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BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) METHODS TO STUDY G PROTEIN-COUPLED RECEPTOR - RECEPTOR TYROSINE KINASE HETERORECEPTOR COMPLEXES

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Abstract

A large body of evidence indicates that G protein-coupled receptors (GPCRs) and Receptor tyrosine kinases (RTKs) can form heteroreceptor complexes. In these complexes, the signalling from each interacting protomer is modulated to produce an integrated and therefore novel response upon agonist(s) activation. In the GPCR-RTK heteroreceptor complexes, GPCRs can activate RTK in the absence of added growth factor through the use of RTK signalling molecules. This integrative phenomenon is reciprocal, and can place also RTK signalling downstream of GPCR. Formation of either stable or transient complexes by these two important classes of membrane receptors is involved in regulating all aspects of receptor function, from ligand binding to signal transduction, trafficking, desensitization and down regulation among others. Functional phenomena can be modulated with conformation-specific inhibitors that stabilize defined GPCR states to abrogate both GPCR agonist- and growth factor-stimulated cell responses or by means of small interfering heteroreceptor complex interface peptides. The bioluminescence resonance energy transfer (BRET) technology has emerged as a powerful method to study the structure of heteroreceptor complexes closely associated with the study of receptor-receptor interactions in such complexes. In this work we provide an overview of different BRET² assays that can be used to study the structure of GPCR-RTK heteroreceptor complexes and their functions. Various experimental designs for optimization of these experiments are also described.

Keywords

G protein-coupled receptors (GPCRs); Receptor tyrosine kinases (RTK); heteroreceptor complexes; bioluminescence resonance energy transfer (BRET); GPCR-RTK heteroreceptor complexes; receptor-receptor interactions; allosteric modulation; homodimerization; heterodimerization; BRET saturation assays; BRET competition assays; BRET kinetics and dose-response assays

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I. INTRODUCTION

It is now several years since a general dogma established that growth-promoting activity of many GPCR ligands involves activation of RTKs and their downstream signalling cascades (Luttrell *et al*, 1999). Such observations led to the emergence of the 'transactivation' concept, which refers to the activation of RTK signalling pathways by GPCR ligands, see (Fuxe *et al*, 2007). However, over the past few years this concept appears to have become increasingly complex since pharmacological, biochemical and biophysical studies provided evidence for an engagement of GPCR signalling molecules (e.g. heterotrimeric G proteins and arrestins) in signal transduction generated by various RTK subtypes, which reveals a bidirectional cross-communication between RTKs and GPCRs (Dalle *et al*, 2002; Lin *et al*, 1998; Pyne and Pyne, 2011).

Perhaps an even more interesting concept relates to the apparent ability of different GPCRs to associate with RTKs and forming large receptor complexes (GPCR-RTK heteroreceptor complexes) (Borrito-Escuela *et al*, 2012a; Borrito-Escuela *et al*, 2012b; Flajolet *et al*, 2008; Fuxe *et al*, 2010). Such a higher molecular organization brings new alternatives in term of physiological functions for each of these two receptor families but it also increases the possibilities regarding therapeutic targeting (Alderton *et al*, 2001; Delcourt *et al*, 2007; Pyne *et al*, 2011; Waters *et al*, 2006). Biochemical and/or pharmacological properties reported for GPCR-RTK heteroreceptor complexes are often distinct from those of the corresponding protomers (Borrito-Escuela *et al*, 2012a; Delcourt *et al*, 2007; Pyne *et al*, 2011; Waters *et al*, 2006). Thus, heteromerization represents an important mechanism for modulating and integrating the physiological functions of GPCRs and RTKs. This can take place at different stages of the receptor's life: biosynthesis, ligand binding, G protein activation, desensitization, internalization and degradation. Also GPCR-RTK heteroreceptor complexes allow flexibility in terms of programming of spatially controlled signalling pathways and/or increasing signalling gain (Fuxe *et al*, 2010).

The existence of conformation-specific GPCRs present in the GPCR-RTK heteroreceptor complexes may offer unique opportunities to modulate pathophysiology driven by growth factors. It is likely that long term and global inhibition of RTK in associated diseases will not be well tolerated due to the role of RTK signalling in normal physiology (Andrae *et al*, 2008; Takeuchi and Ito, 2011). Therefore, GPCR-RTK partnership may lead to novel therapeutic strategies based on specific blockade of RTK signalling via GPCR trans-inhibition by antagonists and/or inverse agonists of the corresponding GPCR (Pyne *et al*, 2011). Such strategies are less likely to produce side effects than approaches based on direct RTK inhibition. This could potentially extend the repertoire of biological actions of a large number of compounds and drugs that bind to GPCRs. Combinations of RTK inhibitors and GPCR-specific ligands that reduce G protein or β -arrestin function may be an effective way of abrogating signalling from these heteroreceptor complexes.

In order to progress in our understanding of the functional role of GPCR-RTK heteroreceptor complexes as well as their potential role as therapeutic targets, we need to continuously look for methods and technologies that can be used to demonstrate and evaluate such heteroreceptor complexes and their receptor-receptor interactions. The bioluminescence resonance energy transfer (BRET) technology has emerged as a powerful and straightforward biophysical technique for studying receptor heteromers and heteroreceptor complexes and their receptor-receptor interactions. The present study focuses on recent work illustrating the power of BRET² for the study of GPCR-RTK interactions, using A2A-FGFR1 (Flajolet *et al*, 2008) and 5-HT1A-FGFR1 (Borrito-Escuela *et al*, 2013; Borrito-Escuela *et al*, 2012a; Borrito-Escuela *et al*, 2012b) heteroreceptor complexes as examples. We highlight the current ways in which the BRET-based methodology is being

used to establish the existence of GPCR-RTK heteroreceptor complexes and their specificity. Furthermore we describe how BRET may be used to establish the involvement of a bidirectional cross-communication mechanism between GPCRs and RTKs.

