Chapter 10

Cell-Surface Protein–Protein Interaction Analysis with Time-Resolved FRET and Snap-Tag Technologies: Application to G Protein-Coupled Receptor Oligomerization

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Abstract

G protein-coupled receptors (GPCRs) are key players in cell–cell communication, the dysregulation of which has often deleterious effects leading to pathologies such as psychiatric and neurological diseases. Consequently, GPCRs represent excellent drug targets, and as such are the object of intense research in drug discovery for therapeutic application. Recently, the GPCR field has been revolutionized by the demonstration that GPCRs are part of large protein complexes that control their pharmacology, activity, and signaling. Moreover, in these complexes, one GPCR can either associate with itself, forming homodimers or homooligomers, or with other receptor types, forming heterodimeric or heterooligomeric receptor entities that display new receptor features. These features include alterations in ligand cooperativity and selectivity, the activation of novel signaling pathways, and novel processes of desensitization. Thus, it has become necessary to identify GPCR-associated protein complexes of interest at the cell surface, and to determine the state of oligomerization of these receptors and their interactions with their partner proteins. This is essential to understand the function of GPCRs in their native environment, as well as ways to either modulate or control receptor activity with appropriate pharmacological tools, and to develop new therapeutic strategies. This requires the development of technologies to precisely address protein–protein interactions between oligomers at the cell surface. In collaboration with Cisbio Bioassay, we have developed such a technology, which combines TR-FRET detection with a new labeling method called SnapTag. This technology has allowed us to address the oligomeric state of many GPCRs.

Key words: Fluorescence resonance energy transfer, G protein-coupled receptor, Dimerization, SnapTag, $GABA_B$ receptor

1. Introduction

An increasing number of G protein-coupled receptors (GPCRs) have been shown to form oligomers (1–3). The first well-characterized functional homodimeric receptor complex was demonstrated for

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the metabotropic glutamate (mGlu) receptors, in which both protomers are linked by a disulfide bond in their extracellular domains (4). The $GABA_B$ receptor, also a Class C GPCR, was the first to be described as a functional obligatory heterodimer (5). The two homologous subunits, $GABA_{B1}$ which binds the endogenous ligand GABA, and $GABA_{B2}$ that couples to G proteins, are required for the formation of an active receptor at the cell surface. Thus, these GPCRs have been models of choice for developing technological tools to study in detail their dimerization states and their roles in receptor function.

Recently, several biophysical methods based on resonance energy transfer (RET), like Bioluminescence Resonance Energy Transfer (BRET) and Förster Resonance Energy Transfer (FRET) have been developed for the analysis of protein–protein interactions in living cells, notably GPCR interactions (6–8). However, these techniques suffer a number of limitations, especially when studying receptors at the cell surface. First, the receptors of interest needs to be fused to fluorescent proteins [such as the green fluorescent protein (GFP) family proteins], meaning that all receptors contribute to the recorded fluorescent signal. This includes receptors that are expressed at the cell surface as well as those localized to intracellular compartments, e.g., within the synthetic, endocytic, and degradation pathways. Second, because absorption and emission spectra of the donor and acceptor fluorophores are not well separated, the excitation of the donor fluorophore leads to a contaminating excitation of the acceptor fluorophore, and the recorded emission spectra of the acceptor fluorophore is contaminated by the emission of the donor fluorophore.

In order to avoid these two major problems, a new technology called Homogeneous Time Resolved FRET (HTRF®) has been developed based on an energy transfer between an europium cryptate (or a terbium cryptate, see Note 1) as the donor fluorophore and the Cy5-like dye (d2) as acceptor (9–11). Thanks to the peculiar properties of these rare earth cryptates, which display a long fluorescence emission lifetime in the millisecond range, compared to the nanosecond range for standard fluorophores, it is possible to record the FRET signal in a time resolved manner (Time Resolved-FRET or TR-FRET). The application of a 50 μs delay between the excitation of the donor and the TR-FRET measurement (emission of the acceptor) allows the investigator to remove the background that arises from either the autofluorescence of the cells or the free acceptor, as their fluorescence is quickly switched off. Moreover, europium cryptate and d2 present optimal spectral properties, such that d2 emits in a spectral range where the emission of the europium cryptate is barely detectable. Each of these features allow a large increase in the signal to noise ratio when compared to the classical RET methods.

