Published in final edited form as:

Mol Cell Endocrinol. 2011 January 15; 331(2): 185–193. doi:10.1016/j.mce.2010.07.011.

Fluorescent protein complementation assays: new tools to study G protein-coupled receptor oligomerization and GPCR-mediated signaling

Pierre-Alexandre Vidi¹, Karin F.K. Ejendal², Julie A. Przybyla², and Val J. Watts^{2,†}

¹ Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University

² Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Purdue University

Abstract

G protein-coupled receptor (GPCR) signaling is mediated by protein-protein interactions at multiple levels. The characterization of the corresponding protein complexes is therefore paramount to the basic understanding of GPCR-mediated signal transduction. The number of documented interactions involving GPCRs is rapidly growing, and appreciating the functional significance of these complexes is clearly the next challenge. New experimental approaches including protein complementation assays (PCAs) have recently been used to examine the composition, plasma membrane targeting, and desensitization of protein complexes involved in GPCR signaling. These methods also hold promise for better understanding of drug-induced effects on GPCR interactions. This review focuses on the application of fluorescent PCAs for the study of GPCR signaling. Potential applications of PCAs in high-content screens are also presented. Non-fluorescent PCA techniques as well as combined assays for the detection of ternary and quaternary protein complexes are briefly discussed.

1 Introduction

A palette of techniques has been used to detect protein-protein interactions in GPCR signaling pathways (Table 1). Immunoprecipitation (IP) remains a good option for native tissue experiments. It allows for probing physical interactions but requires disruption of biological samples, a possible cause of artifacts (Milligan and Bouvier 2005). Non-invasive techniques based on resonance energy transfer (RET) have been developed to measure protein-protein interactions in the cellular context (see Marullo and Bouvier 2007; Gandia et al. 2008 for comprehensive reviews). Using fluorescence resonance energy transfer (FRET) experiments, protein-protein interactions can be followed in time and space whereas saturation bioluminescence resonance energy transfer (BRET) assays allow to measure relative affinities of GPCR-GPCR interactions and to control for nonspecific ('bystander') interactions (Mercier et al. 2002). Multiple strategies of protein complementation assays (PCAs) have also been applied in the study of GPCR signaling (Vidi and Watts 2009). Fluorescent PCAs (hereafter termed bimolecular fluorescence complementation, BiFC)

[†]Correspondence should be addressed to Dr. Val J. Watts, Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Purdue University, 575 Stadium Mall Drive, West Lafayette, IN 47907-2051. Phone: (765) 496-3872, Fax: (765) 494-1414 watts@purdue.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

allows for visualization of either single or dual protein-protein interactions at the sub-cellular level and only require basic experimental setups.

This review focuses on applications of BiFC to study GPCR signaling networks. Recent studies describing the techniques and methodologies to examine drug modulation of GPCR homo- and heteromerization will be presented. New methods combining PCAs and RET techniques for the detection of multi-protein complexes, as well as possible applications for high-content screens are also discussed.

2 Principle of fluorescent PCAs

The general principle of protein complementation assays is the splitting of a reporter protein in two inactive fragments. Two proteins of interest (*X* and *Y*) are expressed as fusions to the PCA fragments in cells or animal models. Interaction (or very close proximity) between *X* and *Y* brings the reporter moieties in close contact, leading to the spontaneous reconstitution of an active reporter. Reporter proteins used in PCAs include the β -galactosidase (PathHunter™ β -Arrestin Assay, DiscoveRx Corporation), luciferases from *Renilla reniformis* or *Gaussia princeps* (RLuc and GLuc) (Remy and Michnick 2006; Stefan et al. 2007), and fluorescent proteins (FPs) derived from the green fluorescent protein (GFP) of *Aequorea victoria* (Hu et al. 2006; Kerppola 2006; Shyu and Hu 2008). FPs with different spectral characteristics have been used in BiFC assays (Fig. 1) (Shyu et al. 2006; Fan et al. 2008; Vidi and Watts 2009). Examples of FPs include the yellow Venus (Nagai et al. 2002), the cyan Cerulean (Rizzo et al. 2004), and the red mCherry (Shaner et al. 2004). Here, we will use a two-letter nomenclature to designate FP fragments: the FP followed by the position of the fragment. For example “Vn” refers to the N-terminal fragment from Venus and “Cc” to the C-terminal fragment from Cerulean. Venus and Cerulean have been split at different positions with similar result in terms of complementation and specificity (Shyu et al. 2006). For example, Vn155/Vc155, Vn173/Vc173, and Vn173/Vn155 complement Venus (fragment overlap such as in Vn173/Vc155 has been shown to stabilize complementation; C.-D. Hu, personal communication). Complemented fluorescent signals can be visualized by fluorescence microscopy in live cells or in animals (Shyu et al. 2008a). Fluorescence-activated cell sorting (Giese et al. 2005; Watanabe et al. 2008) and fluorometry (Hynes et al. 2008; Vidi et al. 2008a) can also be used to quantify fluorescent PCA signals in cell populations.

FPs with distinct excitation and emission spectra can be combined in multicolor BiFC assays that allow the detection of multiple protein-protein interactions in a single cellular field (Hu and Kerppola 2003). Spectral properties of FPs derived from GFP are mostly determined by their N-terminal portion (residues 1–154). In a multicolor BiFC assay, the N-terminal fragments from two different FPs (e.g. Venus and Cerulean) are co-expressed with a single C-terminal fragment (Fig. 1). Signals from the two complementation combinations can be distinguished based on their distinct excitation and emission spectra. For example, if interactions between proteins *X* and *Y* and between *X* and *Z* are to be studied simultaneously, cells are transfected with *Y*-Vn + *X*-Cc + *Z*-Cn. The *X*-*Y* interaction results in yellow Vn/Cc complementation, whereas the detection of a cyan Cn/Cc signal indicates *X*-*Z* interaction.

3 Combining techniques to monitor higher-order oligomers

Most techniques only allow the detection of divalent protein-protein interactions. BiFC and RET techniques can however be combined in multiple ways to measure ternary or quaternary complexes (Vidi and Watts 2009). For example, yellow and cyan fluorescent proteins are compatible with both BiFC and FRET; Cerulean and Venus are popular FRET pairs. Moreover, Venus can serve as an acceptor for RLuc in BRET¹ assays. Signals in BiFC-BRET (Rebois et al. 2006) or BiFC-FRET (Shyu et al. 2008b) assays reflect trivalent

protein complexes. Sequential RET approaches including sequential FRET (3-FRET) (Galperin et al. 2004) and BRET-FRET (SRET) (Carriba et al. 2008) also allow to probe for trivalent complexes. Different types of PCAs can also be combined; for example BiFC and bimolecular luminescence complementation (BiLC) for the detection of tetravalent protein complexes (Guo et al. 2008; Rebois et al. 2008).

4 Strengths and limitations of BiFC in comparison with alternative techniques

As highlighted in Table 1, different techniques available to measure GPCR interactions have distinct strengths and limitations. Compared to biochemical assays such as native PAGE analysis or IP, and to BRET, BiFC (as well as FRET) offers the tremendous advantage of allowing studies of the sub-cellular localization of GPCR interactions. In contrast to FRET, implementation of BiFC measurements is simple as it only requires an epifluorescence microscope or a multiwell plate fluorescence reader. A limitation of the BiFC technique is the irreversible nature of fluorophore complementation (Hu et al. 2002), rendering the measurement of dynamic changes in GPCR interactions difficult. However, multicolor BiFC can be applied to quantitatively measure drug-induced changes in GPCR interactions. As described above, multicolor BiFC allows the study of up to three receptors that complement two unique fluorescent proteins (i.e. Venus; V and Cerulean; C and see Fig 1B). A ratiometric analysis (V/C ratio) is applied following (transient) expression of BiFC fusions in absence or presence of drug (see section 5). Microscopy can be used to study the relative signals at the membrane and the intracellular domain. A fluorescence plate reader can also be used to measure changes in the total signal of V relative to C under each drug condition. The drug-induced changes in the V/C ratio are both time- and dose-dependent (Przybyla and Watts 2010).

