Chapter 21

Time Resolved FRET Strategy with Fluorescent Ligands to Analyze Receptor Interactions in Native Tissues: Application to GPCR Oligomerization

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Abstract

G protein-coupled receptors (GPCRs) play a key role in the regulation of physiological functions. Deregulation of their activities often results in pathological disorders and therefore these receptors constitute major targets for drug development. The emergence of new concepts such as GPCR oligomerization has modified our understanding of these proteins, and identifying the role of receptor complexes is probably a major challenge for the next decade. Various experimental strategies have been developed to study GPCR oligomers and energy transfer experiments between partners within a complex constitute one of the most convenient approaches. These experimental strategies usually require receptor fusion to tags or fluorescent or luminescent proteins and therefore cannot be easily applied to native tissues. We developed a new experimental approach based on the labeling of receptors with high affinity fluorescent ligands compatible with time-resolved energy transfer measurements. Because of the very high signal-to-noise ratio of the time-resolved fluorescent energy transfer (TR-FRET) signals, this approach constitutes a breakthrough since it allows the direct identification of wild-type GPCR oligomers in native tissues.

Key words: Fluorescent ligands, G protein-coupled receptor, GPCR, Time-resolved FRET, Europium, Terbium

1. Introduction

Our understanding of the functioning of G protein-coupled receptors (GPCR) has evolved during the last two decades with the emergence of new concepts. Among them, receptor oligomerization (1, 2) is still the subject of intense investigation since the functional consequences of this are far from being well identified. Oligomerization was first reported as the dimerization of two receptors of the same type leading to the

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formation of receptor homodimers. The concept has evolved in two ways. Firstly, interactions between different types of receptors leading to the formation of heterodimers have been demonstrated. More recently, the existence of larger receptor complexes have been described suggesting the existence of higher-order oligomers.

The investigation of receptor oligomerization is crucial since it has been shown to play a role in receptor targeting and internalization and seems to be at the origin of some variations in pharmacological and coupling profiles. Most of the studies focused on receptor oligomerization have consisted of the identification of receptor complexes and various strategies such as co-immunoprecipitation (3), atomic force microscopy (4) and binding or functional assays (5–8) that have been developed. The most convenient experimental methods to demonstrate receptor proximity are those based on resonance energy transfer since the amplitude of the signal is dependent on the distance between the markers. Various versions of resonance energy transfer technologies have been developed (9), including bioluminescent resonance energy transfer (BRET) in which the donor is a luminescent molecule (generally luciferase), and fluorescent resonance energy transfer (FRET) based on the use of green fluorescent protein (GFP) variants, most often yellow fluorescent protein (YFP) as fluorescent donor and CFP (cyan fluorescent protein) as acceptor fused to the receptor. One of the most important drawbacks of these strategies is that the signal does not only result from receptors targeted to the cell surface but also from those trapped inside cells. The inability of these methods to distinguish between surface receptors participating in cell stimulation and intracellular receptors that cannot be activated by ligands restrains the interest of these strategies, unless sophisticated microscopy approaches are used. Moreover, these strategies are not easily applicable to native tissues since they are based on chimeric receptor expression, which would ultimately require the generation of knock-in mice expressing the desired fluorescent fusion receptors.

More recently, time-resolved FRET (TR-FRET) strategies based on the use of lanthanide (essentially terbium or europium) cryptates have opened new perspectives since they exhibit a very high signal-to-noise ratio (10) and allow the labeling of only the receptors targeted to the cell surface. TR-FRET strategies take advantage of the long-lasting fluorescence of the lanthanides. The fluorescence lifetime of terbium and europium is in the millisecond range, and is about 100,000 times longer than those of classic fluorophores (about 10 ns). Therefore, fluorescent measurement after a time delay (50 μs) allows the measurement of acceptor fluorescence only if it is engaged in a FRET since it will exhibit in this case a long-lasting fluorescence. Fluorescent

