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Application of BRET for Studying G Protein-Coupled Receptors

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Abstract: G protein-coupled receptors (GPCRs) constitute one of the largest classes of cell surface receptors. GPCR biology has been a subject of widespread interest owing to the functional relevance of these receptors and their potential importance in the development of new drugs. At present, over 30% of all launched drugs target these receptors. GPCRs have been considered for a long time to function as monomeric entities and the idea of GPCR dimerization and oligomerization was initially accepted with disbelief. However, a significant amount of experimental and molecular modeling evidence accumulated during the last several years suggests that the process of GPCRs dimer or oligomer formation is a general phenomenon, in some cases even essential for receptor function. Among the many methods to study GPCR dimerization and oligomerization, modern biophysical techniques such as those based on resonance energy transfer (RET) and particularly bioluminescence resonance energy transfer (BRET) have played a leading role. RET methods are commonly applied as non-destructive indicators of specific protein-protein interactions (PPIs) in living cells. Data from numerous BRET experiments support the idea that the process of GPCR oligomerization may be relevant in many physiological and pathological conditions. The application of BRET to the study of GPCRs is not only limited to the assessment of receptor oligomerization but also expands to the investigation of the interactions of GPCRs with other proteins, including G proteins, G protein-coupled receptor kinases, β-arrestins or receptor tyrosine kinases, as well as to the characterization of GPCR activation and signaling. In this review, we briefly summarize the fundaments of BRET, discuss new trends in this technology and describe the wide range of applications of BRET to study GPCRs.

Keywords: BRET, G protein-coupled receptors, G protein-coupled receptor dimers.

INTRODUCTION

G protein-coupled receptors (GPCRs) are membrane proteins that sense a variety of signaling molecules such as hormones and neurotransmitters, thus regulating myriad aspects of cell physiology, [1] and are one of the most important classes of drug targets [2]. GPCRs involve receptors for odorous substance and light and are involved in the processes of vision, olfaction and taste. Furthermore, four GPCRs are protease-activated receptors. Proteases generate and destroy receptor agonists and activate and inactivate receptors, so they make significant contribution to signal transduction. At present, over 30% of launched drugs target these receptors. All GPCRs share common structural features: they have an extracellular N-terminal domain and an intracellular C-terminal domain that are connected by seven transmembrane-spanning helices (Fig. (**1**)). According to phylogenetic criteria, GPCRs in the human genome can be classified into five families; namely the Rhodopsin, Secretin, Glutamate, Adhesion, Frizzled/Smoothened families [3]. The Rhodopsin, Secretin, and Glutamate receptor families equate, respectively, to the classes A, B, and C recognized by the International Union of Pharmacology, Committee on Receptor Nomenclature and Classification (NC-IUPHAR). Rhodopsin family (or class A) is the one that has been most extensively studied in drug discovery and around 25% of marketed small molecule drugs are considered to bind one of its members [4]. Receptors in this family have their orthosteric binding site in the transmembrane (TM) helical bundle. Conversely, receptors from other families either contact ligands both with the helix bundle and with the N-

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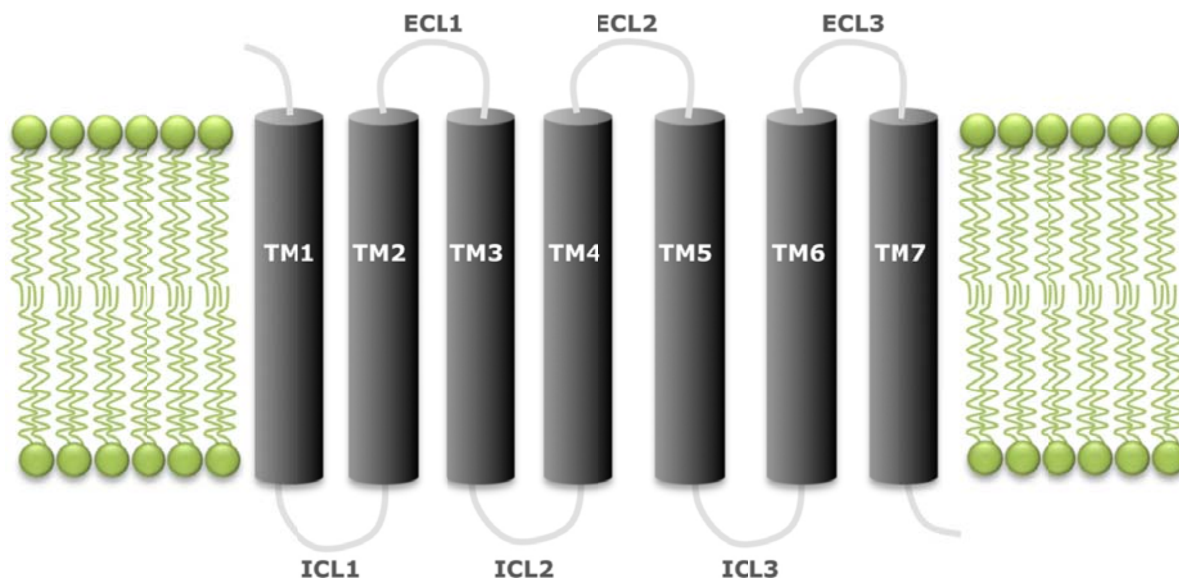

Fig. (1). The topology of GPCRs. TM – transmembrane regions; ECL – extracellular loops; ICL – intracellular loops.

terminus (such as in the Adhesion or Secretin receptor families) or they have their orthosteric binding site in a particular N-terminal domain such as receptors in the Glutamate family [5]. Secretin family, also termed class B GPCRs, is constituted by receptors for 15 peptide hormones, which can be grouped into five subfamilies with different physiological roles [6]. Class C GPCRs are involved in synaptic transmission, taste sensation, and calcium homeostasis and they include metabotropic glutamate receptors, γ-aminobutyric acid B receptors, calcium-sensing (CaS) receptors, and taste 1 receptors, as well as a few orphan GPCRs [7]. The adhesion GPCRs are a family of more than 30 receptors that are characterized by extremely large N termini featuring various adhesion domains capable of mediating cell-cell and cell-matrix interactions [8]. Regarding the Frizzled/ Smoothened family, in mammals, there are 10 Frizzled and 1 Smoothened protein showing low but significant sequence similarity to family B receptors [9]. Frizzled proteins have key significance in governing cell polarity, embryonic development, formation of neural synapses, cell proliferation, and many other processes in developing and adult organisms. The smoothened receptor, a key signal transducer in the hedgehog signaling pathway, is responsible for the maintenance of normal embryonic development and it is implicated in carcinogenesis [10]. Structural information on the diversity of GPCR topology has dramatically increased in the past years, in part thanks to the development of original crystallographic strategies [11].

Nowadays, several representatives of GPCRs of the Rhodopsin family have been crystallized in complex with ligands covering a wide range of activities [12]. Furthermore, recent crystallization of smoothened [10] and corticotropin releasing factor [13] receptors has shed light into receptor diversity among GPCR families. Finally, publication of the structure of the activated β_2-adrenergic receptor in complex with a G protein has given novel insight into the basis of receptor activation and signal transduction [14].