II- THE METHOD PRINCIPLE

BRET is a natural phenomenon found in some marine species (for instance in the sea pansy, *Renilla reniformis*) resulting from the non-radiative energy transfer between a luminescent donor (*Renilla* luciferase - Rluc) and a fluorescent acceptor protein (green fluorescent protein - GFP). Originally developed to study the interactions of circadian clock proteins in bacteria (Xu *et al.*, 1999), BRET has subsequently been applied to study receptor-receptor interactions in living cells. It has become a powerful method to assess such interactions at the molecular level. The method is based on the principle of Förster resonance energy transfer, which postulates that the efficacy of energy transfer between a donor and an acceptor is inversely proportional to the sixth power of the distance between them.

When studying receptor-receptor interactions, one protomer is fused to the donor (Rluc) and the other protomer to the acceptor (fluorescent protein). If the two fusion protomers interact and the distance between the energy donor and acceptor is less than 10 nm, a resonance energy transfer occurs and the emission signal from the acceptor protein can be detected. However, the energy transfer process not only depends on the distance between donor and acceptor, it also relies on the overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor and the relative spatial orientation of donor and acceptor. Therefore, the absence of BRET signal between two receptors does not necessarily mean that these receptors do not interact with each other. Thus, all such factors must be taken into account. On the other hand, increasing the acceptor/donor ratio may lead to a detectable but non-specific BRET signal. This can be illustrated in BRET saturation assays, where a fixed amount of donor is co-expressed with increasing amount of the acceptor. Nonspecific BRET signals tend to increase linearly with increasing acceptor concentrations. In contrast, a progressive increase of the BRET signal in a hyperbolic manner represents the complete saturation of all donors with acceptor molecules and a specific BRET signal (Marullo and Bouvier, 2007; Pflieger and Eidne, 2006b). Over the last years different generations of BRET (BRET¹, BRET², eBRET², BRET³ and QD-BRET) have been developed, depending on the type of enzyme substrate and the nature of donor/acceptor pairs (Kocan *et al.*, 2008; Pflieger *et al.*, 2006b; Xing *et al.*, 2008). As a result, the nomenclature for describing each of the BRET forms has not followed a unique rigorous pattern. The original BRET method using coelenterazine h (benzyl-coelenterazine) as substrate is nowadays called BRET¹ (Marullo *et al.*, 2007). In BRET¹, the maximal emission of Rluc is observed at 480 nm, a wavelength that is appropriate for excitation of a yellow fluorescent protein (enhanced YFP: EYFP), which subsequently reemits light at 530 nm. Several other variants of the YFP with identical excitatory properties are YFP topaz, YFP citrine, YFP venus and YPet (Bacart *et al.*, 2008). BRET¹ is characterized by strong signals and long life-time making it one of the most suitable approaches for BRET saturation assays. Changes in the Rluc substrate used resulted in the second generation of BRET methods, the BRET². In the BRET² is used as Rluc substrate the bisdeoxycoelenterazine (DeepBlueC™) or didehydrocoelenterazine (coelenterazine-400a) resulting in different donor emission spectra that shift the maximal light emission of Rluc to 395 nm. Appropriate acceptors are GFP2 and the GFP10 with excitation and emission maxima of 400 and 510 nm, respectively. BRET² in comparison to BRET¹ has an improved separation of donor and acceptor emission peaks. This makes this form a better choice for screening assays where high signal to noise ratios are required. However, a clear limitation of BRET² is the low light emission (DeepBlueC™ as a substrate leads to up to 300 times less light emission) and the short life-time. More recently, the introduction of a mutated version of the Rluc enzyme, the Rluc8 variant (Rluc mutant

containing eight amino acid substitutions), leads to an approximately 5–30 fold increase in the original BRET² signal, which results in a new BRET form, the so called enhanced BRET² (eBRET²) (Kocan *et al*, 2008). In the eBRET² we can combine the advantage of greater spectral resolution of the original BRET² with a higher quantum yield when using Rluc8. The introduction of a third Rluc substrate, called Endu-Ren, the protected form of the coelenterazine, resulted in a form of BRET¹, known as extended BRET (eBRET) (Pfleger *et al*, 2006a). This enables real-time monitoring of receptor-receptor interactions for extended periods of time and provides stability over time that is logistically advantageous for high throughput screening applications. The eBRET name can result in a nomenclature confusion with the previous name eBRET².

A similar confusion arises with the introduction of the BRET³. For some authors BRET³ refers to a BRET form that results from a combination of a red-shifted fluorophore (e.g mOrange) with Rluc8, using as substrate EnduRen, although the system was validated with coelenterazine h (De *et al*, 2009). However, for other authors (Bacart *et al*, 2008), BRET³ refers to a donor/acceptor pair formed by a firefly luciferase (from *Photinus pyralis*) and acceptors whose excitation peaks overlap with the emitted light at 565 nm (for instance the 24-kDa DsRed fluorescent protein; peptides labeled with Cy3 or Cy3.5) using as a substrate the D-luciferin developed by Gammon *et al*. (Gammon *et al*, 2009). The Firefly luciferase in BRET³ shows lower cellular autofluorescence at the emission wavelength (565nm) and a more sustained light emission by firefly luciferase compared to Rluc. However, disadvantages are weak signals and overlap between donor and acceptor emission peaks. In addition, the tendency of DsRed to oligomerize has to be considered in these BRET experiments for proper data analysis. It is therefore highly recommended to use instead a DsRed-monomeric variant (Clontech, USA). Finally, a new BRET version has been introduced: the Quantum Dot-BRET (QD-BRET) (Bacart *et al*, 2008; Xing *et al*, 2008). QDs are semiconductor nanocrystals excited at any wavelength ranging from UV to 530 nm, and their light emission wavelength, which depends on their diameter, can cover the spectrum from blue to near infrared. They are then suitable energy acceptors for BRET¹- and BRET²-based assays as their broad interval of excitation wavelengths overlaps the currently used luciferase emitted light. It should be mentioned that based on the work of Medintz and Mattoussi, it seems that QD are not so good as energy acceptor but rather used so far as energy donor (Medintz and Mattoussi, 2009). So far, QDs have only been used in BRET¹-based assays, where QDs, directly linked to Rluc, were injected into mice and energy transfer monitored in the presence of coelenterazine h (Xing *et al*, 2008). Even though not really useable at this time, The self-illuminating feature of QD-BRET makes imaging technically possible and could be optimized in the future to work in conditions where the generation of photon is limited such as in tissues. The emission peaks are clearly separated which makes QD-BRET ideal for screening applications. But its major disadvantages are the large size of QD molecules, ranging from 1.5 to 6 nm, and the fact that QD are semiconductor nanocrystals. Thus, it is not a genetically coded protein that can be synthesized by living cells and must therefore be added.

