Lighting up multiprotein complexes: lessons from GPCR oligomerization

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Abstract

Spatiotemporal characterization of protein–protein interactions (PPIs) is essential in determining the molecular mechanisms of intracellular signaling processes. In this review, we discuss how new methodological strategies derived from non-invasive fluorescence- and luminescence-based approaches (FRET, BRET, BiFC and BiLC), when applied to the study of G protein-coupled receptor (GPCR) oligomerization, can be used to detect specific PPIs in live cells. These technologies alone or in concert with complementary methods (SRET, BRET or BiFC, and SNAP-tag or TR-FRET) can be extremely powerful approaches for PPI visualization, even between more than two proteins. Here we provide a comprehensive update on all the biotechnological aspects, including the strengths and weaknesses, of new fluorescence- and luminescence-based methodologies, with a specific focus on their application for studying PPIs.

Background

Biological processes proceed through a sequence of specific protein–protein interactions (PPIs) along intracellular signaling cascades. Characterization of these interactions is thus essential to the understanding of cellular mechanisms. Using genetic approaches (e.g. yeast two-hybrid screens) [1,2], it is possible to reveal new PPIs, which are subsequently confirmed and validated by additional biochemical approaches, such as immobilized PPI assays (e.g. co-immunoprecipitation and pull-down assays) [3]. The combination of genetic and biochemical approaches is therefore a powerful tool for the identification of novel PPIs. However, this combined approach suffers from two major drawbacks: first, it does not provide spatiotemporal information on a specific PPIs occurring within live cells; and second, it requires mechanical-, chaotropic- or detergent-based cell lysis, which can alter native PPIs [4]. To overcome these drawbacks, new imaging technologies have been described recently that are based on the detection of resonance energy transfer (RET) between fluorescent or luminescent proteins (FPs or LPs) (Box 1) [5] and on protein fragment complementation approaches [6,7], both of which facilitate real-time characterization and visualization of PPIs in live cells.

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Box 1

The RET principle

In the late 1940s, Theodor Förster described the basic principle of RET [81], which consists of nonradiative (dipole–dipole) transfer of energy from an excited chromophore, known as the donor, to an acceptor molecule [82]. This transfer of energy – called Förster resonance energy transfer (FRET) – results in a decrease in donor emission paralleled by an increase in acceptor emission. The efficiency of this energy transfer (E_{RET}) is inversely proportional to the sixth power of the distance (R) between donor and acceptor molecules according to the following equation,

$$E_{RET} = 1/(1 + R^6/R_0^6),$$

where R_0 is the distance for 50% energy transfer from the donor to the acceptor, which is typically approximately 5 nm (the effective range for energy transfer is <10 nm). RET depends not only on the distance between the donor and acceptor molecules, but also on the orientation of their polarization [83]. The sensitivity of RET measurements is within the boundary for conventional protein dimensions [84] and for the distances described for multimeric protein complexes observed in biological systems [85].

RET efficiency also depends on other variables: (i) the orientation angle between donor and acceptor molecules; (ii) the degree of spectral overlap between donor emission and acceptor excitation; (iii) the quantum yield of the donor; and (iv) the extinction coefficient of the acceptor [81,83]. Thus, when choosing two chromophores for a RET process, it is necessary to select those with the highest donor-quantum yield and absorbing acceptor that have significant spectral overlap [86]. For inter- or intramolecular FRET between CFP and YFP, for example, FRET can be monitored as the YFP/CFP emission intensity ratio on excitation at 436 nm according to the following equation:

$$\text{Ratio}\left(\frac{F_{YFP}}{F_{CFP}}\right) = \frac{F_{YFP}^{ex436/em535} - b \times F_{YFP}^{ex500/em535}}{F_{CFP}^{ex436/em480}},$$

where $F_{YFP}^{ex436/em535}$ and $F_{CFP}^{ex436/em480}$ represent the emission intensity for YFP at 535 nm and CFP at 480 nm on excitation at 436 nm; a and b are correction factors for CFP bleedthrough into the 535-nm channel and crosstalk due to the direct YFP excitation by light at 436 nm, respectively. $F_{YFP}^{ex500/em535}$ is the YFP emission intensity at 535 nm on excitation at 500 nm, which is recorded at the beginning of each experiment.

Progress in various fluorescence- and luminescence-based approaches has paralleled the study of G protein-coupled receptor (GPCR) oligomerization. GPCRs are the largest family of cell surface receptors and are the targets of a large array of extracellular chemical (e.g. neurotrans-mitters, hormones) and sensory (light, taste and odorant molecules) stimuli, as well as many clinical drugs (e.g. β-blockers and antipsychotics). Several biochemical and crystal structure studies [8,9] have confirmed that GPCRs comprise seven α-helical transmembrane structures connected by intra- and extracellular loops, with N-terminal extracellular and C-terminal intracellular domains. Receptor activation through binding of a ligand agonist proceeds by conformational rearrangement within the transmembrane helical domain as the receptor switches from an inactive to an active state, which in turn activates heterotrimeric G proteins (Gαβγ) [10,11]. Activated G proteins regulate the levels of intracellular second-messenger molecules (Ca^{2+}, cAMP, phoshoinositides, cGMP), which

modulate signaling cascades involving kinases such as PKA, PKC, PKG and CMKII, among others [12].