The irreversible complementation allows for the detection of weak or transient interactions during screening approaches, however, it may pose complications when monitoring dynamic interactions or potentially lead to false-positives requiring follow-up studies using additional approaches. In some instances, irreversible FP complementation may decrease signal-to-noise by stabilizing nonspecific interactions. Thus, appropriate controls including the expression of GPCRs with point-mutations in the interaction domain or non-dimerizing GPCRs as BiFC fusions should be included in the absence of a confirmed interaction (see http://sitemaker.umich.edu/kerppola.lab/kerppola.bifc/bifc_protocols_ and Vidi et al. 2010). The risk of nonspecific interactions increases when fusion proteins are overexpressed (Shyu et al. 2006) making it is important to achieve physiological expression levels of BiFC-receptor fusions. Similar to other techniques (FRET, BRET, and often IP), the presence of a fusion tag may influence GPCR interactions and function through steric hindrance. Thus, the expression and function of tagged GPCRs should be validated by binding and functional assays. BiFC has the potential to be used *in vivo* with the development of far-red or infra-red FPs that can be visualized in a living animal (Lin et al. 2009; Shu et al. 2009), however, it would require the construction of transgenic animals using endogenous promoters, making the strategy difficult to use in native tissues.

5 Application of BiFC to study GPCR signaling

5.1 GPCR oligomerization

Although initially controversial, GPCR oligomerization is now widely accepted and the number of documented oligomeric GPCR combinations is extensive. The earliest biochemical evidence for GPCR dimers was reported in 1996 and involved homodimers of the β_2 adrenergic receptor and the mGlu5 glutamate receptor (Pin et al. 2007). It was later

demonstrated *in vivo* that heterodimerization was required for function of the GABA_B receptor as well as the umami and sweet taste receptors (Pin et al. 2007). Those seminal reports prompted additional studies supporting the concept that GPCR homo- and heteromerization confers unique functional and pharmacological properties to the receptors (Pin et al. 2007; Milligan 2009). Further contributing to the complexity of GPCR oligomers, the composition of the GPCR complexes, i.e. the fraction of monomeric receptors and hetero- and homomeric complexes, is largely unknown. It appears that most GPCRs exist as, or are capable of forming, oligomeric complexes containing multiple GPCRs. For a recent review on GPCR-GPCR interactions, (see Milligan 2009). To date, limited data exist on the effect of persistent receptor activation or antagonism on GPCR oligomer populations. Furthermore, it has been proposed that alterations in GPCR oligomer formation may be linked to neurological disorders such as Parkinson's disease, schizophrenia, and depression (Fuxe et al. 2007). Thus, being able to measure the relative GPCR oligomer species will be critical for understanding normal and pathological states and possibly for developing new diagnostic and treatment strategies. As discussed in the examples below, newly developed PCA techniques as well as RET assays have recently been applied to the study of GPCR dimers and higher-order oligomers and are providing insight on drug-mediated effects on GPCR signaling and oligomerization.

Homo-dimerization of dopamine D₂ receptors (D₂R) is well documented (Lee et al. 2000; Armstrong and Strange 2001; Gazi et al. 2003; Guo et al. 2005). Recently, experiments using a combination of BiFC and BiLC demonstrated the existence of D₂R complexes with four protomers (Guo et al. 2008). The existence of D₂R tetramers was also supported by *in silico* modeling of the two different interaction interfaces as well as cross-linking experiments (Guo et al. 2008).

Adenosine A_{2A} receptors (A_{2A}R) have also been shown to homo-dimerize (Canals et al. 2004) and two recent reports suggest that, similar to D₂R, A_{2A}R form higher-order oligomers. Gandia and colleagues used a BiFC-BRET combination and showed the existence of A_{2A}R oligomer species containing at least three receptors (Gandia et al. 2008). In parallel, the application of BiFC-FRET combined techniques demonstrated that higher-order A_{2A}R oligomers accumulate at the plasma membrane in a neuronal cell model (Vidi et al. 2008b). Although GPCRs are believed to dimerize early during their synthesis at the endoplasmic reticulum (ER) (Bulenger et al. 2005), it is not yet clear where higher-order oligomers (i.e. assemblies of GPCR dimers) are formed. Our preliminary results suggest that A_{2A}R dimers are exported from the ER and subsequently assemble in higher-order oligomers at the plasma membrane as complexes consisting of at least three A_{2A}R were detected at the plasma membrane but not in intracellular compartments upon blocking ER export (Vidi and Watts, unpublished observations, Fig. 2).

The formation of higher-order oligomers is not restricted to D₂R and A_{2A}R. The assembly of β_2 adrenoceptors (β_2 ARs) in tetravalent complexes has been shown using BiFC-BiLC assays (Rebois et al. 2008). Higher-order α_{1b} adrenoceptor oligomers were also identified by 3-FRET (Lopez-Gimenez et al. 2007). The concept of higher-order GPCR oligomers (initially described as the receptor mosaic hypothesis (Fuxe et al. 2007), directly demonstrated for several GPCRs using PCA and RET combinations, is also supported by earlier biochemical and pharmacological data (e.g. Wreggett and Wells 1995; Nimchinsky et al. 1997; Lee et al. 2003; Park and Wells 2004). GPCR congregations constituted of multiple protomers may therefore be predominant at the cell plasma membrane.

In addition to assembling in homomeric complexes *in situ* (see above), A_{2A}R-D₂R have been shown to heteromerize in cell culture models (Hillion et al. 2002; Canals et al. 2003; Kamiya et al. 2003). D₂R and A_{2A}R are both highly expressed in the striatum and colocalize