In agreement with the classical thought that GPCRs function as monomeric entities, recent studies demonstrated that monomeric GPCRs embedded in phospholipid bilayers constitute a minimal functional unit able to couple to and activate G proteins and to recruit arrestin proteins [15-19]. Yet, the data from these studies can still be compatible with a model of pentameric receptor-G protein complexes (R:R:Gα;Gβ:Gγ) with only one protomer within the receptor dimer being capable of coupling to the G protein, and they coexist with a wealth of experimental and molecular modeling data indicating that GPCRs can form dimers and higher-order oligomers (Fig. (**2**)) [20]. Different studies documented that interactions between GPCR monomers are crucial for cell surface delivery [21]. It was also observed for some receptor systems that the structural organization of the formed molecular complexes determines the mechanisms of binding and activation of G proteins [22, 23]. Assembly of different GPCR types can result in alterations of ligand pharmacology as well as distinct and unique signaling properties [24]. Besides that, it has been proposed that targeting GPCR complexes may lead to more selective and safer drugs [25].

A number of experimental and computational approaches have been used to study the phenomenon of GPCR oligomerization [26, 27]. Evidence supporting this idea includes early reports on the quaternary structure of rhodopsin in native membranes assessed by atomic force microscopy [28], as well as studies on other GPCRs by means of cysteine crosslinking [29, 30], co-immunoprecipitation [31, 32], radioligand binding [33, 34], single point and scanning fluorescence correlation spectroscopy diffusion [35], dual color fluorescence recovery after photobleaching (FRAP) [36], or time-resolved fluorescence resonance energy transfer (TR-FRET) at the cell surface [32, 37, 38], among other techniques. A comprehensive discussion of over 20 different biochemical and biophysical methods applied to the study of GPCR oligomerization was recently elaborated by Kaczor

and Selent [26]. Among those techniques, BRET, being a relatively new technique, has been extensively applied to uncover interactions within GPCR dimers and oligomers [39]. One of the advantages of BRET is its compatibility with physiological conditions. Indeed, BRET is currently a method of choice for detecting transient or stable protein-protein interactions in living cells and monitoring them in real time.

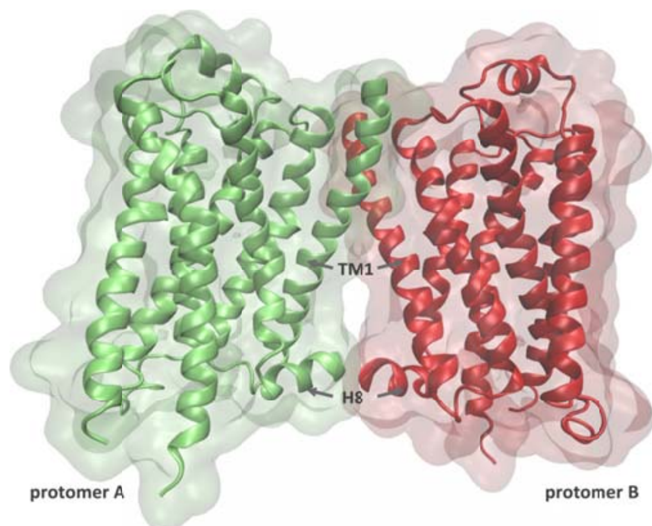

Fig. (2). High-resolution crystallographic structures of β_1 adrenergic receptor (PDB ID: 4GPO) [20].

Although homo- and heteromeric interactions were found between a number of different GPCRs using BRET and other resonance energy transfer (RET)-based methods, it is important to emphasize that these methods sense molecular proximity and relative orientation between the donor and acceptor RET pairs, and the need for proper controls and careful experimental designs cannot be overestimated in order to extract accurate information from these measurements. Hence, the usage of BRET to investigate GPCR oligomerization is not limited to the simple measurement of energy transfer between a donor and an acceptor fused to the receptors. In order to discriminate between specific physical interactions of two receptors and random collisions, different experimental designs are used (e.g. BRET saturation assays and BRET competition assays). The high sensitivity that characterizes BRET measurements enables to use this technology not only to detect intermolecular interactions between GPCRs engaged in dimeric complexes, but also to monitor the dynamic interactions of these receptors with their associated G proteins or with other signaling and effector proteins modulated upon ligand binding [40, 41]. Moreover, intramolecular BRET-based biosensors have allowed monitoring different GPCR-mediated molecular events indicative of the cellular response to external stimuli [42]. BRET is also widely used today to screen for novel GPCR agonists and antagonists and to search for new signaling pathways activated by well-known drugs targeting these receptors (see chapter Receptor-G protein coupling and G protein activation) [43]. Contrary to FRET (fluorescence resonance energy transfer), another RET-based technique, for a long time the BRET signals have been too dim and the instrumentation available was not sufficiently sensitive for single cell imaging or 'high content' settings. However, based on the recent development of new BRET systems more suitable for imaging [44] and considering some advantages of this technique over FRET or other bioluminescence-based imaging methodologies, one could hypothesize that BRET will allow imaging GPCRs at work in living animal disease models or under *in vivo* pharmacological and therapeutic manipulations in the near future. In this review, we shortly summarize the fundaments of BRET and then describe the range of its applications to the study of GPCRs.

FUNDAMENTS OF BRET

RET-based techniques such as BRET or FRET enable to detect signaling processes involving changes in the relative distance between two labels (donor and acceptor) at the nanometer scale, and to monitor the dynamics of these processes in living cells [41, 45]. RET techniques involve the non-radiative long-range transfer of energy between a donor and an acceptor based on the Förster mechanism [46, 47]. A donor chromophore, which is initially in its electronic excited state, can transfer energy to an acceptor chromophore through non-radiative dipole-dipole coupling [46]. Energy is transferred by resonance, what means that the electron of the excited molecule induces an oscillating electric field that excites acceptor electrons, which reach an excited state [48]. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor. The return of the acceptor to a ground state (de-excitation) occurs primarily by a photon emission, if the acceptor is fluorescent, or as a result of its interaction with the solvent, if it does not fluoresce, where the energy is dissipated as heat [48, 49]. There are two conditions that must be fulfilled for the resonance energy transfer to occur: (a) an overlap between the emission spectrum of the donor molecule and the excitation spectrum of the acceptor molecule and (b) that the donor and acceptor must be at a distance below 100 Å and favorably oriented. In a typical BRET experiment for monitoring protein-protein interactions (i.e. intermolecular BRET), two proteins of interest are co-expressed in cells, one as a fusion protein with a variant of the *Renilla reniformis* luciferase (RLuc), and the other fused to a compatible BRET acceptor, such as a variant of the green fluorescent protein from *Aequorea victoria* (GFP). Upon addition of a luciferase substrate to the cells, the enzyme will catalyze the oxidation of the substrate yielding BRET donor energy that can be transferred to the BRET acceptor (Fig. (**3**)) [50].