Table 1
Donor and acceptor fluorophores compatible with time-resolved FRET experiments, with their typical excitation and emission wavelengths

Donor				Acceptor	
	Name	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ (nm)	Name	$\lambda_{emission}$ (nm)
Europium-cryptate	Eu – PBBP	320–340	620	d2 like fluorophores (d2, d1 Cy5, Alexa 647, DY647…)	665
Terbium- cryptate	Lumi4-Tb®	335–340	620	-d2 like fluorophores -fluorescein like fluorophores	665 520

acceptors exhibiting fluorescein-like properties (with Lumi4-terbium as donor) or emitting in the near infra-red range around 665 nm (d2-like fluorescent properties, with europium-cryptates or Lumi4-terbium as donors), are compatible with TR-FRET experiments (Table 1).

Various strategies can be developed to label receptors targeted to the cell surface with TR-FRET compatible fluorophores. For some of them, receptor modifications are mandatory and are achieved by fusing it to tags or to suicide enzymes recognized by specific antibodies (11, 12) or specific substrates (Tag-lite® strategy) (13), respectively. Such techniques cannot be used in native tissues. To overcome this limitation, we developed an alternative strategy allowing the labeling of GPCRs targeted to the cell surface. It is based on the binding of specific fluorescent ligands compatible with time-resolved FRET experiments (14).

This strategy of GPCR labeling has been used to study homodimers. In that context, two fluorescent versions of the same ligand (donor-ligand and acceptor-ligand) have to be developed and optimal TR-FRET signal is observed when all receptors are labeled, 50% with the donor-ligand and 50% with the acceptor-ligand. Of note, in these conditions, and when considering only dimers and not higher-order oligomer complexes, 25% of the dimers are labeled with two donors, 25% with two acceptors, and 50% with a donor and an acceptor. Only these last complexes contribute to the FRET signal. The optimization of the labeling with donor and acceptor, first to determine the best conditions for receptor labeling and second to obtain a donor and acceptor well-balanced labeling, is thus crucial for appropriate homodimer detection.

The same strategy could also be used to detect GPCR heterodimers although it has not been done yet. In such a context, the combination of a donor-labeled ligand targeting the first GPCR with a second ligand targeting the second GPCR labeled with an acceptor should allow the labeling of 100% of these heterodimers.

This receptor labeling strategy is really efficient since it does not require modification of the receptor sequence. However, it is noteworthy that TR-FRET signal amplitude is dependent on ligand binding and therefore on the affinity of the ligands. For the same receptor occupation, low affinity ligands (Ki > 20 nM) have to be used at higher concentrations leading to lower signal-to-noise ratio.

2. Materials

2.1. Cells

Stable cell lines expressing the receptor of interest or cell lines which can be transiently transfected (see Note 1).

2.2. Cell Culture

1. Phosphate buffered saline (PBS) prepared from 10× stock solution (Invitrogen, Cergy Pontoise, France).
2. Trypsin–EDTA solution (Invitrogen, Cergy Pontoise, France).
3. Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Verviers, Belgium).
4. Fetal calf serum (FCS) (Lonza, Verviers, Belgium).
5. Penicillin/streptomycin (Invitrogen, Cergy Pontoise, France).
6. Electroporation buffer (EB): 250 mM KH_2PO_4, 100 mM CH_3COOK, and 100 mM KOH prepared from a 5× solution (EB 5×).
7. Solution of $MgSO_4$ (1 M).
8. Cell electroporator (e.g., Gene pulser, Bio-Rad Laboratories, Marnes-La-Coquette, France).
9. Black 96-well plate (e.g., Greiner Cell Star 96-well plate, Dominique Dutscher, Brumath, France).
10. Lipofectamine™ 2000 (Invitrogen, Cergy Pontoise, France).