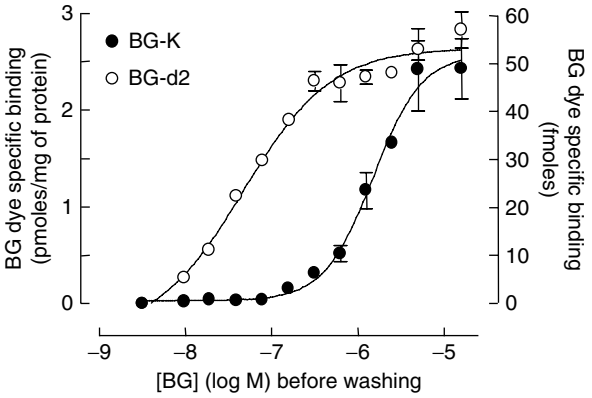

Fig. 1. Cell surface-specific binding with increasing concentrations of either BG-K or BG-d2 for cells expressing constant amount of ST-GB1 and GB2. Data are represented as the number of mol of either BG-K or BG-d2 fixed to the GB1 subunit per well (*right scale*) or per milligram of protein (*left scale*). Reproduced from (13) with permission from Nature Publishing Group NPG.

In our laboratory, a recent improvement of this technology has been the coupling of the TR-FRET approach to the Snap-tag labeling method (12), which allows the covalent labeling of one protein with one fluorophore at the cell surface (see Fig. 1 and Note 1) (13). The Snap-tag (ST) 20 kDa derives from the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), that recognizes O^6-alkylated guanines in DNA and irreversibly transfers the alkyl group to one of its reactive cysteine residues. The Snap-tag corresponds to a modified AGT that displays faster reaction kinetics with O^6 benzylguanine (BG) substrates, and no longer interacts with DNA. Practically, the Snap-tag protein is fused to the extracellular extremity of the protein of interest and expressed in cells. Cells are then incubated with BG labeled with either cryptate donor or d2 acceptor fluorophores. Thus, it is possible to perform FRET experiments between adjacent Snap-tagged proteins covalently labeled with a fluorophore (11). Incubation with a precise ratio of donor and acceptor BG-fluorophores leads to several possible dimer combinations: 25% of dimers containing both receptors labeled with the donor, 25% of the dimers containing both receptors labeled with the acceptors, and 50% of the dimers containing one receptor labeled with the donor and one receptor labeled with the acceptor, which will provide the FRET signal. Methodology for performing TR-FRET, as well as the analysis and the interpretation of the data is described below (13). The newly commercial version of the technology, using reagents based on Terbium crytates and known as the Tag-Lite substrates (Cisbio Bioassay; Bagnols/Cèze, France)

are referenced (see Note 1). An alternative method to the Snap-tag is the use of antibodies conjugated to HTRF fluorophores to specifically label receptors at the cell surface. This approach is also described at the end of the present article (11).

2. Materials

2.1. Cell Culture and Transfection with Electroporation

1. HEK 293 or COS7 cells.
2. 100 mm tissue culture dishes.
3. 96-well plates (black plate with black bottom) (CellStar®; Greiner).
4. Phosphate-Buffered Saline (PBS): 1× prepared from PBS 10× (Lonza).
5. Trypsin/EDTA solution: 0.05% trypsin and 0.53 mM EDTA (Gibco/BRL-Life Technologies).
6. Complete Dulbecco's Modified Eagle's Medium (DMEM) medium: DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% nonessential amino acids. All the products used for cell culture are purchased from Gibco/BRL-Life Technologies.
7. Braun water (Braun).
8. 5× Electroporation buffer (EB): 250 mM KH_2PO_4, 100 mM CH_3COOK, 100 mM KOH. Prepare a concentrated buffer stock (5× EB) that will be diluted with water for the buffer (1× EB).
9. 1 M $MgSO_4$ solution.
10. Electroporator (Gene pulser®; Biorad) and adapted electroporation cuvettes.
11. 2.53 mM polyornithine solution diluted in PBS and kept at 4°C.

