Using Quantitative BRET to Assess G Protein-Coupled Receptor Homo- and Heterodimerization. Methods Mol Biol

ARTICLE *in* METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.) · JANUARY 2011

Impact Factor: 1.29 · DOI: 10.1007/978-1-61779-160-4_9 · Source: PubMed

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Chapter 9

Using Quantitative BRET to Assess G Protein-Coupled Receptor Homo- and Heterodimerization

Lamia Achour, Maud Kamal, Ralf Jockers, and Stefano Marullo

Abstract

Over a period of 15 years the concept of G protein-coupled receptor (GPCR) dimerization moved from a challenging hypothesis to a scientific fact, which is now accepted by the vast majority of the scientists working in the field. However, several important issues remain debated such as the biological function of dimerization, or the actual complexity of the oligomeric organization. Because of its major potential implications in physiology and pharmacology, the question of GPCR heterodimerization (or hetero-oligomerization) is currently one of the most central. Several complementary experimental approaches are used to investigate these novel important aspects of GPCR biology. In this context, Bioluminescence Resonance Energy Transfer-based techniques are extremely powerful, provided that they are conducted with the appropriate (numerous) controls and correctly interpreted.

Key words: G protein-coupled receptor, Resonance energy transfer, Bioluminescence resonance energy transfer, Oligomerization, Endoplasmic reticulum, Quality control, Biosynthetic pathway, Allostery, Conformational change

1. Introduction

Studying interactions among proteins is one of the most important and challenging tasks of post-genomic biology. Among the available approaches to study these phenomena in living cells, Resonance Energy Transfer (RET)-based techniques have become increasingly popular over the past few years, in particular when investigating G protein-coupled receptor (GPCR) signaling and oligomerization. Indeed, these approaches do not necessitate any separation or purification, and are sensitive enough to allow studies at physiological concentrations of proteins. RET consists of the non-irradiative energy transfer between a donor and an acceptor.

Louis M. Luttrell and Stephen S.G. Ferguson (eds.), *Signal Transduction Protocols*, Methods in Molecular Biology, vol. 756,
DOI 10.1007/978-1-61779-160-4_9, © Springer Science+Business Media, LLC 2011

Because the efficacy of the energy transfer varies inversely with the sixth power of the distance between the donor and acceptor molecules, a signal is obtained only if the two molecules are in close proximity (1–10 nm). Thus, the detection of an energy transfer between two proteins fused, respectively, to an energy donor and an energy acceptor, often reflects the existence of molecular interaction between the proteins of interest. In contrast, an absence of signal does not exclude the possibility that two proteins interact. It may be due to the particular conformations of the interacting partners that maintain the acceptor too distant from the donor or cause inappropriate relative orientation of the donor to acceptor molecules.

Bioluminescence RET (BRET) has been inspired by a natural phenomenon observed in glowing marine organisms. In the presence of its substrate, coelenterazine, *Renilla* luciferase (Rluc, the luminescent energy donor) transfers some energy to a GFP variant (the energy acceptor) (1). No excitation of the donor is required and the substrate, which is membrane permeable, can be added to the supernatant of cultured cells. BRET-based protocols have been designed to monitor and quantify both regulated and constitutive molecular interactions in intact cells, such as GPCR oligomerization (2). In this context, performing experiments in intact cells avoids possible artifacts due to receptor solubilization, an obligate step for other biochemical assays such as coimmunoprecipitation.

Most (if not all) GPCRs may exist as either homo- or heterodimers or as higher-order oligomers (3). Oligomerization is not an absolute prerequisite for proper signaling, as shown by functional studies on purified monomers (4, 5). Dimerization, instead, could have an important role during biosynthesis for the quality control of newly synthesized receptors (6, 7). Moreover, ligand-driven transactivation or inhibition, between protomers within a receptor dimer or between adjacent dimers in larger complexes, has been reported for an increasing number of receptors representing additional functions for GPCR oligomerization (8).

Except in few specific cases, such as the extensively investigated example of $GABA_B$ receptors (9), the issue of GPCR heterodimerization is a complex phenomenon to analyze experimentally and to interpret functionally, even with well-controlled BRET experiments. The hydrophobic properties of GPCR transmembrane domains may lead to false-positive interactions between different protomers in reconstituted systems. In this context, quantitative issues are critical to consider, since nonspecific interactions tend to increase with rising concentrations of the protein of interest. Also, because reconstitution systems used for BRET analysis artificially drive the synthesis of the receptors to be studied in the same cell at the same time, specific interactions may not be representative of a physiological situation simply because in real life the receptors are not synthesized in the same cell and/or

at the same moment. The literature reports numerous examples of functional cooperativity between different GPCRs (reviewed in (10) and other articles of the same issue), which are attributed to GPCR heteromerization. However, at least in some cases, this functional cooperativity is more likely due to the interaction of distinct receptor homodimers within higher-order oligomers (11, 12). The protocols detailed below will allow the reader to quantitatively investigate the oligomerization of their favorite GPCRs using BRET and will provide some guidelines for the appropriate interpretation of their results.