Traditionally, it has been considered that GPCRs signal exclusively as single monomeric entities [13]. However, emerging studies have revealed the existence of receptor homo- and heterodimers in many cell types (neuronal, cardiac, endocrine) [14,15]. For example, adenosine A_{2A} and dopamine D_2 receptor heterodimers at the cell surface of striatopallidal GABAergic neurons have opposite effects. Adenosine inhibits dopamine-induced locomotor activity in the basal ganglia [16], thereby exerting finely tuned control of neural activity in response to adenosine and dopamine. The detection of specific receptor–receptor interactions is therefore a first step in understanding how GPCR oligomers modulate cell signaling in response to multiple ligands. Hence, here we review recent developments in FP- and LP-based technologies in the context of GPCR oligomerization, specifically focusing on cases where the receptor oligomers accomplish established recognition and acceptance NC-IUPHAR (the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification) criteria, including evidence for physical association in native tissue or primary cells, demonstration of a specific functional property for the oligomer, and *in vivo* evidence of its existence [17]. The ensuing discussion of GPCR oligomer function in living cells is extended to the study of mutiprotein complexes in their physiological environments.

Applying intermolecular RET to the study of PPIs

Fluorescence RET (FRET)

Classical RET techniques, including fluorescence RET (FRET) and bioluminescence RET (BRET), use the non-radiative transfer of energy (Box 1) between donor and acceptor fluorescent molecules as a measure of their proximity. For example, cyan (CFP) and yellow (YFP) variants of the green fluorescent protein (GFP) can be used for FRET in live cells. When CFP is excited and FRET occurs, CFP emission decreases and YFP emission increases (Figure 1a). The presence of specific receptor homo- or heterocomplexes in transfected cells can therefore be detected by FRET measurements between two receptors, usually tagged at the C-terminus with either CFP (R1^{CFP}) or YFP (R2^{YFP}), because dimer formation between R1^{CFP} and R2^{YFP} causes a RET process between fluorophores (Figure 1a).

For specific receptor–receptor interactions, the efficiency of energy transfer between CFP and YFP can be measured by donor fluorescence recovery after acceptor photobleaching (DFRAP). The efficiency increases as a hyperbolic function of the concentration of R2^{YFP}, as determined by the YFP emission intensity (Figure 1b). However, nonspecific FRET due to random distribution and collisions between CFP and YFP molecules in the plasma membrane can be assessed by the expression of pairs of N-terminal membrane-tagged CFP and YFP molecules [18]. In this case, the FRET efficiency increases as a function of the acceptor molecule concentration; consequently, high non-physiological concentrations of the acceptor are needed to achieve saturation. Thus, prompt saturating behavior is expected for proteins forming specific oligomers, which prevents nonspecific FRET resulting from random collisions of overexpressed fluorescent proteins [19]. Interestingly, the latter issue constitutes a common problem in most RET-based methods. With respect to FRET, several techniques can overcome this drawback; for instance, fluorescence lifetime imaging (FLIM) is largely independent of fluorophore concentrations [20,21], and DFRAP is less sensitive to unbalanced expression levels of fluorescent proteins [22].

Time-resolved FRET

The major limitations encountered with experiments using CFP–YFP as a FRET pair – specifically, crosstalk (direct acceptor excitation by light used to excite the donor; Box 1), bleedthrough (partial overlap of donor and acceptor emission wavelengths) and photobleaching [5] (Table 1) – can be circumvented by time-resolved FRET (TR-FRET) or BRET approaches. TR-FRET uses Eu^{3+} cryptate as a donor molecule and either Alexa Fluor 647 or allophycocyanin (APC) as an acceptor molecule. The advantage of this approach is the long-lived (300–1000 μs) emission fluorescence of Eu^{3+} cryptate, which facilitates prolonged excitation of the acceptor molecule in the absence of an external continuous excitation source [23,24]. In addition, the very weak emission of Eu^{3+} at the acceptor emission wavelength (e.g. 665 nm for APC) considerably reduces bleedthrough [23]. Consequently, this approach can largely increase the signal-to-noise ratio owing to both the long lifetime of Eu^{3+} and negligible bleedthrough emission at the acceptor emission wavelength.

