai et al., 2002) and Cerulean (Rizzo et al., 2004), respectively. Venus is also less sensitive to high temperatures and matures faster than YFP at 37°C. It is therefore recommended to use Venus or Cerulean in BiFC assays (Shyu et al., 2006). Several amino acid cut sites in Venus and Cerulean, including 154-155 and 172-173, have been used in BiFC assays. The authors of this unit have used overlapping -VN173/-VC155 fragments (Shyu et al., 2006) for the study of adenosine and dopamine receptor oligomerization (Vidi et al., 2008a,b). The -VN173/-VC173 and -VN155/-VC155 combinations may also be used. For the detection of multiple protein interactions, a combination of yellow and cyan FP fragments are used in multicolor BiFC assays (Fig. 1, See Alternate Protocol 1, Hu and Kerppola, 2003). The use of red fluorescent proteins (mRFP1-Q66T, Jach et al., 2006 and mCherry, Fan et al., 2008) have also been explored for BiFC assays. However, their general applicability to mammalian cell studies remains to be established.

Previous studies have used GPCRs tagged at their C-termini with BiFC fragments (Vidi and Watts, 2009). Receptor fusion expression may be determined using radioreceptor binding assays, as well as by immunodetection. When using the pBiFC vectors (Shyu et al., 2006), an N-terminal epitope tag (FLAG, MYC or HA) is typically fused to the receptor in addition to the C-terminal BiFC fragment, facilitating immunodetection. Finally, the importance of the length and composition of the linker sequence separating the GPCR coding sequence and the BiFC fragment should be stressed since it may affect the efficiency of fluorescence complementation (Chen et al., 2006; Vidi and Watts, unpublished data). A 15- to 20-amino acid linker composed of small, uncharged residues is recommended. The authors have generally used the GGGGSGGGGSGGGGS sequence to obtain optimal results.

Use of BiFC to Evaluate Receptor Oligomers—The use of BiFC shows significant promise to provide further insights into the investigation of GPCR oligomerization. However, investigators are urged to carefully consider the choice of the GPCRs used for BiFC studies, paying attention to other evidence implicating the physiological relevance for such interactions. Studies of hetero-oligomers should examine the report from the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) who developed a set of criteria for the identification and evaluation of novel GPCR hetero-oligomers (Pin et al., 2007). It is proposed that two of

three criteria should be met in order for a receptor dimer to be accepted and recognized. First, there must be "evidence for physical association in native tissue or primary cells" (Pin et al., 2007). In other words, both receptor partners must be present in the same cell in native tissue. Second, "a specific functional property for the heterodimeric receptor will be critical to identify such receptors in native tissue" (Pin et al., 2007). The receptor dimer must possess unique signaling, regulation, and/or localization that is "truly a result of receptor dimerization and not merely from the coexpression." Lastly, "the use of knockout animal models and/or RNAi technology may provide important information about the receptor dimer *in vivo*." However, this criterion may only be met if the first two criteria are fulfilled. Along with other complementary experimental techniques that explore protein-protein interactions, BiFC can be a very useful tool to evaluate GPCRs that oligomerize in a physiologically relevant manner.

Receptor pharmacology function—The modification of a protein's primary structure may impact its biochemical properties. This also applies in BiFC assays where receptor sequences are fused to FP fragments. A modified receptor may for instance be retained in the ER, have an abnormal internalization dynamic, or have impaired interactions with regulatory or signaling components, which in turn may affect oligomerization. C-terminal BiFC tags are commonly used for GPCRs, but they may affect receptor function in some cases because the intracellular C-terminal domains of GPCRs interact with G proteins as well as GPCR kinases, and arrestins. It is therefore essential to ensure that GPCR-BiFC fusions retain their proper pharmacological characteristics, function, and localization. Since receptor function may be influenced by expression density, it is desirable to express the BiFC-tagged receptors at physiologically relevant levels when possible. The pharmacological profile of the GPCR-BiFC fusion is usually determined using radioreceptor binding and functional assays. Assays assessing receptor modulation of second messengers or downstream signaling (e.g., cAMP accumulation or ERK phosphorylation) are typically used to determine if receptor functionality is retained.

Expression systems—Common mammalian cell lines including HEK293, COS-1, and HeLa cells have been used to express BiFC fusions. Cells of neuronal origin may be also be used and may be more appropriate to study the oligomerization of GPCRs relevant to the nervous system. For example, the neuronal CAD cell line has been used to study adenosine and dopamine receptor oligomerization (Vidi et al., 2008a,b).