in spiny neurons. The interaction between D₂R and A_{2A}R was recently also confirmed by BiFC measurements in a neuronal cell model (Vidi et al. 2008a). To measure heteromeric A_{2A}R-D₂R and homomeric receptor complexes (A_{2A}R-A_{2A}R or D₂R-D₂R), multicolor BiFC assays were implemented (Fig. 1). Fluorescent complementation signals reflecting A_{2A}R-A_{2A}R, A_{2A}R-D₂R, and D₂R-D₂R were detected at the cell surface as well as in intracellular compartments. A ratiometric approach, in which the relative intensities of the two distinct fluorescence complementation signals were calculated, allowed for comparison of homo- and heteromeric receptor pools after prolonged drug treatments. Prolonged stimulation of A_{2A}R resulted in increased A_{2A}R-D₂R heteromer relative to A_{2A}R-A_{2A}R homomer populations. Moreover, persistent stimulation of D₂R with its agonist quinpirole leads to a reduction of A_{2A}R-D₂R heteromers relative to A_{2A}R-A_{2A}R homomers. These phenomena could not be explained by drug-induced changes in receptor expression levels. Therefore, it was proposed that drug-induced changes either in the conformation of the receptor or in their localization in the membrane microenvironment impact oligomer formation (Vidi et al. 2008a). Persistent D₂R activation may for instance cause enhanced D₂R homooligomerization by increasing the affinity of D₂R for other D₂R molecules or by decreasing A_{2A}R-D₂R interactions. Persistent D₂R activation causes sensitization of A_{2A}R receptor-stimulated cyclic AMP accumulation (Vortherms and Watts 2004). A decrease of A_{2A}R-D₂R heterooligomerization resulting from prolonged D₂R stimulation may reduce D₂R antagonism on A_{2A}R signaling, hence leading to increased A_{2A}R signaling. The cell's GPCR oligomer repertoire has been proposed to be altered in certain pathologies or as a consequence of prolonged therapies, for example L-DOPA replacement treatment for Parkinson's disease (Fuxe et al. 2007). It is well known that L-DOPA treatments often lead to the development of dyskinesias (uncontrolled tremor) in patients. Recently, A_{2A}R agonists used in conjunction with L-DOPA were shown to reduce L-DOPA-induced dyskinesias (Schwarzschild et al. 2006; Morelli et al. 2007). Long-term L-DOPA exposure may alter A_{2A}R and D₂R homo- and heteromerization in striatal neurons (Antonelli et al. 2006). Although still speculative, the possible D₂R agonist-induced up-regulation of A_{2A}R-A_{2A}R homooligomerization could result in enhanced A_{2A}R signaling. This would rationalize the therapeutic use of A_{2A}R antagonist in combination with L-DOPA in treatments of Parkinson's disease.

More recently, multicolor BiFC was used to study drug modulation of cannabinoid 1 receptor (CB₁R) and D₂R heterodimers (Przybyla and Watts 2010). These studies were based on a series of observations suggesting that CB₁R and D₂R form heterooligomers displaying unique pharmacological properties *in vitro* as well as in native tissues. Specifically, it was suggested that the interaction between CB₁R and D₂R causes CB₁R to switch coupling from G_{α_{i/o}} to a G_{α_s}, leading to robust AC stimulation (Glass and Felder 1997; Jarrahian et al. 2004; Kearn et al. 2005). Despite these intriguing reports and potential implications in CNS disorders (Ferre et al. 2009), studies exploring the regulation of CB₁R and D₂R receptor oligomers are sparse. Multicolor BiFC was used to examine the subcellular localization and regulation of CB₁R and D₂R oligomers in a neuronal cell model (Przybyla and Watts 2010). Persistent D₂R stimulation with quinpirole increased CB₁R-D₂R heteromer formation and interestingly, the effect was restricted to intracellular compartments. Persistent CB₁ receptor stimulation with its agonist CP55,940 also increased the formation of CB₁R-D₂R heteromers relative to the D₂R-D₂R homomers, this time both at the plasma membrane and in intracellular compartments (Fig. 1D). These effects were time-dependent and attenuated by antagonist treatment. The effect of CB₁ receptor activation on oligomer formation was also dose-dependent. However, the associated EC₅₀ values (ca. 200–300 nM) were higher compared to other CP55,940 signaling events such as cyclic AMP inhibition (EC₅₀ ca. 5 nM). Since the drug treatments did not affect CB₁R expression and modestly decreased D₂R levels, the observed alteration in oligomer populations were unlikely to reflect changes in CB₁R densities. Instead, the data suggest that

GPCR activation may modulate GPCR-GPCR interactions. Consistent with this hypothesis is the increased intracellular CB₁R-D₂R heteromer formation observed upon expression of a constitutively active CB₁R receptor mutant (CB₁T210I). There are still many questions to be addressed regarding the physiological implications of drug-induced modulation of CB₁R-D₂R oligomerization in CNS disorders such as Parkinson's disease and drug abuse. For example, it has been suggested that antagonistic interactions between CB₁R and D₂R (and possibly their oligomerization) can be exploited for pharmacological benefits. CB₁R antagonists have been shown to enhance the anti-Parkinson's activity of L-DOPA and may have beneficial effects in managing L-DOPA induced dyskinesias (van der Stelt et al. 2005; Cao et al. 2007). Similar to our observation revealing reduced D₂R expression following persistent CB₁R agonist treatment (Przybyla and Watts 2010), studies in rats and humans have shown that chronic exposure to marijuana decreases D₂R levels in the CNS (Walters and Carr 1986; Wang et al. 2004).

A_{2A}, CB₁, and D₂ receptors are often co-expressed on the same striatal neurons. Such expression patterns provide opportunities for a large number of drug-modulated divalent interactions and raise the possibility of higher-order GPCR assemblies of CB₁R, D₂R, and A_{2A}R. Recent BiFC-BRET data support the existence of such complexes (Navarro et al. 2008). The work from our laboratory suggests that both persistent agonist and antagonist treatment could promote or modulate a number of GPCR oligomers including A_{2A}R-A_{2A}R, A_{2A}R-D₂R, CB₁R-D₂R, and D₂R-D₂R (Vidi et al. 2008a; Przybyla and Watts 2010). Biochemical and behavioral studies have also suggested physiological relevant interactions between CB₁, D₂, and A_{2A} receptors (Marcellino et al. 2008) highlighting the potential added complexity of drug effects on GPCR oligomerization. Additional GPCRs may also associate with CB₁R-D₂R-A_{2A}R complexes. For instance, higher-order oligomers of A_{2A}R, D₂R, and mGluR5 receptors have been shown using BiFC-BRET and SRET in live cells, as well as coimmunoprecipitation in rat striatal homogenates (Cabello et al. 2009).

5.2 Interactions involving GPCRs and modulator proteins

Modulators of GPCR signaling can influence receptor expression, targeting to the plasma membrane, receptor internalization, and desensitization. Single transmembrane domain receptor activity-modifying proteins (RAMPs) modulate the trafficking of the calcitonin receptor-like receptor (CRLR) (McLatchie et al. 1998). Using BiFC assays, homomers of either RAMP1 or CRLR were shown to accumulate at the endoplasmic reticulum (ER), whereas CRLR/RAMP1 heteromers (that constitute the functional receptors) were localized at the plasma membrane (Heroux et al. 2007). Information on the stoichiometry of CRLR/RAMP1 complex was gained in BiFC-BRET experiments which suggested that a single RAMP1 molecule associates with a minimum of two CRLR molecules (Heroux et al. 2007).

Arrestins have been shown to modulate GPCR desensitization, notably by promoting ligand-mediated receptor internalization following receptor activation (Gurevich et al. 2008). Ligand-induced interaction between β_2 AR and β -arrestins were detected using BiFC (MacDonald et al. 2006), which demonstrates the potential of the technique to measure receptor activation and subsequent desensitization (see section 6 below). These initial studies were based on the observation that a key step in GPCR desensitization is the phosphorylation of the receptor by G protein-coupled receptor kinases (GRK). The phosphorylated GPCR amino acid residues serve as binding sites for arrestins. BiFC measurements recently confirmed the association of the G protein-coupled receptor kinase 4 (GRK4) with dopamine D₃ receptors (D₃R). It was revealed that the D₃R-GRK4 interaction was initiated at the cell membrane upon D₃R activation and subsequently was found intracellularly after receptor internalization (Villar et al. 2009).

The results of the studies highlighted above show that PCAs provide powerful tools to study not only fundamental interactions involved with GPCR signaling, but provide important information about stoichiometry, localization, and drug modulation of protein-protein interactions.