There are two BRET assay variants, referred to as $BRET^1$ and $BRET^2$, which have been widely applied over the last decade, both using RLuc and hydrophobic, cell membrane-permeable luciferase substrates. In the $BRET^1$ system coelenterazine *h* is used as the substrate of luciferase giving a peak donor emission at 475-480 nm that excites the enhanced yellow fluorescent protein variant (eYFP), with an emission peak at 525-530 nm [51, 52]. One disadvantage of the $BRET^1$ system is that the broad emission spectrum of RLuc leads to a considerable overlapping of donor and acceptor emissions, which accounts for a high background signal. This was improved in the $BRET^2$ system that makes

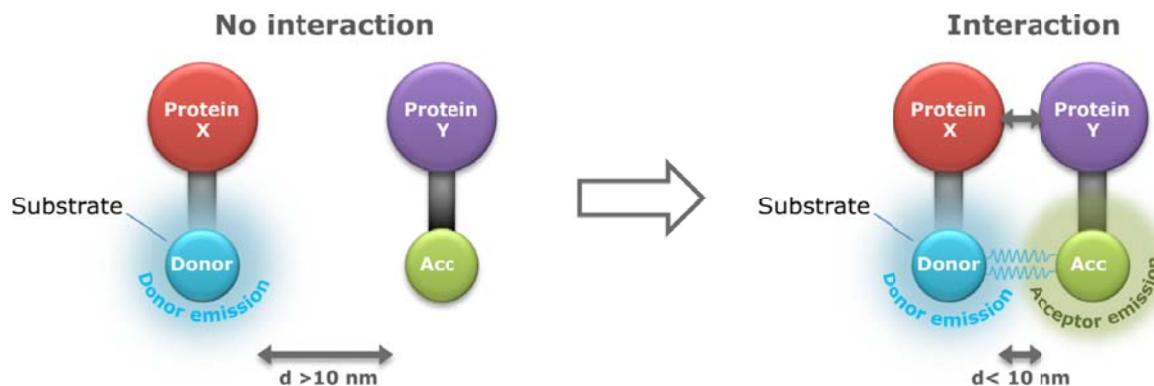

Fig. (3). Principle of BRET. The *Renilla reniformis* luciferase (RLuc), genetically fused to a Protein X of interest, acts as BRET donor catalyzing a chemical reaction on its substrate that yields BRET donor energy susceptible of being transfer by RET to a suitable BRET acceptor genetically fused to Protein Y, when donor and acceptor are in close proximity and favorably oriented. This will result in the excitation of the acceptor and the detection of an increase in emission light at the wavelength characteristic of the acceptor emission. The figure adopted from reference [50].

use of the coelenterazine derivative coelenterazine 400a (DeepBlueC^{TM}) as substrate. This shifts the peak emission of RLuc to 395 nm, adequate for exciting GFP^2 [53] or GFP10 [54] (excitation peak at 395-400 nm, emission peak at 509-510 nm) as BRET acceptors. These combinations result in lower background signal that allows detection of protein-protein interactions at lower concentrations of the interacting proteins. Yet, the luminescence emitted by RLuc using DeepBlueC^{TM} as substrate experiences a faster signal decay and lower quantum yield (~200-fold lower) than that emitted using coelenterazine *h* [55]. Both BRET^1 and BRET^2 systems are extensively used today for the study of protein-protein interactions, and they coexist with some recent enhanced variations of both systems (see below).

BRET offers the high sensitivity of RET techniques to relative changes in distance or orientation together with their ratiometric nature, minimizing the impact of artifacts. The fact that the luminophores and fluorophores can be genetically tagged to the proteins of interest avoids the requirement of introducing subsequent protein labeling steps, including the use of indirect approaches involving e.g. specific antibodies. Opposite to biochemical approaches for the assessment of protein/protein interactions such as co-immunoprecipitation, RET-based techniques allow the real-time monitoring of these processes in the native environment of living cells. In addition, interactions between membrane proteins or those occurring in intracellular compartments can be specifically investigated, an advantage of RET-based techniques over other protein/protein interaction technologies such as the yeast two-hybrid systems. The RET-techniques can report the dynamics of reversible or transient interactions, opposite to fluorescent- or bioluminescent-complementation techniques that report the proximity of two proteins of interest by the irreversible assembling of a new flurorophore or luminophore molecule. Being a bioluminescence-based technique and contrary to FRET, BRET does not suffer from a number of issues related to the requirement of external excitation illumination, and the tendency of donor/acceptor self-association observed between green-fluorencent protein variants is absent in the case of the RLuc donor. Yet, the BRET methodology also suffers from some limitations, particularly those common to FRET. Among them, the potential impact of tagging the proteins on their folding, maturation, sub-cellular localization or functionality. RET assays often involve the transient co-transfection of donor + acceptor proteins, which are in general tedious experimental protocols with a high inter-assay variability. Another drawback of the RET-techniques is the lack of information that can be extracted from experiments where a RET signal is not detected and the associated risk of false negatives, which can be due to improper orientation or relative positioning of the donor and/or acceptor within an interaction protein complex.

Novel luminophores and Fluorophores and Current Trends in their Applications

New luciferase and fluorophore variants as well as luciferase substrates have been recently introduced that lead to improvements in the sensitivity of the BRET assays [44, 56, 57], allowing researchers to expand the range of BRET applications. Hence, the original BRET^1 and BRET^2 systems, which are commonly applied nowadays to investigate protein-protein interactions, coexist today with some recent enhanced variations of both systems using optimized forms of RLuc as BRET donors, such as the codon-humanized form of RLuc (hRLuc), RLuc2 or RLuc8 [56, 57]. In the hRLuc cDNA, the codons of the native RLuc have been humanized in order to favor higher expression levels of the fusion proteins in mammalian cells, resulting in a modest improvement in BRET signal. Codon optimization, i.e., exchanging the native codons rarely found in the host organism with more frequently found codons, is currently applied to most optimized luciferases in order to improve translational efficiency and therefore, bioluminescence signal in mammalian cells. RLuc2 results from the introduction of a double-mutation (C124A/M185V) in native RLuc and shows both increased stability and high quantum yield [58]. RLuc8 is a mutant of RLuc incorporating eight point mutations, with markedly increased stability and even higher quantum yield than RLuc2. RLuc2 and RLuc8 showed enhanced quantum yield and light output as luminophores both using

BRET[1] and BRET[2] substrates [58], and they led to an increase in the Förster distance in combination with the yellow fluorescent protein variant Venus in the BRET[1] system, and to an increase of ~30 fold in brightness compared to the original BRET[2] system in combination with GFP[2] [59, 60]. Venus (excitation peak at 515 nm, emission peak at 528 nm) is an acceptor for BRET[1] with improved protein maturation and increased resistance to the environment [61]. The combination of RLuc2 and particularly of RLuc8 with Venus or with other BRET acceptor variants (YPet, *Renilla* GFP) led to enhanced BRET systems with improved sensitivity for measurements of GPCR/β-arrestin interactions in live cells [62-64]. Hence, RLuc8 combinations favored the detection of weak or transient protein-protein complexes as in the case of constitutive (agonist-independent) GPCR/β-arrestin interactions [56]. Regarding luciferase substrates, Enduren is a coelenterazine derivative more stable in aqueous solutions employed also in the so-called extended BRET (eBRET) [65]. This substrate must be metabolized by intracellular esterases in order to become intracellularly available for the BRET reaction, while unprocessed substrate remains in equilibrium in the extracellular medium [65], which enables the monitoring of BRET signals over extended periods of time [65, 66]. Hence, eBRET might be of particular interest for high-throughput BRET screening applications.