Several of these variants of the BRET technology have or can be utilized to provide evidence for receptor-receptor interactions in living cells. In line with the huge potential of BRET technology, bimolecular fluorescence complementation (BiFC) (Cabello *et al*, 2009) and bimolecular luminescence complementation (BiLC), see (Vidi *et al*, 2011), have recently been developed and combined with BRET to study more complex receptor-receptor interactions in higher-order receptor oligomers. Several studies continue to improve the potential use of other luciferases and new coelenterazine derivatives have been developed with brighter (ViviRenTM) or extended (EnduRenTM) light emission. Furthermore, the development of the BRET³ opens the door to the characterization of receptor-receptor interaction *in vivo*.

III. SETTING UP A BRET ASSAY

Performing a BRET assay to investigate a potential GPCR-RTK interaction involves several steps:

1. Selection and generation of the donor/acceptor and substrate combination. Generation of the two proteins of interest genetically fused with either donor luciferase protein or acceptor fluorescent protein depending of the chosen BRET approach (see Table 1) at either the N- or C-terminus. For a membrane-linked receptor it is more intuitive to fuse the donor/acceptor protein to the intracellular C-terminal tail of the receptor. A large proportion of GPCRs and RTKs require the presence of a N-terminal signal peptide for correct cell surface expression. This signal is susceptible to proteolytic cleavage and therefore precludes the use of the N-terminal position for the fusion. To avoid to lose the N-terminal moiety after cleavage of the peptide signal the only alternative is to insert the cDNA of the fluorescent protein downstream of the peptide signal. While this has been done successfully with smaller tags (e.g. myc, HA) it does not seem to be as practical with larger cDNAs.
2. Design and validation of the receptor of interest, including suitable controls. For a given BRET assay, the most adequate control protein has to be determined since the inclusion of a negative/positive control is crucial to determine the specificity of the studied interaction. As a positive control one can generate a double fused chimeric protein where the donor and acceptor proteins have been linked together. Also when studying GPCR-RTK interactions the use of the corresponding homodimeric pair of receptors can be considered as positive control(s). Thus, for both GPCRs and RTKs it has been extensively documented that they can exist as homodimers. In the case of RTKs this process takes place upon agonist induced receptor activation. As negative controls, receptors presenting a similar topology and subcellular localization as the receptor of interest should be considered. With the development of molecular dynamic simulation of receptor-receptor interactions, it is nowadays possible to predict the receptor interface. Interesting interaction hot spot(s) can be identified and mutated to serve as negative control(s) (Borrito-Escuela *et al*, 2010b).
3. Selection of the cell system and coexpression of the two BRET fusion proteins at a relevant physiological level of expression. Transient transfection (e.g. calcium phosphate, Lipofectamine, FuGENE HD) or virus-based transfection systems have been used successfully. Common mammalian cells (e.g. HEK293T, COS-7, CHO) as well as primary neuronal culture cells are compatible with BRET assays. Depending on the type of BRET assay, different ratios of donor/acceptor should be used/tested (see Subheading 4.1.1 and 4.2.1). For some assays we may consider the use of bi-cistronic vectors, which may guarantee a more homogeneous receptor ratio expression level.
4. Detection of the BRET signal and design of an appropriate assay. The BRET signal can be measured from adherent cells, cell suspensions, subcellular fractions, purified proteins, and also from culture medium in the case of secreted proteins using a white plate to avoid light absorption and in parallel a black plate to record total fluorescence values. The biological material (50–100 μ l) is then dispatched into the 96-well plate and 10–20 μ l substrate is added (5 μ M final concentration for coelenterazin). BRET signals are measured immediately after substrate addition using a BRET reader capable of measuring light emitted at donor and acceptor wavelengths in a quasi-simultaneous manner. The use of the BRET method to study GPCR-RTK interactions goes further than the simple measurement of energy

transfer between the donor/acceptor pair together with the use of proper positive and negative controls. If a BRET signal is observed, the proper cellular localization and function of the fusion proteins have to be verified. If the functional validation step is successful, the BRET signal can be studied further to evaluate its specificity. In fact, several assays have been developed with the aim of providing evidence for the existence of specific receptor-receptor interactions and to discriminate between genuine physical interactions between the GPCR and the RTK versus a random collision due to overexpression of the receptor pairs. Therefore, in addition to the classical negative control used, BRET saturation, competition and ligand-promoted (kinetic and dose-response) assays have been developed. In addition, each of these assays can shed light on the stoichiometry, conformation and dynamic changes that may take place in GPCR-RTK heteroreceptor complexes.

5. **Analysis of the BRET signal.** The BRET signal or BRET ratio is defined as the light signal of the acceptor emission relative to the light signal of the donor emission. This proportion is corrected for the background signal due to the overlap of donor emission at the acceptor wavelength, always determined in parallel for cells expressing the donor alone. Then the BRET net value is calculated by subtracting this BRET background ratio from the BRET ratio obtained in cells coexpressing the two partners. The amount of donor can be estimated from the maximal luciferase values measured separately after the BRET reading. The amount of acceptor has to be determined in an independent reading by recording fluorescence values of the acceptor pair in a fluorometer (using a black microplate to avoid light scattering). The calculated acceptor/donor ratio can be used to compare different experiments. To obtain acceptor/donor ratios that correspond to real protein quantities, luciferase and fluorescence values have to be converted into protein amounts using independently established standard correlation curves with real protein quantities (e.g., determined in radioligand binding experiments for each receptor fusion protein).