2. Materials

2.1. Cell Culture and Transfection

1. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 4.5 g/L glucose, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1 mM glutamine (Invitrogen).
2. Solution of trypsin-EDTA (0.05%).
3. PBS-EDTA solution: Phosphate-buffered saline (PBS; 1×) without $CaCl_2$ and $MgCl_2$ (Invitrogen) plus 2 mM ethlenediaminetetraacetic acid (EDTA).
4. 6- and 12-well plates for cell culture, White 96-well plates with clear well sterile and tissue culture treated (Perkin Elmer).
5. Poly-L-lysine used to coat 96-white well plates for adherent cell experiments.
6. Transfection reagents: JetPEI (Polyplus transfection), GeneJuice (Novagen), FuGENE6 transfection reagent (Roche Applied Science).

2.2. Chemiluminescence, Fluorescence, and BRET-Ratio Measurements

1. Coelenterazine-h powder (Uptima; Interchim) or Deepblue C (Coelenterazine 400a; Uptima, Interchim) are dissolved in 100% ethanol at 1 mM (stock solution) and stored at –20°C in opaque microcentrifuge tubes (see Note 1).
2. Hank's balanced salt solution (HBSS; 1×) containing $CaCl_2$ and $MgCl_2$ (Invitrogen).
3. PBS (1×) containing $CaCl_2$ and $MgCl_2$ (Invitrogen).
4. Multi-mode microplate reader: Mithras (LB 940) (Berthold) or equivalent.
5. White 96-well plates (Perkin Elmer Optiplate™-96HB or equivalent) for BRET measurements.
6. Black 96-well plates (Perkin Elmer Optiplate™-96HB #6005279 or equivalent) for fluorescence measurements.

2.3. SDS-Polyacrylamide Gel Electrophoresis

1. Separating buffer (4×): 1.5 M Tris–HCl, pH 8.8, 0.4% SDS. Store at room temperature (RT).
2. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8, 0.4% SDS. Store at RT.
3. Forty percent (w/v) acrylamide/bisacrylamide solution (Sigma). Acrylamide is a neurotoxin when unpolymerized and so care should be taken not to receive exposure.
4. *N,N,N,N′*-Tetramethyl-ethylenediamine (TEMED).
5. Ammonium persulfate solution: Prepare 10% solution in water and immediately freeze in single use (200 μL) aliquots at –20°C.
6. Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a glass bottle and allow separation. Use the top layer. Store at RT.
7. Running buffer (5×): 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS. Store at RT.
8. Prestained molecular weight markers: e.g., BenchMark Prestained Protein Ladder (Invitrogen).

2.4. Western Blotting for Quantitative Assessment of GPCR Expression in BRET Experiments

1. BCA protein assay reagent.
2. Lysis Buffer: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2ethanesulfonic acid), pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.5% NP40, 10% glycerol, and complete protease inhibitors.
3. Laemmli sample buffer (5×): 312.5 mM Tris–HCl, pH 6.8, 50% glycerol (v/v), 10% SDS (w/v), 8% Dithiothreitol DTT (w/v), 1% Bromophenol blue (w/v).
4. Setup buffer: 25 mM Tris–HCl (do not adjust pH), 190 mM glycine, 20% (v/v) methanol.
5. Transfer buffer: Setup buffer plus 0.05% (w/v) SDS. Store in the transfer apparatus at RT.
6. Nitrocellulose Transfer Membrane: e.g., PROTRAN (Whatman).
7. PBS-Tween 0.2%: 500 ml PBS with 1 ml Tween-20.
8. Blocking buffer: 5% (w/v) nonfat dry milk in PBS-Tween 0.2%.
9. Secondary antibodies: Peroxydase-conjugated Affinipure antibodies (Jackson Immuno-research).
10. ECL™ western blotting detection reagent (Amersham™, GE Healthcare).
11. High performance chemiluminescence film: e.g., Amersham Hyperfilm™ ECL from GE Healthcare.

2.5. Radioligand Binding Assays for Quantitative Assessment of GPCRs in BRET Experiments

1. TEM lysis buffer: 25 mM Tris pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, containing protease inhibitors (2 μg/ml, benzamidine 1 mM AEBSF, 1 μg/ml pepstatin A and 1 μg/ml leupeptin).
2. Ultra Turrax® tissue disperser (Janke and Kunkel).

3. Methods

3.1. Preliminary Notions

To monitor the homo- or heterodimerization of GPCRs, one GPCR protomer is fused to Rluc (BRET-donor) and the other to a fluorescent BRET-acceptor (YFP for BRET1 or GFP2 for BRET2; see Note 2). The energy transfer requires that both the donor and the acceptor be on the same side of the cellular membrane. In theory, it should be possible to fuse Rluc or fluorescent proteins to the extracellular (or intraluminal) extremity of a GPCR, but in this case signal peptides should be added N-terminally to facilitate receptor export (these type of constructs have been used for FRET experiments). In general, for BRET experiments, the donor and the acceptor are fused at the C-terminal tail of a GPCR and are thus located in the cytosol. Depending on the type of BRET assay to be used (BRET1 or BRET2), the appropriate fusion protein constructs are generated using classical cloning methods (see Note 3). BRET measurements can be performed in various types of intact mammalian cell lines; either adherent (e.g., HEK-293 T, CHO, COS, Hela cells) or in suspension (e.g., THP-1, Jurkat). Optimization of the conditions of transfection of BRET constructs is necessary (see Note 4). BRET measurements consist of calculated ratios of the light emitted by the luciferase (donor) over the fluorescence emitted by the YFP (acceptor). They require BRET readers that can measure rapidly, simultaneously (at both wavelengths) and repetitively luminescence (produced by the BRET-donor) and fluorescence (emitted by the BRET-acceptor) values in the same well. These readers are generally controlled by software, programmed by the user to accomplish the measurements, and also calculate the BRET ratios. Both raw data and calculations are saved on a spreadsheet compatible with Microsoft Excel or similar.