Very notably, the recent advances in enhanced bioluminescent and fluorescent proteins and substrates, together with new and improved instrumentation, make *in vivo* imaging become an area of current progress in BRET application [44]. Bioluminescence imaging is distinctively characterized by the low background, high signal-to-noise ratio, and lack of requirement of external light illumination, therefore being free from probe photobleaching, phototoxicity, photo-induced physiological reactions, autofluorescence from the specimen or tissue attenuation of the excitation light. These characteristics make bioluminescence applications potentially superior to fluorescence for noninvasive live cell imaging and deep tissue imaging in living animals, even though the weakness of the bioluminescent signals requires in general longer exposure time than fluorescence imaging. Being BRET a bioluminescence-based technique, BRET imaging shares with bioluminescence imaging those advantages related to the absence of external excitation. Moreover, the transfer of the bioluminescence energy occurring in BRET results in an increase of the total number of photons emitted, increasing the intensity of the readout signal [67]. Additionally, as a ratiometric measurement, BRET facilitates canceling out signal drifts in live cell imaging as those due to changes in cell shape or focus shift, while it does not present the issue of direct excitation of the acceptor common to FRET.

An ideal BRET imaging system for translation to living animals would desirably possess good spectral resolution without compromising RET efficiency, a BRET donor with high bioluminescence quantum yield, and a red light-emitting BRET acceptor. Aiming at this, the system currently designated as BRET[3] was developed [68]. It combines RLuc8 with a red-shifted acceptor, the fluorescent protein mOrange (excitation peak at 548 nm, emission peak at 562 nm), a variant of the red fluorescent protein DsRed2 [69], and it uses coelenterazine *h* or its long-acting derivative Enduren as substrates [68]. The spectral shifting to the red that minimizes light absorption from live specimen components and autofluorescence, together with a good light intensity and donor/acceptor spectral resolution, help to improve spatial and temporal resolution and make this system particularly advantageous for BRET imaging in living animals [68].

Further improved red-shifted BRET systems have also been evaluated in imaging applications for detection of protein-protein interactions in deep tissues of living subjects [70]. Among them, the systems designated as BRET6 (RLuc8.6-native coelenterazine-TurboFP635) and BRET6.1 (RLuc8.6-coelenterazine-v-TurboFP635) displayed high sensitivity for BRET imaging, holding the greatest potential for *in vivo* application. These systems combine RLuc8.6 (a green-emitting RLuc variant with peaked emission at 535nm) [71] as BRET donor, with TurboFP635 (a red variant of the wildtype *Entacmaea quadricolor* red fluorescent protein (RFP) with excitation peak at 588 nm and emission peak at 635 nm) [72] as acceptor. As luciferase substrates, these systems make use of either native coelenterazine or coelenterazine-v [73], a synthetic derivative that further red-shifts the emission peak of RLuc8.6 to 570 nm [74].

Recent achievements in BRET imaging of molecular and cellular events in cell lines and particularly in animal models include the visualization of Ca^{2+} dynamics at the single-cell level as well as *in vivo* by using BRET-based biosensors [75-77], and in particular upon stimulation of HeLa cells with the GPCR agonist histamine [75]. Other bioluminescence-based technique, the split luciferase complementation technology, was previously used for real time imaging of GPCR-β-arrestin interactions at the plasma membrane in single living cells [78, 79] and in deep tissues of living animals [79] This technology implies the formation of a bioluminescent protein when two non-luminescent luciferase fragments are brought into proximity due to the interaction of two proteins of interest tagged with those fragments [80]. A drawback of this technology is that the resultant luciferases exhibit only 40–60% of the activity of an intact luciferase. Additionally, false-negative and false-positive signals can arise from misfolding of the reconstituted reporter and from nonspecific association of the split fragments, respectively. Some potential advantages of BRET over split luciferase for monitoring protein-protein interactions in living animals are the higher light output of BRET that translates into enhanced sensitivity; its reversible nature that allows monitoring the dynamics of the interactions; and its ratiometric character that eliminates the need of introducing internal reference reporters. The overall conclusion from the research in this field is that BRET imaging might become a key technique for the visualization of protein-protein interactions (e.g. receptor oligomerization) and of the consequences of protein–drug interactions in living animals in the near future, allowing the acquisition of highly relevant physio-pathological data [81].

RANGE OF APPLICATIONS-GPCR DIMERIZATION AND INTERACTION WITH G PROTEINS

RET efficiency is inversely proportional to the sixth power of the distance between the donor and acceptor moieties. This, together with its dependence on the relative orientation of the donor and acceptor dipoles, makes RET techniques

very sensitive to small changes in the relative distance between two proteins or in their relative orientation when assessing intermolecular RET. Similarly, RET efficiency is very sensitive to changes in the relative distance or orientation of different domains of a protein upon conformational rearrangement when assessing intramolecular RET. The investigation of GPCRs in terms of oligomerization, interaction with other proteins, and function in their natural environment remains a challenge. However, both intermolecular and intramolecular BRET (and FRET) approaches have remarkably contributed to the progress in the GPCR field.

Assessment of GPCR Dimerization

The application of RET techniques to detection of protein-protein interactions that occur constitutively (i.e. not regulated by a triggering event that could be introduced during the RET measurement protocol) requires experimental designs able to discriminate between specific signals and those coming from random collision between the labeled proteins or from bystander RET (RET between donor and acceptor proteins that co-localize in close proximity), which can be particularly high for membrane proteins. In RET type 1 experiments, the combined number of donors and acceptors is kept constant while the acceptor to donor ratio is increased by substituting donor molecules for acceptor molecules (Fig. (**4a**)) [82]. In the case of a dimeric protein, an increase in the acceptor to donor ratio increases the likelihood of all donors being engaged with an acceptor, therefore generating effective BRET pairs, which would result in a fast increase in BRET efficiency particularly at low acceptor concentrations. However, in the case of monomeric proteins and once a certain total expression level is achieved, the increase in random interactions expected from an increase in acceptor concentration will be compensated by the reduction in donor concentration. Hence, by replacing one donor with one acceptor, the “acceptor environment” randomly experienced by each donor will not be altered significantly, and neither will be the BRET efficiency (Fig. (**4b**)) [82]. In type 2 experiments [82], the total expression level of the labeled proteins is increased while keeping the acceptor to donor ratio constant. Under these conditions, BRET signals coming from random collisions will approach zero at very low expression levels, increasing linearly as a function of total protein density. However, BRET signals coming from dimeric proteins will be notable even at very low densities, reflecting the specific affinity of the dimer partners to associate. Hence, BRET efficiency of type 2 reference experiments involving monomeric proteins will increase as a function of donor + acceptor expression with a slope higher than that observed in experiments involving dimeric proteins.

In particular for the detection of GPCR dimerization by RET techniques, two experimental designs have proven to be very useful. Saturation experiments, where the donor concentration is kept constant while the acceptor expression levels are increased, evidence the hyperbolic fast increase in specific RET signals as opposed to the quasi-linear slow increase in RET efficiency for unspecific RET (Fig. (**4c**)) [83]. In competition experiments, a third unlabeled GPCR is co-transfected together with the BRET pair constituted by the interacting partners labeled GPCR A and labeled GPCR B under study. The co-expression of unlabeled GPCR B will result in a decrease of the BRET signal to an extend that will not be reached upon co-expression of an unlabeled GPCR C that does not interact with any of the two dimer partners, or co-expression of a dimerization-deficient GPCR B mutant [39]. This is a consequence of unlabeled GPCR B diluting the labeled GPCR B that can effectively contribute to the BRET signal. In this sort of experiments it is important to monitor that co-expression of the unlabeled receptor does not alter the expression levels of the labeled proteins.

The concept of homo- and heterooligomers formed between various GPCRs has been extensively studied over the past two decades. During the last decade it was found that the process of heterodimerization influences ligand specificity and signaling at GPCRs, being essential for the function of class C GPCRs [84, 85]. The capacity of one GPCR to modify its binding and coupling properties by interacting with a second receptor opens new perspectives regarding the regulation of GPCR patho-physiological functions [86].