IV. METHODS AND DETAILED PROTOCOLS

In recent years a number of excellent papers have well documented and provided detailed discussions on different forms and approximations to the BRET assay methodology and its straightforward use to study receptor-receptor interactions mainly focused on GPCR-GPCR interactions (Ayoub and Pfleger, 2010; Hamdan *et al*, 2006; Marullo *et al*, 2007). However, in this field, works describing in detail the potential use of BRET methodology to study GPCRRTK heteroreceptor complexes and their dynamics are still missing. Consequently, we have used here the A2A-FGFR1 and 5-HT1A-FGFR1 heteroreceptor complexes as examples to illustrate the potential use of different BRET² assay formats to monitor GPCR-RTK interactions in living cells.

We chose the BRET² variant assay with Rluc8 as a donor and GFP2 as an acceptor with improved spectral separation of the donor and acceptor emission peaks. This implies less bleed-through at the acceptor emission maximum and lower background. Also, the rationale for the use of mutated Rluc (Rluc8, Genbank: EF446136) instead of the non-mutated Rluc is mainly based on the fact that Rluc8 gives a higher quantum yield compared to the non-mutated Rluc. Therefore, we do not need to overexpress the luciferase fusion receptor that is detrimental for interaction specificity.

Performing a BRET assay to investigate a potential GPCR-RTK interaction and in more general terms any receptor-receptor interaction involves several steps:

1. **Generation and validation of BRET Fusion Constructs.** The two receptors of interest must be genetically fused to either *Renilla* luciferase (Rluc) or GFP variants at either the N- or C-terminus. The choice of the fusion protein (N- vs C-terminus) depends on the nature of the studied proteins. For RTK and GPCR it is for instance more advisable to fuse the donor and acceptor protein to the C-terminal tail (see **III.1**). It is important to consider pre-existing information concerning specific domains of the receptor of interest, like for example the tyrosine kinase domains in RTK and their potential binding sites to regulatory/adaptor proteins. Also posttranslational modification sites, like palmitoylation at the C-terminal of some Class A GPCR, can induce a restricted conformation of the C-terminal tail of the receptor and longer polylinker spacers should be used in order to unmask the donor/acceptor protein and give it a proper orientation. Finally, it is important to ensure that the insertion of the donor/acceptor protein does not interfere with the proper folding of the receptor and insurance of a correct functionality and localization deserves a particular attention. Below are listed the 5 categories of constructs generated for this study. 1) BRET acceptor constructs: 5-HT1A-GFP2 and A2A-GFP2 for expression of both the 5-HT1A and A2A receptors, C-terminally tagged with GFP2 (PerkinElmer, Sweden). 2) BRET donor constructs: FGFR1-Rluc8 and 5-HT1A-Rluc8 for expression of the FGFR1 and 5-HT1A, C-terminally tagged with Rluc8. 3) Positive control: BRET fusion construct for expression of the Rluc8-GFP2 fusion protein. 4) Negative control BRET constructs: pcDNA3.1-Rluc8 and pGFP2 for expression of Rluc8 and GFP2 alone, respectively. 5) Specificity controls: 5-HT2A-GFP2, and D2mutantR-Rluc8 for expression of other GPCRs were C-terminally tagged with either Rluc8 or GFP2. Each fusion construct was tested for detectable luminescence (using a luminometer following addition of coelenterazine 400a or DeepBlueC™ substrate) or fluorescence (using a fluorometer following direct laser excitation). The fusion receptor was also validated with respect to their function by means of radioligand binding assay and/or gene reporter assays to ensure that the addition of the donor or acceptor molecule has not altered ligand affinity/efficacy/potency. The use of confocal microscopy is highly recommended to ensure correct protein localization.
2. **Cell transfection and co-expression of the BRET fused receptors in mammalian cells.** For BRET experiments, we have mostly used human embryonic kidney cells (HEK293T-27). Independently of the kind of BRET assay to be conducted, it is always recommended to perform two sets of transfections: (a) Transfection of one set of cells only with donor fused construct, which will allow to correct for the luciferase signal; (b) Co-transfection of another set of cells with both the cDNAs coding for the donor and acceptor fused constructs, which correspond to the BRET pair for the signal measurement. Dependent on the BRET assay to be performed, different or constant donor/acceptor plasmid ratios must be used (see **4.1.1** and **4.2.1** respectively).
3. **Harvesting the transfected cells.** BRET² measurement can be performed in two different ways: (a) 24h after transfection cells are detached using trypsin-EDTA or Versene (for adherent cell layer). Cells are then microcentrifuged 5 min at 300 × g at room temperature, the supernatant discarded and the cells washed and resuspended in HEPES-buffered DMEM without phenol red. 40–100 µl of cells are distributed per well of a 96- well white cell culture plate and maintained at 37°C with 5% CO₂ in a humidified incubator for a further 24h to allow attachment before BRET² recording. (b) A second approach consists in detaching the cells 48h after transfection and microcentrifuge for 5 min at 300 × g at room temperature. The cell pellets are washed once with 1 ml PBS containing 0.5 mM MgCl₂, PBS removed

and the cells resuspended in 1 ml of PBS containing 0.5 mM MgCl₂ and 0.1% glucose. The cell number in the cell suspension is determined either by measuring the OD₆₀₀ or by protein concentration (e.g. BCA method). Equal amounts of cell suspension are distributed per well of a 96-well white cell culture plate and then proceeded to BRET² measurement.

4. **BRET measurement.** BRET² measurements are performed under temperature-controlled conditions to obtain reproducible results. BRET² is initiated by adding the luciferase substrate coelenterazine400a (5 μ M final concentration) and detected using a luminometer (e.g. POLARstar Optima plate reader; BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings 410 $\pm$ 80nm and 515 $\pm$ 30nm when using coelenterazine400a as substrate, Rluc8 as donor and GFP2 as acceptor. Light from each well is measured simultaneously through each filter. BRET², where the rapid decay in the emitted luminescence is faster than in BRET¹, presents some limitations and the interval time for measurement must be taken into account. BMG POLARstar is equipped with two online injectors that can deliver compounds and substrate while simultaneously measuring luminescence at two different wavelengths. This significantly increases the reading time per plate. Alternatively, the substrate can be added manually to a maximum of 12 wells at a time, followed by the BRET² readings. However, this can become easily inconvenient when large numbers of samples are considered.
5. **Calculating BRET ratio.** Data are then represented as a normalized BRET² ratio, which is defined as the BRET ratio for coexpressed Rluc and GFP2 constructs normalized against the BRET ratio found for the Rluc expression construct alone in the same experiment:

$$\text{BRET}^2 \text{ ratio} = \left[\left(\text{GFP}^2 \text{ emission at } 515 \pm 30\text{nm} \right) / \left(\text{Rluc emission } 410 \pm 80\text{nm} \right) \right] - \text{cf.}$$

The correction factor, cf, corresponds to (emission at 515 $\pm$ 30nm)/(emission at 410 $\pm$ 80nm) found with the Receptor-Rluc construct expressed alone in the same experiment.