TR-FRET has been used extensively to validate the existence of GPCR oligomers at the surface of living cells using fluorescently labeled antibodies specific for target receptors. This method has provided evidence of constitutive oligomerization of the δ-opioid receptor, with expression of combinations of N-terminal c-Myc- and Flag™-tagged forms of the δ-opioid receptor in HEK293 cells, such as $R1^{Myc}$ and $R2^{Flag}$, respectively (Figure 1c); a Eu^{3+}-labeled (K) anti-c-Myc antibody was used as an energy donor with an APC-labeled anti-Flag™ antibody as an acceptor [25]. It is noteworthy that the major potential limitation for the TR-FRET approach is related to the use of antibodies. Antibody size (150 kDa) can either increase the FRET signal due to random collisions or hamper oligomer assembly. In addition, it is difficult to ascertain if oligomerization of the partners is not promoted by the bivalent nature of the antibodies [26].

Bioluminescence RET

Bioluminescence RET (BRET) depends on the bioluminescent enzyme luciferase from *Renilla reniformis* (*R*luc), rather than CFP, to produce emitted light compatible with YFP excitation [27]. The interactions between receptors R1 and R2 tagged with *R*luc and YFP ($R1^{RLuc}$ and $R2^{YFP}$, respectively) can thus be detected by BRET in a similar manner as described for FRET (Figure 1a). Saturation BRET experiments have been extensively used to characterize specific interactions between GPCRs [26]. Typically, these experiments consist of expression of a constant amount of the donor-labeled protein ($R1^{Rluc}$) with increasing amounts of the acceptor-labeled protein (e.g. $R2^{YFP}$), followed by incubation with the luciferase substrate and detection of the resulting BRET signal [27]. The acceptor concentration that generates a half-maximal BRET signal ($BRET_{50}$) is often used as a parameter to express the relative binding affinity between two receptors. However, $BRET_{50}$ is difficult to interpret if the association between receptors is irreversible.

Although promising single-cell BRET imaging has been recently performed [28,29], subcellular BRET is difficult to detect because the luciferase substrate cannot be dispensed to a specific cellular domain. This issue is highly important when RET-tagged proteins are overexpressed, such as in transient transfection experiments; accumulation of these proteins in intracellular organelles (e.g. endoplasmic reticulum, Golgi apparatus and trafficking vesicles) can lead to nonspecific energy transfer by random collisions, thus hindering positive identification of RET events resulting from direct interaction between proteins at the cell surface [30–32].

PPIs at the surface of a living cell

The difficulties encountered with classical RET techniques for analysis of GPCR homo- and heterocomplex assembly at the cell surface can be circumvented using the SNAP-tag technology (Table 1) [33]. The SNAP-tag method is based on irreversible and specific reaction of the DNA repair protein *O*6-alkylguanine-DNA alkyltransferase (AGT) with *O*6-benzylguanine (BG) derivatives, which can be selectively labeled with a variety of chemical fluorophores (Figure 1c). The first remarkable characteristic of this tool is its high versatility; multiple types of experiments can be performed, such as fluorescence microscopy applications, protein purification, and PPI analysis, because AGT can be fitted with various labels. Second – and even more advantageous in the field of GPCR oligomerization – this tool can overcome some limitations of the classical fusion of auto-fluorescent proteins to the protein of interest. Thus, the small size of AGT (24 kDa) affects protein function in living cells to a lesser extent than GFP, especially when oligomerization occurs. Furthermore, fluorescent labeling is achieved after protein expression in the membrane and it has been demonstrated that fluorescently labeled AGT-fusion proteins are very stable, thus facilitating analysis of the dynamics of cell-surface protein complexes in living cells [33–35].

The SNAP-tag methodology has recently been used to study GPCR oligomerization [36]. In this study, distinct GPCRs (e.g. GABA$_B$ receptors) were tagged at the N-terminus with AGT (R1^{AGT}). BG derivatives were then prepared, carrying Eu^{3+} cryptate as a donor and d2 (an organic moiety that emits at approx. 665 nm) as an acceptor on the benzyl group. Alternatively, TR-FRET was measured between Flag-GABA$_B$ receptors (R2^{Flag}) labeled with d2 antibodies and SNAP-tag fusion proteins labeled with BG-Eu^{3+}-cryptate (BG^K) (Figure 1c). This SNAP-tag and TR-FRET combined approach facilitated rapid, easy and quantitative assessment of cell surface interactions and confirmed the oligomeric assembly of distinct GPCRs at the cell surface [36].

Another technique for specific labeling of multiple proteins in a single cell has recently been described [37]. This study used the CLIP-tag tool (based on the SNAP-tag technology) for which an AGT mutant was generated to selectively accept a substrate other than BG. The new substrate was *O*6-benzylcytosine (BC), which has sufficient specificity for orthogonal labeling; thus, BC only reacted with the mutant AGT version (CLIP-tag) and not with the wild-type AGT (SNAP-tag), which could be labeled specifically with BG. Therefore, the presence of specific receptor homo- or heterocomplexes in transfected cells could be detected by FRET measurements between two receptors fused at the N-terminus with the two AGT variants (i.e. SNAP-tag and CLIP-tag). Once expressed at the plasma membrane, these receptor constructs were specifically labeled with BG and BC derivatives carrying distinct FRET-compatible fluorophores (BG^{Cy3} and BC^{Cy5}, respectively) (Figure 1c).