Regarding heterodimerization of class A GPCRs, aminergic receptor complexes were the most extensively investigated, including adrenergic and dopaminergic, dopaminergic and serotoninergic, adrenergic and peptide receptors and many other heterodimeric combinations [26]. One of the latest examples of the application of BRET to the study of GPCRs dimerization or oligomerization is the dimerization process of purinergic receptors, reported by Suzuki *et al.*, who used BRET in transfected human embryonic kidney 293T cells together with other strategies to prove receptor dimerization both in these cells and in native brain tissue [87]. They found that the pharmacology of the homo- and heterooligomers formed between purinergic receptors is exceptional in this receptor group and it could be presumably exploited in the process of the “fine-tuning” of purinergic receptor signaling.

In another example, investigations of the relative propensity of melatonin receptors to homo- and heterodimerize, based on the $BRET_{50}$ values determined in BRET saturation assays, were presented by Ayoub *et al.* [88]. In this work, a correlation was observed between the binding affinity of ligands and their capacity to promote changes in the BRET signal coming from the dimers, attributed to conformational changes within the dimer upon ligand binding. These measurements served to study the specific ligand binding properties of the MT_1/MT_2 heterodimers. The fact that ligands might trigger changes in BRET signals coming from a dimer due to events different to dimer assembly or disruption (such as conformational changes in the protomers, dimer redistribution and trafficking or alterations in the environment of the luminophores and fluorophores affecting their properties) has hampered the study of the ligand-dependency of the receptors oligomeric state. Nevertheless, RET saturation experiments proved to be valuable for the assessment of ligand-dependent dimerization of GPCRs under well-controlled experimental settings in recent studies [89].

BRET also proved to be a valuable technique for the investigation of the structural basis of dimerization/ oligomerization in other class A GPCRs. A work of Berthouze *et al.*, combining BRET and co-immunoprecipitation and docking experiments, identified two cysteine residues in

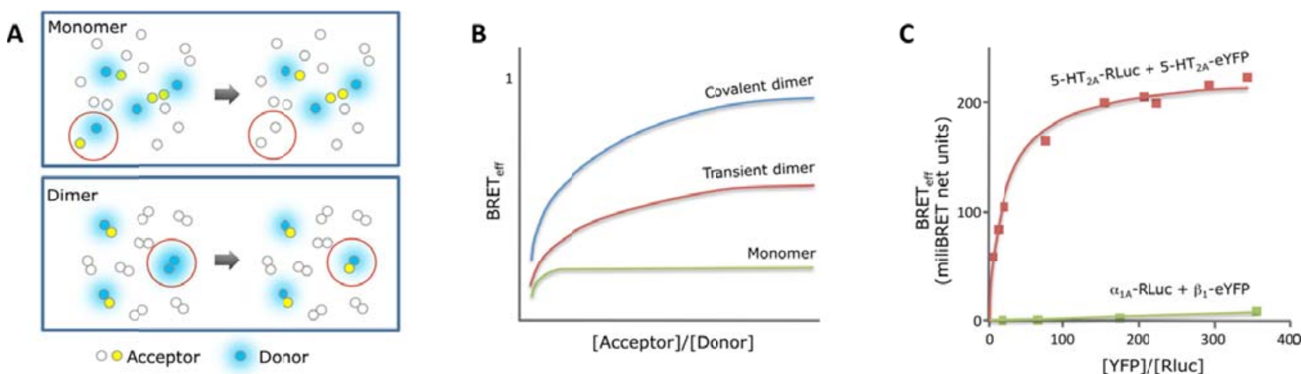

Fig. (4). Scheme of the fundaments of a 'type 1' BRET experiment and differences in the behavior of specific and unspecific BRET signals in BRET experimental settings for the assessment of protein-protein interactions. A - In the scheme shown, the acceptor/donor ratio is increased by replacing one donor with one acceptor, keeping the overall donor + acceptor expression level constant. For monomeric proteins (upper panel), the BRET efficiency ($BRET_{eff}$, which could be expressed as the number of total donors experiencing an acceptor close enough for the RET process to occur) remains constant as the 'acceptor environment' experienced by each of the remaining donors is unchanged. Conversely, for dimeric proteins (lower panel), $BRET_{eff}$ increases as more donors are now engaged in RET-productive dimers. B - Graphical representation of the expected behavior of $BRET_{eff}$ for monomeric, transient dimeric and covalent dimeric proteins as a function of acceptor/donor ratio when this ratio in progressively increased. The figure adopted from reference [82]. C - Experimental determination of $BRET_{eff}$ in a BRET saturation experiment for different GPCR pairs: serotonin 5-HT_{2A} receptors that are reported to form homodimers in transfected cells [83] (5-HT_{2A}-RLuc + 5-HT_{2A}-eYFP), and adrenergic receptors α_{1A}-Rluc + β_1-eYFP, a pair of non-interacting GPCRs used here as negative control. The net BRET ratio was defined as [(emission at 510–590)/(emission at 440–500)]–Cf, where Cf corresponds to (emission at 510–590)/(emission at 440–500) for the 5-HT_{2A}-RLuc or β_{1A}-RLuc receptors expressed alone in the same experimental conditions. Curves were fitted to a non-linear regression equation, assuming a single phase, with GraphPad Prism software (San Diego, CA, USA). $BRET_{eff}$ is expressed as miliBRET net units and is the net BRET ratio x 1000 (RAF and MC, unpublished results).

TM3 and TM4 forming disulfide bonds critical for the constitutive homodimerization of serotonin 5-HT_4 receptors [90]. More recently, a systematic and unbiased analysis focused on the outward-facing residues of the TM helical bundle involved in the formation of receptor dimers (oligomers) of the M_3 muscarinic acetylcholine receptor (on the basis of BRET data together with molecular modeling) suggested the existence of multiple dimeric/oligomeric receptor arrangements, possibly transient and existing in a dynamic equilibrium [91]. The results indicated the participation of multiple TM regions including TM1, 2, 4, 5 and 7, in dimer formation for this class A GPCR, and pointed to the involvement of the intracellular loop 2 and of helix 8 of both protomers in two structurally different dimer interfaces, respectively. These structural insights were later confirmed by other experimental approaches [92].

In spite of the extensive study of the structural aspects of dimerization among class A GPCRs, the outcomes linking oligomerization and functional impact were not always consistent [93, 94]. On the other hand, in case of the class B of GPCRs, less examined comparing to the class A, the results are well demonstrated [95]. An example of such research was presented by Harikumar *et al.*, who explored the occurrence and functional importance of human and rabbit calcitonin receptor oligomerization [96]. In earlier studies, they proved that the human secretin receptor produces an inter-receptor BRET signal with most other human class B peptide receptors, except for the calcitonin receptor [97]. Additional data indicated that the human receptor (hCTR) yielded no significant homodimeric BRET signal above background, while the rabbit receptor (rCTR) elicited a significant static BRET signal indicative of homodimerization. This work identified lipid-exposed residues in TM4 important for the dimerization of the calcitonin receptor. Further experiments were performed employing the peptide representing TM4 of the calcitonin receptor, which acts as competitor disrupting the homodimeric BRET signals from rCTR. The TM4 peptide altered cAMP signaling at both rCTR and hCTR, exposing functional differences between receptor monomers and dimers and indicating homodimerization of hCTR despite the absence of a BRET signal. Since the rabbit and human calcitonin receptor constructs bound calcitonin similarly, the results suggest that differences in BRET could result from differences in the orientation or in the stability of homo-dimeric receptor complexes, which were nevertheless similarly effective in producing the functions attributed to that complex.

