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Fluorescent and Bioluminescent Protein-fragment Complementation Assays in the Study of G Protein-coupled Receptor Oligomerization and Signaling*

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Abbreviations: AR, adrenoceptor; BiFC, bimolecular fluorescence complementation; BiLC, bimolecular luminescence complementation; BRET, bioluminescence resonance energy transfer; CRLR, calcitonin receptor-like receptor; ER, endoplasmic reticulum; FP, fluorescent protein; FRET, fluorescence (Förster) resonance energy transfer; GPCR, G protein-coupled receptor; PCA, protein complementation assay; RAMP, receptor activity-modifying protein; RET, resonance energy transfer

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Abstract

Most cellular functions, including signaling by G protein-coupled receptors (GPCRs), are mediated by protein-protein interactions, making the identification and localization of protein complexes key to the understanding of cellular processes. In complement to traditional biochemical techniques, non-invasive resonance energy transfer (RET) and protein-fragment complementation assays (PCAs) now allow protein interactions to be detected in the context of living cells. In this review, fluorescent and bioluminescent PCAs are discussed and their application illustrated with studies on GPCR signaling. Newly developed techniques combining PCA and RET assays for the detection of ternary and quaternary protein complexes are also presented.

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Introduction

The identification and localization of protein-protein interactions is central to the understanding of biological processes such as extracellular signal integration, gene expression regulation, as well as most cellular metabolic pathways. Immunoprecipitation (IP) and pull-down assays have been extensively used to demonstrate protein-protein interactions. Although these techniques remain very useful, they necessitate the disruption of biological samples, the solubilization of membrane proteins, and they generally provide little information on the sub-cellular localization of the protein complexes (Milligan and Bouvier, 2005). Fluorescence and bioluminescence resonance energy transfer (FRET and BRET), as well as reporter complementation assays, are non-invasive approaches that overcome some limitations of the classical biochemical techniques. They allow the examination of protein-protein interactions in their native context: in living cells or even in living animals. Moreover, information on the sub-cellular localization of the interaction can be gained with microscopic FRET or bimolecular fluorescence complementation (BiFC) analysis.

Multiple protein-protein interactions play essential roles in signaling events mediated by GPCRs. It is now recognized that oligomerization between GPCRs can modulate the pharmacological characteristics of the receptors and influences their coupling to G proteins (Fuxe et al., 2007; Milligan, 2007; Pin et al., 2007). The interaction between GPCRs and G proteins is also well documented. GPCR activation upon ligand binding induces conformational changes in the receptor structure that promotes a GDP to GTP nucleotide exchange to the α subunit of the interacting trimeric G protein. GTP-bound Gα subunits then dissociate from (or change conformation relative to) the receptor and the β/γ subunits, allowing interactions between Gα as well as Gβ/γ with effector proteins (Lambert, 2008). In contrast to early models where individual components of the system were envisioned as free-diffusing and random-colliding (see Rebois and Hebert, 2003 and references herein), recent evidence indicates the existence of signaling complexes comprising GPCRs, G proteins, and effector proteins such as adenylyl cyclase, phospholipase C, or ion channels (Gales et al., 2006; Rebois and Hebert, 2003; Rebois et al., 2006).

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However, the extent to which these observations may be generalized to the highly diverse repertoire of GPCR signaling remains to be determined. This review focuses on the recent application of PCAs to study GPCR oligomerization as well as interactions involving effector or regulatory proteins. Although the principle of RET techniques is not covered (see Gandia et al., 2008; Marullo and Bouvier, 2007 for recent reviews), new methods combining PCAs and RET techniques for the detection of multi-protein complexes are discussed.