Recently, a non-RET-based technique based on fluorescence recovery after photobleaching (FRAP) was used to investigate GPCR oligomerization at the plasma membrane [38]. In brief, after immobilization of a defined fraction of receptors with antibodies, the lateral mobility of the non-immobilized fraction was measured by FRAP [38]. Thus, this FRAP approach can be used to determine the stability of oligomers at the plasma membrane and to quantify and distinguish between heteromerization and higher-order oligomerization of human β-adrenoceptors [38].

Protein-fragment complementation assays toward the study of protein oligomerization

Protein-fragment complementation assays (PCAs) facilitate direct detection of PPIs and the study of their dynamic events in living cells [39,40]. In brief, PCAs consist of the structural and functional reconstitution of an active protein, typically an enzyme or FP, from two inactive halves that are genetically fused to the interacting proteins of interest. When FP or LP fragments are used for complementation, these assays are known as bimolecular fluorescence or luminescence complementation (BiFC/BiLC). Both BiFC and BiLC rely on the generation of a fluorescent or luminescent signal from two non-fluorescent or non-luminescent fragments of an FP or LP when brought in close proximity by fusion partners [4]. It is important to note that BiLC, in contrast to BiFC, generates complemented LPs (e.g. Rluc) that assemble mostly in a reversible fashion, thus facilitating real-time measurements of protein–protein association and dissociation and providing dynamic information on the interaction [41,42]. It is important to consider that not all complemented LPs are reversible and that the affinity and kinetics of the BiLC process might interfere with and change the PPI under study. BiLC assays, in contrast to BiFC, are not suitable enough to provide information about the subcellular localization of the interaction. Indeed, luciferases have a low quantum yield, so a specialized detection system is required to record luminescence images, such as a microscope coupled to an intensified charge-coupled device (CCD) camera, in addition to long exposure times.

To date, the BiFC approach has been successfully used to visualize more than 200 PPIs in different models [40,43–45]. BiLC has also been used successfully in the detection of protein interactions in living animals, taking advantage of its high signal-to-noise ratio [46,47]. Interestingly, fluorescence- and luminescence-based approaches can also be used in the study of the GPCR downstream machinery (i.e. G proteins or scaffolding proteins) [48,49]. Toward this end, the BiFC assay was used to visualize specific dimers between the β and γ subunits of G proteins. Briefly, an N-terminus fragment of YFP was fused to Gβ (R1^{N-YFP}) and a C-terminus fragment of YFP to Gγ (R2^{C-YRP}); when expressed together in HEK-293 cells, a fluorescent signal was obtained (Figure 2a). These experiments demonstrated the functionality of the Gβγ complex in the plasma membrane and revealed the role of the different subunits in subcellular targeting [50].

The BiFC method, which facilitates direct subcellular visualization of protein interactions in living cells, has several advantages compared to the RET techniques described above (Table 1). Essentially, BiFC is a more sensitive technique because the complementation process produces a new fluorescent signal, whereas RET methods produce changes in existing fluorescence, and thus have some detection drawbacks (Box 1). Although BiFC assays are powerful tools in the study of PPIs, they have limitations that might preclude their extensive use: the inherent irreversible association of the fluorescent-protein fragments [44]; the intrinsic ability of the FP halves to spontaneously associate under certain circumstances [45,51]; and the time required for fluorophore maturation (Table 1). RET approaches can be used for reversible analysis of protein complex formation (protein–protein association and dissociation), so they should be ideal choices for PPI kinetic studies. RET approaches and BiFC methods are complementary and consequently an experimental tradeoff exists between the dynamism of RET and the sensitivity of BiFC when analyzing PPIs (Table 1). Interestingly, some investigators have tried to combine both approaches. As an example, BRET and BiFC combined results led to the proposal of a new protein composition for the calcitonin gene-related peptide receptor. The authors concluded that dual BRET/BiFC is a powerful tool for analysis of constitutive and dynamically regulated multiprotein complexes [52].

Multicolor BiFC has also been developed to simultaneously visualize several interactions within one cell [53]. This assay is based on the generation of complemented FPs by the association of fragments originating from different FPs. These new complemented FPs possess distinct spectral properties when compared to the native FPs [53,54]. For instance, Venus and Cerulean FPs (which are GFP derivatives of yellow and cyan FPs, respectively) can be used in this approach. The complementation between Venus N-terminal (VN) and Cerulean C-terminal (CC) fragments results in a green-shift in emission compared with reconstitution of the original Venus or Cerulean FPs [53,54].

