

## Time-Resolved FRET Strategy to Screen GPCR Ligand Library

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### Abstract

Screening chemical libraries to find specific drugs for G protein-coupled receptors is still of major interest. Indeed, because of their major roles in all physiological functions, G protein-coupled receptors remain major targets for drug development programs. Currently, interest in GPCRs as drug targets has been boosted by the discovery of biased ligands, thus allowing the development of drugs not only specific for one target but also for the specific signaling cascade expected to have the therapeutic effect. Such molecules are then expected to display fewer side effects. To reach such a goal, there is much interest in novel, efficient, simple, and direct screening assays that may help identify any drugs interacting with the target, these being then analyzed for their biased activity. Here, we present an efficient strategy to screen ligands on their binding properties. The method described is based on time-resolved FRET between a receptor and a ligand. This method has already been used to develop new assays called Tag-lite® binding assays for numerous G protein-coupled receptors, proving its broad application and its power.

**Key words** Tag-lite® screening, G protein-coupled receptor, Fluorescent ligand, Time-resolved FRET, Lanthanide, Terbium, Self-labeling enzyme, Binding experiment

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## 1 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of membrane proteins, and about 400 receptors (excluding chemosensory receptors) have been identified. They can be activated by a large variety of stimuli, from photon to large proteins, and participate in the regulation of many physiological functions. Thus, they constitute very important targets for drug development representing 30 % of the therapeutic drugs on the market [1]. By contrast only 15 % of all GPCRs are the target of actual drugs indicating that screening for new drugs is far from being completed and is still an actual challenge.

Recently, the emergence of the concept of receptor functional selectivity opened up new perspectives in drug development. Indeed, it was found that some ligands activate only a subset of the signaling pathways of one receptor. This offers the possibility of designing ligands that not only are specific for one target but also have the desired effect (agonist or antagonist) on the single signaling cascade expected to have the therapeutic effect. This renewed the interest for screening new drugs for “old” receptor targets.

Ligand-binding screening has been done using radioactive tracers, but their use is less and less frequent because these strategies are generally not homogeneous and can be hazardous although displaying high sensitivity. By contrast, strategies based on fluorescence tools generally exhibit low sensitivity because of a high non-specific signal leading to a low signal-to-noise ratio. We have developed various time-resolved FRET strategies to perform high-throughput screening-binding assays in various contexts, either on cell lines or on membrane preparations. Their sensitivities and the easiness to carry out these assays make them efficient for either high- or low-throughput screenings and thus very attractive both for big pharmaceutical companies and for academic laboratories.

### **1.1 Principle of Time-Resolved FRET**

Förster (or fluorescence) resonance energy transfer (FRET) has been described in the early 1940s, but its use in biology remained quite restricted until the 1990s. The synthesis of more stable and brighter fluorophores combined with the development of more sensitive fluorescence detectors now makes FRET techniques a standard in biological studies.

FRET consists of non-radiative energy transfer from one fluorophore, a donor, to another, the acceptor, the excitation of the donor leading to the fluorescence emission of the sensitized acceptor. The fluorophores should fulfill at least three criteria to generate an important FRET [2]: (1) they should exhibit energy compatibility – the greater the spectral overlap between the donor emission and the acceptor excitation spectra, the more efficient the transfer; (2) the orientation of the fluorophores should be compatible, optimal FRET being obtained when dipole transition moments of the donor and the acceptor are parallel; and (3) the distance between the fluorophores should not exceed about 1.5 of the Förster distance ( $R_0$ ),  $R_0$  being defined as the distance for which 50 % of FRET efficacy is measured. Although  $R_0$  depends on the pair of fluorophores engaged in FRET, it is usually between 40 and 80 Å. Since FRET efficiency varies as a function of the inverse of the distance to the 6th power, a distance between the fluorophores greater than  $1.5 \times R_0$  or less than  $0.5 \times R_0$  results in an absence or a maximal FRET, respectively.