5.3 Measuring G protein subunit composition and trafficking

A large number of combinations are possible between members of the four families of $G\alpha$ subunits ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12/13}$), the five $G\beta$ and the twelve $G\gamma$ subunits in mammalian cells. Preferential associations between $G\beta$ and $G\gamma$ subunits were evidenced in multicolor BiFC experiments. For instance, a strong $G\beta_5/\gamma_2$ interaction that correlated with robust phospholipase C activity was detected (Yost et al. 2007). It is also well established that the activity of G proteins can be regulated by a class of multifunctional proteins known as regulator of G protein signaling (RGS) proteins. Multicolor BiFC experiments revealed a stronger $G\beta_5$ - $G\gamma_2$ compared to $G\beta_5$ -RGS7 interaction that was reverted in the presence of the R7 family binding protein (R7BP) (Yost et al. 2007). Insight regarding G protein subunit trafficking has also been gained using BiFC. The results show that association of G protein subunits is necessary for their targeting to the plasma membrane. Expression of BiFC-tagged $G\beta_1$ and $G\gamma_7$ dimers promoted plasma membrane targeting of CFP-tagged $G\alpha_s$ (Hynes et al. 2004; Mervine et al. 2006). Moreover plasma membrane localization of BiFC-tagged $G\beta_5/\gamma_2$ dimers was observed upon co-expression of $G\alpha_o$ or $G\alpha_q$ subunits (Yost et al. 2007). Recent work has suggested a significant signaling and functional specificity for individual $G\beta$ and $G\gamma$ subunits (Dupre et al. 2009; McIntire 2009). Moreover, it is increasingly clear that the localization of $G\beta\gamma$ subunits has important implications for their cellular functions including their ability to modulate GPCR signaling (for review, see Dupre et al. 2009; McIntire 2009). These recent discoveries involving the $G\beta\gamma$ subunits signaling highlight the numerous applications of BiFC or other PCAs to study the role(s) of $G\beta\gamma$ subunit in GPCR-mediated signaling.

5.4 Interactions involving adenylyl cyclases

As presented above, BiFC and other fluorescent techniques have been used extensively to probe for interactions between GPCRs as well as between G proteins. However, the opportunities of using BiFC to explore multi-protein complexes involved in GPCR-mediated signaling are immense. It has been observed that the β_2 AR can form a complex with multiple signaling components like G proteins, adenylyl cyclases (ACs), and ion channels by co-IP (Davare et al. 2001; Lavine et al. 2002) as well as BRET (Lavine et al. 2002; Rebois et al. 2008). Additionally, scaffolding proteins have been shown to tether multiple signaling proteins such as GPCRs, ACs, G proteins and ion channels in signaling complexes to coordinate GPCR mediated signaling (reviewed in Dessauer 2009). In a study examining the membrane distributions of β_2 AR signaling molecules, the interactions between β_2 AR and AC5 were measured using BRET (Pontier et al. 2008). In a very recent study, FRET was employed to measure the interaction between fluorescently tagged AC5 and $G\beta\gamma$ (Sadana et al. 2009). Collectively, GPCR-mediated signaling can be facilitated by the formation of multi-protein signaling complexes or signalosomes where the ACs may play a central role as a “signal integrator” between surface receptors, G proteins and appropriate intracellular pathways. Notably, several of the studies mentioned above have demonstrated the use of fluorescent techniques to study protein-protein interactions involving the ACs, which provides an excellent foundation for additional studies using fluorescently tagged ACs. In preliminary studies, we have used BiFC to detect interactions both between AC9 and different GPCRs as well as AC9 and G-protein subunits (Przybyla, Ejendal and Watts, unpublished observations). Possible future avenues include using BiFC to detect changes and the localization of protein-protein interactions involving ACs in response to GPCR modulation or under conditions invoking heterologous sensitization (see below).

Several lines of evidence suggest that ACs can dimerize and that such dimers or oligomers may be homomeric or composed of more than one AC isoform. In an early study, AC1 was shown to homodimerize, as inactive epitope-tagged AC1 can co-IP with active AC1 (Tang et al. 1995). Further, homodimers of truncated AC8 have been detected by FRET and homodimerization of AC8 has been shown indirectly by functional assays, where non-functional AC8 inhibit the function of full-length AC8 (Gu et al. 2002). Furthermore, the study also showed that co-expression of the inactive AC8 attenuate the activity of AC5 and AC6, suggesting that AC8 can form heterodimers with both AC5 and AC6 (Gu et al. 2002). Similar to GPCRs, oligomerization of ACs offers an additional mode of regulation of AC activity, which may be crucial during certain conditions for the cell to integrate and respond to various stimuli. In a recent study using BRET, it was observed that AC2 and AC5 can heteromerize, and that heteromerization confers unique features to the AC2-AC5 complex that differ from the corresponding homomers (Baragli et al. 2008). Further, in a study of both endogenously expressed and overexpressed ACs in a neuronal cell model, it was observed that increased expression of AC9 correlates with the inhibition of AC6 activity, suggesting that AC9 may be a negative regulator of AC6, possibly by heteromer formation (Johnston et al. 2004). The finding that AC9 may function as a negative regulator is in agreement with an earlier report (Hacker et al. 1998). To directly examine AC-AC interactions, we have used AC9 fused to either the Vc or Vn fragments of Venus (see section 2). When co-expressed, these BiFC tagged fusion proteins result in robust complementation of the Venus signal, indicating AC9-AC9 homodimerization (Ejendal and Watts, unpublished data). These preliminary studies show that in addition to BRET, BiFC is a powerful method to detect AC-AC interactions, and set the stage for future BiFC studies with additional AC isoforms.

6 Applications of BiFC in high content screens

In addition to being a useful tool to detect interactions between known (or identified) partners, the BiFC assay has the potential to be used to screen libraries for novel interacting partners of a protein of interest (Ding et al. 2006; MacDonald et al. 2006; Morell et al. 2008). Our laboratory has had a long standing interest in understanding the potential protein-protein interactions involved D₂R induced heterologous sensitization of AC (Watts and Neve 2005). Heterologous sensitization (a.k.a. cyclic AMP overshoot, supersensitivity, superactivation, and supersensitization) of AC was first described in 1975 in the laboratory of Dr. Marshall Nirenberg as a mechanism of opiate tolerance and dependence (Sharma et al. 1975). Briefly, heterologous sensitization of AC occurs following prolonged activation of inhibitory receptors (G $\alpha_{i/o}$ -coupled receptors such as the D₂R or μ opioid receptors) and leads to sensitized or exaggerated cyclic AMP signaling following removal of the inhibitory signal. Using traditional evidence-based approaches, significant, but limited successes have been documented in the search for the mechanism of heterologous sensitization during the last 35 years. The use of a BiFC-based screen would represent a novel approach to identify the proteins involved heterologous sensitization (i.e. the “sensitization interactome”). The sensitization interactome would be defined as protein-protein interactions with AC that occur following persistent activation of D₂ receptors. As illustrated in Figure 3, the AC would be the bait protein tagged with Vn and a Vc-tagged library would be used to screen for interacting proteins. These assays would be completed in living cells and under the conditions (i.e. during drug treatment) in which heterologous sensitization is observed. There are a vast number of drug-modulated protein-protein interactions that could be studied using a very similar approach.

Collectively, high content screening using BiFC could be particularly useful for identification of novel GPCR- or AC- interacting proteins under specific conditions such as the response to acute or persistent GPCR activation. Compared to other screening

approaches such as yeast two-hybrid screening, BiFC-based screens can be conducted under physiological conditions enabling the identification of inducible interactions. Furthermore, the stability of the BiFC complex allows the BiFC-based screening approach to identify transient as well as weak interactions. Obviously one must also consider limitations associated with BiFC for screening such as false positives and the need for appropriate controls (see section 4).