Principle of fluorescent and luminescent PCAs

PCAs have been developed in which fluorescent or luminescent protein-fragments are used for complementation. These assays are also known as bimolecular fluorescence/luminescence complementation (BiFC/BiLC). They both rely on the ability of non-fluorescent/luminescent protein fragments to reconstitute functional fluorescent/luminescent proteins when brought in close contact by fusion partners (Hu et al., 2006; Kerppola, 2006; Remy and Michnick, 2007; Shyu and Hu, 2008). Several fluorescent proteins (FPs) have been successfully split and used in BiFC assays (Fan et al., 2008; Shyu et al., 2006) (Fig. 1A, Table 1). These include Venus (Nagai et al., 2002), Cerulean (Rizzo et al., 2004), and mCherry (Shaner et al., 2004). The BiFC technique is very straightforward in that protein-protein interactions are revealed by simple fluorescence intensity measurements in live cells or in live animals (Shyu et al., 2008a). These may be performed using fluorescence-activated cell sorting (Giese et al., 2005; Watanabe et al., 2008), fluorometry (Hynes et al., 2008; Vidi et al., 2008a), or microscopy (e.g. Hu et al., 2002), the latter providing insights into the sub-cellular localization of the interacting proteins.

Similar to other recombinant protein tagging approaches, the addition of the BiFC/BiLC fragments may disrupt or alter the function and/or localization of their fusion partner. It is therefore essential to test these possibilities with functional assays (e.g. by measuring second messenger levels upon ligand exposure in the case of GPCR-BiFC/BiLC fusions) and by comparing the sub-cellular localization of tagged versus untagged proteins. FP complementation has proven to be irreversible so far and it appears that BiFC essentially locks the protein-protein interaction (Hu et al., 2002). Although this may be an advantage in

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some cases, it is also a major limitation of the technique because dynamic changes in protein complexes can not be examined. Therefore, when designing experiments where BiFC is combined with other methods to detect multiple proteins in a complex (see below); BiFC is preferable for the detection of the more stable interaction. Another potential pitfall is that in some conditions certain FP fragments may reconstitute a FP without an interaction of their fusion partners. To rule out the possibility of nonspecific interactions, negative controls included in BiFC experiments need therefore to be carefully chosen (see http://sitemaker.umich.edu/kerppola.lab/kerppola.bifc/bifc_protocols_ for guidelines). For example, transfections omitting one of the two FP-fragment fusion proteins may serve to determine background fluorescence but does not indicate specificity of BiFC signals. As a suitable negative control, one of the FP-fragment fusion proteins should be replaced by a functional protein with sub-cellular distribution and expression levels similar to that of the tested protein.

Several unique split positions have been used for YFP and CFP (or their enhanced versions, see Table 1) and a similar effectiveness of complementation is achieved with different FP fragment combinations (e.g. VenusN155/VenusC155, VenusN173/VenusC173, or VenusN173/VenusC155) (Shyu et al., 2006). The intensity of the fluorescence complementation signal is, however, consistently weaker than the signal from the corresponding full-length FP under similar transfection conditions, typically 2.5 to 5.5 fold (Vidi and Watts, unpublished observations). This reduction in signal intensity may hinder the detection of receptor-BiFC fusions that are not expressed at sufficient levels. Finally, the composition and length of the linker sequence separating the protein of interest and the FP fragment, as well as the design of the assay (which fragment fused to which partner), may affect the efficiency of fluorescence complementation (Chen et al., 2006; Vidi and Watts, unpublished data).

Multicolor BiFC assays, in which two FPs with distinct excitation and emission spectra are reconstituted, have been implemented for the detection of multiple protein-protein interactions (Hu and Kerppola, 2003). N-terminal BiFC fragments from GFP-derived FPs contain the chromophore, as well as most amino acid residues important for the spectral properties of the FPs (with the notable exception of residue 203 in the C-terminal region). As a consequence, N-terminal fragments from two different FPs, upon

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complementation with a C-terminal FP fragment (e.g. Cerulean C-terminus), reconstitute FPs with distinct excitation and emission maxima, which, as illustrated in Fig. 1B, allows the simultaneous detection of two protein-protein interactions in live cells. Complementation between Venus N-terminal and Cerulean C-terminal fragments (VN/CC) results in green-shifted FPs compared to Venus (Table 1). The absence of tyrosine at position 203, characteristic of Venus and other yellow-shifted GFP-variants (Tsien, 1998), in VN/CC is likely responsible for this spectral shift.