BRET-based studies of receptor-receptor interactions are particularly prone to false-positive signals and require multiple controls. BRET² saturation and competition assays have been developed to extend the information obtained from basic BRET² experiments toward a more quantitative and detailed analysis of the BRET² signal.

4.1. Protocol 1: Saturation Assay

In titration or saturation BRET² experiments cells are transfected with a constant amount of BRET²-donor in presence or absence of increasing amounts of the acceptor. Theoretically, for any specific interaction between the Receptor-donor and Receptor-acceptor fusions, the BRET² ratio increases hyperbolically as a function of increasing GFP/Rluc value, to reach an asymptote (saturation) when all donor molecules are associated with acceptors (BRET_{max}, Figure 1). By contrast, in the case of nonspecific interactions (bystander BRET), a quasi linear plot is expected or eventually reaches a plateau for higher values of receptor density. Nevertheless, the saturation curve should be independent of the total expression level of receptors and the BRET² configuration used. However, BRET_{max} values cannot be used as a quantitative measure of the relative number of homo/heteromers formed for each combination because they are not only a function of the dimer numbers but also depends on the distance between the energy transfer partners as well as their relative orientation within the receptor complex. BRET² saturation curves have been particularly

used with the aim to establish the oligomeric order of receptor complexes, as well as the proportion of receptors engaged in dimers or oligomers. They are also used to determine whether ligand-induced BRET² signals depend on conformational changes or association/dissociation of interacting receptors. Also, saturation assay has been used to compare the relative affinity of receptors for each other and their probability to form a complex, the so called BRET50, which represents the acceptor/donor ratio giving 50% of the maximal signal (Figure 1). The BRET50 value is often compared between the two different homomer subtypes and their corresponding heteromer. Many GPCR/GPCR heteromers show no difference in the relative affinity between their receptor homomers and their specific heteromers. Furthermore, neither BRETmax nor BRET50 values may be modified following the agonist activation of the heteromer, consistent with the general consensus that GPCR homo- and heteromerization is often constitutive. In contrast, GPCR/RTK heteroreceptor complexes show more dynamic features where agonist treatment markedly affect BRETmax, BRET50 or both these values (Figure 1).

Details are shown below for typical BRET² assays for detection and analysis of GPCR-RTK (A2A-FGFR1) heteroreceptor complexes using either adherent cells or cells in suspension.

1. Several independent transfections should be performed using a constant amount of cDNA coding for the BRET donor (10–100 ng, FGFR1-RLuc8) and increasing quantities of cDNA coding for the BRET acceptor (i.e., 0, 10, 20, 50, 100, 200, 300, 500, 1000 ng, A2A-GFP2) and sufficient “empty” vector (such as pCDNA3.1 or any other cloning vector) to bring the total amount of cDNA in the transfection to 1000 ng/well in a 6-well plate.
2. 24 hours (preferred option -a-, see **IV.3**) or 48 hours (preferred option -b-, see **IV.3**) after transfection cells are washed, detached and distributed into white opaque 96-well microplates: 40–100 μ l per well, incubated as described below (**4.1.3**) or moved directly to the BRET ratio measurements.
3. The cells are pre-incubated in the absence or presence of agonist/antagonist drugs if ligand-induced BRET² signals will be analyzed. Otherwise, proceed directly to BRET ratio measurements.
4. BRET² ratio measurements are performed after adding coelenterazine-400a diluted in HBSS or PBS CaCl₂/MgCl₂ to each well in order to reach a final concentration of 5 μ M. BRET² ratio readings are performed using a lumino/fluorometer that allows sequential integration of luminescence signals detected with two filter settings (see **IV.4**). The specific BRET² ratio is calculated by subtracting from the mean BRET² ratio value above the background BRET² ratio, which corresponds to the signal obtained with cells expressing the BRET-donor alone (see **IV.5**).
5. In saturation assays specific BRET² ratio values are plotted as a function of the GFP2/RLuc fusion protein ratio. Therefore, the total amount of luminescence (BRET donor amount) and fluorescence (BRET acceptor amount) must be determined for each transfection. It is important that the BRET-donor levels are relatively constant throughout the experiment. In case of significant variation (difference of 20% or more from the average value) the corresponding points should be excluded from the final plot or the experiment repeated again. To quantify the amount of BRET-acceptor in each well, the fluorescence is measured at 510 nm after external excitation at 410 nm in black 96-well microplates. Background fluorescence is obtained by determining fluorescence in wells containing untransfected cells (GFP2 zero (0) value) and subtracted from the fluorescence values measured in cells expressing increasing amounts of BRET-acceptor (GFP2) to obtain the specific GFP2 values. Often when distributing cell

suspensions into white opaque 96-well microplates (see above **4.1.2**) a parallel and similar procedure is performed in black 96-well microplates in order to quantify the amount of the acceptor by fluorescence measurements. Otherwise, cells are detached from spare wells of the white 96-well plate using 100 μ l PBS-EDTA or Versene, washed twice with PBS and collected by centrifugation for 5 min at $300 \times g$ at RT. The pellet is resuspended in 150–200 μ l PBS and transferred to a black 96-well plate for fluorescence measurements.