The growing recognition of altered GPCR oligomerization in pathological situations (see discussion above for A_{2A}, D₂, and CB₁R receptor examples) is prompting the need for drugs targeting specific GPCR oligomers. We propose that BiFC, and in particular multicolor BiFC, may be used in high-content screens for small molecules influencing the composition and sub-cellular localization of specific GPCR assemblies.

7 Non-fluorescent complementation assays to study GPCR oligomerization

Similar to complementation of fluorescent proteins, luminescent proteins can be split in two nonfunctional fragments that complement to form a functional luciferase (Remy and Michnick 2006; Paulmurugan and Gambhir 2007; Stefan et al. 2007; Rowe et al. 2009). Using fragments of *R. reniformis* luciferase, BiLC, was employed to detect D₂R homodimers, and BiLC in combination with Venus fluorescent protein or Vn and Vc BiFC fragments, BiLC was used to demonstrate D₂R trimeric and tetrameric complexes, respectively (Guo et al. 2008). Dimers of β_2 AR have also been detected using complementing fragments of *G. princeps*, and BiLC in combination with BiFC were used to detect homotetramers of β_2 AR (Rebois et al. 2008).

Furthermore, non-fluorescent enzyme fragment complementation (EFC) assays have also been used to study activation and heterodimerization of GPCRs as well as high content screens (Eglen 2002, 2007; Zhao et al. 2008). This technology relies on complementation of two fragments of the β -galactosidase enzyme, which yields an active enzyme that converts a non-fluorescent substrate to a fluorescent product. A small fragment of β -galactosidase is fused to one of the GPCRs of interest and the other part of β -galactosidase is fused to β -arrestin. Upon activation of the GPCR, β -arrestin is recruited and complementation of the two fragments of β -galactosidase yields a functional enzyme that is detected. Heterodimerization of GPCRs is studied by introducing an unlabeled GPCR into the cell-based system. Stimulation with an agonist of the second GPCR will activate it, and if it heterodimerizes with the GPCR carrying the fusion tag, activation will result in β -galactosidase activity. This technique allows for a quantitative and specific measure of GPCR activation and heterodimerization. However, compared to multicolor BiFC, EFC does not allow for simultaneous visualization of several dimer compositions. One advantage of EFC over BiFC, FRET and BRET, is that it only one of the GPCRs requires a fusion tag.

8 Concluding remarks and outlooks

GPCR signaling is mediated by protein-protein interactions at multiple levels involving receptors as well as the entire signal transduction cascade. The appreciation of the functional significance of protein-protein interactions within GPCR signalosomes (i.e. complexes of GPCRs, G proteins, and downstream effectors) is growing. Recent studies based on RET and PCA techniques are shedding new light on the composition, localization, and drug-induced changes of protein complexes involved in GPCR signaling. Although the physiological and pharmacological significance of higher-order GPCR complexes remains to be fully elucidated, newly discovered complexes involving GPCRs and their signaling partners (e.g. G proteins, arrestins, and ACs) offer new targets with unique pharmacological properties for drug discovery. PCA techniques, which are amenable to high throughput

screening, represent new tools for the identification of novel pharmacological probes targeting GPCR signalosomes.

Acknowledgments

This work was supported by Purdue University and by the National Institute of Mental Health [Grant MH060397]. We apologize to authors whose work was omitted due to space limitations. The Rab GTPase Sar1 H79G construct was generously provided by Dr. Terence Hebert. We thank Dr. Catherine Berlot for providing the mCherry-MEM plasmid and Dr. Cheng-Deng Hu for providing the BiFC and fluorescent vectors.

Abbreviations

AC	adenylyl cyclase
AR	adrenoceptor
A_{2A}R	adenosine 2A receptor
BiFC	bimolecular fluorescence complementation
BiLC	bimolecular luminescence complementation
BRET	bioluminescence resonance energy transfer
CB₁R	cannabinoid 1 receptor
CNS	central nervous system
CRLR	calcitonin receptor-like receptor
D₂R	dopamine 2 receptor
EFC	enzyme fragment complementation
ER	endoplasmic reticulum
FLIM	fluorescence lifetime imaging
FP	fluorescent protein
FRET	fluorescence (Förster) resonance energy transfer
GFP	green fluorescence protein
GPCR	G protein-coupled receptor
PCA	protein complementation assay
RAMP	receptor activity-modifying protein
RET	resonance energy transfer

References

- Antonelli T, Fuxe K, Agnati L, Mazzoni E, Tanganelli S, Tomasini MC, Ferraro L. Experimental studies and theoretical aspects on A_{2A}/D₂ receptor interactions in a model of Parkinson's disease. Relevance for L-dopa induced dyskinesias. *J Neurol Sci.* 2006; 248:16–22. [PubMed: 16765381]
- Armstrong D, Strange PG. Dopamine D₂ receptor dimer formation: evidence from ligand binding. *J Biol Chem.* 2001; 276:22621–9. [PubMed: 11278324]
- Baragli A, Grieco ML, Trieu P, Villeneuve LR, Hebert TE. Heterodimers of adenylyl cyclases 2 and 5 show enhanced functional responses in the presence of Galphas. *Cell Signal.* 2008; 20:480–92. [PubMed: 18164588]
- Bulenger S, Marullo S, Bouvier M. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci.* 2005; 26:131–7. [PubMed: 15749158]