Luminescence complementation is analogous to BiFC in that split, non-luminescent, fragments from *Renilla reniformis* or *Gaussia princeps* luciferases (*R*Luc and *G*Luc) reconstitute luminescent proteins upon interaction of their fusion partners (Remy and Michnick, 2006; Stefan et al., 2007) (Table 1). In contrast to BiFC, BiLC biosensors do not assemble irreversibly and thus the association as well as the dissociation of protein complexes may be detected (Remy and Michnick, 2006; Stefan et al., 2007). Moreover, due to its low background, the technique is well suited for the detection of protein interactions in living animals (Paulmurugan and Gambhir, 2007). In contrast to BiFC, however, BiLC does not currently provide information on the sub-cellular localization of protein complexes.

Application of PCA to study GPCR oligomerization and interactions with modulators

In recent applications of PCAs to GPCR interactions, FP fragments have been genetically fused to the carboxyl end of the receptors (Fig. 1A). Using this approach, homo-oligomers of the α_{1b} adrenoceptor (AR) were detected, consistent with FRET and IP results (Lopez-Gimenez et al., 2007). Interestingly, point mutations in the transmembrane domains I and IV of the α_{1b} AR (predicted to constitute the dimer interface) prevented export to the plasma membrane without completely suppressing α_{1b} AR dimerization as monitored by BiFC, in line with the notion that GPCR oligomerization occurs during their synthesis (Bulenger et al., 2005).

The well-studied interaction (Fuxe et al., 2007) between the adenosine A_{2A} and the dopamine D_2 receptors was also recently examined using BiFC (Vidi et al., 2008a). In this case, multicolor BiFC allowed the

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simultaneous detection of A_{2A} and D_2 homo- and heteromers in neuronal cells. Quantification of fluorescent signals at the cell surface or in intracellular compartments revealed the co-existence and co-localization of A_{2A}/D_2 heteromers and A_{2A} homomers. A ratiometric approach was used to quantitate changes in GPCR oligomerization and it was observed that the relative abundance of A_{2A}/D_2 heteromers and A_{2A} or D_2 homomers was influenced by prolonged stimulation of either receptor (Vidi et al., 2008a) (Fig. 1C).

Proteins interacting with GPCRs have been shown to modulate receptor expression, membrane targeting, and desensitization. Among these, arrestins are well-characterized scaffolding proteins that are notably involved in receptor internalization following ligand activation (Gurevich et al., 2008). As part of a high-content PCA study based on FP complementation, interaction between β-arrestins and the β_2 AR was detected in cells treated with β_2 AR agonists, thus providing a readout of receptor activation and internalization (MacDonald et al., 2006). These studies further demonstrate the applicability of fluorescent PCAs for the detection of drug-induced changes in GPCR interactions. Similarly, non-fluorescent PCAs based on ß-galactosidase fragment complementation (PathHunter™ ß-Arrestin Assay, DiscoveRx Corporation) can also be used for the detection GPCR-β-arrestin interactions.

Single transmembrane domain receptor activity-modifying proteins (RAMPs) are essential modulators of the calcitonin receptor-like receptor (CRLR) functional activity. The association between RAMP1 and CRLR produces the calcitonin gene-related peptide (CGRP) receptor. Both RAMP1 and CRLR homomers were shown to accumulate at the endoplasmic reticulum (ER) in BiFC assay, whereas CRLR/RAMP1 heteromers were localized at the plasma membrane (Heroux et al., 2007), consistent with the involvement of RAMP1 in receptor trafficking (McLatchie et al., 1998). This work highlights the power of fluorescent PCAs to identify the sub-cellular localization of protein-protein interactions. Further insight into the composition of the CGRP receptor was gained by combining BiFC with BRET (see below).