6. GFP2-GFP20/Rluc8 fusion protein ratio is calculated for each data point. Depending on the application, it may be necessary to convert luminescence and fluorescence values into absolute amounts of interacting partners using standard curves correlating luminescence and fluorescence signals with amounts of proteins (see below **4.1.7**).
7. Correlations are assessed between fluorescence or luminescence values and receptor expression levels in BRET² experiments. When appropriate radioligands are available determination of receptor expression levels in BRET² assays can be relevant to ascertain that the expression level of fusion proteins falls within the physiological range. It can also be useful to determine the true acceptor/donor ratio in BRET² saturation experiments. Luminescence and fluorescence levels of several receptor-donors and receptor-acceptors are found to be linearly correlated with receptor densities (Borrito-Escuela *et al*, 2010a). This correlation is an intrinsic characteristic of each fusion protein, and therefore correlation curves have to be established for each construct. Cells are transfected (a reasonable number of different independent transfections should be performed) with different quantities of either the BRET-donor or the BRET-acceptor fusion protein plasmid. Then the luciferase activity (for the BRET-donor receptor) and fluorescence values (for the BRET-acceptor receptor) are determined as described above. In parallel, saturation radioligand binding experiments are performed using appropriate assay conditions for each receptor. The number of radioligand binding sites is then plotted against fluorescence or luminescence values determined in the same sample; and a linear correlation is expected and obtained. These standard curves can be used to transform fluorescence and luminescence values into fmol of receptor. Thus, the fluorescence/luminescence ratios can be transformed into (receptor-GFP²)/(receptor-Rluc8) ratios, which allows to determine accurate BRET_{max} and BRET₅₀ values.
8. BRET ratio values from **4.1.4** can be plotted as a function of the (GFP2-GFP0)/Rluc8 ratio (**4.1.6**) or (receptor-GFP²)/(receptor-RLuc) ratio as described in **4.1.7**. Data are fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism) and BRET_{max} and BRET₅₀ values can be determined (Figure 1).

4.2. Protocol 2: Competition Assays

BRET² displacement experiments can also be performed to shed some light on the specific nature of a given receptor-receptor interaction. In a BRET² competition or displacement assay, the BRET² ratio is measured at a fixed ratio of donor and acceptor in the presence of increasing concentrations of a non-tagged native partner. Over the last few years, in order to test the ability of a GPCR to form heteromers and to further investigate the specificity of BRET signals, competition experiments have often been carried out. It has been demonstrated that the use of an untagged Receptor X or Y co-expressed with the Receptor X-Rluc and Receptor Y-GFP2 decreases the BRET ratio signal as a consequence of the ability of the untagged receptor to interact with one or both fusion proteins and compete for the complementary BRET fusion protein. In a GPCR-RTK heteroreceptor complex a true

interacting partner (e.g an excess of one of them or a competitive interacting receptor) would be also capable of reducing the BRET signal of the receptor complex, whereas a non-interacting partner would not. However, in spite of this theoretically reasonable and until now well supported concept that reflects true receptor-receptor interactions (at least for several GPCR-GPCR interactions) this does not necessarily represent an unequivocal interpretation. It could be that, for instance in the case of GPCR-RTK interaction analysis, the results will not be in line with the expected idea that a true interacting partner would be capable of reducing the BRET signal of the receptor complex. GPCRs are seven helix transmembrane receptors and RTKs are single helix transmembrane receptors. The specificity and diversity of the interface interaction between these two classes of receptor families may allow for a wider plasticity or diversity of the receptor-receptor interaction. Instead of observing a reduction of the BRET ratio upon untagged receptor co-expression?, we can still observe a similar or even increased BRET ratio. This behaviour could be the result of a reorganization/reconfiguration of the heteroreceptor complex, where more than two receptors could be accommodated, sharing different receptor-receptor interface interactions.

1. Before running a BRET² displacement assay, it is highly recommended to conduct a classical BRET² saturation experiment as described in **4.1**. Using around 50–60% of the (GFP2-GFP20)/Rluc8 BRET_{max} values in the saturation experiment, will give a better idea of the amount of donor and acceptor needed for transfection in the BRET competition experiments.
2. Perform several independent transfections using a constant ratio amount of cDNA coding for the BRET² donor and acceptor (as selected in **4.2.1**). Increasing amounts of the untagged receptor are used together with sufficient “empty” vector (such as pCDNA3.1 or any other cloning vector) to keep equal total amount of cDNA /well.
3. Incubate the cells and measure the BRET² ratio (see **IV.4**).
4. Calculate BRET² ratios (see **IV.5**) and plot them as a function of the expression of native receptor determined by binding experiments or as a function of the total untagged cDNA amount employed (Figure 1).

4.3. Protocol 3: Kinetics and dose-response assays

In addition to monitoring constitutive receptor-receptor interactions, BRET² can be used to measure ligand-dependent induction of facilitatory or inhibitory receptor-receptor interactions and also to follow the kinetics of these interactions in real time. Indeed, ligand modulation of the BRET² signal may also prove the specificity of the examined interaction. Until now, for most GPCR heteromers studied by BRET, homo- and heteromerization appear to occur constitutively and independently of receptor activation state. However, the dependence of the energy transfer process on the distance between the donor and acceptor fused to the receptors as well as on their relative orientation suggests that movements within receptor complexes as a consequence of ligand-induced conformational changes may result in detectable changes in FRET/BRET signals. This does not necessarily mean changes in the association–dissociation between the receptors.

Many interesting examples where specific ligand-induced conformational changes translate into changes in BRET² signal were observed in most studies on RTK-RTK interactions and on GPCR-RTK heteroreceptor complexes (Borrito-Escuela *et al*, 2012b; Romero-Fernandez *et al*, 2011). This illustrates the usefulness of the BRET² technology as a read-out to characterize GPCR-RTK heteroreceptor complexes and their pharmacological features. This is in addition to the more specific outcome of opening up novel avenues for GPCR-RTK drug discovery.

As an example, this protocol describes the use of BRET² to measure the kinetics and dose-responses of the agonist-promoted FGFR1 activation (homodimerization) and the effects of combined and single agonist treatment with 8-OH-DPAT and FGF2 on FGFR1 homodimerization in cells containing FGFR1-5-HT1A heteroreceptor complexes (Figure 2). For this type of experiments where we intend to test how the GPCR-RTK heteroreceptor complexes may induce modulation of the ligand-dependent BRET² RTK-homodimerization signal, cells are co-transfected at a fixed ratio with a plasmid coding for the BRET-donor (RTK-Rluc8) in the presence of a single concentration of the plasmid encoding the BRET-acceptor (RTK-GFP2) and the plasmid encoding for the WT-GPCR protomer. BRET measurements are performed over time (up to 20–30 min) after incubation with a RTK-specific agonist or upon combined agonist treatment (RTK and GPCR agonist).