2.2. Snap-Tag and Antibody Labeling of Receptors

1. Benzylguanine conjugated with fluorophores: europium cryptate for donor (BG-K) and d2 for acceptor (BG-d2) calibrated at 1 mM each (see Note 1 for the commercially available version of these reagents (Cisbio Bioassay)).
2. Tris KREBS buffer (TK buffer): 20 mM Tris, 118 mM NaCl, 5.6 mM Glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$, pH 7.4.
3. Monoclonal anti-HA (12CA5) and anti-Flag (M2) antibodies conjugated with europium cryptate and d2 provided (Cisbio Bioassay). See manufacturer recommendations for preparation and storage of the different compounds.

2.3. FRET Measurements

1. Time-resolved fluorimeter, e.g., RUBYStar® plate reader (BMG Labtechnologies).
2. An adapted plate reader is required when reading the specific d2 fluorophore signal at 680 nm, e.g., Analyst® plate reader (Molecular Devices).

3. Methods

The analysis of receptor oligomerization relies on the capacity of the methods to detect proximity between proteins at the cell surface. Thus, it is crucial to know the percentage of proteins labeled with the fluoropohores. Usually, it is necessary to perform experiment with near 100% of the proteins of interest labeled by the fluorophores you intend to use.

3.1. Preparation of Expression Plasmids

The plasmid used for mammalian cell expression should contain several specific restriction sites upstream of the receptor cDNA sequence to allow extracellular localization of labeling proteins at the amino-terminus of the GPCRs.

1. Subclone the Snap-tag cDNA sequence (obtained from the pSST26m plasmid from Covalys) upstream of the cDNA sequence encoding the protein of interest.
2. Upstream of the Snap-tag, a tag (e.g., HA, Flag or myc) is added in order to perform ELISA experiments to allow the determination of cell surface expression of labeled receptors.
3. The HA, Flag, and myc tags can also be useful to perform HTRF technology with commercially available fluorophores conjugated antibodies against these tags. This will also permit orthogonal labeling between a Snap-tag and an epitope-tag to measure TR-FRET.

3.2. Preparation of the Cells and Transfection

The method below describes the transfection of 1×10^7 cells by electroporation (HEK 293 or COS-7 cell lines). To electroporate 5×10^6 cells, all the quantities mentioned bellow should be divided by two.

1. HEK 293 or COS7 cells are cultured in complete DMEM incubated at 37°C, 5% CO_2. Cells are split into 100-mm dishes when approaching confluence to provide new cell cultures.
2. Greiner CellStar® 96-well plates should be treated with 50 µl per well of 2.53 mM polyornithine and incubated at 37°C in 5% CO_2 for at least 30 min.
3. Prepare the plasmid cDNA mix encoding the proteins of interest and completed to a total amount of 10 µg plasmid cDNA

with pRK5 empty vector. For example, to obtain maximal expression of the $GABA_B$ receptor, use 1 μg of pRK-$GABA_{B1}$ (GB1 subunit) and 1 μg of pRK-$GABA_{B2}$ (GB2 subunit) supplemented with 8 μg of pRK5. For FRET experiments, a mock with only pRK5 empty vector must be included.