BiFC-fusion receptors can be studied using both stable and transient expression techniques. GPCR expression levels can be adjusted in both strategies. Transient transfections are very rapid to set up and allow for a variety of test combinations of receptor fusions to be examined. They are recommended for initial establishment of the BiFC assay for interacting receptors. Higher reproducibility may be achieved using stable cell lines with defined pharmacological profiles and more constant receptor expression levels. Stable cell lines may also be suitable for high-content applications. The irreversible nature of BiFC should also be considered when choosing between stable and transient expression strategies. If the influence of a drug treatment on GPCR interactions is of interest, rapid turnover of stably expressed GPCR-BiFC fusion is required to observe significant effects on fluorescence complementation. Alternatively, cells may be treated before or during transient transfection procedures (see Support Protocol 2). In transient assays, the incubation time following transfection should be optimized, paying close attention to the incubation leading to robust BiFC signals. It is advantageous, at least for some receptors and cell systems, to avoid incubations greater than 24 hours because signal intensity may decrease or nonspecific interactions may develop.

Data collection and signal quantification—A simple fluorescence microscope is sufficient to monitor BiFC signals, however, a confocal microscope can offer higher resolution and additional functionality. For quantitative measurements, the acquisition settings (such as the exposure time) should be kept rigorously constant during image acquisition or differences should be accounted for in the intensity calculations. Background fluorescence needs to be carefully determined and subtracted from BiFC signals. Microscopic measurements may be complemented with unbiased whole cell fluorometric measurements (see Alternate Protocol 2).

Controls—Choosing the appropriate negative control is arguably the most difficult task in BiFC experiments (Hu et al., 2006). Ideally, if information on the dimerization interface is available, receptors with point mutations predicted to disrupt the interaction should be used as negative controls. Since multiple transmembrane domains are usually involved in GPCR oligomerization, and structural information on oligomers is not typically available, design of a control mutated receptor may be difficult. As an alternative, a related noninteracting receptor may be used as negative control. The BiFC fusion construct used as a negative control should be expressed at similar levels and have a similar sub-cellular distribution as the tested GPCR. In addition, control receptors should be tested for functionality and should display the expected pharmacological profile.

Interpretation of lack of fluorescence complementation—The detection of a BiFC signal is consistent with interactions between the proteins of study. In contrast, a negative BiFC result is more difficult to interpret because a lack of fluorescence complementation does not necessarily reflect the absence of interaction between the proteins fused to the BiFC fragments. Conformational constraints placing the BiFC fragments far apart or in incompatible orientations within a protein complex will prevent fluorescence complementation. If fluorescence complementation between the proteins of interest is not detected in initial experiments, it is recommended to test different BiFC assay combinations (e.g., modify BiFC fragments fused to receptor) and/or to modify the linker. Finally, it should be noted that the fluorescent signal intensity following complementation is markedly reduced (2.5–5.5 fold) when compared to signals from full length FPs (Vidi and Watts, unpublished observations).

Interpretation of changes in BiFC ratios—Multicolor BiFC allows visualization of two distinct types of protein-protein interactions in living cells. Quantification of the multicolor BiFC signals provides information on the relative proportion of the distinct receptor dimers. The ratiometric BiFC technique may be used to examine the effect of receptor ligands or biochemical reagents on GPCR oligomerization (see Alternate Protocol 2). If changes in the BiFC fluorescence ratio are observed (relative to the control) following a treatment, it is likely that the treatment influenced receptor oligomerization, favoring the formation of one type of interaction over the other. Expression levels of BiFC fusions may also influence overall BiFC signal strength and thus, it is recommended to assess the effect of the drug treatments on receptor density/expression as well as BiFC fluorescence ratios. For several GPCRs, both agonists and antagonists have been shown to increase receptor density (Bernier et al., 2004). A potential pharmacological chaperone effect should be taken into consideration in experiments with prolonged ligand treatments. Changes in multicolor BiFC fluorescence ratios may also result from agonist-induced conformational changes within GPCR oligomers as a consequence of ligand binding. It is anticipated that such agonist-induced changes in multicolor BiFC fluorescence ratio would be prevented by co-application of an appropriate antagonist. The results obtained with a multicolor BiFC combination can be further substantiated by using complementary BiFC assay combinations. For example, if a treatment leads to decreased Venus/Cerulean fluorescence with the [A-

VN/B-CC/B-CN] combination, the opposite (increased Venus/Cerulean) may be observed for the [A-VN/A-CC/B-CN] combination.

Further applications and developments—The uses of BiFC continue to grow for the study of protein-protein interactions as well as drug discovery. For example, approaches combining BiFC with fluorescence or bioluminescence resonance energy transfer (FRET and BRET) now allow interactions of up to four different partners to be detected within a complex (for review see Vidi and Watts, 2009). MacDonald and colleagues (MacDonald et al., 2006) have also demonstrated the applicability of BiFC for high-content screens. It is expected that other BiFC-based small compound screens will be developed in the near future and it possible that the ratiometric multicolor BiFC analysis described here may be useful for the identification of compounds influencing GPCR oligomerization (Vidi et al., 2008a).