1. HEK-293T cells are prepared and transfected using a constant ratio amount of cDNA coding for the BRET RTK-donor and RTK-acceptor. This ratio should be selected from a previously performed BRET saturation assay. Otherwise different plasmid DNA concentrations should be tested to determine the optimal ratio of receptor-donor to receptor-acceptor which gives the highest BRET² signal following receptor activation. Also a constant amount of the untagged GPCR protomer and sufficient “empty” vector (such as pCDNA3.1 or any other cloning vector) will be used to maintain equal total amount of cDNA per well during transfection.
2. The next day cells are harvested and dispensed in 50–100 μ l of cell suspension containing 20,000 to 30,000 cells into a white opaque 96-microplate in HEPES-buffered DMEM without phenol red.
3. *For agonist-induced dose response BRET:* 24 hours after plating cells are incubated with different concentrations of the selected receptor agonist or PBS alone (basal control condition) for a fixed time. Then coelenterazine-400a solution at a final concentration of 5 μ M is added and the BRET² signal is measured (Figure 2). The agonist and substrate can be added manually or injected if the plate reader is equipped with built-in online injectors. *For kinetic BRET experiments:* cells are incubated for different time periods with a fixed concentration of the selected receptor agonist or PBS alone (basal control condition). Then coelenterazine-400a solution at a final concentration of 5 μ M is added and the BRET² signal is measured. We highly recommend when using BRET² assay to incubate first the cells with the selected agonist at different times ranging in the order of minutes (if it is possible) and then read all together instead of performing a more quick kinetic analysis with continuous measurements in view of the fast kinetic delay of substrate when coelenterazine-400a is used (Figure 2).
4. Calculation and interpretation of the BRET² signal. In agonist-induced BRET² signalling, the BRET² ratio is defined as: *ligand-promoted BRET² ratio* = *BRET ratio (in presence of a ligand)* – *BRET ratio (in absence of a ligand or in presence of PBS)*. Plot the BRET data against the logarithm of ligand concentration and analyze it by nonlinear curve fitting (sigmoidal dose response) using GraphPad PRISM, which will allow to obtain the concentration eliciting a half-maximal response (EC50 value). When analyzing the kinetic BRET² data, the BRET² signal can be plotted against time to produce kinetic profiles, examples of which are shown in Figure 2. Changes in the time-course profile and apparent association (or dissociation) rate constants can be calculated from such data.

VI. DISCUSSION AND NOTE ON CRITICAL PARAMETERS

In spite of the increased understanding of the role of GPCR transactivation by RTK ligands and visa versa and their existence as heteroreceptor complexes, only few examples have until now been validated using the BRET² methodology (Borrito-Escuela *et al*, 2012a; Borrito-Escuela *et al*, 2012b), most likely due to the particularly difficult nature of such receptor interactions. We believe that a well controlled and carefully analysed BRET assay has a great potential to identified and/or study GPCR-RTK heteroreceptor complexes (Figure 3). It also seems to have a high value in investigating the mechanism of action and the pharmacological properties of drugs acting on these important therapeutic targets. The present work gives a step-by-step description of the BRET² methodology using specific examples, confronting the pros and cons of various protocols.

BRET² assays have been used to study GPCR heteromerization for about a decade now and as the field has matured, we have a better understanding of the underlying technological limitations and an improved ability to interpret BRET data. The method is particularly suited for the molecular characterization of GPCR-RTK heteroreceptor complexes, but also for the screening of new compounds that bind specifically to these complexes, with the ultimate goal of identifying novel therapeutic drugs.

Progressive improvements and diversification of the BRET-based assay place this technology among the most powerful methods for the study of GPCR-RTK heteroreceptor complexes and their conformational changes in living cells. Also, new perspectives of this methodology could include the monitoring of these interactions at the subcellular level to unravel the molecular mechanisms underlying GPCR-RTK heteroreceptor complex trans-activation/trans-inhibition processes, internalization and signaling.

However, as any other methodologies, it has its limitations and drawbacks and is constantly being improved with regard to experimental design, instrumentation and reagents. For example, in some receptor-receptor interaction studies, because the efficiency of energy transfer is tightly dependent on proper orientation of the donor and acceptor dipoles, conformational states of the fusion proteins may fix the dipoles into a geometry that is unfavourable for energy transfer. Thus, two receptors may form heteroreceptor complexes without producing any significant BRET signal. Thus, a negative result with BRET does not necessarily mean absence of the heteroreceptor complex. Furthermore, the luminescent and fluorescent protein fused to the candidate protomers may affect their interaction, alter their subcellular localisation, protein folding and receptor function. Moreover, as with any technique that involves transient transfection, overexpression of the protomers may bring misleading results. Therefore, such drawbacks limits its use today and leads to a demand for proper controls. It is now easier to keep the receptor protomer expression levels within physiologically relevant ranges thanks to the improvement of donor/acceptor protein quantum yield (e.g. the new mutated Rluc8). On the other hand, BRET² presents various advantages compared to standard biochemical procedures to study receptor-receptor interactions that require cell-invasive processes such as solubilisation and co-immunoprecipitation, and which includes even the previously developed FRET technique. BRET², as opposed to FRET, does not require the excitation of the donor with an external light source. Therefore, it does not suffer from problems usually associated with autofluorescence, light scattering, photobleaching and/or photoisomerization of the donor moiety which results in an overall improved signal-to noise-ratio when compared to earlier versions of the resonance energy transfer technologies. Also the absence of contamination of the light output by the incident light results in a very low background in BRET² assays, thereby permitting the detection of small changes in the BRET² signal as compared to FRET. Also, the BRET² signal is a ratiometric measurement which can help to reduce data

variability caused by fluctuations in light output due to variations in assay volume, cell types, number of cells per well and/or signal decay across a plate. Finally, the coelenterazine derivative DeepBlueC or coelenterazine-400a used in BRET² is membrane permeable and non-toxic, which makes BRET² an ideal assay technology for live cell assays. DeepBlueC penetrates the cell membrane in seconds to activate Rluc8 emission. With respect to receptor-receptor interaction mechanisms and signalling research, its capabilities have now significantly reached beyond studying GPCRs. The BRET² assay technology is used successfully for a wide range of assay types including GPCR-beta-arrestin assay for monitoring G-protein coupled receptor activity, tyrosine kinase receptor activation, Ca²⁺ and cAMP detection, apoptosis assay, kinase activity, and protease activity.