3.2. Setting Up a BRET Donor Saturation Experiment

The BRET-donor saturation assay has been developed from original basic BRET experiments for a more quantitative and precise interpretation of BRET signals (13, 14). The level of expression of the BRET-donor used in saturation experiments (GPCR-Rluc, in the present case) should correspond to the lowest amount of protein required to obtain a detectable and robust BRET signal.

Cells are transfected with a constant amount of plasmid DNA coding for the BRET-donor fusion protein in the presence or absence of increasing concentrations of the plasmid for the BRET-acceptor fusion protein. The BRET signal will increase with the concentration of the acceptor up to a maximum that is achieved when all BRET-donor molecules are in proximity of BRET-acceptor molecules. In case of a specific interaction, the BRET signal increases hyperbolically and reaches an asymptote. In contrast, in case of nonspecific interaction resulting from random proximity, the "bystander BRET" signal increases almost linearly and eventually saturates at very high expression levels of the BRET-acceptor protein (15) (see Note 5).

3.2.1. Detailed Protocols to Detect GPCR Dimerization in HEK-293T Cells

We will detail below typical BRET1 assays for detection and analysis of GPCR dimers using either adherent cells or cells in suspension.

3.2.2. BRET Measurements on Adherent Cells

1. HEK-293T cells are seeded 24 h before transfection in 12-well plates ($250–500 \times 10^3$ cells per well in 2 mL of complete DMEM medium).
2. Cells are transfected with 1 μg of total DNA per well using one of the transfection reagents indicated in Subheading 2.1, according to the manufacturer's instructions. This DNA quantity comprises a fixed amount of plasmid encoding the BRET-donor (GPCR1-Rluc: 10–100 ng, depending on the obtained expression level); increasing concentrations of the energy acceptor (GPCR2-YFP: 0, 25, 50, 100, 200, 300 … 990 ng) and sufficient "empty" vector (such as pCDNA3.1 or any other cloning vector) to bring the total amount of DNA in the transfection to 1 μg.
3. White 96-well plates are incubated with poly-L-lysine (50 μL per well) for 10 min at 37°C. The poly-L-lysine solution is then removed and the wells washed once with complete medium. Note that this step can be skipped when using cell lines that adhere tightly to the plastic.
4. Twenty-four hours after transfection, the cells of each well of the 12-well plates are washed with PBS, detached in 200 μL PBS-EDTA (5 min at 37°C) and resuspended in 2 ml of complete medium. Cells in suspension are distributed into white 96-well plates: 200 μl per well (corresponding to about 50,000 cells) and incubated at 37°C for 24 h before BRET measurements.
5. The next day, cells are washed with PBS containing $MgCl_2$ and $CaCl_2$ (see Note 6). The BRET signal is measured after adding 50 μl of a mixture containing 40 μL of PBS-$CaCl_2$/$MgCl_2$ and 10 μL of a freshly prepared solution of 25 μM coelenterazine-h diluted in HBSS or PBS-$CaCl_2$/$MgCl_2$ to each well.

6. After 10 min incubation at RT, BRET readings are started using a lumino/fluorometer that allows sequential integration of luminescence signals detected with two filter settings (Rluc Filter: 485 ± 10 nm; YFP filter: 530 ± 12.5 nm) (see below and Note 7 for the detailed procedure).
7. Fluorescence measurements to quantify the amount of expressed acceptor are performed using the same equipment. Cells are detached from spare wells of the white 96-well plate using 100 μL PBS-EDTA as described, washed twice with PBS and collected by centrifugation for 5 min at 300 × *g* at RT. The pellet is resuspended in 200 μL PBS and transferred to a black 96-well plate for fluorescence measurement at 530 ± 12.5 nm.

3.2.3. BRET Measurements on Cells in Suspension

1. Forty-eight hours after transfection (performed as described above), cells are detached from 12-well plates with PBS-EDTA (5 min at 37°C), transferred to a microcentrifuge tube, and collected by centrifugation for 5 min at 300 × *g* at RT.
2. Pellets are resuspended in 250 μl HBSS (or PBS-$CaCl_2$/$MgCl_2$) and 40 μl of the cell suspension are distributed into each well of a white 96-well plate.
3. BRET measurements are performed directly after adding 10 μl of 25 μM coelenterazine-h solution.
4. For YFP fluorescence measurements, 50 μl of the cell suspension is distributed into black 96-well plates and readings are performed as described above.

3.3. Calculating the BRET Ratio and Data Analysis

1. The BRET ratio is the fluorescence signal (filter 530 ± 12.5 nm) over the Rluc signal (filter 485 ± 10 nm) measured simultaneously. This ratio (automatically calculated by the software of BRET readers) is obtained by measuring each well for 1 s. The readings are repeated three to six times to obtain average values and all data are saved on a spreadsheet. The specific BRET ratio is calculated by subtracting from the mean BRET ratio value above the background BRET ratio, which corresponds to the signal obtained with cells expressing the BRET-donor alone (not expressing the BRET-acceptor). Results are expressed in milli-BRET units (mB) by multiplying the values × 1,000 to avoid the need to manipulate decimal numbers.
2. To quantify the amount of BRET-donor in each well, the average luminescence values at 485 ± 10 nm are calculated (see Note 8). It is important that BRET-donor levels are relatively constant throughout the experiment. In case of significant variation (difference of 30% or more from the average value) the corresponding points should be excluded from the final plot.