- Cabello N, Gandia J, Bertarelli DC, Watanabe M, Lluís C, Franco R, Ferre S, Lujan R, Ciruela F. Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. *J Neurochem*. 2009; 109:1497–507. [PubMed: 19344374]
- Canals M, Burgueno J, Marcellino D, Cabello N, Canela EI, Mallol J, Agnati L, Ferre S, Bouvier M, Fuxe K, Ciruela F, Lluís C, Franco R. Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Neurochem*. 2004; 88:726–34. [PubMed: 14720222]
- Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Agnati LF, Woods AS, Ferre S, Lluís C, Bouvier M, Franco R. Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem*. 2003; 278:46741–9. [PubMed: 12933819]
- Cao X, Liang L, Hadcock JR, Iredale PA, Griffith DA, Menniti FS, Factor S, Greenamyre JT, Papa SM. Blockade of cannabinoid type 1 receptors augments the antiparkinsonian action of levodopa without affecting dyskinesias in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated rhesus monkeys. *J Pharmacol Exp Ther*. 2007; 323:318–26. [PubMed: 17630359]
- Carriba P, Navarro G, Ciruela F, Ferre S, Casado V, Agnati L, Cortes A, Mallol J, Fuxe K, Canela EI, Lluís C, Franco R. Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat Methods*. 2008; 5:727–33. [PubMed: 18587404]
- Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW. A beta2 adrenergic receptor signaling complex assembled with the Ca²⁺ channel Cav1.2. *Science*. 2001; 293:98–101. [PubMed: 11441182]
- Dessauer CW. Adenylyl cyclase--a-kinase anchoring protein complexes: the next dimension in cAMP signaling. *Mol Pharmacol*. 2009; 76:935–41. [PubMed: 19684092]
- Ding Z, Liang J, Lu Y, Yu Q, Songyang Z, Lin SY, Mills GB. A retrovirus-based protein complementation assay screen reveals functional AKT1-binding partners. *Proc Natl Acad Sci U S A*. 2006; 103:15014–9. [PubMed: 17018644]
- Dupre DJ, Robitaille M, Rebois RV, Hebert TE. The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. *Annu Rev Pharmacol Toxicol*. 2009; 49:31–56. [PubMed: 18834311]
- Eglen RM. Enzyme fragment complementation: a flexible high throughput screening assay technology. *Assay Drug Dev Technol*. 2002; 1:97–104. [PubMed: 15090161]
- Eglen RM. Assessing GPCR activation using protein complementation: a novel technique for HTS. *Biochem Soc Trans*. 2007; 35:746–8. [PubMed: 17635139]
- Fan JY, Cui ZQ, Wei HP, Zhang ZP, Zhou YF, Wang YP, Zhang XE. Split mCherry as a new red bimolecular fluorescence complementation system for visualizing protein-protein interactions in living cells. *Biochem Biophys Res Commun*. 2008; 367:47–53. [PubMed: 18158915]
- Ferre S, Goldberg SR, Lluís C, Franco R. Looking for the role of cannabinoid receptor heteromers in striatal function. *Neuropharmacology*. 2009; 56(Suppl 1):226–34. [PubMed: 18691604]
- Fuxe K, Canals M, Torvinen M, Marcellino D, Terasmaa A, Genedani S, Leo G, Guidolin D, Diaz-Cabiale Z, Rivera A, Lundstrom L, Langel U, Narvaez J, Tanganelli S, Lluís C, Ferre S, Woods A, Franco R, Agnati LF. Intramembrane receptor-receptor interactions: a novel principle in molecular medicine. *J Neural Transm*. 2007; 114:49–75. [PubMed: 17066251]
- Galperin E, Verkusha VV, Sorkin A. Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nat Methods*. 2004; 1:209–17. [PubMed: 15782196]
- Gandia J, Galino J, Amaral OB, Soriano A, Lluís C, Franco R, Ciruela F. Detection of higher-order G protein-coupled receptor oligomers by a combined BRET-BiFC technique. *FEBS Lett*. 2008; 582:2979–84. [PubMed: 18675812]
- Gazi L, Lopez-Gimenez JF, Rudiger MP, Strange PG. Constitutive oligomerization of human D2 dopamine receptors expressed in *Spodoptera frugiperda* 9 (Sf9) and in HEK293 cells. Analysis using co-immunoprecipitation and time-resolved fluorescence resonance energy transfer. *Eur J Biochem*. 2003; 270:3928–38. [PubMed: 14511374]
- Giese B, Roderburg C, Sommerauer M, Wortmann SB, Metz S, Heinrich PC, Muller-Newen G. Dimerization of the cytokine receptors gp130 and LIFR analysed in single cells. *J Cell Sci*. 2005; 118:5129–40. [PubMed: 16254248]

- Glass M, Felder CC. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J Neurosci.* 1997; 17:5327–33. [PubMed: 9204917]
- Gu C, Cali JJ, Cooper DM. Dimerization of mammalian adenylate cyclases. *Eur J Biochem.* 2002; 269:413–21. [PubMed: 11856299]
- Guo W, Shi L, Filizola M, Weinstein H, Javitch JA. Crosstalk in G protein-coupled receptors: changes at the transmembrane homodimer interface determine activation. *Proc Natl Acad Sci U S A.* 2005; 102:17495–500. [PubMed: 16301531]
- Guo W, Urizar E, Kralikova M, Mobarec JC, Shi L, Filizola M, Javitch JA. Dopamine D2 receptors form higher order oligomers at physiological expression levels. *Embo J.* 2008; 27:2293–2304. [PubMed: 18668123]
- Gurevich VV, Gurevich EV, Cleghorn WM. Arrestins as multi-functional signaling adaptors. *Handb Exp Pharmacol.* 2008;15–37. [PubMed: 18491047]
- Hacker BM, Tomlinson JE, Wayman GA, Sultana R, Chan G, Villacres E, Distech C, Storm DR. Cloning, chromosomal mapping, and regulatory properties of the human type 9 adenylyl cyclase (ADCY9). *Genomics.* 1998; 50:97–104. [PubMed: 9628827]
- Heroux M, Hogue M, Lemieux S, Bouvier M. Functional calcitonin gene-related peptide receptors are formed by the asymmetric assembly of a calcitonin receptor-like receptor homo-oligomer and a monomer of receptor activity-modifying protein-1. *J Biol Chem.* 2007; 282:31610–20. [PubMed: 17785463]
- Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluís C, Franco R, Ferre S, Fuxe K. Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem.* 2002; 277:18091–7. [PubMed: 11872740]
- Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell.* 2002; 9:789–98. [PubMed: 11983170]
- Hu CD, Grinberg AV, Kerppola TK. Visualization of protein interactions in living cells using bimolecular fluorescence complementation (BiFC) analysis. *Curr Protoc Cell Biol.* 2006; Chapter 21(Unit 21):3. [PubMed: 18228482]
- Hu CD, Kerppola TK. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol.* 2003; 21:539–45. [PubMed: 12692560]
- Hynes TR, Mervine SM, Yost EA, Sabo JL, Berlot CH. Live cell imaging of Gs and the beta2-adrenergic receptor demonstrates that both alphas and beta1gamma7 internalize upon stimulation and exhibit similar trafficking patterns that differ from that of the beta2-adrenergic receptor. *J Biol Chem.* 2004; 279:44101–12. [PubMed: 15297467]
- Hynes TR, Yost E, Mervine S, Berlot CH. Multicolor BiFC analysis of competition among G protein beta and gamma subunit interactions. *Methods.* 2008; 45:207–13. [PubMed: 18586104]
- Jarrahan A, Watts VJ, Barker EL. D2 dopamine receptors modulate Galpha-subunit coupling of the CB1 cannabinoid receptor. *J Pharmacol Exp Ther.* 2004; 308:880–6. [PubMed: 14634050]
- Johnston CA, Beazely MA, Bilodeau ML, Andrisani O, Watts VJ. Differentiation-induced alterations in cyclic AMP signaling in the Cath.a differentiated (CAD) neuronal cell line. *J Neurochem.* 2004; 88:1497–508. [PubMed: 15009651]
- Kamiya T, Saitoh O, Yoshioka K, Nakata H. Oligomerization of adenosine A2A and dopamine D2 receptors in living cells. *Biochem Biophys Res Commun.* 2003; 306:544–9. [PubMed: 12804599]
- Kearn CS, Blake-Palmer K, Daniel E, Mackie K, Glass M. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol Pharmacol.* 2005; 67:1697–704. [PubMed: 15710746]
- Kerppola TK. Visualization of molecular interactions by fluorescence complementation. *Nat Rev Mol Cell Biol.* 2006; 7:449–56. [PubMed: 16625152]
- Lavigne N, Ethier N, Oak JN, Pei L, Liu F, Trieu P, Rebois RV, Bouvier M, Hebert TE, Van Tol HH. G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. *J Biol Chem.* 2002; 277:46010–9. [PubMed: 12297500]