The multicolor BiFC approach has also been used to simultaneously visualize the relative formation of distinct GPCR oligomeric species and monitor the effect of receptor ligands on the oligomeric status via ratiometric fluorescence [55,56]. For instance, the drug-induced changes in adenosine A_{2A} receptor and dopamine D_2 receptor homo- and heterodimers were assessed in living cells [56]. In brief, the D_2R fused to the $R1^{VN}$ was co-transfected in a variant of the Cath.a CAD cell line with $A_{2A}R$ fused to the N- ($R2^{CN}$) or C-terminal ($R2^{CC}$) fragment of the Cerulean FP. Venus ($A_{2A}R$–D_2R heterodimer, $R1^{VN}$–$R2^{CC}$) and Cerulean ($A_{2A}R$–$A_{2A}R$ homodimers, $R2^{CN}$–$R2^{CC}$) fluorescent signals coexisted and were largely co-localized at the plasma membrane and in intracellular vesicular structures, facilitating simultaneous visualization of the distinct receptor oligomers within a cell (Figure 2b). Interestingly, when these cells were treated with the D_2R agonist quinpirole, Venus fluorescence was decreased compared with Cerulean fluorescence in the plasma membrane, indicating a ratiometric decrease in the $A_{2A}R$–D_2R heterodimer with respect to the $A_{2A}R$–$A_{2A}R$ homodimer (Figure 2c). Overall, these results demonstrate one of the first BiFC applications in monitoring drug-mediated GPCR oligomerization modulation. Multicolor BiFC could theoretically shed light on the earlier proposed paradigm that GPCR oligomerization is altered in pathological conditions such as Parkinson's disease [57–59]. Consequently, this approach could potentially facilitate screening of drugs that impinge on the stoichiometry of a named GPCR oligomer (e.g. $A_{2A}R$–D_2R heterodimer), which would have high clinical impact [59].

Detection of higher-order protein complexes

The RET techniques described above are well-suited for detection of interactions between two proteins that form homo- or heterodimer complexes; however, a given protein can also be part of multiprotein complexes involving numerous interactions with different receptor partners. The formation of highly organized protein structures can be extremely specific, especially if they dictate the final functional output of a specific receptor oligomer. Thus, the existence of high-order oligomer complexes has been detected for some GPCRs [60–62], including rhodopsin (by means of atomic force microscopy [63–65]) and the α_{1b}-adrenoceptor, for which high-order oligomer formation is required for receptor maturation, surface delivery and function [61]. Notably, such highly organized protein structures have not been found for some other GPCRs. In fact, rhodopsin oligomerization remains controversial and it has been suggested that the native protein might not form a high-order complex [66]. It has also been demonstrated that metabotropic glutamate receptors (mGluR) homodimers are not in contact with each other at the surface of HEK-293 cells and a higher-order oligomer is not formed [67].

It has been postulated that to modulate GPCR function, GPCR homo- and heterodimers can be interconnected at the plasma membrane, forming higher-order oligomers, which are also termed receptor mosaics (RMs) [57,68,69]. In a named RM, the interconnected receptors would display cooperativity phenomena; thus, these high-order oligomers behave as computational processors that directly integrate and transmit assorted simultaneous signals into the cell [70]. Consequently, RMs constitute potential drug targets in the treatment of

some diseases. A high-order oligomer, $A_{2A}R$-D_2R-$mGlu_5R$, has been described [71] in which D_2R actions are allosterically modulated by the other two receptors within the oligomer hub, which could explain, for instance, the observed positive action of $A_{2A}R$ antagonists combined with D_2R agonists in the management of Parkinson's disease [59].

Sequential RET (SRET)

Fluorescence-based techniques have played valuable roles in characterizing higher-order receptor complexes. One of the first approaches described for analysis of interactions between several proteins by means of fluorescence microscopy was called three-chromophore fluorescence resonance energy transfer (3-FRET) [72]. This method was used to detect trivalent α_{1b} adrenoceptor complexes in living cells [60–62]. Later, SRET was developed in which the acceptor from a first RET pair serves as the donor for a second RET process. Therefore, in the SRET experimental approach shown in Figure 3a, the bioluminescence emission produced by catabolism of the *R*luc substrate ($R1^{Rluc}$) provides the first energy for transfer (BRET) to a proximal FP acceptor ($R2^{GFP2}$), assuming the RET principle is achieved (Box 1). The initial FP acceptor can then engage in a second RET process, thus becoming a donor FP in the transfer of energy (FRET) to a downstream acceptor FP ($R3^{YFP}$). This elegant SRET approach has revealed the existence of distinct higher-order GPCR oligomers in living cells, including the $A_{2A}R$-D_2R-CB_1R complex and the $A_{2A}R$-D_2R-$mGlu_5R$ oligomers mentioned previously [71,73].

Interestingly, two variations of the SRET approach, $SRET^1$ and $SRET^2$, have been described. $SRET^1$ involves catabolism of coelenterazine H by *R*luc and the luciferase-mediated emission (475 nm) provides energy for transfer to a nearby YFP ($BRET^1$ process); finally, YFP emission (527 nm) can result in a second energy transfer to DsRed. During $SRET^2$, the luciferase-mediated emission after *R*luc catabolism of DeepBlueC (395 nm) is able to excite GFP^2 ($BRET^2$ process) and the resulting FP emission (510 nm) can engage in a second energy transfer to YFP (Figure 3a) [73]. Overall, the SRET method represents a valuable technique for the study of trimeric PPIs and can further our understanding of how proteins assemble and how these assemblies are governed by specific allosteric modulators acting on their interfaces.