2.3. Membrane Preparation

1. PBS without calcium and magnesium (Lonza, Levallois-Perret, France).
2. Ice-cold lysis buffer: 15 mM Tris-HCL, 2 mM $MgCl_2$, 0.3 mM EGTA, pH 7.4.
3. Ice-cold 10% sucrose buffer: 10% (w/v) sucrose, 10 mM Tris-HCL, 1 mM EDTA, pH 7.4.
4. Ice-cold 35% sucrose buffer: 35% (w/v) sucrose, 10 mM Tris-HCL, 1 mM EDTA, pH 7.4.
5. Membrane suspension buffer: 50 mM Tris, 5 mM $MgCl_2$, pH 7.4.
6. An Ultra-Turrax homogenizer (Janke Kunkel IKA-Labortechnik, Staufen, Germany).

7. An ultracentrifuge with a swing SW-28-out bucket rotor (Beckman Coulter, Roissy, France).
8. Bradford protein assay (BioRad protein assay laboratories, Marnes-La-Coquette, France).

2.4. Ligands

1. GPCR ligands derivatized with TR-FRET acceptors (fluorescein, AlexaFluor 488, d2, d1, AlexaFluor 647 or Cy5 can be found in the literature (15) and can therefore be synthesized (see Note 2)).
2. Ligands derivatized with lanthanide cryptates were synthesized by Cisbio Bioassays (Cisbio Bioassays Drug Discovery, Bagnols-sur-Cèze, France).
3. Labeling buffer: 20 mM Tris-HCL, 118 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.8 mM $CaCl_2$, pH 7.4.

2.5. Reading of the Signal

TR-FRET signals can be read on any microplate reader compatible with HTRF (see www.HTRF.com) (see Note 3).

3. Methods

The method should be first optimized on cell lines transiently expressing the receptor of interest and then applied to native tissues. Because experiments can be performed both on intact cells and membrane preparations, the protocol used for membrane preparation is indicated afterwards.

3.1. Expression of the GPCR in a Cell Line

Electroporation was initially used to transiently express Snap-tag receptors. Recently, we have also used Lipofectamine transfection according to the protocol provided by the manufacturer. We indicate below the protocol followed for electroporation (see Subheading 2.2).

1. COS-7 are kept in culture in DMEM medium supplemented with Fetal calf serum (10%), penicillin/streptomycin antibiotics (1%) at 37°C in an atmosphere of 95% air and 5% CO_2. Cells are split before they reach confluence.
2. The plasmid mix (152 µL) is prepared in sterile water from plasmid encoding the Snap-tag receptor of interest and supplemented with empty vector to 10 µg (see Note 4).
3. 40 µL EB 5× and 8 µL 1 M $MgSO_4$ are added to the plasmid mix.
4. Cell dishes are washed once with PBS (10 mL) and cells are dissociated with prewarmed trypsin–EDTA solution. (4 mL/dish) for about 3 min at 37°C.

5. To neutralize trypsin/EDTA solution activity, 6 mL/dish of prewarmed DMEM supplemented with 10% FCS is added and cells are harvested, counted on a Malassez cell and centrifuged at 160 × *g* for 5 min.
6. Supernatant is removed and cells are resuspended in EB 1× buffer at a density of 100 million cells/mL. Of note, at this density, cell volume represents about 1/3 of the final volume.
7. 100 µL of cells are added to 200 µL of the plasmid mix. Solution is gently mixed by pipetting and incubated at room temperature for 5 min.
8. Each mix preparation is transferred to an electroporation cuvette and placed in the electroporator (see Note 5).
9. After electric shock delivery, cells are resuspended in 10 mL of fresh complete culture medium and are seeded in 96-well plate at a density of 100,000 cells/well.
10. Cells are incubated for 48 h at 37°C, 5% CO_2.

3.2. Membrane Preparation

See Subheading 2.3.