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PCA as readout of G protein subunit composition and trafficking

The functional roles of G proteins in GPCR recognition and effector regulation is largely determined by their α subunits. In addition, specific combinations among the 5 mammalian G protein β and 12 γ subunits may be required for certain GPCR signaling events (Robishaw and Berlot, 2004). Further insight into G protein sub-cellular localization and subunit association preferences and was recently gained with the application of BiFC and multicolor BiFC techniques (see Hynes et al., 2008) for methodological aspects). Dynamic trafficking studies of GPCRs and G protein subunits revealed that both the β_2 AR and the Gαs/β1/γ7 complex are internalized as a consequence of receptor activation, however, the mechanisms and vesicular compartments to which they were trafficked differed (Hynes et al., 2004). The requirement of G protein subunit association for plasma membrane targeting was also addressed using BiFC. Co-expression of BiFC-tagged β1/γ7 dimers lead to plasma membrane localization of CFP-tagged Gαs that otherwise accumulated in the cytosol (Hynes et al., 2004; Mervine et al., 2006). Conversely, expression of Gαo or Gαq was required for plasma membrane targeting of BiFC-tagged β5/γ2 dimers (Yost et al., 2007).

The formation and composition of β/γ complexes was also analyzed using BiFC. Different efficiencies of β5 complex formation with different γ subunits were observed that correlated with signal transduction efficiencies as measured by phospholipase C activity (Yost et al., 2007). The β5/γ2 interaction was found to be the strongest, a finding corroborated with multicolor BiFC competition experiments used to measure the association preference of β and γ subunits (Yost et al., 2007). A similar approach was taken to examine interactions between G protein subunits and alternative binding partners such as the R7 family regulator of G protein signaling 7 (RGS7). A preference of β5 for γ2 over RGS7 was observed which was abolished upon co-expression of the R7 family binding protein (R7BP) (Yost et al., 2007). These experiments highlight the usefulness of BiFC for the detection of less stable protein complexes such as β5/γ2 and to address the role of co-expressed proteins on G protein subunit association.

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Combining techniques to monitor interactions between multiple proteins

A large number of protein-protein interactions involving GPCRs have been documented, but little is known about the relative stoichiometry of GPCR oligomers or of GPCR/G protein/effector complexes. Whereas only two interacting proteins can be detected with most established methods, combining them allows in principle the detection of multiple proteins within a complex. YFP, which has frequently been used in BiFC assays, also constitutes a very popular FRET pair with CFP and serves as an acceptor for *R*Luc in BRET[1] assays, thus allowing BiFC and RET assays to be combined (Fig. 2A and B). In BiFC-BRET assays (Rebois et al., 2006), RET between *R*Luc and complemented YFP indicates a very close proximity of three tagged proteins that can be interpreted as a trivalent protein complex. Similarly, BiFC can be combined with FRET (Shyu et al., 2008b) and in this case, the sub-cellular localization of trivalent complexes can be determined using microscopic FRET measurements.

A BiFC-BRET approach implemented by the Bouvier lab was used to examine the composition of CRLR/RAMP1 complexes and provided evidence for the association of a single RAMP1 molecule with a CRLR homomer (Heroux et al., 2007). BiFC-BRET measurements also demonstrated the existence of A_{2A} homo-oligomers with at least three receptors (Gandia et al., 2008). Subsequent localization studies used BiFC-FRET to demonstrate that higher-order oligomers of A_{2A} receptors occur at their site of action, the plasma membrane (Vidi et al., 2008b). Moreover, inhibiting anterograde protein transport from the ER to the golgi apparatus by treatment with brefeldin A or by expression of a dominant-negative SarI GTPase lead to the accumulation of A_{2A} dimers but not of higher A_{2A} oligomers in intracellular compartments (Vidi and Watts, unpublished). These latter observations suggest the possibility that GPCR dimers are trafficked from the ER to the plasma membrane followed by higher-order oligomerization at the plasma membrane.

Sequential RET approaches have been developed as an alternative to BiFC-RET and applied to GPCR oligomerization. In these approaches, the acceptor from a first RET pair serves as donor for a second RET pair (Fig. 2C and F). By implementing a three-chromophore FRET (3-FRET) protocol (Galperin et al., 2004), Lopez-Gimenez and colleagues (Lopez-Gimenez et al., 2007) showed that recombinant α_{1b} AR

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exists as higher-order oligomers in HEK293 cells. Similarly, complexes of A_{2A}, D_2, and cannabinoid CB_1 receptors were identified using sequential BRET-FRET (SRET) assays (Carriba et al., 2008).