Integrating PCA assays and RET techniques

Recently, a new set of techniques combining PCA and RET assays has been developed for the detection of trimeric and quaternary protein complexes. These approaches are based on the use of a reconstituted YFP (BiFC) as an acceptor FP in a RET process. Thus, BiFC-BRET and BiFC-FRET assays have been developed to demonstrate the existence of trimeric GPCR complexes with spatial (BiFC-FRET) and temporal (BiFC-BRET and BiFC-FRET) resolution [71,74–76]. The existence of higher-order oligomers is not restricted to GPCRs, and these techniques can be used to investigate heterotrimeric G proteins or other trimeric complexes comprising downstream effectors [52,77,78].

To demonstrate the existence of quaternary structures, it is possible to combine bioluminescence or fluorescence complementation (BiLC or BiFC) and energy transfer (RET). Receptors genetically fused to N- or C-terminal fragments of an LP or FP are coexpressed in the same cell and if these form a tetravalent protein complex they can engage in a RET process (Figure 3b). This approach led to detection of β_2 adrenoceptor tetramers [79]. It was also demonstrated that at least four D_2Rs are located in close molecular proximity when expressed in living cells, a phenomenon that is consistent with the notion that they might be organized as higher-order oligomers or RMs at the plasma membrane [80]. Identification of ternary and quaternary protein complexes has been possible by combining PCA and RET approaches, catalyzed, in part, by research into GPCR

oligomerization. Thus, the combination of these techniques will undoubtedly evolve as widely used biotechnological tools for the identification and monitoring of PPIs not only at the surface of living cells, but also at intracellular locations.

Concluding remarks

Fluorescence- and luminescence-based assays are useful tools for the visualization and characterization of noncovalent PPIs in many cell types and organisms. In recent years, new optical techniques based on RET and protein-fragment complementation have enabled researchers to detect specific PPIs in diverse biological fields. Thus, as the use of these approaches increases dramatically, it is timely to revise their significant strengths and weaknesses to encourage reliable biotechnological exploitation of their present capabilities and future potential. Fluorescence- and luminescence-based methods have been successfully applied to the discovery of multimeric GPCR complexes in living cells; indeed, some of these technical approaches are already patented because it is predicted that they will evolve into powerful biotechnological tools in several research areas, such as drug discovery.

Despite their initial promise, it is becoming evident that these approaches need to pass some technological barriers before they are fully incorporated into the biotechnological toolbox as, for example, non-invasive techniques for discovering native PPIs *in vivo*. One obvious drawback to these fluorescence- and luminescence-based methods is the ectopic expression and/or overexpression of fusion proteins often required, which can lead to artefacts (e.g. promotion or inhibition of PPIs). Consequently, when applied to the study of physiologically relevant PPIs, physiological expression levels of the proteins under study should be considered. The use of fluorescence- and luminescence-based approaches for PPI detection in native tissue is still scarce and restricted to certain methods, namely the RET-based techniques using antibodies against native proteins (i.e. TR-FRET), thus relying in the specificity of the antibodies to success (Table 1). In conclusion, the discovery of GPCR oligomerization exemplifies the power of the fluorescence/luminescence methods, and our progressive understanding of these techniques will ensure further incorporation into the 'everyday' biotechnological toolbox.