3. To quantify the amount of BRET-acceptor in each well, the fluorescence is measured at 530 ± 12.5 nm after external excitation at 480 nm. Background fluorescence measured in cells not expressing the BRET-acceptor (YFP_0) is subtracted from fluorescence values measured in cells expressing the increasing amounts of BRET-acceptor (YFP) to obtain YFP-YFP_0 values. Depending on the application, it may be necessary to convert luminescence and fluorescence values into actual protein expression levels using standard curves correlating luminescence and fluorescence signals with protein amounts (see Note 9).
4. From the values of *Points 2* and *3*, YFP-YFP_0/Rluc values are calculated. BRET ratios from *Point 1* are then plotted as a function of YFP-YFP_0/Rluc values. A slightly different calculation method can be used (see Note 10), which is of interest when comparing experiments conducted at different times. Data are fit using a nonlinear regression equation assuming a single binding site (GraphPad Prism) to estimate BRETmax and BRET50 values (see Note 11 and Fig. 1).

3.4. BRET Displacement Assays

A hyperbolic BRET-saturation curve using two different GPCRs, as BRET-acceptor and BRET-donor, does not have an unequivocal interpretation. It may reflect true heterodimerization or proximity of distinct homodimers. BRET competition experiments can be used to assess GPCR heterodimerization. If two GPCRs do form heterodimers, an excess of one of them should be capable of displacing the homodimerization of the other (Fig. 2).

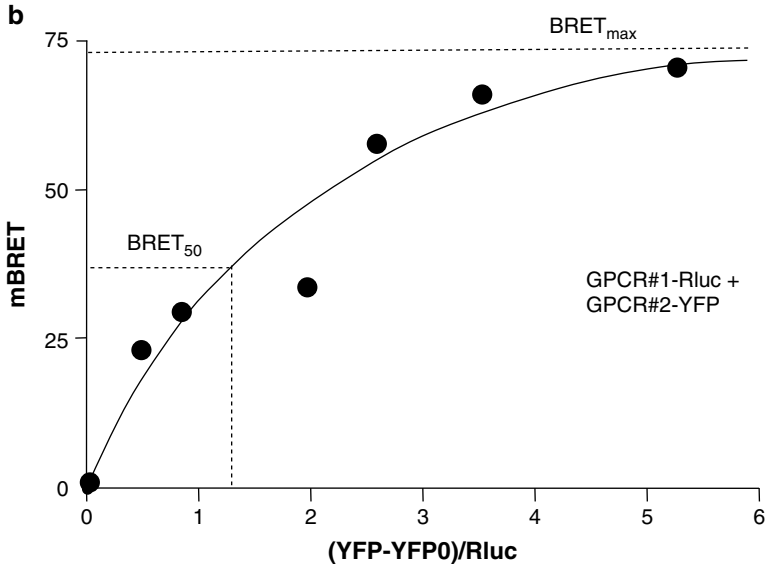
1. A typical BRET-saturation experiment is conducted, as described in Subheading 3.2, using the GPCR1 as both BRET-donor and BRET-acceptor.

Fig. 1. Prototypical BRET donor saturation experiment to study GPCR heterodimerization. (**a**) BRET data acquisition and calculations. In the presented experiment, cells have been transfected with a constant amount of GPCR1-Rluc : (10 ng; column 1) and increasing amounts of GPCR2-YFP (25, 50, 100, 250, 500, 1000; columns 2–7). Cells from each transfection have been distributed into three wells (triplicate) of a white 96-well plate and BRET measurements have been repeated six times for each well. Because of the limited space, the values obtained for only one well are shown in the figure, although the values in lines 23–32 have been calculated from the average values of triplicates. The six measurements of the Rluc signal are in lanes 3–8, those for the YFP signal in lanes 10–15. The mean BRET ratio calculated from values of lanes 17–22 is shown in lane 23. The background ratio, which corresponds to the BRET signal obtained in samples expressing the GPCR1-Rluc alone (column 1), is shown in lane 24. Specific BRET ratios (lane 25) are calculated by subtracting from each mean BRET ratio (lane 23) the background BRET ratio (lane 24); values are then multiplied × 1,000 to obtain mBU (lane 26). The average of the specific BRET ratio (lane 27) represents the average of specific BRET ratios from the triplicate multiplied by 1,000. The average of luminescence values from triplicates is used to quantify the BRET donor (GPCR1-Rluc) in the transfection (lane 28). The quantity of BRET acceptor (GPCR2-YFP) expressed in each transfection (lane 31), is then calculated by subtracting the background fluorescence (YFP_0, lane 30) measured in cells expressing the BRET-donor alone (column 1), from fluorescence values of lane 29. YFP-YFP_0/Rluc are then calculated and multiplied ×100 (lane 32) to avoid the manipulations of decimal numbers. (**b**) A BRET-saturation curve is obtained by plotting BRET ratios from A (lane 27) as a function of YFP-YFP_0/Rluc (lane 32) and fitting the data with a hyperbolic equation. Two important parameters are defined from the curve: the $BRET_{max}$, which represents the maximal signal reached at saturation, and the $BRET_{50}$, which corresponds to the BRET ratio giving 50% of the maximal BRET signal.