Another example of class B GPCR dimerization is that of PAC1 receptor of PACAP (pituitary adenylate cyclase-activating polypeptide), which has been reported for the first time by Yu *et al.* by means of BRET and other complementary techniques [98]. Their findings suggest that the cysteine residue in the N-terminal HSDCIF (His-Ser-Asp-Cys-Ile-Phe) motif of the receptor, homologous to PACAP, plays a crucial role in cell surface trafficking and signaling as well as in the dimerization of PAC1. Moreover, an exogenous HSDCIF oligopeptide significantly modulated the signaling, trafficking and dimerization of the PAC1 receptor.

Recently, class B GPCR oligomerization has been reviewed in the literature [99, 100]. In their review, Roed *et al.* collected the currently accessible data on Secretin family GPCR homo- and heteromerization, including the most studied class B GPCRs - which comprise the SECR receptor (found in gastrinoma), GLP-1R, GCGR and GIPR (receptors involved in the intricate control of blood-glucose levels), as well as GLP-2R, PTHR1, VPAC1 and VPAC2, GHRHR or CALCR receptors - with a special focus on BRET studies [99]. For their part, Ng *et al.* reviewed this subject enunciating the advantages of the BRET technique over traditional biochemical methods as well as its drawbacks applied to those studies [100]. Both articles discuss the functional consequences of receptor oligomerization in ligand binding and cooperativity, allosterism, signaling, and trafficking for class B GPCRs. The current knowledge on oligomerization in the particular case of class C GPCRs, which form obligatory dimers, has been also recently reviewed elsewhere [101].

The theoretical background of BRET assays, presenting mathematical models for the quantitative interpretation of BRET saturation and competition assay results, was summarized by Drinovec *et al.* [102]. They reviewed not only the assessment of the specificity of the BRET signals, but also the possibilities of quantitative analysis of the data generated by RET-based techniques. Rigorous RET saturation experiments have been applied to assess the number of protomers per oligomer [54, 89, 103]. However, and consistently with the principle of RET, resolving the exact quaternary structure of GPCRs by using standard two-component RET approaches is considered experimentally difficult [104]. Sequential RET approaches such as three-color FRET (3-FRET) have allowed the assessment of the existence of higher order oligomers of the α_{1B}-adrenoceptor [105]. Nevertheless, perhaps the most significant contributions to this matter will come in the near future from the application of quantitative FRET approaches such as those based on spectral analysis [106, 107] and linear unmixing FRET (lux-FRET) [108].

Although the process of GPCR dimerization has been already accepted as a scientific fact, there are still some aspects waiting to be further investigated, such as the biological function of dimerization, the actual complexity of oligomeric organization or the nature of GPCR dimer coupling to G proteins and dimer stability [109, 110]. These phenomena are generally regarded as controversial primarily due to technical limitations for easy and precise detection of the oligomeric state of membrane proteins in living cells. Difficulties in proper interpretation of BRET experiments are also associated with functional limitations of the proteins fused with fluorescent or luminescent probes (because of the considerable size of the label), the artificial oligomerization and/or random transfer in the aftermath of the process of over-expression, the difficulty in controlling the donor/ acceptor expression ratios, the detection of signals from not only plasma membrane but also intracellular receptors, as well as a lack of validation of the method with standard membrane proteins [111]. However, according to Kawano *et al.* [111] the improvement of BRET analysis reliability could be achieved in the context of cell-surface-specific labeling, precise control of the labeling stoichiometry of the energy donor and acceptor, and diminution of the label. An auspicious tool to meet these prerequisites is the use of posttranslational labeling methods [37, 112].

In light of the presented facts about BRET, alone or in concert with complementary methods (sequential resonance energy transfer (SRET), bimolecular fluorescence complementation (BiFC), SNAP-tag technologies, TR-FRET) [86, 113], this technique seems to be, after all, capable of yielding answers for the remaining questions and doubts concerning the physio- and pathological role of GPCR dimerization and oligomerization.

Receptor-G Protein Coupling and G Protein Activation

GPCR interactions with G proteins have been monitored in real time in living cells by measuring $BRET^2$ between RLuc-tagged GPCRs and different constructs of Gα, $G\beta_1$, or $G\gamma_2$ individually tagged with GFP10 [114]. This is an example of how BRET can be used to identify new signaling pathways. i.e. by investigation of interactions of GPCRs with different G proteins. Since this early work, similar bimolecular BRET approaches have allowed the detection of constitutive GPCR/G protein coupling, the discrimination between full agonists, partial agonists and antagonists, and the characterization of specific receptor conformations promoted by different ligands through a unique receptor that could lead to ligand bias (reviewed in [41, 115]). A strategy to directly assess G protein activation is to follow Gα and Gβγ dissociation by monitoring BRET signals between both protein subunits, although decreases in BRET might reflect conformational rearrangements rather than complete dissociation. This line of work led to the description of the $G\alpha_{i1}$-91-RLuc/GFP10-$G\gamma_2$ $BRET^2$ sensor that monitors the separation of the Gα helical domain and the Gγ N-terminus occurring during G protein activation, allowing the characterization of ligand efficacy [116]. The molecular organization and dynamics of ternary complexes between GPCRs, regulators of G protein signaling (RGS) and G proteins have been investigated also by BRET approaches [117], and the same is true for GPCR signaling downstream events such as the interaction of Gβγ subunits with their effector adenylate cyclase II [118].

INTERACTION OF GPCRS WITH OTHER PROTEINS

GPCR/G Protein-Coupled Receptor Kinases (GRKs) Interactions

The interaction of GPCRs with proteins that regulate their function, such as the G protein-coupled receptor kinases (GRKs), has been assessed by BRET approaches among other techniques. GRKs phosphorylate the agonist-activated receptor, which accordingly to the accepted paradigm, determines the uncoupling of the receptor and the G protein, leading to homologous desensitization of classical G protein-dependent receptor signaling and binding of β-arrestins [119]. This would trigger receptor internalization and the initiation of non-classical signaling [120]. GPCR/ GRK2 interaction seems to be fast and transient, peaking after seconds of agonist application and declining within minutes [121-123], while GRK5, contrary to GRK2, appeared to be pre-associated to non-stimulated neurokinin-1

receptor [123]. More recently, and measuring either BRET[1], BRET[2] or FRET in a combined approach, Breton *et al.* determined the dynamics of pairwise interactions between α_{2A}-adrenoceptors, G protein ($G\alpha_{i1}\beta_1\gamma_2$) and GRK2 [40] in basal conditions and upon agonist stimulation, in different samples of the same population of cells expressing three distinct fusion proteins labeled with RLuc, GFP[2] and eYFP, together with the complementary unlabeled interacting partners. The results suggested that GRK2 interacts with the receptor while both Gα and Gβγ are still forming a complex.