Acknowledgments

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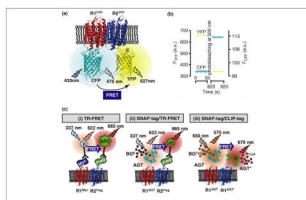
Figure 1.
Fluorescence-based methods used in the study of protein–protein interactions. (**a**) Schematic representation of the basic FRET principle applied to the study of GPCR oligomerization. Two putative receptors, R1 and R2, bear a donor CFP (R1^{CFP}) and an acceptor YFP fluorophore (R2^{YFP}). If R1 and R2 interact, then the donor and acceptor fluorophores might be in close proximity (≤10 nm) and energy transfer between the two fluorophores can occur after donor excitation at 433 nm and emission at 475 nm and acceptor emission at 527 nm. The schematic GPCR and FP diagrams were prepared using the PyMOL molecular graphics system (DeLano Scientific), with the crystal structure of the sensory rhodopsin II and GFP from *Aequorea victoria* (PDB 1JGJ and 1EMA, respectively) as models. (**b**) Example of the DFRAP effect. Emission intensities of YFP (527 nm, yellow line) and CFP (480 nm, cyan line) recorded from cells coexpressing the μ-opioid and α$_{2A}$ adrenergic receptors tagged at the C-terminus with YFP and CFP, respectively. Emission intensities were recorded before and after YFP (the acceptor fluorophore) was photobleached via 5-min exposure to light at 500 nm. Adapted with permission from [18]. (**c**) Schematic representations of various technologies applied to the study of GPCR oligomerization. Two putative GPCRs, (i) bearing a Myc and a Flag epitope tag on their N-terminal domains or fused to (ii) *O*6-alkylguanine-DNA alkyltransferase (AGT; SNAP tag) or (iii) mutant *O*6-alkylguanine-DNA alkyltransferase (AGT*; CLIP tag), are labeled with specific antibodies against these tag sequences (i.e. Myc or Flag) or with specific substrates for either AGT or AGT*. The antibodies are conjugated to the acceptor (APC; or d2 molecule) and the donor (K, Eu^{3+}-cryptate) FRET-pair fluorophore molecules. The SNAP-tag substrate *O*6-benzylguanine (BG) is conjugated to the donor (K or Cy3) and the CLIP-tag substrate *O*6-benzylcytosine (BC) to the acceptor (Cy5). The schematic AGT diagram was prepared using PyMOL (PDB 1EH6).
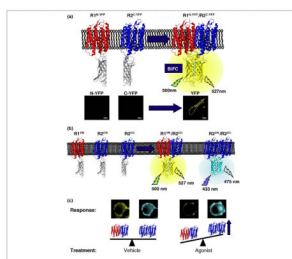
Figure 2.
Schematic representation of the basic principle of BiFC and multicolor BiFC. (**a**) Two putative receptors, R1 and R2, each carry one half of YFP: R1 carries an N-terminal YFP (R1$^{N\text{-}YFP}$); R2 carries a C-terminal YFP (R2$^{C\text{-}YFP}$). The interaction of R1 and R2 generates a fluorescent complex formed by the two halves of YFP (N and C) and YFP fluorescence can therefore be directly visualized after excitation at 500 nm. Corresponding confocal microscopy images of HEK cells expressing the respective receptor constructs are shown below the schematic. Scale bar, 10 μm. Adapted with permission from [87]. GPCR and FP diagrams were prepared as described for Figure 1. (**b**) Application of multicolor BiFC to the simultaneous detection of receptor homodimers and heterodimers. Receptor R1 carries an N-terminal fragment of the Venus FP (R1^{VN}), and receptor R2 carries either the N-terminus of the Cerulean FP (R2^{CN}) or the C-terminus of the Cerulean FP (R3^{CC}). The relative amount of heterodimer (R1/R2) versus homodimer (R2-R2) can be simultaneously visualized after excitation at 500 nm (Venus) or 433 nm (Cerulean). (**c**) Receptor oligomerization in response to drugs can be monitored using the approach in (b). For example, prolonged treatment with a receptor agonist resulted in a decrease in Venus fluorescence at 527 nm as measured against Cerulean fluorescence at 475 nm, thus indicating an agonist-induced change in the GPCR homo-/heterodimer ratio (R2/R2 vs R1/R2). Adapted with permission from [56], copyright © 2008 American Society of Pharmacology and Experimental Therapeutics.
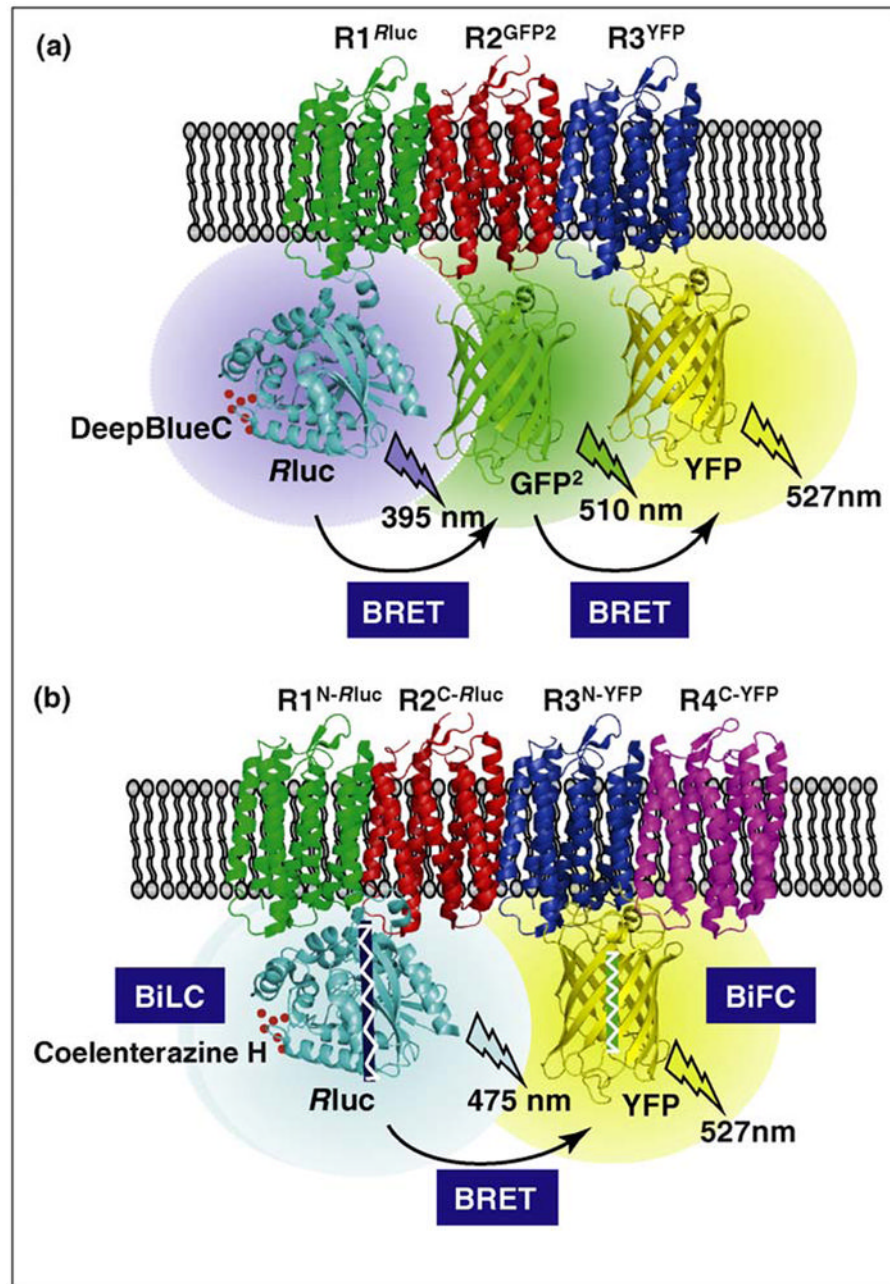
Figure 3.
Fluorescence-based methods used in the study of higher-order protein complexes. (**a**)
Schematic representation of the basic SRET principle applied to the study of GPCR
oligomerization. Three putative receptors, R1, R2 and R3 bearing a donor (*R*luc), an
acceptor and donor (GFP^2) and acceptor (YFP) chromophores, are depicted as R1^{Rluc},
R2^{GFP2} and R3^{YFP}, respectively. When the three receptors are in close proximity, a
sequential double energy transfer (BRET-FRET) might occur: *R*luc emission at 395 nm
excites GFP^2; GFP^2 emits at 510 nm, in turn exciting YFP, which then emits at 527 nm. The
schematic GPCR diagrams were prepared as described in Figure 1. The crystal structure of
luciferase from *Renilla reniformis* (PDB 2PSD) is shown. (**b**) The combination of PCA and
RET techniques allows for the detection of tetrameric receptor complexes (R1-R2-R3-R4).