3.2.1. Membrane Preparation from Cultured Cell Line

1. Cells cultured in 150-mm culture dishes are rinsed twice with 10 mL of PBS without calcium or magnesium.
2. 4 mL of ice-cold lysis buffer are added and cells are scraped with a rubber policeman.
3. The cells are transferred to a 50-mL tube.
4. Culture dishes are rinsed with 4 mL of ice-cold lysis buffer and the solution is pooled with the previously collected fraction.
5. Cells are homogenized for 30 s with an Ultra-Turrax homogenizer, and centrifuged at 100 × *g* for 5 min at 4°C.
6. Supernatants are recovered and centrifuged at 44,000 × *g* for 30 min at 4°C.
7. Pellets are resuspended in suspension buffer and centrifuged at 44,000 × *g* for 30 min at 4°C.
8. Pellets are resuspended in a small volume of suspension buffer. For each membrane preparation, protein concentration is estimated by colorimetric assay (e.g., Bradford assay).
9. Membrane preparation can either be immediately used or stored as aliquots (100 µL containing 100–300 µg membrane protein) in liquid nitrogen.

3.2.2. Membrane Preparation from Native Tissue

Preparation from native tissue is dependent on the tissue collected. Here we indicate the protocol we used to prepare membranes from mammary gland.

1. After sacrifice of 14–18 day lactating rats, mammary glands are collected and kept in ice-cold PBS without calcium or magnesium.
2. Dissected mammary glands are freed of connective tissue and cut into small pieces in ice-cold lysis buffer.
3. Tissues are homogenized for 30 s with an Ultra-Turrax homogenizer (see Note 6).
4. The homogenate is spun at 500 × *g* for 10 min at 4°C.
5. Supernatants are recovered and centrifuged at 12,000 × *g* for 30 min at 4°C.
6. Pellets are homogenized in 90 mL of ice-cold 10% sucrose buffer and 15 mL of the homogenate are layered onto 15 mL 35% sucrose buffer in each centrifuge tube.
7. The preparation is centrifuged at 100,000 × *g* for 2 h at 4°C in a swing SW-28-out bucket rotor.
8. The membranes of mammary gland are collected at the 10–35% sucrose interface.
9. Membranes are dispersed in ice-cold membrane suspension buffer (25–30 mL).
10. Membranes are centrifuged at 40,000 × *g* for 30 min at 4°C and pellets are resuspended in a small volume (3–5 mL) of the same buffer. For each membrane preparation, protein concentration is estimated by colorimetric assay (e.g., Bradford assay).
11. Membranes are immediately used or aliquots (100 µL containing 100–400 µg of membrane protein) are stored in liquid nitrogen (see Note 7).

3.3. Labeling of Receptor: Optimization of the FRET Signal

Three parameters can vary in order to optimize the FRET signal (see Note 8).

See Note 9 and Table 2 for experimental conditions which have to be included in the experiments in order to properly analyze the results.

Table 2
Experimental controls required for the analysis of FRET signals

Samples	Negative FRET control	Dynamic FRET control	Receptor specificity
Donor-ligand + acceptor-ligand	Donor-ligand	Donor-ligand + acceptor-ligand	Donor-ligand + acceptor-ligand
Expressed receptor (cell or membrane)	Expressed receptor (cell or membrane)	No cell or membrane	Mock cell or membrane or expressed receptor (cell or membrane) + excess unlabeled ligand