4. To the cDNA mix, add 142 μl of Braun water, 40 μl of 5× EB, and 8 μl of 1 M $MgSO_4$.
5. Take a 100-mm dish of cells from the incubator. Wash the cells once with PBS solution (10 ml/dish) and then dissociate the cells using prewarmed trypsin/EDTA solution (5 ml/dish) for 10 min at 37°C in 5% CO_2.
6. Neutralize the trypsin by adding 5 ml of prewarmed complete DMEM to each dish. Cells are then transferred to a 50 ml conical polypropylene tissue culture tube and gently triturated. A small volume is removed and used to count the cells with a Malassez cell/hemocytometer.
7. Centrifuge the cells for 5 min at $167 \times g$ and remove the supernatant.
8. Resuspend the cell pellet in 1× EB. The volume of 1× EB to add should be previously determined in order to obtain a concentration of 1×10^7 cells per 100 μl.
9. Add 100 μl of cell suspension to each cDNA mix (total volume for electroporation: 300 μl) and gently resuspend cells using a pipette. Incubate the cDNA/cell mix for 10 min at room temperature.
10. During this time, select the electroporation parameters for the pulse mode: for 1×10^7 HEK 293 or COS7 cells, the electroporation parameters are 250 V–1,000 μF and 280 V–1,000 μF, respectively.
11. Transfer each eletroporation mix to an electroporation cuvette and place the cuvette in the electroporator. Deliver the electric shock for about 40 ms in the pulse mode.
12. Remove the cells from the cuvette and resuspend in 10 ml of fresh complete DMEM.
13. Take out the Greiner CellStar® 96-well plates from the incubator, remove the polyornythine, seed wells with 100 μL of cell suspension (1×10^5 cells per well) and incubate for 24 h at 37°C in 5% CO_2.

3.3. Optimization of Snap-Tag Labeling

First, it is necessary to determine the optimal concentration of fluorophore to use in order to achieve 100% protein labeling, which is required for reliable interpretation.

1. Cells are transiently transfected with plasmids encoding the protein of interest fused in N-terminal end with the Snap-tag, such as ST-GB1 and ST-GB2 as described in Subheading 3.2.

2. Prepare BG-fluorophores (both K and d2) in prewarmed complete DMEM at concentrations ranging between 10 nM and 10 µM. Each concentration of fluorophore is distributed to three wells in a 100 µL volume.
3. Wash the cells once with prewarmed complete DMEM.
4. Add each concentration of BG-fluorophores in triplicate wells of the 96-well plate which is subsequently incubated for 1 h at 37°C in 5% CO_2 (see Note 2).
5. Following the incubation step, each well of the 96-well plate is washed four times with 100 µL of TK buffer, which can be removed by aspiration, but care should be taken not to aspirate the cells (see Notes 3–5).
6. Add 100 µl per well of TK buffer and measure the specific fluorescence emission of the BG-K and BG-d2 at 620 and 682 nm, with excitation at 337 and 640 nm, respectively (total fluorescence minus that measured with mock transfected cells) (Fig. 1) on a fluorimeter. The fluorescence signal should become saturated with increasing fluorophore concentration.
7. To ensure that 100% of the proteins of interest are labeled, an additional experiment is performed. For cells expressing increasing amounts of ST-receptors, the specific labeling of the ST-receptor with the BG-dye is plotted as a function of the receptor density at the cell surface. Receptor density can be determined by radioligand binding. The slope of the line provides the labeling efficiency, which is defined as the ratio between the number of labeled ST-receptors to the total population of ST-receptors. If the slope of the straight line is equal to one, it is concluded that 100% of the ST-receptors expressed at the cell surface are labeled each with one fluorophore.

After the Snap-tag labeling optimization step, two options are possible to perform FRET assay: a Snap-tag/Snap-tag labeling (Subheading 3.4) or Snap-tag/Antibody labeling (Subheading 3.5).

3.4. Snap-Tag/Snap-Tag TR-FRET Assay After Optimization of the Snap-Tag Labeling

To perform TR-FRET experiments between two ST-GPCRs, like ST-$GABA_{B1}$ and ST-$GABA_{B2}$, it is necessary to determine the labeling conditions that will ensure equivalent labeling of the Snap-tags with either fluorophore (50% of each protein labeled with the donor and 50% with the acceptor). This is necessary as the BG-K and the BG-d2 have slightly different labeling kinetics depending on their chemical environment.