a

1	Transfection	1	2	3	4	5	6	7
2	Rluc Signal							
3	00:00:000	106030	93920	98140	95920	102750	91520	73370
4	01:56:330	117770	118040	114230	121720	118970	92560	91520
5	03:52:690	112280	119730	112120	121790	115300	87300	92560
6	05:48:990	102790	112360	102770	114430	104200	84910	87300
7	07:45:270	93260	103570	94120	105140	94650	87850	79640
8	09:41:630	86790	95140	86310	96950	86610	83220	72770
9	YFP signal							
10	00:00:000	68460	62530	65910	65328	72620	64594	52639
11	01:56:330	75290	78840	76290	82419	82468	65385	65152
12	03:52:690	71310	79780	75340	82474	80722	61937	66311
13	05:48:990	65760	75060	68830	76536	72711	60024	61936
14	07:45:270	59830	68180	62740	70300	65954	61702	57655
15	09:41:630	55720	64030	57520	65761	60146	59024	49998
16	BRET ratio							
17	00:00:000	0,6457	0,6658	0,6716	0,6811	0,7068	0,7058	0,7174
18	01:56:330	0,6393	0,6679	0,6679	0,6771	0,6932	0,7064	0,7119
19	03:52:690	0,6351	0,6663	0,6720	0,6772	0,7001	0,7095	0,7164
20	05:48:990	0,6398	0,6680	0,6697	0,6688	0,6978	0,7069	0,7095
21	07:45:270	0,6415	0,6583	0,6666	0,6686	0,6968	0,7024	0,7239
22	09:41:630	0,6420	0,6730	0,6664	0,6783	0,6944	0,7093	0,6871
23	Mean BRET ratio	0,6406	0,6653	0,6696	0,6746	0,6989	0,7062	0,7158
24	Background BRET ratio	0,6416						
25	Specific BRET ratio	0	0,0247	0,0280	0,0330	0,0573	0,0646	0,0742
26	Specific BRET ratiox1000	0,00	24,71	27,96	32,98	57,34	64,60	74,24
27	Average specific BRET ratio	0,00	25,03	28,71	34,36	56,84	64,47	70,62
28	Average luminescence (Rluc)	106217	106803	102238	100998	106536	95820	79932
29	Fluorescence (YFP)	692	1250	1570	1900	2650	3362	4130
30	YFP0	692						
31	YFP-YFP0	0	558	878	1208	1958	2670	3438
32	(YFP-YFP0/Rluc)x100	0,00	0,52	0,86	1,88	2,49	3,51	5,17

b



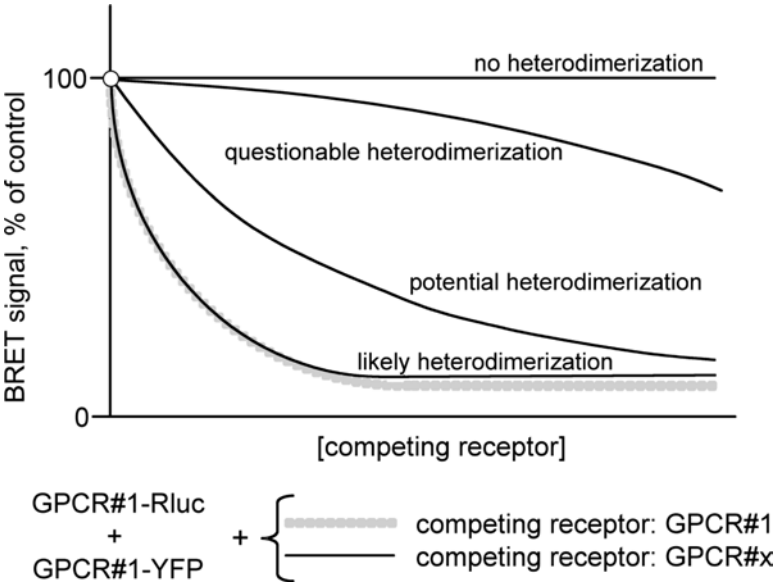


Fig. 2. BRET competition experiments to assess GPCR heterodimerization. The possibility of true heterodimerization between 2 GPCRs, GPCR1 and GPCRX, which interact in a BRET-donor saturation experiment, is investigated in this example by studying the displacement of the interaction between the protomers of one receptor (GPCR1-Rluc and GPCR1-YFP) in the presence of excess untagged GPCR1 or GPCRX. Cells are co-transfected with a constant amounts of GPCR1-Rluc and GPCR1-YFP (giving BRET signals falling in the ascending portion of the saturation curve, just before the plateau) in the presence of increasing amounts of plasmid coding for untagged receptor 1 or receptor X. BRET ratios are calculated and expressed as a function of the concentration of the competing receptor, determined by immunoblot or using biding experiments. Depending on the aspect of the competition curve, the indicated conclusions can be drawn.

2. The actual amount of donor and acceptor plasmids giving an YFP-YFP_0/Rluc value in the ascending portion of the curve before the plateau is selected for the displacement assay.
3. HEK-293T cells are then transfected with the selected amount of plasmids for BRET-donor and acceptor in the presence of increasing quantities of plasmid coding for native GPCR2 or GPCR1. As in classical saturation experiments, the total amount of transfected DNA is maintained constant using appropriate amounts of "empty" cloning vector.
4. BRET ratios are calculated as described in Subheading 3.3 and plotted as a function of the expression of native receptor determined by Western blot or binding experiments (as detailed in Subheading 3.6). The interpretation of the data is described in Note 12.