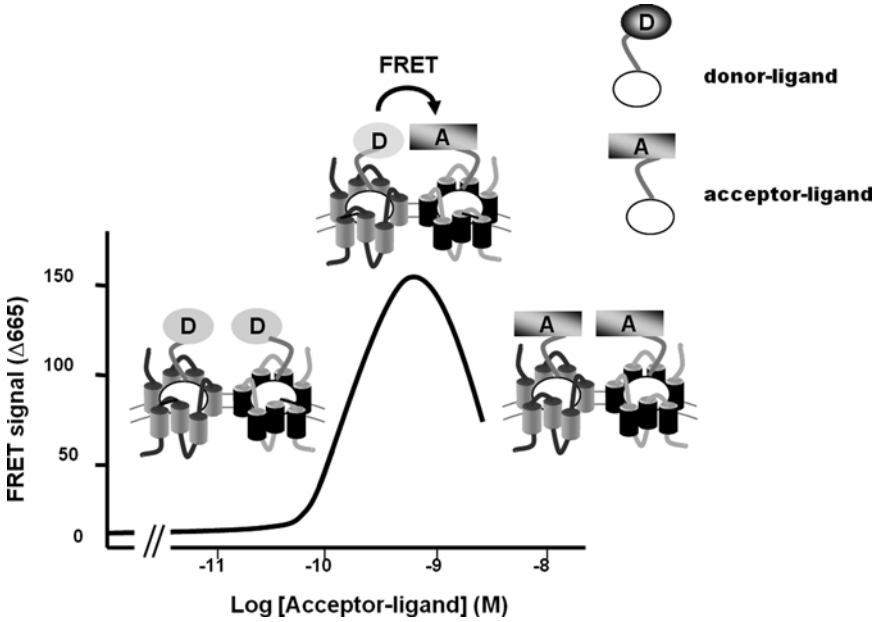

Fig. 1. Optimization of the TR-FRET signal by variation of the ligand concentrations. Different donor-ligand/acceptor-ligand ratios are tested by varying the acceptor concentration while the concentration of the donor remains constant. The optimized ratio corresponds to the peak of the bell-shaped curve. Various curves can be established to determine the optimal concentration of donor.

3.3.1. Optimization of the Ligand Concentrations

As mentioned above, the aim is to optimize the concentration of donor and acceptor labeled ligands to obtain an optimal TR-FRET signal. As a prerequisite, affinities of the ligand and more specifically of the donor-ligand should have been determined. To determine the donor-ligand/acceptor-ligand ratio, we keep the donor concentration constant and vary the acceptor concentration from picomolar to micromolar levels (Fig. 1). Various curves are established with different concentrations of donor-ligand. These experiments can either be performed on cells expressing the receptor of interest or on membrane preparations.

Experiments on Cells

1. Cells are transiently transfected with plasmids encoding the protein of interest in black 96-well plates and incubated for 48 h at 37°C, 5% CO_2 (see Subheading 3.1).
2. The different ligands are prepared separately in ice-cold labeling buffer complete with 0.1% BSA and 0.1% glucose, and kept on ice (by working below 15°C, all internalization phenomena are avoided). At this point, all ligand solutions are prepared at four times the desired final concentration (see Note 10).
3. Premixes of the solutions are performed according to the conditions tested and kept on ice. Cells are placed on melting

ice to cool down. The medium is then removed and replaced with 100 µL of cold mixed ligand solutions.

4. Plates are incubated overnight at 4°C with gentle rocking.
5. The signals are recorded at 620 nm for the donor and at 520 nm (e.g., fluorescein, alexa488) or 665 nm (e.g., d2) depending on the acceptor (see Note 11).
6. Data analyses are performed as indicated in the data analysis section.

Experiments on Membrane Preparations

1. Experiments are performed in a final volume of 200 µL/well. 100 µL of membranes are distributed in each well, in a black 96-well plate, and placed on melting ice.
2. 100 µL of premixed ligand solution (prepared as described previously for experiments on cells, except with 8× initial solutions, due to additional dilution by the membranes) is distributed in each well.
3. Plates are incubated overnight at 4°C with gentle rocking.
4. Fluorescence and TR-FRET signals are recorded as previously described.

3.3.2. Optimization of the Membrane Concentration

After determining the optimal fluorescent ligand concentrations, a second step of optimization of the FRET signal can be performed on the quantity of membrane used in the assay. Indeed the FRET signal is in theory proportional to the receptor labeling. However, different parameters can reduce the FRET signal. For example, if fluorescent ligands are not in excess regarding the number of receptor ÿimmers, the receptor dimer will be labeled with only one fluorescent ligand, either a donor or an acceptor, resulting in the absence of FRET. Therefore, variation of the amount of membrane per assay will result in a biphasic curve, with the ascending portion proportional in the initial phase to the receptor concentration and a descending portion which could be explained by an insufficient occupation of the ÿimmers by the ligands (Fig. 2).