Anticipated Results

If the receptors under investigation interact, fluorescence complementation signals are likely to be detected in cells expressing the corresponding BiFC fusions. Positive BiFC results can strengthened by literature support as well as the inclusion of appropriate negative controls (see Critical Parameters). Caution is warranted in the absence of fluorescence complementation because the lack of efficient and detectably complementation could result from a variety of experimental confounds (see Critical Parameters). Multicolor BiFC experiments allow investigators to examine multiple receptor complexes in living cells and can provide information on the sub-cellular distribution of the two complexes. Quantitative multicolor BiFC measurements may also reveal novel information on the effect of pharmacological or biochemical treatments on receptor oligomerization patterns.

Time Considerations

Molecular cloning of the receptors of interest into pBiFC vectors can be achieved in 1 to 2 weeks. The Basic Protocol and the Alternate Protocols 1 and 2 can be completed in 3 days: day 1 for plating the cells, day 2 for cell transfection, and day 3 for the analysis of BiFC signals. Day 1 and 2 time commitments include approximately 1 hour for cell culture and transfection. Image acquisition (microscopic BiFC analysis, 1–2 hours) is performed on day 3. The image acquisition process may be expedited with automation functions of software packages such as Metamorph. Additional time needs to be dedicated for image analysis (1–4 hours) and for the measure of receptor-BiFC densities by radioreceptor binding or immunoblot analysis. Time considerations for fluorometric BiFC measurements (Alternate Protocol 2) only differ for the microscopic analysis performed on day 3. Cell suspensions are prepared within 1.5 hour and approximately 1 hour is required to measure and analyze the BiFC signals.

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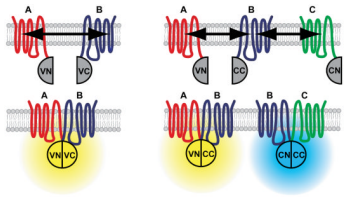

Figure 1.

Schematic representation of BiFC and multicolor BiFC principles. Fragments from the Venus fluorescent protein (VN and VC) or from the Cerulean fluorescent protein (CN and CC) are fused to putative interaction partners (A, B and C). Physical association of A with B (left) allows the formation of a bimolecular fluorescent complex. In multicolor BiFC assays (right), two distinct interactions (A-B and B-C) are simultaneously visualized by the reconstitution of two fluorescent complexes of distinct spectral properties. For the color version of this figure, go to http://www.currentprotocols.com.

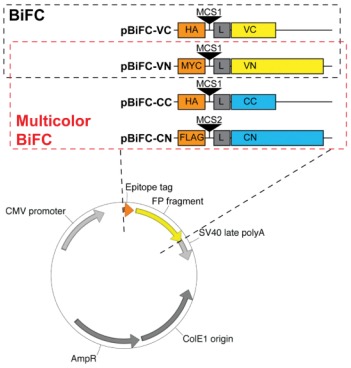

Figure 2.

Schematic representation of DNA vectors for BiFC and multicolor BiFC experiments. The coding sequence of the receptor of interest is ligated into the multiple cloning sites (MCS) from the pBiFC vectors, in the same reading frame as the epitope tags (HA, MYC or FLAG), the linkers (L), and the fluorescent protein (FP) fragments. Expression of the BiFC fusion proteins is driven by the cytomegalovirus (CMV) promoter. Unique restriction sites include MscI; EcoRI; SalI; BglII; XhoI; KpnI (MCS1) and HindIII; NotI; EcoRI; ClaI; BglII; EcoRV; KpnI; SalI; XbaI (MCS2). SV40 polyA, simian vacuolating virus 40 poly-adenylation site; AmpR, ampicillin resistance gene. The pBiFC vectors and their sequences may be requested from Dr. C.-D. Hu (email: cdhu@pharmacy.purdue.edu). For the color version of this figure, go to http://www.currentprotocols.com.

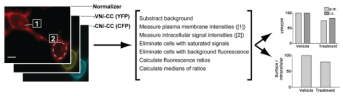

Figure 3.

Flowchart of the analysis of microscopic multicolor ratiometric BiFC measurements. Fluorescent BiFC signals (-VN/-CC and -CN/-CC), as well as normalizer fluorescent signals, are imaged by epifluorescence or confocal microscopy. Pixel intensities are measured and processed as indicated in brackets. BiFC fluorescence ratios, as well as BiFC signal localization are compared between treatments and controls. Scale bar, 5μm. For the color version of this figure, go to http://www.currentprotocols.com.

Table 1

Characteristics of fluorescent proteins used in BiFC assays

Fluorescent protein (fragments)	Split position	Ex/Em (filter)	Application
Venus (-VN / -VC)	154–155;172–173	515/528 (YFP)	BiFC analysis
Cerulean (-CN / -CC)	154–155;172–173	452/478 (CFP)	BiFC analysis
Venus/Cerulean (-VN/ -CC)	154–155;172–173	504/513 (YFP)	Multicolor BiFC
Cerulean	NA	452/478 (CFP)	Normalization of BiFC signals, colocalization
mCherry	NA	587/610 (TR)	Normalization of BiFC signals, colocalization

Ex/Em, maximal excitation/emission wavelengths (nm); YFP and CFP, yellow and cyan fluorescent proteins; TR, Texas red