3.5. Measuring the Kinetics of BRET Changes Upon Ligand Binding to GPCRs

For this type of experiment, cells are transfected with a plasmid coding for the BRET-donor in the presence of a single concentration of the plasmid encoding the BRET-acceptor and BRET measurements are performed over time (up to 20–30 min).

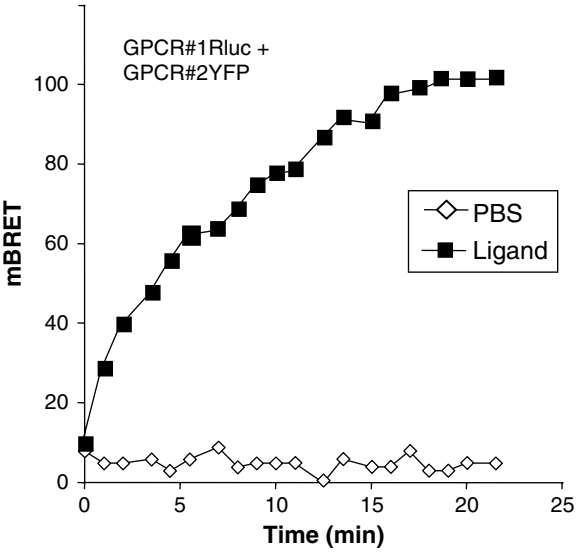


Fig. 3. BRET changes induced by ligand binding. Cells are transfected with a constant amounts of GPCR1-Rluc (10 ng) and GPCR2-YFP (500 ng). BRET measurements are carried out at the indicated times after addition of the appropriate ligand or buffer alone (PBS).

Detailed below is an example of a ligand-dependent BRET signal modulation for a GPCR heterodimer in adherent HEK-293T cells.

1. HEK-293T cells are prepared and transfected as in Subheading 3.2.
2. The BRET signal is directly measured in the same plate. Forty microliter of a mixture composed of 30 µL of PBS-$CaCl_2$/$MgCl_2$ and 10 µL of 25 µM coelenterazine-h solution is distributed in each well, then 10 µL of ligand (at saturating concentration) in PBS or of PBS alone (basal control condition) is added to each well.
3. BRET measurements are started and repeated for the desired duration in order to measure the evolution of the BRET ratio with time. BRET ratios are then plotted over time (Fig. 3).

3.6. Assessing GPCR Expression Levels in BRET Experiments

Determination of GPCR expression levels in BRET experiments 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 BRET-donor saturation experiments. Relative expression levels can be obtained by western blotting using specific antibodies, whereas actual receptor levels can be determined using radioligand binding assays.

3.6.1. Preparation of Samples for Western Blotting

1. Cells from BRET experiments are collected in a microcentrifuge tube by centrifugation at 300 × *g* for 5 min at RT.

2. The supernatant is aspirated and the pellet resuspended in 300 µl of lysis buffer for 15–30 min in ice.
3. Cell lysates are then centrifuged at 12,000 × *g* for 15 min at 4°C to remove debris.
4. Supernatants are transferred in clean microcentrifuge tubes and 5 µl of each sample is used to determine its protein concentration (with the BCA protein Assay).
5. 50–100 µg of protein in 40 µl is mixed with 10 µl of Laemmli 5× buffer and denatured for 1–6 h at RT. Samples can then be stored at −20°C or used immediately for separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using appropriate antibodies.

3.6.2. SDS-PAGE and Western Blotting

1. The glass plates for the gels are cleaned with ethanol and rinsed extensively with distilled water.
2. The separating gel is prepared. For example, to prepare a 10% gel; mix 7.5 ml of 4× separating buffer, with 10 ml acrylamide/bis solution 40% (w/v), 12.5 ml water, 100 µl ammonium persulfate solution and 20 µl TEMED. Pour the gel, leave space for a stacking gel, and overlay with water-saturated isobutanol. The gel should polymerize in about 20–30 min. The isobutanol is then poured off and the gel is rinsed twice with water.
3. The stacking gel is prepared by mixing 2.5 ml of 4× stacking buffer with 1.3 ml acrylamide/bis solution, 6.1 ml water, 50 µl ammonium persulfate solution, and 10 µl TEMED. The stacking gel is then poured and the comb is inserted.
4. Once the stacking gel is polymerized, the comb is removed carefully and the gel is rinsed with water.
5. The gel is then ready and 50 µl of each sample are loaded into each well (10 µl of prestained molecular weight markers is added in one well).
6. After separation by SDS-PAGE, samples are transferred to nitrocellulose membranes electrophoretically (a gel of 15 × 6 cm requires a transfer at 100 mA for 2 h).
7. Once the transfer is completed, the nitrocellulose membrane is incubated in 10 ml of blocking buffer for 1 h at RT on a rocking platform.
8. The nitrocellulose membrane is then incubated with the primary antibody needed to detect the specific GPCR, washed three times in blocking buffer and then incubated with the appropriate secondary antibody.
9. The membrane is next washed three to four times for 30 min with PBS-Tween 0.2% before incubation in the ECL detection reagent.