1. The different ligands are prepared separately and ligand premixes are made as described above.
2. Various quantities of membranes (0–300 µg) are distributed in the wells from a stock solution and volumes in the well are completed with the suspension buffer to a final volume of 100 µL.
3. For each membrane concentration, negative controls have to be performed (see Note 9).
4. Plates are incubated overnight at 4°C with gentle rocking.
5. FRET signals are measured as described above.

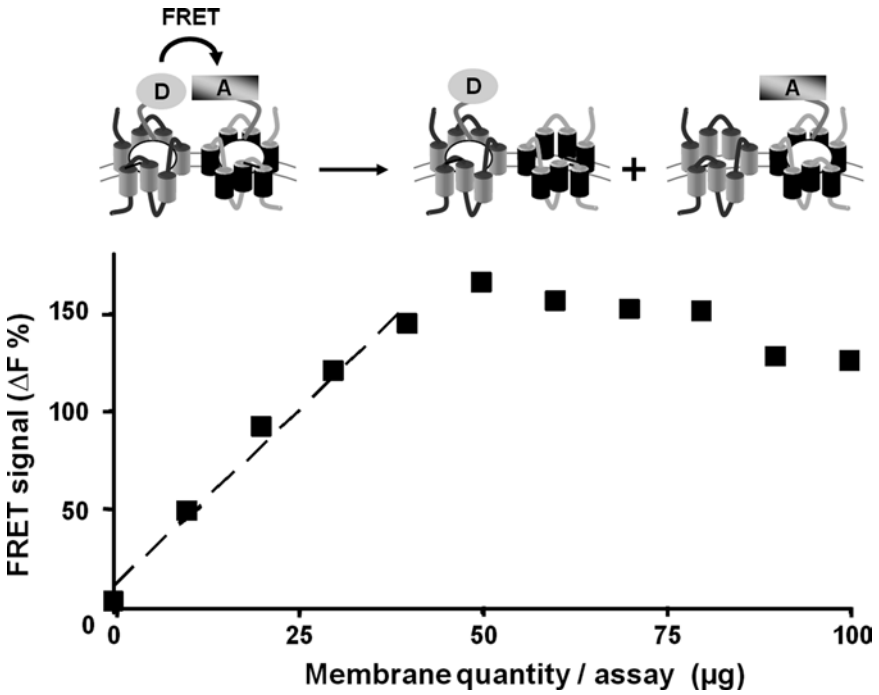

Fig. 2. Optimization of the TR-FRET signal by variation of the membrane quantity. The donor-ligand/acceptor-ligand ratio being established, the amount of membrane per assay can be optimized.

3.3.3. Optimization of the Fluorescent Ligands

Various FRET signal intensities can be measured depending on the nature of the ligand. Indeed, crosstalk between binding sites of protomer within a dimer can lead to positive or negative cooperativity of binding. Positive cooperativity favors the binding of two ligands per dimer leading to a greater FRET signal. Testing various pairs of fluorescent ligands can therefore be relevant to optimize the FRET signal. Using various GPCRs, we repeatedly observed a larger signal with fluorescent antagonists than with fluorescent agonists (see Note 12).

3.4. Data Analysis

Two parameters are classically used to measure the FRET signal.

1. Δ665 (for d2-like acceptor) or Δ520 (for fluorescein-like acceptor) corresponding to variation of the fluorescence emission intensity of the acceptor. This parameter is the simplest one but intensity variations can also reflect variations in the experimental conditions.
2. Δ*F*% (see the formula below): this parameter takes into account the variation of the donor concentration and corresponds to a percentage of the FRET increase compared to a negative control. However, the Δ*F*% parameter does not allow the comparison of experiments that are not performed with the same donor-ligand concentrations.