BRET-based GPCR/β-Arrestin Interaction Assays

Agonist-promoted β-arrestin translocation is considered a generic assay for GPCR activation. Most GPCRs interact with β-arrestins, and particularly with β-arrestin2 [124], therefore this assay can be applied without prior knowledge of the receptor-G protein coupling. Typical experiments consist on the co-transfection of a GPCR tagged at the C-terminus with either a BRET donor or a BRET acceptor, and β-arrestin2 tagged either at the N- or C-terminus with the complementary BRET partner [125]. Agonist potency and efficacy at promoting β-arrestin translocation can be determined by constructing BRET dose-response curves for extraction of EC_{50} (agonist concentration eliciting a half-maximal response) and E_{max} (maximal response) values. Particularly, BRET-based assays for the detection of GPCR/β-arrestin interactions probed to be robust for drug discovery applications. Hence, the reliability of this assay for different BRET systems, estimated in terms of Z′ values (a statistical parameter for assessment of the quality of an assay itself taking into account only data from control wells within the assay) [126], is well compatible with high throughput screening (HTS) performance standards [127, 128] (Fig (**5**)), and its sensitivity allowed for example the detection of inter-species selectivity of ligands at GPCR orthologues [127].

A first study describing a BRET[1]-β-arrestin recruitment assay in stable mammalian cells and its successful application in HTS for GPCRs antagonists is the work of Hamdan *et al.* [55]. HEK293 cell lines were developed stably co-expressing optimal amounts of β-arrestin2-Rluc and Venus fusions of GPCRs belonging both to class A or class B, which included receptors that interact transiently or stably with β-arrestins. One of these cell lines was used for HTS of antagonists of the chemokine receptor CCR5, the primary co-receptor for HIV. After screening a total of 26,000 compounds, 12 compounds were found to specifically inhibit the agonist-induced β-arrestin2 recruitment to CCR5, and three of them were confirmed in other secondary functional assays of CCR5 signaling [55]. The increasing feasibility of generating stable cell lines for this type of BRET assay [55, 129, 130] will foster its implementation in HTS campaigns. A step further in the application of BRET-based GPCR/β-arrestin interaction assays was the successful monitoring of the dynamics of this interaction for the β_2-adrenergic receptor in a cell line derived from transgenic mice expressing the BRET[2] donor and acceptor transgenes at levels within physiological range [131].

Interactions of GPCRs with Receptor Tyrosine Kinases

It has been demonstrated that GPCRs and receptor tyrosine kinases (RTKs) can form heteroreceptor complexes within the cell membrane [132]. In these assemblies, the signaling from each receptor is modulated to produce an integrated, and therefore novel, response upon agonist activation [132, 133]. GPCRs and RTKs can form stable or transient complexes that are involved in modulating different aspects of receptor function, including ligand binding, signal transduction and receptor trafficking, desensitization and down-regulation, among others [132]. BRET was successfully applied to the study of protein-protein interactions in these complexes. In particular, Flajolet *et al.* [134] used BRET to identify a direct physical interaction between the adenosine A_{2A} receptor and the receptor tyrosine kinase fibroblast growth factor receptor1 (FGFR1). Also by means of BRET, Borroto-Escuela *et al.* [135] showed that this receptor can form complexes with serotonin 1A (5-HT_{1A}) receptors and they analyzed how these complexes are modulated by agonists. Their results demonstrate that neurotrophic and antidepressant effects of serotonin in the central nervous system might be partially mediated by the activation of the 5-HT_{1A} receptor protomer within the hippocampal FGFR1-5-HT_{1A} receptor complex, which enhances the FGFR1 signaling [135].

BYSTANDER BRET AS A READOUT OF SUB-CELLULAR LOCALIZATION OF MEMBRANE PROTEINS

By detecting bystander BRET between membrane proteins of interest and compartment-targeted BRET partners, one can obtain indirect information about the subcellular location of membrane proteins as well as their transit through the biosynthetic secretory pathway or their recruitment to membrane compartments in live cells, with subsecond temporal resolution and without involving imaging [136]. The information extracted from such experiments is qualitative and limited to proteins associated with membranes at a density of donors of ~200 copies, while acceptors (preferably the localization markers) must be expressed at relatively high densities. However, it is a sensitive and high resolution strategy (enough to resolve membrane topology and discriminate noncontiguous membrane compartments) that demands little user intervention for data acquisition or analysis. For this reason, it allows the scale-up of the assays without requirement of automated imaging instrumentation but standard plate readers. An early work employed bystander FRET measurements for reporting the activation of endogenous Ras on different cell membranes [137]. More recently, bystander BRET, with its advantages over FRET of little user intervention and low background signals allowing low levels of donor expression, was probed valuable to resolve the location of outer and inner mitochondrial membrane proteins, as well as the preferential association of the small GTPases H-Ras and K-Ras to different cell membrane compartments and the altered distribution of H-Ras as a consequence of preventing its palmitoylation [136]. The different subcellular distributions of two mature forms of the brain-specific splice variant of Cdc42 (bCdc42) were characterized by measuring bystander BRET between donor-labeled bCdc42 and acceptors directed to different membrane compartments [138]. This approach was also applicable to monitor the rapid redistribution from plasma membrane to endoplasmic reticulum (ER) of a fusion protein containing