10. The membrane is then removed from ECL and placed in an X-ray film cassette with film for the suitable exposure time (typically from 30 s to a few minutes).

3.6.3. Establishing Correlation Curves Between Fluorescence or Luminescence Values and Receptor Expression by Radioligand Binding

As an alternative to western blotting, true GPCR expression levels can be determined for each BRET experiment by radioligand binding when appropriate radioligands are available. If BRET-donor and acceptor receptors both bind the same radioligand, determination of the expression level of each fusion protein can be challenging. We therefore recommend the generation of correlation curves between luminescence (Rluc fusion protein) or fluorescence (YFP fusion proteins) values and radioligand binding sites in independent sets of experiments (16).

1. Cells are transfected with different quantities of either the BRET-donor or the acceptor fusion protein plasmid. At least 10 different independent transfections should be performed for each fusion protein to obtain a sufficient number of data points for correlation curves.
2. Luciferase activity (for the BRET-donor GPCR) and fluorescence values (for the BRET-acceptor GPCR) are determined as described above.
3. Radioligand binding experiments using saturating ligand concentrations are performed in parallel using appropriate assay conditions for each GPCR (see Note 13).
4. The number of radioligand binding sites is then plotted against fluorescence or luminescence values measured in the same sample; a linear correlation is expected. Provided that the settings for fluorescence and luminescence measurements are identical to those used in BRET experiments, these curves can be used to convert fluorescence and luminescence values measured in BRET experiments into actual receptor amounts (see Note 14).

4. Notes

1. Coelenterazine-h is sensitive to degradation by light and oxygen. Working solutions are freshly prepared, typically at 25 μM in HBSS or PBS-$CaCl_2$ + $MgCl_2$. Coelenterazine-h solutions should be protected from light during long incubation periods. Whereas coelenterazine-h is used for routine BRET experiments, other *Renilla* luciferase substrates can be used for more specific applications. For BRET2 assays (see Note 2) use of Deepblue C (Coelenterazine 400a, UPBB8392; Uptima, Interchim) is recommended. The recently developed ViviRen™ substrate (Promega) generates a rapid light burst

(three- to fivefold increase of the luminescence peak) followed by accelerated decline. On the other hand, EnduRen™ (Promega) generates long-lasting luminescence signals (up to 24 h) but with 10–25 times lower amplitude. All three substrates are used at working concentrations of 60 μM. ViviRen™ and EnduRen™ can only be used for BRET measurements in intact cells because they are pro-substrates necessitating transformation by cellular esterases to become effective *Renilla* substrates.

2. BRET1 is the most widely used BRET assay. The energy donor in this assay is the luciferase of *Renilla reniformis* (Rluc) and the energy acceptor, the enhanced yellow fluorescent protein (YFP), a codon-humanized enhanced and yellow-shifted mutant of the *Aequorea victoria* GFP (green fluorescent protein). The substrate for BRET1 assays is coelenterazine-h. Rluc variants, such as Rluc8, and the YFP variant YPet have been obtained by random mutagenesis (17, 18). Superior BRET signals have recently been reported with this optimized Rluc8/YPet BRET pair, compared to the "classical" Rluc/YFP pair, due to increased assay sensitivity (19). In the BRET2 assay, the YFP is replaced by GFP2, a GFP mutant that can be excited at 400 nm, and the Rluc substrate is the Deepblue C (also known as coelenterazine 400a) (see Note 1). The advantage of BRET2 is a superior separation of donor and acceptor peaks. Indeed, in the presence of Deepblue C, Rluc emits light at 400 nm, a wavelength that excites GFP2, which, in turn, emits light at 510 nm. The recommended filter sets for BRET2 are therefore 400 ± 10 nm and 515 ± 10 nm. The disadvantage of BRET2, compared to BRET1 is the 100–300 times lower intensity of emitted light. To compensate this loss of amplitude, fusion proteins are often overexpressed, which may cause problems for interpreting the results, in particular when GPCR oligomerization is the object of the study.

3. For BRET experiments, C-terminal fusion proteins are used. To generate fusion constructs, the entire GPCR coding sequence is typically amplified by PCR without its stop-codon using sense and antisense primers harboring unique restriction sites. The sequence is then subcloned upstream and in frame with YFP (or GFP2) and Rluc, resulting in GPCR-YFP and GPCR-Rluc, respectively. Fusion of Rluc and YFP at the N-terminal end of GPCRs is also possible but interferes in many cases with proper export of receptors to the cell surface and requires addition of signal peptide sequences.

4. Depending on each construct and on the quality of the plasmid preparation, the actual amount of expressed fusion protein after transfection of a given amount of DNA may change. It is recommended to establish experimentally the amount of

BRET-donor DNA leading to a suitable luminescence signal. This signal should correspond to GPCR levels compatible with physiological conditions and to luminescence counts that are sufficiently high over background. Once the conditions for the BRET-donor are established, a range of different BRET-acceptor protein levels are coexpressed in order to determine the conditions that correspond to the highest specific BRET signals.