A proof-of-concept for the detection of tetravalent protein complexes with a BiFC-BiLC combination (Fig. 2D) was given by Rebois et al. (Rebois et al., 2008): the β_2 AR was fused to N- or C-terminal fragments from both Venus or *G*Luc and BRET signals were detected in cells expressing the four receptor fusion constructs (Rebois et al., 2008). A similar approach was used to demonstrate D_2 homo-oligomeric complexes with four protomers (Guo et al., 2008), a finding supported by modeling of the two interaction interfaces and confirmed by cross-linking experiments (Guo et al., 2008). In line with earlier pharmacological and biochemical data (Lee et al., 2003; Nimchinsky et al., 1997; Park and Wells, 2004; Wreggett and Wells, 1995), these results suggest that higher-order GPCR oligomers, likely constituted of four protomers, may be a general rule. The physiological and pharmacological significance of tetrameric GPCR complexes remains however largely to be determined.

New evidence for GPCR signaling complexes has also emerged from recent BiFC-BRET studies. The assembly of trimeric G proteins was shown in cells expressing $G\alpha i_1$-*R*Luc and $G\beta_1/G\gamma_2$ fusions to YFP fragments (Dupre et al., 2006). Similarly, the association of the type II adenylyl cyclase (ACII) or the inward-rectifier potassium channel $Kir_{3.1}$ with $G\beta_1/\gamma_2$ was demonstrated in cells expressing ACII-*R*Luc or $Kir_{3.1}$-*R*Luc and $G\beta_1/\gamma_2$ fusions to YFP fragments (Rebois et al., 2006; Rebois et al., 2008). In cells expressing ACII-*R*Luc and $G\beta_1/\gamma_2$ fusions to YFP fragments, activation of co-transfected β_2 AR resulted in stronger BRET signals, possibly reflecting conformational changes within the complex (Rebois et al., 2006). The association between a GPCR, a G protein subunit, and an effector was also revealed in BiFC-BRET assays using cells co-expressing ACII-*R*Luc with FP-fragment fusions to β_2 AR and $G\gamma_2$ (Rebois et al., 2008). These experiments highlight the use of BiFC-BRET as a novel method to study and examine the existence of GPCR signaling complexes.

Despite the strengths of the studies described above, some caution is warranted in the interpretation of results from PCA, RET or combined assays for a variety of reasons. All of these approaches are proximity

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indicators and do not provide direct proof of physical contact between proteins. Such methodologies do not define the total number of interacting proteins, leaving open the possibility of higher-order oligomers. Finally, in combined RET/PCA assays, increasing the number of interacting partners increases the number of combinations for possible interactions (many of which may be "non-productive", i.e. not leading to RET or PCA readouts) and possibly weaker overall RET/PCA signals. This consideration highlights the need for more sensitive RET/PCA techniques and the importance to develop brighter and more stable fluorescent/luminescent proteins.

Conclusions and outlooks

Recent studies applying PCA to GPCR signaling highlight the usefulness of the technique and new investigations using PCA, alone or in combination with other approaches are soon to follow. As discussed above, PCA can be readily combined with RET measurements, but also with fluorescence correlation spectroscopy (Briddon et al., 2008), or with biochemical techniques such as IP (Heroux et al., 2007). In addition, a score of unexplored possibilities can be envisaged. The incorporation of BiFC-tagged proteins in sequential RET experiments would allow the detection of tetrameric complexes (Fig. 2C and F). Moreover, dynamic interactions between GPCR dimers and their localization may be followed with a BiFC-BiFC-FRET experimental setup (Fig. 2E). We also predict the usefulness of BiFC and multicolor BiFC, not only for the visualization and quantification of multiple GPCR interactions (Vidi et al., 2008a), but also for the isolation of oligomeric complexes, because the complementation of fluorescent proteins is likely to stabilize protein-protein interactions (Hu et al., 2002). These efforts are likely to bring new insights into the functional and pharmacological significance of GPCR interactions. Finally, we anticipate a growing importance of PCAs in drug screening efforts (MacDonald et al., 2006) and propose that multicolor BiFC assays may be used to screen for compounds that selectively alter the composition of GPCR oligomers in living cells.