3.4.1. Determination of the Δ665 Specific FRET Signal (or Δ520, Depending on the Acceptor Fluorophore Used)

The calculation of the Δ665 (for example) allows the representation of the specific FRET signal from which has been subtracted non-specific FRET (bleed-through from the donor, FRET resulting from random donor-acceptor collision: dynamic FRET). It corresponds to Δ665 = signal at 665 nm of the sample − signal at 665 nm of the negative control.

The negative control can include:

1. Sample with only the donor-ligand.
2. Mock cells or membranes with donor and acceptor labeled ligands (gives an estimation of the dynamic FRET, especially if labeled ligands are used at concentrations higher than 10 nM).
3. Sample with donor and acceptor labeled ligands and an excess of unlabeled ligand (e.g.: 1 μM of unlabeled ligand).

3.4.2. Calculation of the ΔF%

The FRET ratio is simply calculated for each well as the ratio between the acceptor FRET signal (at 665 or 520 nm, depending on the acceptor used) and the donor emission at 620 nm:

Ratio: (signal at 665 nm/signal at 620 nm) × 10,000 (see Note 13).

The resulting data represents the FRET efficiency.

The calculation of the Δ*F*% is based on the different FRET ratios:

$$\Delta F\% = \frac{\text{Ratio}_{\text{sample}} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

As mentioned above, it is important to be very careful when comparing Δ*F*% values obtained with different donor-ligand concentrations. If variations in the $\text{Ratio}_{\text{neg}}$ are observed, it may not be relevant to compare Δ*F*% values. In such a case, it is preferable to compare between Δ665 values.

FRET values are plotted against the acceptor-ligand concentration (for the FRET optimization assay) or against receptor expression/membrane quantity (for the dimerization assay).

4. Notes

1. Two cell lines have been used: CHO and COS-7 cell lines.
2. A large collection of these ligands are now commercially available from Cisbio Bioassays (Cisbio Bioassays Drug Discovery, Bagnols-sur-Cèze, France) since they are used in Tag-lite® binding assays (see www.HTRF.com).
3. We read TR-FRET signal on a Rubystar reader (BMG Labtechnologies) and on a Tecan Infinite F500 microplate reader (Tecan).

4. Because receptor expression is extremely variable from one receptor to another, the quantity of plasmid encoding for one receptor has to be optimized. For example, we generally use between 200 ng and 1 μg of vectors coding vasopressin V_2 or dopamine D_2 receptors and between 9 and 9.8 μg of empty vector.
5. Parameters of electroporation differ slightly from one cell line to another. For COS-7 cell lines, we use 270 V and 1,000 μF.
6. We recommend homogenizing the tissue little-by-little to avoid the formation of aggregates and to keep the preparation on ice.
7. The dissection of mammary gland of one lactating rat routinely gives between 10 and 40 mg of membrane protein.
8. Three parameters can vary in order to optimize the FRET signal:
   (a) The concentrations of the donor-ligand and the acceptor-ligand: the concentration of donor-ligand and acceptor-ligand should be high enough to label receptors. The donor/acceptor ratio has to be determined in order to get 50% of the receptors labeled with donor-ligand and 50% with acceptor-ligand. Finally the lower the concentration of donor-ligand and acceptor-ligand, the lower the nonspecific FRET signal resulting from nonspecific dynamic FRET signal (FRET resulting form random collision of donors and acceptors in suspension).
   (b) The amount of membrane: the FRET signal intensity varies with the number of receptors labeled with ligands. However, increasing the amount of membrane per well increases the signal to a maximum and then the signal decreases. This signal decrease can be explained by an excess of receptor amount compared to the available ligand.
   (c) The nature of the labeled ligand (e.g., agonist or antagonist): we observe that the TR-FRET signal intensity can vary depending on the nature of the ligand and the interaction between the receptors.
9. The following conditions have to be included in the experiments in order to analyze the results:
   (a) A negative control obtained in the presence of donor-ligand and the absence of acceptor-ligand.
   (b) A positive control based on the combination of donor and acceptor labeled ligands.
   (c) A nonspecific control obtained with an excess of unlabeled ligand, in the presence of donor and acceptor labeled ligands.