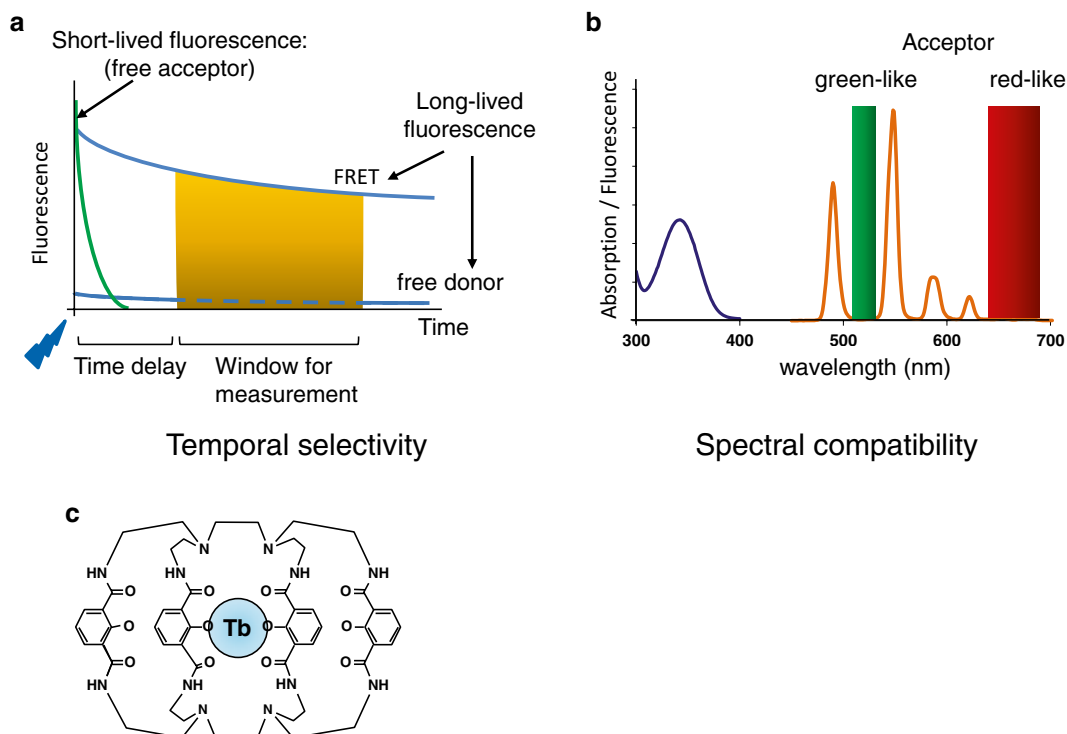
Various criteria are used to choose a pair of fluorophores to perform FRET experiments. First, the fluorophores have to fulfill the criteria mentioned above. Second, easiness to label the molecules

of interest has to be considered: organic fluorophores can be convenient to label small molecules during their synthesis or purified proteins (e.g., antibodies), but their use to label intracellular or membrane-targeted protein is more difficult. By contrast, fluorescent proteins such as green fluorescent protein (GFP) or its derivatives can be easily used to label cellular proteins by molecular engineering but are not adapted to label small ligands.

Different strategies have been developed to measure FRET signals. The simplest one consisting in the measurement of the fluorescence of the acceptor is however limited because of a low signal-to-noise ratio. This is generally due in part to a direct excitation of the acceptor at the excitation wavelength of the donor but also to the emission of the donor and to a high autofluorescence of the biological preparation or of the medium at the acceptor emission wavelength. The mathematical analysis (various steps of normalization and background subtraction) required to separate the FRET signal from nonspecific fluorescence makes this approach fastidious, with a little sensitivity, and incompatible with high-throughput screening [3]. By contrast, time-resolved FRET strategies [4] exhibit high signal-to-noise ratios, up to 100 times greater than conventional FRET. Their high sensitivity is due to the physical properties of lanthanides complexes (Fig. 1). First, these fluorophores exhibit long-lasting emission (luminescence lifetime greater than 1 ms) by contrast to conventional fluorophores (fluorescence lifetime less than 20 ns). The measurement of the fluorescence after a time delay (usually about 50  $\mu$ s), during which all short-lived fluorescences responsible for the high background are extinguished, allows a specific detection of fluorescence emission resulting from a FRET process (Fig. 1a). Second, lanthanide derivatives exhibit a large pseudo-Stokes shift and have atomic-like emission spectra which leave the spectral windows to measure green or red emission from acceptor species with low background from the donor (Fig. 1b). Third, since luminescence from lanthanides is nonpolarized, time-resolved FRET is thus far less sensitive to the fluorophore's relative orientation [4].