BiLC generates a complementary luminescent protein (*R*luc) that acts as a donor in a BRET process, with a complementary fluorescent acceptor protein (YFP) generated by BiFC. If these receptors are in close proximity, then donor–acceptor energy transfer can occur after *R*luc substrate (coelenterazine H) oxidation. Excitation (475 nm) and emission (527 nm) wavelengths are indicated.

Table 1

Comparison of different fluorescence- and luminescence-based approaches for monitoring PPIs

	FRET	TR-FRET	BRET	SNAP-tag[a]	BiFC/BiLC
Spatial resolution (subcellular localization)	+	+	−	+	+/−
Temporal resolution (interaction dynamics)	+	+	+	+	−/+
Distance measured	~2–5 nm	~10 nm[b]	~2–5 nm	~2–5 nm[c]	~2–5 nm
Regular equipment required	Microscope or microplate reader	Microscope or microplate reader	Microplate reader or scanning spectroscope	Microscope or microplate reader	Microscope or microplate reader
Suitability for monitoring multiple distinct interaction pairs (multicolor)	−	−	−	−	+/−
Suitability for detecting higher-order interactions	+ (SRET and PCA/RET combination)	−[d]	+ (SRET and PCA/RET combination)	−[d]	+/+ (BRET/BiFC and BiLC/BiFC combination)
Specific disadvantages	Donor bleedthrough, crosstalk, photobleaching	Antibody specificity	Overexpression (random collision by intracellular accumulation)	Donor bleedthrough, crosstalk and photobleaching[e], antibody specificity[f]	False positives (irreversibility)

[a]In a RET process.

[b]The lanthanides used in TR-FRET (e.g. europium) have much larger R_0 values (Box 1), as great as 90 Å, which increases the effective range for energy transfer [24]. In addition, the presence of an antibody between the fluorophore molecules and the proteins of interest increases the operating distance for the protein–protein interaction, thus facilitating detection of larger protein complexes, but making determination of the precise distance between interacting partners difficult.

[c]Closer to 5 nm in SNAP-tag-TR-FRET.

[d]Might be possible if in a SRET or PCA-RET combination process.

[e]In SNAP-tag-CLIP-tag FRET.

[f]In SNAP-tag-TR-FRET.