Table 3
Example of ligand dilutions for triplicates performed in 96-well plate formats

	Cells/membranes	Donor-ligand 4× (e.g., 4× 1 nM)	Acceptor-ligand 4× (e.g., 4× 1 pM to 1 μM)	Cold ligand 4× (e.g., 4× 1 μM)	Labeling buffer qsp 400 μL
Mock	Mock	100 μL	100 μL	–	200 μL
Donor only	Expressing receptor	100 μL	–	–	300 μL
Nonspecific		100 μL	100 μL	100 μL	100 μL
Experiment		100 μL	100 μL	–	200 μL

(d) Another nonspecific control can be performed using cells transfected with an empty vector or membrane preparation from cells or tissues that do not express the receptor of interest. Such controls can replace the control performed with an excess of unlabeled ligand. We strongly recommend this control for each donor and acceptor ligand concentration since mixtures of donor and acceptor can lead to nonspecific dynamic TR-FRET signal.

10. For the calculation of volumes, the assay is performed in triplicates, with a final concentration in 100 μL/well. For an example of ligand dilutions see Table 3).
11. Since the plate readers are generally not thermostated, plates should be keep on ice as much as possible.
12. Although a general rule cannot be drawn from experiments performed on only a few types of GPCRs, we think that the design and the synthesis of fluorescent antagonists are potentially more relevant (Fig. 3).
13. The factor 10,000 just allows an acceptable scale on the Y axis.

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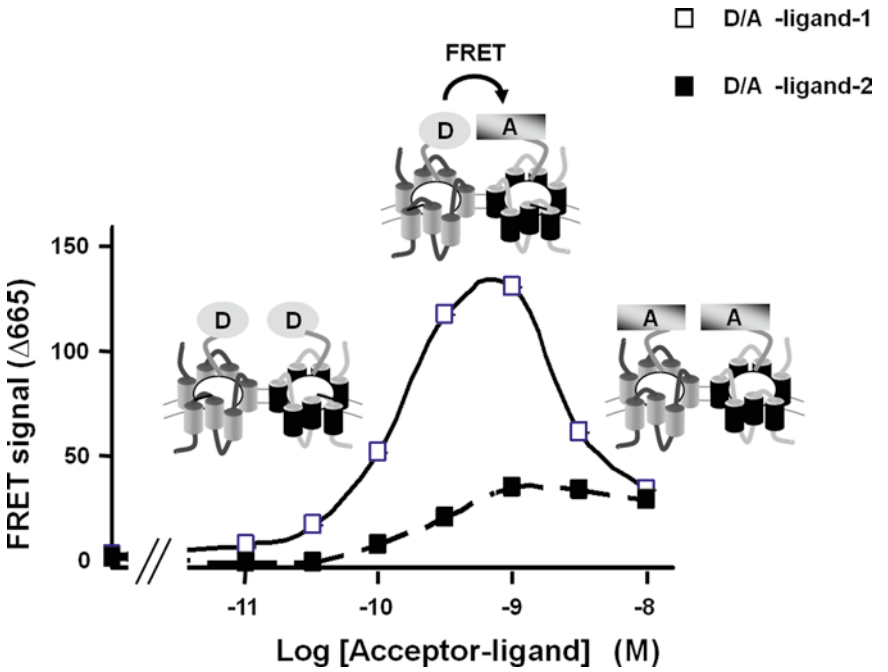

Fig. 3. Optimization of the TR-FRET signal by testing various ligands. TR-FRET signal amplitude can vary depending on the nature of the ligand. As illustrated, we observed on significant variation of the FRET signal between fluorescent agonists on the one hand, and fluorescent antagonists on the other.

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