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Footnotes

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Legends for Figures

Figure 1. Application of BiFC to monitor GPCR interactions. **A**, BiFC principle. FP fragments (e.g. Venus N- and C-termini, VN and VC) are fused to the carboxy-termini of the receptors (*a* and *b*). Upon interaction of the tagged receptors, the protein fragments reconstitute a functional FP. **B**, Multicolor BiFC principle. Different receptor interactions lead to the complementation of distinct fluorescent protein variants. Complex formation between receptor *a* (tagged with VN) and *b* (tagged with the C-terminal moiety of Cerulean, CC) reconstitutes a yellow fluorescent protein, whereas interaction between *b* and *c* reconstitutes Cerulean (see Kerppola, 2006 for detailed protocols and general technical considerations). **C**, Application of multicolor BiFC to the simultaneous detection of A_{2A} homomers and A_{2A}/D_2 heteromers in neuronal cells. Prolonged treatment with the D_2 agonist quinpirole resulted in decreased Venus (VN/CC) over Cerulean (CN/CC) fluorescence, interpreted as an alteration in GPCR homo-/heteromer abundance (right). Adapted from Vidi et al., 2008a.

Figure 2. Combination of PCA and RET techniques. Trimeric protein complexes (*a*, *b*, *c*) detected by BiFC-FRET (**A**), or BiFC-BRET (**B**). In both approaches, a reconstituted FP serves as donor or acceptor in a RET pair. In this case, complemented Venus serves as acceptor for Cerulean (**A**) or *R*Luc (**B**). Coelenterazine is used as a substrate for *R*Luc. Maximum excitation and emission wavelengths are indicated. **C**-**F**, Experimental approaches to detect tetrameric protein complexes (*a*, *b*, *c*, *d*; see text for examples). Complemented fluorescent (or luminescent) proteins replace a donor or acceptor in sequential FRET (**C**) or BRET-FRET (**F**) assays. Bioluminescence (**D**) or fluorescence (**E**) resonance energy transfer between two complemented luminescent or fluorescent proteins also allows the detection of tetramers. The approaches illustrated in **C**, **E**, and **F** are theoretical and have, to our knowledge, not been implemented yet.

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Table

Table 1. Fluorescent and bioluminescent protein fragments used in PCA assays.

	Protein	Split position	Ex/Em (nm)	References
BiFC	GFP	157-158	485/500	(Ghosh et al., 2000)
	YFP, Citrine, Venus	154-155; 172-173	515/528	(Hu et al., 2002; Shyu et al., 2006)
	CFP, Cerulean	154-155; 172-173	452/478	(Hu et al., 2002; Shyu et al., 2006)
	Venus/Cerulean	154-155; 172-173	504/513	(Hu and Kerppola, 2003; Shyu et al., 2006)
	mRFP1-Q66T	154-155; 168-169	549/570	(Jach et al., 2006)
	mCherry	159-160	587/610	(Fan et al., 2008)
BiLC	*R*Luc	110-111	Ch/485	(Stefan et al., 2007)
	*G*Luc	93-94	Ch/480	(Remy and Michnick, 2006)