5. Negative control fusion proteins should be included in BRET-donor saturation assays to verify the specificity of the generated BRET. Ideally, negative control proteins should have a similar topology as the GPCR of interest (i.e., another GPCR) and should be localized in the same subcellular compartment. As many GPCRs have a natural tendency to associate (see Subheading 1), finding a true negative control may be challenging. Therefore, membrane proteins with different topologies (the single membrane-spanning protein CD4, for example) can be used as negative control protein.
6. To perform measurements on attached cells, the culture medium has to be removed by washing once with PBS (containing $CaCl_2/MgCl_2$), as the phenol red, present in most culture mediums, quenches the luciferase signal measured at 485 nm.
7. Improved BRET1 filter sets (480 ± 10 nm and 540 ± 20 nm) have been described recently. In our hands, BRET values determined with optimized filter sets were increased by approximately 50% for various BRET pairs (link to Berthold Application Note: http://www.berthold.com/ww/en/pub/bioanalytik/overview/notes.cfm). These filters are now included in the filter set package of some microplate BRET readers (e.g., Mithras, Berthold).
8. Rluc light emission is transient. The typical profile of light emission in the presence of coelenterazine-h is composed of a rapid raise, a transient stabilization at maximal values and a slow decline in light output. The duration of each phase may vary with assay conditions. Whereas maximal values are reached rapidly (less than 3 min) when working with cell extracts and purified proteins, approximately 5 and 15 min are necessary to complete the rise when experiments are performed with cell suspensions and adherent cells, respectively. According with these considerations, to estimate the amount of expressed donor in BRET donor saturation experiments, we determine the maximal luciferase value obtained by measuring luminescence values repeatedly at 485 nm in the appropriate time window.
9. Conversion of luminescence and fluorescence values into actual receptor amounts depends on the availability of appropriate

tools (antibodies, radioligands) and on the specific question to be answered. Whereas radioligand binding assays provide absolute values, western blot detection using anti-receptor antibodies provides only relative values (i.e., comparison with receptor expression levels in tissue samples examined in parallel). A common question in the context of GPCR homo- and heterodimerization is the relative propensity of formation of such complexes. The most straightforward approach to investigate this issue is based on the parallel study of the coexpression of a given GPCR-Rluc fusion protein with different GPCR-YFP fusion proteins. The $BRET_{50}$ values obtained in BRET-donor saturation assays for each pair of GPCRs provide an estimate for the relative propensity of the corresponding interaction. To obtain meaningful results, the amount of expressed Rluc fusion protein must be equivalent in all assays (a maximal variation of 30% is tolerated). This approach is based on the assumption that the YFP-associated fluorescence of all GPCR-YFP fusion proteins is comparable (which appears to be the case for most, but not all, fusion proteins). If a more precise quantification of fusion proteins is necessary, fluorescence and luminescence values should be converted into absolute expression levels. To determine the physiological relevance of the interactions, conversion into absolute receptor expression levels (or comparison with endogenous expression levels in western blot experiments with tissue lysates) are mandatory. Conversion into absolute expression levels is obligate when comparing GPCR interactions with different BRET-donors. Semi-quantification based on luminescence and fluorescence values are sufficient, however, to analyze BRET changes induced by ligands.

10. We noticed that background fluorescence may vary, not only according to the cell type in which BRET experiments are conducted, but also between two experiments conducted at different times in the same cell type. Moreover, for some constructs, the transfection of the same amount of BRET-donor plasmid can result in interday fluctuation in luminescence signals. In these cases, when data obtained from experiments performed at different times are plotted together, it may be convenient to normalize the background fluorescence and luminescence values. BRET values are thus plotted as a function of $[(YFP\text{-}YFP_0)/YFP_0]/[Rluc/Rluc_0]$ where YFP_0 corresponds to background fluorescence measured in cells not expressing the BRET-acceptor and $Rluc_0$ to the average luminescence value in cells expressing the BRET-donor alone.
11. In the case of specific interactions, hyperbolic BRET-donor saturation curves are expected. Maximal BRET values, which correspond to the saturation of all BRET donors by BRET acceptors, are defined as the $BRET_{max}$. Half-maximal BRET values,

corresponding to the saturation of 50% of BRET donors by BRET acceptors, are defined as the $BRET_{50}$. $BRET_{50}$ values give an approximation of the relative affinity of receptors for each other. When studying heterodimers, $BRET_{50}$ values are indicative of the likelihood of the examined interaction in natural cells. The closer the measured $BRET_{50}$ is to the $BRET_{50}$ values measured between homodimers, the greater the possibility that the heterodimer forms in native cells.

12. In case of true heterodimerization, native GPCR2 is expected to displace the BRET signal. If the BRET signal remains unmodified by increasing concentrations of GPCR2, heterodimerization can be ruled out. If the displacement curve is close to that obtained with untagged GPCR1, one can assume that the heterodimerization can occur in natural cells provided that they express a corresponding level of endogenous receptors synthesised at the same moment. In case of partial displacement by GPCR2 or displacement requiring high concentrations of GPCR2, heterodimerization in native cells is unlikely, but final conclusions will require additional investigation using other approaches.
13. Radioligand binding assays for GPCRs can be performed on crude membranes or intact cells. Assay conditions can vary depending on the specific GPCR and available radioligands, but should always be designed to detect all receptor binding sites, whether they are located at the plasma membrane or on intracellular membranes. Indeed BRET values reflect the entire receptor population expressed in the cell. Fluorescence-labeled ligands can be used to estimate receptor binding sites, provided that interference with the excitation spectrum of BRET-acceptor are avoided.
14. Independent determination of donor (luminescence) and acceptor (fluorescence) quantities in cells coexpressing both fusion proteins, represents a key feature of the BRET assay and is a clear advantage compared to FRET assays based on two GFP variants.

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