- Lee SP, O'Dowd BF, Ng GY, Varghese G, Akil H, Mansour A, Nguyen T, George SR. Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. *Mol Pharmacol.* 2000; 58:120–8. [PubMed: 10860933]
- Lee SP, O'Dowd BF, Rajaram RD, Nguyen T, George SR. D2 dopamine receptor homodimerization is mediated by multiple sites of interaction, including an intermolecular interaction involving transmembrane domain 4. *Biochemistry.* 2003; 42:11023–31. [PubMed: 12974638]
- Lin MZ, McKeown MR, Ng HL, Aguilera TA, Shaner NC, Campbell RE, Adams SR, Gross LA, Ma W, Alber T, Tsien RY. Autofluorescent proteins with excitation in the optical window for intravital imaging in mammals. *Chem Biol.* 2009; 16:1169–79. [PubMed: 19942140]
- Lopez-Gimenez JF, Canals M, Pediani JD, Milligan G. The $\alpha 1b$ -adrenoceptor exists as a higher-order oligomer: effective oligomerization is required for receptor maturation, surface delivery, and function. *Mol Pharmacol.* 2007; 71:1015–29. [PubMed: 17220353]
- MacDonald ML, Lamerdin J, Owens S, Keon BH, Bilter GK, Shang Z, Huang Z, Yu H, Dias J, Minami T, Michnick SW, Westwick JK. Identifying off-target effects and hidden phenotypes of drugs in human cells. *Nat Chem Biol.* 2006; 2:329–37. [PubMed: 16680159]
- Marcellino D, Carriba P, Filip M, Borgkvist A, Frankowska M, Bellido I, Tanganelli S, Muller CE, Fisone G, Lluís C, Agnati LF, Franco R, Fuxe K. Antagonistic cannabinoid CB1/dopamine D2 receptor interactions in striatal CB1/D2 heteromers. A combined neurochemical and behavioral analysis. *Neuropharmacology.* 2008; 54:815–23. [PubMed: 18262573]
- Marullo S, Bouvier M. Resonance energy transfer approaches in molecular pharmacology and beyond. *Trends Pharmacol Sci.* 2007; 28:362–5. [PubMed: 17629577]
- McIntire WE. Structural determinants involved in the formation and activation of G protein betagamma dimers. *Neurosignals.* 2009; 17:82–99. [PubMed: 19212142]
- McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature.* 1998; 393:333–9. [PubMed: 9620797]
- Mercier JF, Salahpour A, Angers S, Breit A, Bouvier M. Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem.* 2002; 277:44925–31. [PubMed: 12244098]
- Mervine SM, Yost EA, Sabo JL, Hynes TR, Berlot CH. Analysis of G protein betagamma dimer formation in live cells using multicolor bimolecular fluorescence complementation demonstrates preferences of beta1 for particular gamma subunits. *Mol Pharmacol.* 2006; 70:194–205. [PubMed: 16641313]
- Milligan G. G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *Br J Pharmacol.* 2009; 158:5–14. [PubMed: 19309353]
- Milligan G, Bouvier M. Methods to monitor the quaternary structure of G protein-coupled receptors. *Febs J.* 2005; 272:2914–25. [PubMed: 15955052]
- Morell M, Espargaro A, Aviles FX, Ventura S. Study and selection of in vivo protein interactions by coupling bimolecular fluorescence complementation and flow cytometry. *Nat Protoc.* 2008; 3:22–33. [PubMed: 18193018]
- Morelli M, Di Paolo T, Wardas J, Calon F, Xiao D, Schwarzschild MA. Role of adenosine A2A receptors in parkinsonian motor impairment and l-DOPA-induced motor complications. *Prog Neurobiol.* 2007; 83:293–309. [PubMed: 17826884]
- Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol.* 2002; 20:87–90. [PubMed: 11753368]
- Navarro G, Carriba P, Gandia J, Ciruela F, Casado V, Cortes A, Mallol J, Canela EI, Lluís C, Franco R. Detection of heteromers formed by cannabinoid CB1, dopamine D2, and adenosine A2A G-protein-coupled receptors by combining bimolecular fluorescence complementation and bioluminescence energy transfer. *ScientificWorldJournal.* 2008; 8:1088–97. [PubMed: 18956124]
- Nimchinsky EA, Hof PR, Janssen WG, Morrison JH, Schmauss C. Expression of dopamine D3 receptor dimers and tetramers in brain and in transfected cells. *J Biol Chem.* 1997; 272:29229–37. [PubMed: 9361002]

- Park PS, Wells JW. Oligomeric potential of the M2 muscarinic cholinergic receptor. *J Neurochem.* 2004; 90:537–48. [PubMed: 15255931]
- Paulmurugan R, Gambhir SS. Combinatorial library screening for developing an improved split-firefly luciferase fragment-assisted complementation system for studying protein-protein interactions. *Anal Chem.* 2007; 79:2346–53. [PubMed: 17295448]
- Pin JP, Neubig R, Bouvier M, Devi L, Filizola M, Javitch JA, Lohse MJ, Milligan G, Palczewski K, Parmentier M, Spedding M. International Union of Basic and Clinical Pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol Rev.* 2007; 59:5–13. [PubMed: 17329545]
- Pontier SM, Percherancier Y, Galandrin S, Breit A, Gales C, Bouvier M. Cholesterol-dependent separation of the beta2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. *J Biol Chem.* 2008; 283:24659–72. [PubMed: 18566454]
- Przybyla JA, Watts VJ. Ligand-induced regulation and localization of cannabinoid CB1 and dopamine D2L receptor heterodimers. *J Pharmacol Exp Ther.* 2010; 332:710–9. [PubMed: 20016021]
- Rebois RV, Robitaille M, Gales C, Dupre DJ, Baragli A, Trieu P, Ethier N, Bouvier M, Hebert TE. Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. *J Cell Sci.* 2006; 119:2807–18. [PubMed: 16787947]
- Rebois RV, Robitaille M, Petrin D, Zylbergold P, Trieu P, Hebert TE. Combining protein complementation assays with resonance energy transfer to detect multipartner protein complexes in living cells. *Methods.* 2008; 45:214–8. [PubMed: 18586102]
- Remy I, Michnick SW. A highly sensitive protein-protein interaction assay based on Gaussia luciferase. *Nat Methods.* 2006; 3:977–9. [PubMed: 17099704]
- Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol.* 2004; 22:445–9. [PubMed: 14990965]
- Rowe L, Dikici E, Daunert S. Engineering bioluminescent proteins: expanding their analytical potential. *Anal Chem.* 2009; 81:8662–8. [PubMed: 19725502]
- Sadana R, Dascal N, Dessauer CW. N terminus of type 5 adenylyl cyclase scaffolds its heterotrimer. *Mol Pharmacol.* 2009; 76:1256–64. [PubMed: 19783621]
- Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci.* 2006; 29:647–54. [PubMed: 17030429]
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol.* 2004; 22:1567–72. [PubMed: 15558047]
- Sharma SK, Klee WA, Nirenberg M. Dual regulation of adenylyl cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A.* 1975; 72:3092–6. [PubMed: 1059094]
- Shu X, Royant A, Lin MZ, Aguilera TA, Lev-Ram V, Steinbach PA, Tsien RY. Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. *Science.* 2009; 324:804–7. [PubMed: 19423828]
- Shyu YJ, Hiatt SM, Duren HM, Ellis RE, Kerppola TK, Hu CD. Visualization of protein interactions in living *Caenorhabditis elegans* using bimolecular fluorescence complementation analysis. *Nat Protoc.* 2008a; 3:588–96. [PubMed: 18388940]
- Shyu YJ, Hu CD. Fluorescence complementation: an emerging tool for biological research. *Trends Biotechnol.* 2008; 26:622–30. [PubMed: 18804297]
- Shyu YJ, Liu H, Deng X, Hu CD. Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *Biotechniques.* 2006; 40:61–6. [PubMed: 16454041]
- Shyu YJ, Suarez CD, Hu CD. Visualization of AP-1 NF-kappaB ternary complexes in living cells by using a BiFC-based FRET. *Proc Natl Acad Sci U S A.* 2008b; 105:151–6. [PubMed: 18172215]
- Stefan E, Aquin S, Berger N, Landry CR, Nyfeler B, Bouvier M, Michnick SW. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. *Proc Natl Acad Sci U S A.* 2007; 104:16916–21. [PubMed: 17942691]
- Tang WJ, Stanzel M, Gilman AG. Truncation and alanine-scanning mutants of type I adenylyl cyclase. *Biochemistry.* 1995; 34:14563–72. [PubMed: 7578062]