In order to obtain bright complexes, the lanthanides are engaged into chelating antenna such as cryptates [5]. Furthermore, these complexes can be bioconjugated to amine or thiol groups. Lumi4-Terbium (Lumi4-Tb), one of the brightest complexes upon excitation at 337 nm, is of particular interest because it is compatible with various acceptors such as fluorescein-like (green acceptors) or d2-like (red acceptors) fluorophores (Fig. 1).

Time-resolved FRET strategy is perfectly adapted for the development of binding assays for G protein-coupled receptors. Such assays are based on TR-FRET between compatible fluorophores carried on the one hand by ligands and on the other hand by tagged receptors. The binding of a fluorescent ligand in the binding pocket of a GPCR results in a close proximity of the

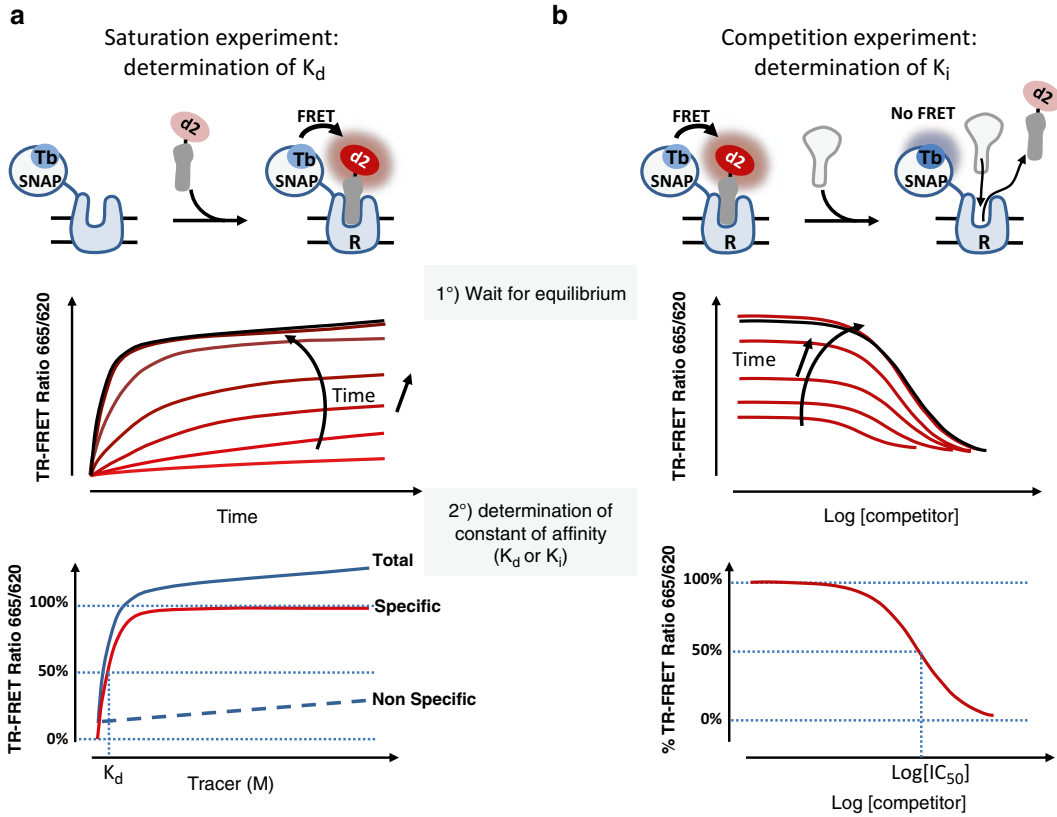


**Fig. 1** Principle of time-resolved FRET technique. **(a)** Temporal selectivity: FRET signal is measured 50  $\mu$ s after the excitation, in a time window of 400–500  $\mu$ s. During the delay, all short-lived fluorescent decreased to zero, and only long-lived time fluorescence FRET and free donor fluorescence can be measured. Because the fluorescence emission of donor is weak at 665 nm (emission wavelength of the acceptor), the contamination to free donor fluorescence is often negligible. **(b)** Absorption (dark blue) and emission (orange) spectra of Lumi4-Tb. Green and red box indicate the emission wavelengths of the acceptors which are compatible with Lumi4-Tb. **(c)** Structure of the Lumi4-Tb

fluorophores leading to a potential FRET between them. Based on this principle, saturation and competition experiments can be carried out (Fig. 2).