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Figure 1. BRET² saturation assay shows specific A2AR and FGFR1 interaction in HEK293T cells

(A) The existence of a A2AR-FGFR1 heteroreceptor complexes and their agonist regulation by CGS21680 and/or FGF2 have been validated using quantitative BRET² saturation curves assay in HEK293T27 cells co-transfected with a constant amount of FGFR1-Rluc8 plasmid and increasing amount of the A2AR-GFP2 plasmid. In the current analysis the amount of each receptor effectively expressed in transfected cells was monitored for each individual

experiment by correlating both total luminescence and total fluorescence to the number of receptor-binding sites (biochemical binding analysis) in permeabilized cells. The linear regression equations derived from these data were thus used to convert fluorescence and luminescence values into femtomoles/mg protein of receptor in order to obtain accurate values. Cells were pre-incubated 10 min with vehicle, CGS21680 (100 nM), FGF-2 (50ng/ml), or with both CGS21680 and FGF-2 (100nM and 50ng/ml, respectively). The A2AR/FGFR1 curve fitted better to a saturation curve than to a linear regression, *F* test ($P < 0.001$). Data are means $\pm$ s.e.m. ($n=5$). The BRETmax values were significantly enhanced by combined, CGS21680 and FGF-2 treatment alone versus vehicle or CGS21680 treatment alone; and FGF2 treatment alone versus vehicle or CGS21680 treatment alone ($P<0.01$). **(B)** The BRET50 values were significantly reduced by combined, CGS21680 and FGF-2 treatment alone versus vehicle ($P<0.001$).

Figure 2. Effects of combined and single treatment with 8-OH-DPAT and FGF2 on FGFR1 homodimerization in HEK293T27 cells containing FGFR1-5-HT1A heteroreceptor complexes
The modulatory effect of 5-HT1A agonist 8-OH-DPAT was studied on the FGF2 induced FGFR1/FGFR1 homodimer formation by means of BRET² analysis. HEK293T27 cells were transiently co-transfected at a constant ratio (1:1:1) with 5-HT1A, FGFR1-Rluc8 and

FGFR1-GFP2. **(A)** A concentration-response curve with FGF-2 was performed on the development of the BRET² signal from the FGFR1 homodimer in the HEK293T27 cells. The cells were transiently co-transfected at a constant ratio (1:1:1) of 5-HT1A, FGFR1-Rluc8 and FGFR1-GFP2 and treated with the agonist ligands for 5 min before BRET² measurement. Treatment with 8-OH-DPAT (with two different concentrations: 50nM and 250nM) shifted the curves of the BRET² signal to the left which indicate an enhanced potency of combined treatment with FGF2 and the 5-HT1A agonist vs FGF-2 treatment alone to promote FGFR1 homodimer formation. **(B)** The kinetics of the FGFR1-Rluc/FGFR1-GFP2 interaction after FGF2 treatment and its modulation by 8-OHDPAT was also studied in transiently transfected HEK293T27 cells using the BRET² assay to study the FGFR1 homodimer over a period of 20 minutes. FGF-2 and the combined FGF-2 and 8-OH-DPAT treatments showed no clear-cut changes of the BRET² value over the 8 min period. However, the combined treatment had a weak tendency to increase the BRET² signal over time whereas the FGF2 alone treatment had a markedly tendency to decrease the BRET₂ signal over time.

Figure 3. Different BRET² strategy assays to study GPCR-RTK heteroreceptor complexes dynamic

BRET² assays seems to be particularly suited for the molecular characterization and study of GPCR-RTK heteroreceptor complexes. Using different combination of receptor donor/acceptor fused receptor, it could be possible to unravel the molecular mechanisms underlying GPCR-RTK heteroreceptor complex trans-activation/trans-inhibition processes, internalization and signalling. (**centre**) For instance, it could be possible to study the existence of the GPCR-RTK heteroreceptor complexes and the pharmacological properties of drugs acting on these important therapeutic targets using as a BRET pairs, the GPCR-fused receptor as a donor and the RTK-fused receptor as acceptor. (**left**) Furthermore, the effects of combined and single treatment with GPCR and RTK agonist on the RTK homodimerization in cells containing the GPCR-RTK heteroreceptor complexes could be feasible. (**right**) In addition, the effects of combined treatment with the GPCR and RTK agonist on GPCR receptor homodimerization in cells containing the GPCR-RTK heteroreceptor complexes could be possible. Each of these different BRET approach could bring new light in the conformation changes and dynamic process that can take place upon agonists treatment in the GPCR-RTK heteroreceptor complexes.

TABLE1

Summary of the different BRET methods.

Method	Donor	Acceptor	Spectral Properties (emission, nm)		Substrate
			Donor	Acceptor	
BRET¹	Rluc	EYFP	480	530	coelenterazine h
Extended BRET	Rluc	EYFP	480	530	Endu-Ren
BRET²	Rluc	GFP2	395	510	DeepBlueC™/coelenterazine-400a
Enhanced BRET²	Rluc8	GFP2	395	510	DeepBlueC™/coelenterazine-400a
BRET³	Firefly	DsRed	565	583	D-luciferin & cofactors
BRET³	Rluc/Rluc8	mOrange	480	564	Endu-Ren / coelenterazine h
QD-BRET	Rluc	QD	480	605	coelenterazine h

Over the last years different generations of BRET (BRET¹, BRET², eBRET², BRET³ and QD-BRET) have been developed, depending on the type of enzyme substrate and the nature of donor/acceptor pairs. As a result, the nomenclature for describing each of the BRET forms has not followed a unique rigorous pattern.