3.4.1. Determination of Optimal Labeling Conditions

1. Transfect either HEK 293 or COS7 cells with plasmid cDNAs encoding the Snap-tagged receptors of interest as described in Subheading 3.1.
2. Twenty-four hours following transfection, dilute BG-K in complete DMEM at a concentration corresponding to the maximal occupancy of the Snap-tag sites as outlined in Subheading 3.3.

3. Using the BG-K solution, prepare BG-d2 and O^6-BG ("cold"-BG, if both BG-d2 and O^6-BG display the same reactivity; otherwise see Note 6) at concentrations ranging from 10 nM to 10 µM (three wells per concentration tested, 100 µl per well).
4. Wash the cells once with prewarmed complete DMEM.
5. 100 µL volumes of either BG-K/BG-d2 or BG-K/O^6-BG mix are added to triplicate wells (for each label) of a 96-well plate at each of the concentrations tested. The 96-well plate is then incubated for 1 h at 37°C, 5% CO_2 (see Note 2).
6. After the labeling, carefully wash the cells four times with the TK buffer (100 µl per well) (see Notes 3–5).
7. Add 100 µL of TK buffer to each well and record the signal at 665 nm using the time-resolved fluorimeter. The specific FRET signal is determined by substracting the signal recorded at 665 nm for cells labeled with BG-K/BG-d2 from the signal recorded at 665 nm for cells labeled with BG-K/O^6-BG.
8. FRET signal is represented as a function of the increasing concentrations of BG-d2 to obtain a bell curve. The ratio BG-K/BG-d2 allowing the equivalent labeling of the Snap-tagged receptors with either fluorophore will correspond to maximal point of the curve (Fig. 2).

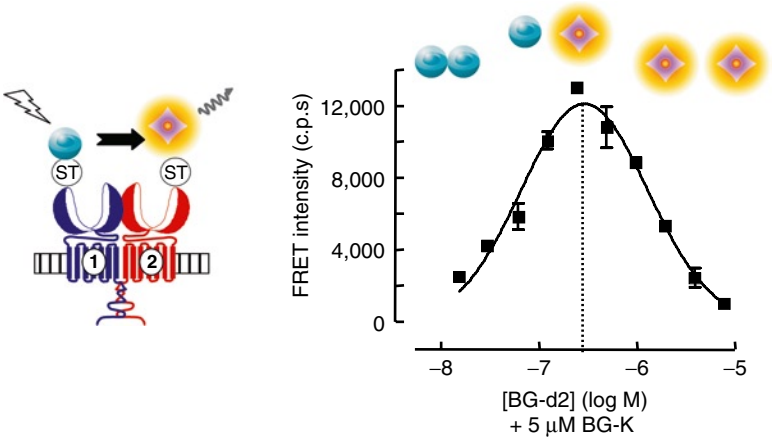

Fig. 2. FRET intensity depending on the ratio BG-K/BG-d2 applied to cells expressing ST-GB1 and ST-GB2. A constant concentration of BG-K is used combined with increasing concentrations range of BG-d2. The ratio given rise to the maximal FRET signal corresponds to the peak of the bell curve. Reproduced from (13) with permission from Nature Publishing Group NPG.