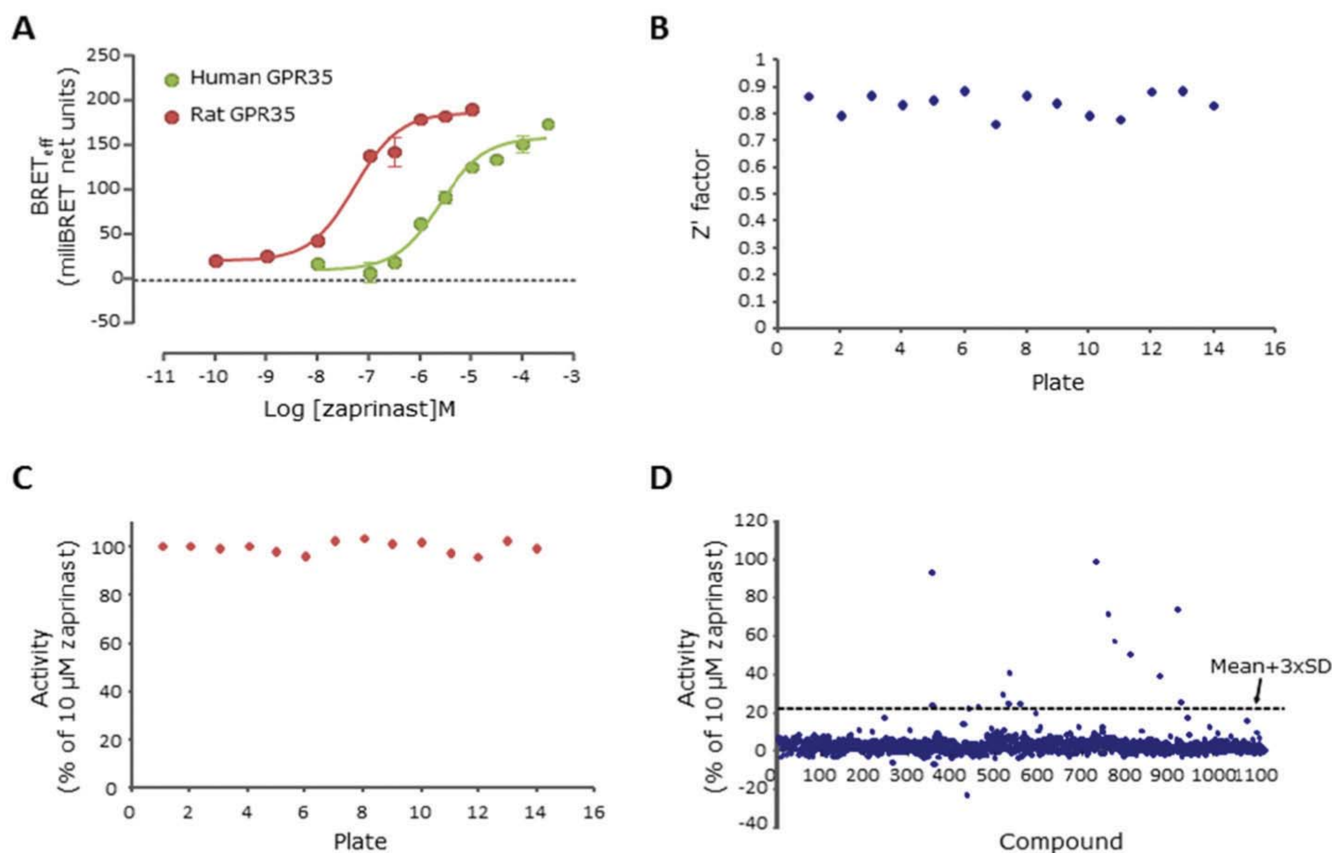

Fig. (5). Performance of a BRET[1]-based GPCR/β-arrestin2 interaction assay in a screening campaign on GPR35. A – Concentration-response curves for the GPR35 reference agonist zaprinast at human (green) and rat (red) GPR35 receptors. $BRET_{eff}$ between β-arrestin2 C-terminally tagged with RLuc and the rat or human orthologues of GPR35 C-terminally tagged with eYFP was defined as indicated in Fig. (**4c**) and it is expressed as miliBRET net units. EC_{50} values of zaprinast at rat and human GPR35 receptors were 6.8×10^{-8} M and 2.6×10^{-6} M, respectively. B – Assessment of the reliability of the assay in a screen of the Prestwick Chemical Library® at the rat GPR35. 1120 compounds were screened in 96 well plates, and each plate incorporated intraplate controls consisting of assay wells containing the highest concentration of zaprinast (1×10^{-5} M) for use as a 100% stimulation reference, and assay wells containing assay buffer only (in place of test compounds) for use as basal reference. Z′ factor was calculated for each individual plate accordingly to the equation $Z' = 1 - \{[(3 \times \sigma_{stim}) + (3 \times \sigma_{basal})]/(\mu_{stim} - \mu_{basal})\}$, where σ_{stim} and σ_{basal} are the standard deviation (S.D.) values of the wells containing 1×10^{-5} M zaprinast and assay buffer respectively and μ_{stim} and μ_{basal} are the means for the wells containing 1×10^{-5} M zaprinast and assay buffer respectively [127]. The graph shows the Z′ obtained for each plate. Plates with Z′ value higher than 0.5 are typically considered as valid in a screen. C – Distribution of the activity of a positive control (5×10^{-6} M zaprinast) included in each plate of the screen, expressed as % of the activity of 1×10^{-5} M zaprinast (100% stimulation reference). D – Results of the screen and selection of actives using an activity threshold based on statistical criteria: compounds showing an activity higher than the mean + (3 × S.D.) of the activity of all the compounds tested in this screen were considered possible hits. The figure adopted from reference [127].

the FKBP-rapamycin binding domain, in response to rapamycin in cells expressing an ER-localized FKBP moiety [136]. In the particular case of GPCRs, this approach allowed monitoring the trafficking of the β_2-adrenergic receptor during biosynthesis and the effect of ligands as pharmacological chaperones [136].

APPLICATION TO CONFORMATIONAL CHANGES IN PROTEINS: INTRAMOLECULAR BRET

Although less implemented than FRET-based biosensors in the GPCR field [139-141], intramolecular BRET approaches have been applied to the study of GPCR function. Monitoring of receptor conformational changes upon ligand binding has been assessed by means of intramolecular BRET between RLuc fused to the C-terminus and eYFP inserted into the ICL3 of the type 1 angiotensin II AT_1 receptor [142], a biosensor analogue to the FRET-based GPCR biosensors first described by Vilardaga *et al.* [139]. This approach allowed the detection of conformational rearrangements in a non-stimulated protomer of the AT_1 homodimer, due to allosterism upon ligand binding to the second protomer [142]. Moreover, a similar biosensor engineered on the nematode odorant GPCR ODR-10, in this case based on BRET[2], was produced in a yeast expression system and resulted in a high sensitivity biosensor for the detection of GPCR ligands in cell-free assays [143].

Other applications of intramolecular BRET biosensors in the GPCR field can be exemplified by the development of

sensors for the detection of cAMP signaling [144, 145] or of the double-brilliance β-arrestin2 biosensor for the monitoring of the conformational changes of β-arrestin2 upon its recruitment to the receptor [146]. These biosensors have been applied to the study of GPCR function in the case of trace-amine associated receptors (TAAR) and dopamine receptors, among others [42]. In particular, the double-brilliance β-arrestin2 biosensor underwent conformational changes that directly correlated with the ligand efficacy at β_2-adrenergic receptors. Moreover, it was able to differentially "sense" β-arrestin-biased or unbiased GPCR ligands [147].

CONCLUSIONS

BRET has significantly contributed to the study of GPCR signaling pathways and regulation. It was one of the technologies that allowed proving the existence of GPCR dimers and higher-order oligomers in living cells. Understanding the process of GPCR oligomerization at the molecular level is crucial for the design of more efficient and safer drugs targeting GPCR complexes. Although bivalent ligand of GPCRs are pharmacological probes rather than potential drugs due to unfavorable pharmacokinetic properties caused by their size, some registered drugs are GPCR monovalent ligands acting on GPCR dimers. This can be exemplified by morphine, fentanyl and other opioid antinociceptive compounds as their mechanism of action is best explained by assumption that they operate on μ opioid receptor – δ opioid receptor heterodimer. Furthermore, BRET has been also applied to uncover the interactions of GPCRs with receptor tyrosine kinases, complexes also with a great potential for the development of better drugs. Finally, the BRET technique made it possible to describe some aspects of the interaction of GPCRs with their respective G proteins, β-arrestin and other protein mediators and regulators of GPCR function. BRET has helped to establish the spatial and temporal patterning of GPCR signals in intact cells. In summary, BRET remains nowadays a method of choice to study protein-protein interactions in living cells and in real time, and we envision that it will contribute notably to the imaging of GPCR function in living animals.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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LIST OF ABBREVIATIONS

BiFC	=	bimolecular fluorescence complementation
BRET	=	bioluminescence resonance energy transfer
eBRET	=	extended bioluminescence resonance energy transfer
ECL	=	extracellular loop
eYFP	=	enhanced yellow fluorescent protein
FRAP	=	fluorescence recovery after photobleaching
FRET	=	fluorescence resonance energy transfer
GFP	=	green fluorescent protein
GPCRs	=	G protein-coupled receptors
GRK	=	G protein-coupled receptor kinases
ICL	=	intracellular loop
NC-IUPHAR	=	International Union of Pharmacology, Committee on Receptor Nomenclature and Classification
PPIs	=	protein-protein interactions
RET	=	resonance energy transfer
RLuc	=	*Renilla reniformis* luciferase
SRET	=	sequential resonance energy transfer
TM	=	transmembrane region
TR-FRET	=	time-resolved-fluorescence resonance energy transfer

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