Ex/Em, excitation/emission wavelengths; Ch, Coelenterazine h

Fig. 1

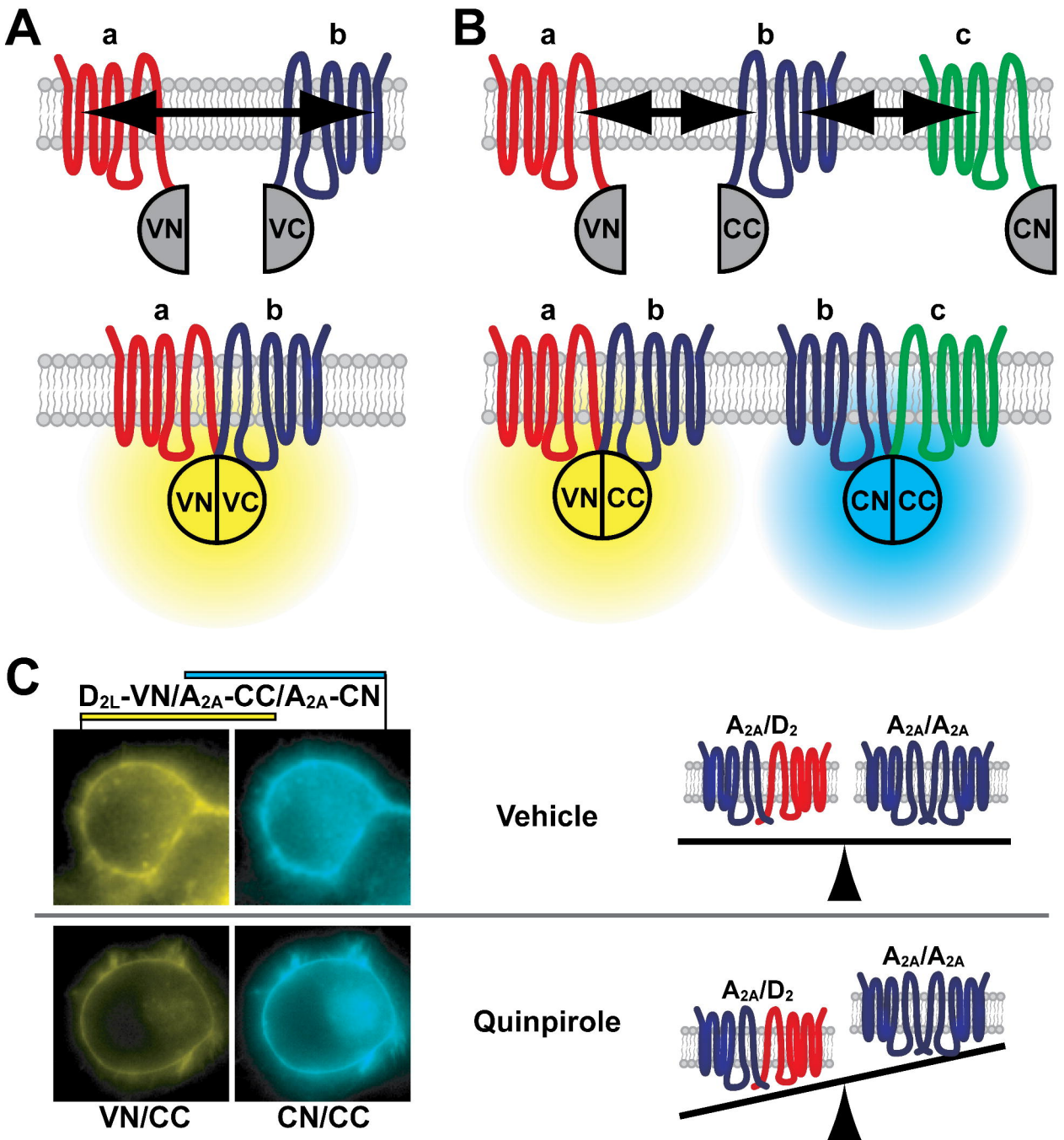
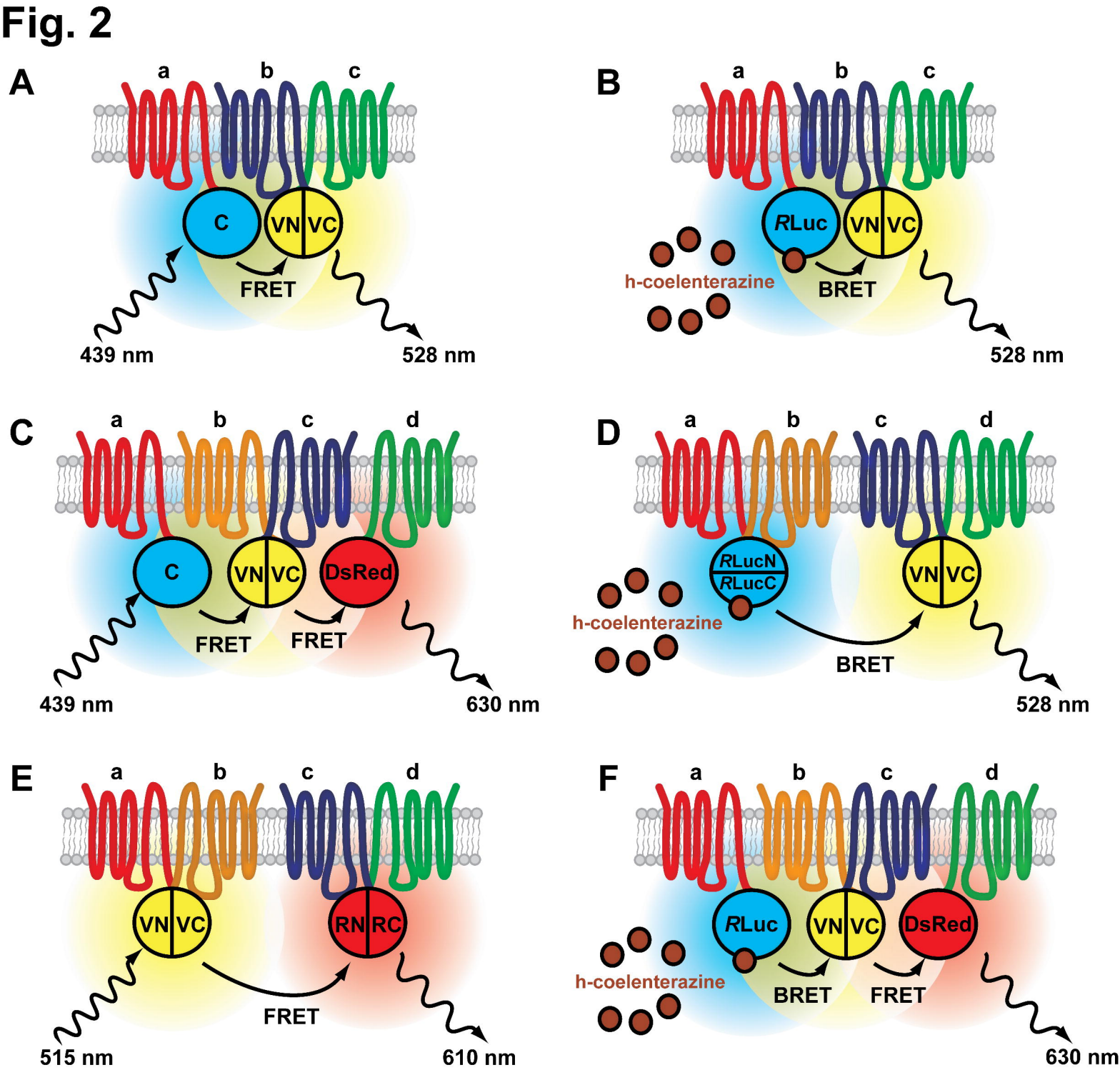

A
a
b
VN
VC
a
b
VN
VC
B
a
b
c
VN
CC
CN
a
b
b
c
VN
CC
CN
CC
C
D2L-VN/A2A-CC/A2A-CN
VN/CC
CN/CC
Vehicle
A2A/D2
A2A/A2A
Quinpirole
A2A/D2
A2A/A2A

Fig. 2


A
a
b
c
C
VN
VC
FRET
439 nm
528 nm
B
a
b
c
RLuc
VN
VC
h-coelenterazine
BRET
528 nm
C
a
b
c
d
C
VN
VC
DsRed
FRET
FRET
439 nm
630 nm
D
a
b
c
d
RLucN
RLucC
VN
VC
h-coelenterazine
BRET
528 nm
E
a
b
c
d
VN
VC
RN
RC
FRET
515 nm
610 nm
F
a
b
c
d
RLuc
VN
VC
DsRed
h-coelenterazine
BRET
FRET
630 nm