3.4.2. FRET Determination Using the Defined Conditions

1. Transfect either HEK 293 or COS7 cells with increasing amounts of plasmid cDNAs encoding the Snap-tagged receptors of interest and empty pRK5 vector as a control, as described in Subheading 3.1. Distribute the electroporated cells in a Greiner CellStar® 96-well plate as described in Subheading 3.2: six wells per transfection. Plate the extra cells on another plate to determine the cell surface expression of the receptors for each transfection (perform either an ELISA assay or radioligand binding).
2. Twenty-four hours after transfection, prepare two mixtures in complete DMEM: either BG-K/BG-d2 or BG-K/cold-BG. The optimal ratio of BG-dyes to use was determined previously (see Subheading 3.4.1). The cold-BG is diluted at the same concentration than the BG-d2.
3. Wash the cells once with prewarmed complete DMEM.
4. Add the BG-dyes to the cells: three wells with BG-K/cold-BG and three other wells with BG-K/BG-d2 per transfection. Incubate the 96-well plate for 1 h at 37°C at 5% CO_2 (see Note 2).
5. Wash the cells four times with TK buffer (see Notes 3–5).
6. Add 100 µl per well of TK buffer and read the fluorescence signal on the time-resolved fluorimeter. The specific FRET signal is determined by the calculation of the Δ665, as detailed below (Subheading 3.6.1).
7. FRET signal is plotted against the cell surface expression of the proteins of interest (Fig. 3).

3.5. Snap-Antibody Labeling Associated with FRET Assay

To perform an orthogonal labeling of receptor, it is possible to combine the Snap-tag and antibody labeling. To do this, cells should be transfected with plasmid cDNA encoding a Snap-tagged receptor and with plasmid cDNA encoding an amino-terminal epitope-tagged (e.g., HA, Flag, or myc) receptor.

1. Transfect either HEK 293 or COS7 cells with increasing amounts of a plasmid cDNA encoding a Snap-tagged receptor and another plasmid cDNA encoding an amino-terminal epitope-tagged receptor as outlined in Subheading 3.1 and plate cells in a Greiner CellStar® 96-well plate, six wells per transfection as outlined in Subheading 3.2 in order to perform TR-FRET analysis. The extra cells are plated in parallel in another plate to determine the cell surface expression of the receptors.
2. Twenty-four hours after transfection, label the Snap-tagged protein with the BG-d2 (for optimal FRET pairs, see Note 7). Note that the concentration of the BG-fluorophore required