- van der Stelt M, Fox SH, Hill M, Crossman AR, Petrosino S, Di Marzo V, Brotchie JM. A role for endocannabinoids in the generation of parkinsonism and levodopa-induced dyskinesia in MPTP-lesioned non-human primate models of Parkinson's disease. *FASEB J*. 2005; 19:1140–2. [PubMed: 15894565]
- Vidi PA, Chemel BR, Hu CD, Watts VJ. Ligand-Dependant Oligomerization of Dopamine D2 and Adenosine A2A Receptors in Living Neuronal Cells. *Mol Pharmacol*. 2008a; 74:544–551. [PubMed: 18524886]
- Vidi PA, Chen J, Irudayaraj JM, Watts VJ. Adenosine A(2A) receptors assemble into higher-order oligomers at the plasma membrane. *FEBS Lett*. 2008b; 582:3985–3990. [PubMed: 19013155]
- Vidi PA, Przybyla JA, Hu CD, Watts VJ. Visualization of G protein-coupled receptor (GPCR) interactions in living cells using bimolecular fluorescence complementation (BiFC). *Curr Protoc Neurosci*. 2010; Chapter 5(Unit 5):29. [PubMed: 20373507]
- Vidi PA, Watts VJ. Fluorescent and bioluminescent protein-fragment complementation assays in the study of G protein-coupled receptor oligomerization and signaling. *Mol Pharmacol*. 2009; 75:733–9. [PubMed: 19141658]
- Villar VA, Jones JE, Armando I, Palmes-Saloma C, Yu P, Pascua AM, Keever L, Arnaldo FB, Wang Z, Luo Y, Felder RA, Jose PA. G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. *J Biol Chem*. 2009; 284:21425–34. [PubMed: 19520868]
- Vortherms TA, Watts VJ. Sensitization of neuronal A2A adenosine receptors after persistent D2 dopamine receptor activation. *J Pharmacol Exp Ther*. 2004; 308:221–7. [PubMed: 14566008]
- Walters DE, Carr LA. Changes in brain catecholamine mechanisms following perinatal exposure to marihuana. *Pharmacol Biochem Behav*. 1986; 25:763–8. [PubMed: 2878443]
- Wang X, Dow-Edwards D, Anderson V, Minkoff H, Hurd YL. In utero marijuana exposure associated with abnormal amygdala dopamine D2 gene expression in the human fetus. *Biol Psychiatry*. 2004; 56:909–15. [PubMed: 15601599]
- Watanabe N, Bodin L, Pandey M, Krause M, Coughlin S, Boussiotis VA, Ginsberg MH, Shattil SJ. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin $\alpha\text{IIb}\beta\text{3}$. *J Cell Biol*. 2008; 181:1211–22. [PubMed: 18573917]
- Watts VJ, Neve KA. Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. *Pharmacol Ther*. 2005; 106:405–21. [PubMed: 15922020]
- Wreggett KA, Wells JW. Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J Biol Chem*. 1995; 270:22488–99. [PubMed: 7673239]
- Yost EA, Mervine SM, Sabo JL, Hynes TR, Berlot CH. Live cell analysis of G protein $\beta\gamma$ complex formation, function, and targeting. *Mol Pharmacol*. 2007; 72:812–25. [PubMed: 17596375]
- Zhao X, Jones A, Olson KR, Peng K, Wehrman T, Park A, Mallari R, Nebalasca D, Young SW, Xiao SH. A homogeneous enzyme fragment complementation-based beta-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. *J Biomol Screen*. 2008; 13:737–47. [PubMed: 18660457]

Figure 1.

BiFC and multicolor BiFC to visualize GPCR oligomerization.

Principle of BiFC (**A**) and multicolor BiFC (**B**). N- or C- terminal fragments (n or c) of fluorescence proteins (e.g. Venus, V and Cerulean, C) are fused to the carboxy-termini of the receptors (X, Y, and Z). Receptor dimerization leads to the complementation of functional FPs. (**C**) Multicolor BiFC for the simultaneous detection of A_{2A}R homomers and A_{2A}R/D₂R heteromers in neuronal cells. Prolonged treatment with quinpirole, a D₂R agonist, leads to decreased Venus (Vn/Cc) over Cerulean (Cn/Cc) fluorescence, interpreted as an alteration in GPCR homo-/heteromer abundance (Adapted from Vidi et al. 2008a). (**D**) Drug-induced changes in the composition and sub-cellular localization of CB₁R homomers and CB₁R/D₂R heteromers following persistent activation of CB₁R with CP55,940 (Adapted from (Przybyla and Watts 2010)).

Figure 2.

Applications of combined fluorescence techniques to study receptor trafficking. **(A)** The expression of a dominant negative COPII Rab GTPase, Sar1 H79G, can be used as a tool to prevent GPCR export from the endoplasmic reticulum (ER). A_{2A}R fused to the Venus fluorescent protein was expressed in the CAD neuronal cell model. Plasma membrane expression of A_{2A}R was inhibited upon coexpression of Sar1 H79G (arrows). The N-terminal fragment of GAP43 fused to mCherry was used to label the plasma membrane (mCherry-MEM). Scale bar, 5 μ m. **(B)** Fluorescence resonance energy transfer (FRET) measured with fluorescence lifetime imaging in cells expressing A_{2A}R-Cerulean (A_{2A}R-(C)) and A_{2A}R-Venus (A_{2A}R-V), or A_{2A}R-Vn/A_{2A}R-Vc BiFC fusions, as well as Sar1 H79G. Cells were selected for analysis that displayed intracellular signals (gray bars) or plasma membrane signals (black bar). FRET evidenced by decreased Cerulean lifetime was measured in cells expressing A_{2A}R-C and A_{2A}R-V and at the plasma membrane from cells expressing A_{2A}R-C and A_{2A}R-Vn/A_{2A}R-Vc ('trimer'). In contrast, no BiFC-FRET signal was detected in intracellular compartments. The data suggest that GPCR dimers may be trafficked from the ER and assemble into higher-order oligomers at the plasma membrane.

Figure 3.

Possible application of BiFC in high-content screening for novel AC interacting proteins. The 'sensitization interactome' of AC will be examined using viral-mediated BiFC. Stable cell lines coexpressing GPCRs and AC-Vn will be infected with a Vc-tagged retroviral cDNA library. Complementation in the absence of GPCR stimulation represents pre-assembled complexes, constitutive interactions, or potential false positives. In contrast, cells revealing drug-induced complementation suggest the presence of a new interaction that occurs as a result of receptor activation and could represent novel AC-protein interactions relevant to the 'sensitization interactome'.

Table 1

Protein interaction assays available for the study of GPCR signaling.

Assay	Localization	Interaction/Proximity	Multiple interactions
coIP	NO	Interaction	(YES)
FRET	YES	Proximity	(YES)
BRET	NO	Proximity	NO
BiFC	YES	Proximity	YES
BiLC	NO	Proximity	YES *
EFC	NO	Proximity	NO

*
in combination with other fluorescence techniques (section 3)