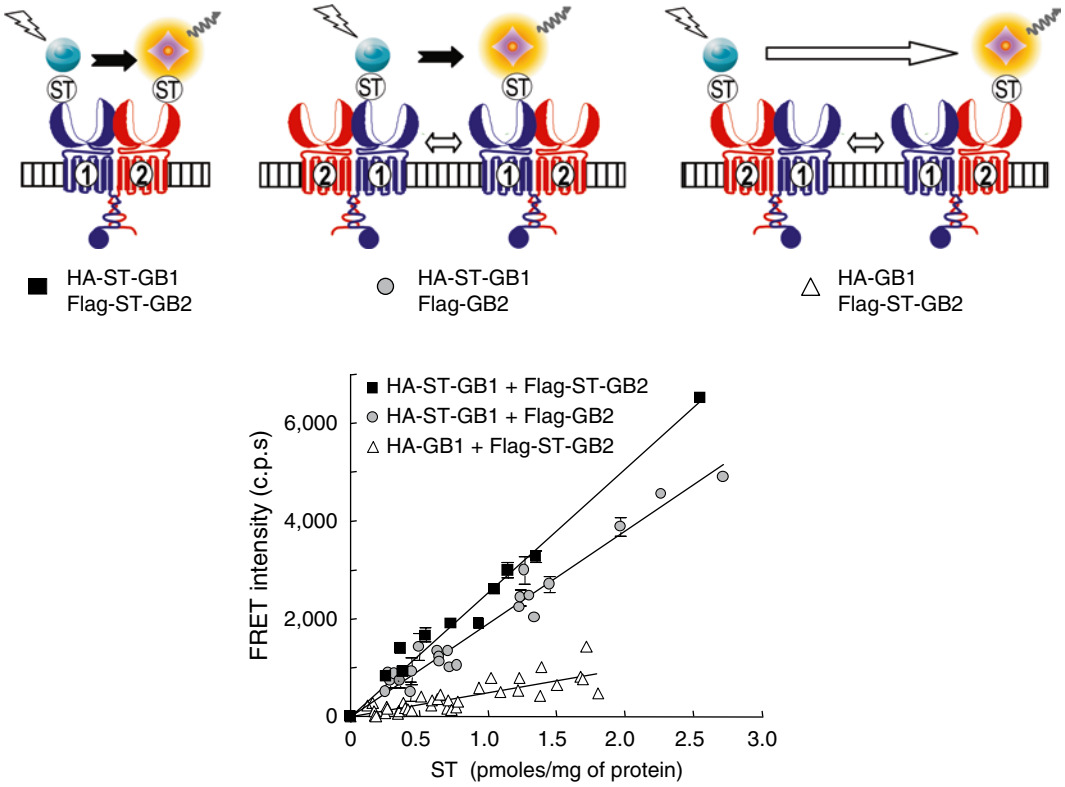

Fig. 3. Detection of higher-order multimers of $GABA_B$ dimers at the cell surface using Snap-tag labeling associated with TR-FRET assay. FRET intensity is recorded on cells expressing increasing amounts of different combinations of ST-$GABA_B$ receptors (*see scheme*): (1) both subunits GB1 and GB2 carry a Snap-tag, (2) only GB1 carries a Snap-tag, and (3) only GB2 carries a Snap-tag. FRET intensity is plotted as a function of the amount of snap-tags at the cell surface. These results support a model of higher-ordered oligomer, whereby $GABA_B$ heterodimers interact each other via the GB1 subunit. Reproduced from (13) with permission from Nature Publishing Group NPG.

for 100% labeling of the Snap-tagged protein is determined as described above in Subheading 3.3.

3. Wash cells four times with TK buffer, add 100 µL of anti-Tag-K antibody diluted in TK buffer to a final concentration of 2 nM, and incubate the 96-well plate overnight at 4°C (see Note 8). We have observed that the FRET signal is higher when the FRET donor is carried by the antibody and the FRET acceptor is carried by the BG rather than the inverse.
4. Read the signal on the time-resolved fluorimeter. The specific FRET signal is determined by the calculation of the Δ665 as detailed below.
5. FRET signal is plotted against the cell surface expression of the proteins of interest (Fig. 3).

3.6. Data Analysis and Presentation

3.6.1. Determination of the Specific Δ665 FRET Signal

The calculation of the Δ665 FRET signal allows the determination of the specific FRET signal and thus removes nonspecific FRET due to random collisions and the weak signal due to the contamination of the donor emission at 665 nm (see Note 9).

1. Δ665 represents the signal at 665 nm measured on cells co-labeled with the donor and the acceptor (positive) from which the signal recorded on cells labeled with the donor in absence of acceptor (negative) is subtracted: Δ665 = (signal at 665 nm from the positive) – (signal at 665 nm from the negative) (Table 1).
   (a) In the case of the Snap-tag/Snap-tag FRET signal, the Δ665 is obtained by subtraction of the signal recorded at 665 nm from cells labeled with BG-K/cold-BG from the one measured from cells labeled with BG-K/BG-D2.
   (b) In the case of the Snap-tag/Antibody FRET signal, the Δ665 is obtained by subtraction of the signal recorded at 665 nm from control cells expressing two proteins that do not interact (one Snap-tagged and one epitope-tagged), labeled with BG-acceptor and donor anti-epitope antibodies from the one measured from cells expressing the proteins of interest labeled with BG-acceptor and the donor anti-epitope antibody. If the control cells are not available, they can be replaced by mock cells treated the same way.

3.6.2. Data Interpretation

1. FRET signal is plotted against the amount of receptor protein of interest that is expressed at the cell surface (Fig. 3). If the receptors interact with each other, the curve should fit with a linear regression analysis.

Table 1
Determination of the positive and negative parameters for calculation of FRET analysis in various conditions

	Positive		Negative	
Fusion proteins	**Cells**	**Fluorophores**	**Cells**	**Fluorophores**
Snap-tag + Snap-tag	Snap-tag + Snap-tag	BG-K + BG-d2	Snap-tag + Snap-tag	BG-K + cold-BG
Snap-tag + Epitope-tag	Snap-tag + Epitope-tag	BG-d2 + anti-Tag-K	Snap-tag + Epitope-tag BUT non interacting proteins (by default use mock cells)	BG-d2 + anti-Tag-K

2. As TR-FRET intensity is a relative unit, the analyzed curve has to be compared with the curves obtained with at least two other conditions, named "Positive control" using two proteins already known to interact with each other, and "Negative control" using two proteins known to not interact. The Positive control cells and Negative Control cells have to be treated the same way and at the same time as the cells expressing the proteins of interest.
3. If the curve does not fit with a linear regression analysis, but rather with a hyperbolic curve analysis, the signal can reflect the clustering of the proteins rather than their oligomerization.

4. Notes

1. Three Tag-lite substrates are commercially available from Cisbio Bioassays to perform GPCR oligomerization assays. The SNAP-Lumi4 Tb bearing a terbium cryptate (5 moles; Reference SSNTBE) can be either associated with the SNAP-red substrate (20 nmoles; Ref SSNPREDE) or with the SNAP-green substrate (5 nmoles; Reference SSNGRNE). These new benzylguanine derivatives conjugated with different fluorophores are significantly more reactive than the reagents used in the present chapter. However, the protocols to be used to carry out experiments with these reagents are very similar to those described in this chapter. Additional information can be found on http://www.htrf.com.
2. If dyes sensitive to the light are used (e.g., fluorescein), protect your 96-well plate from the light with an aluminum foil to avoid the bleaching of dyes.
3. The presence of BSA in TK buffer during the wash steps does not increase or decrease the nonspecific signal.
4. Washes in TK buffer rather than DMEM are more efficient to obtain a higher signal to noise ratio.
5. HEK 293 cells are less adherent than COS7 cells to the 96-well plate even if treated with polyornithine 1×, so the washes should be done carefully. Usually, plates were seeded with a higher number of HEK cells (1.5×10^5 cells) compared to COS7 cells (1×10^5 cells) to obtain comparable results.
6. If the BG-d2 and the O^6-BG do not have the same reactivity, an additional experiment will be required to determine the concentration allowing 50% of the Snap-tag sites to be occupied by the O^6-BG in the presence of the BG-K. On cells expressing the Snap-tagged receptors of interest, increasing concentrations of O^6-BG are added in combination with a

constant amount of BG-K (see Subheading 3.3). The O^6-BG concentration to use is defined as the concentration for which the BG-K emission is half of the maximal emission (as measured in the absence of O^6-BG).

7. Of note, the FRET signal is significantly higher when using BG fused with an acceptor and antibody fused with a donor rather than the inverse. In the current protocol this is the only approach described.
8. Due to the low antibody concentration (2 nM) and the low temperature used, the labeling kinetic is quite slow, hence a long incubation time is required. However, incubating cells with antibodies at low temperature prevents receptors from clustering and internalizing and limits the nonspecific antibody binding.
9. In screening assays, the emission at 620 nm is used as an internal reference to correct the signal at 665 nm from possible interfering artifacts like absorption by the assay medium at the excitation wavelength. By dividing the signal at 665 nm by the signal at 620 nm it is possible to introduce a correction of the FRET signal, which is now independent of the media optical properties. Here, we chose not to represent the TR-FRET signal with a ratiometric representation but with the Δ665. It is more convenient to use this representation as TR-FRET measurement cannot be performed in homogeneous format due to the labeling steps needed to wash out the excess of free benzylguanine derivatives (see Subheading 3.6). In this heterogeneous format the emission of the europium cryptate is not constant and cannot be used as internal reference. It is important to mention here that Δ665 can be used as a TR-FRET representation if experiments have been performed in the same conditions (i.e., same buffers, same plate reader).

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