## 1.2 Ligand Labeling

As mentioned above, ligands can generally be derivatized by fluorophores through well-established chemical approaches. However, with the derivatization position being a particular case for each ligand, general rules to design fluorescent ligands are difficult to be brought out. If fluorophores can be linked in some cases to peptides without any spacer, spacers are required to link bulky fluorophore groups on small ligands such as biogenic amines. The analyses of the structure-activity relationship of ligands, which have been studied on a few receptors, suggest that the affinity of the ligand is not significantly impacted if the fluorophore is brought



**Fig. 2** Time-resolved FRET-based binding assay. Saturation (a) and competition (b) experiments can be performed. Two steps are required to develop a time-resolved FRET-based assay. The first step consists in defining the kinetics to reach equilibrium. Evolution of the signal follows when time of incubation increases. *Lower panels* illustrate saturation and competition curves. Specific signal is obtained after subtracting non-specific signal from total signal

outside the binding pocket through an optimized linker [6]. The hindsight we have proves that derivatization of small ligands to get a high-affinity ligand is feasible [7].

### 1.3 Labeling of G Protein-Coupled Receptor

Various strategies have been used to label GPCRs. First non-covalent labeling has been performed using antibodies against the receptors themselves or against epitope sequences fused to the N-terminus of the receptor. Although positive results have been obtained [8], two major drawbacks have been identified: (1) antibodies are large molecules (150,000 Da) compared to a receptor (about 40,000–65,000 Da) and can generate steric hindrance and (2) the labeling is not covalent but leads to an equilibrium between unlabeled and labeled GPCR. Therefore, the efficacy of the labeling depends

both on the affinity of the antibodies and on their concentration. Labeling efficacy can also be affected by washing steps. Moreover, the kinetics to reach the equilibrium is dependent on at least the bindings of the fluorescent ligand, the competitor, and the antibodies preventing the determination of the affinity of the competitor.

As an alternative, GPCRs can be covalently labeled by resorting to Tag-lite®, a platform developed to accurately label a protein of interest on a targeted site with homogeneous time-resolved fluorescence (HTRF®) dyes that make use of SNAP-tag®, CLIP-tag®, and HaloTag® fusions.

SNAP-tag® is derived from *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT). This wild-type enzyme involved in DNA repair transfers alkyl group inserted in the guanine bases of DNA [9, 10] to itself. Mutations have been performed in order to increase its enzymatic activity and to modify its DNA-interacting site [11–15]. Addition of non-permeant fluorescent benzylguanine (BG) substrates such as SNAP-Lumi4-Tb in the medium results in the fluorescent covalent labeling of the enzyme-GPCR chimera. The SNAP-tag® strategy improves the previous strategy in different ways. First, the size of the enzyme is about 2/3 of GFP and 1/7 of an antibody leading to a reduced steric hindrance. Second we showed that 100 % of receptors targeted to the surface can be labeled, resulting in an increase of the fluorescent signal. Moreover, because the labeling is covalent, the equilibrium of the binding assay is only dependent on the association and dissociation kinetics of the fluorescent tracer and of the competitor, allowing the determination of the affinity of the competitor in competition binding experiments. Finally because of the covalent nature of the labeling, washing steps do not affect the labeling. More recently, other self-labeling proteins such as CLIP-tag® [16] or HaloTag® [17] have been developed to label receptors.

As illustrated in Fig. 2a, b, either saturation or competition binding assays based on the Tag-lite® strategy can be performed. The assay exhibits a number of advantages. First, batches of cells or membrane preparation expressing GPCRs can be labeled and stored frozen because of the covalent labeling. From this ready-to-use cellular material, binding assays are very simple to perform since no washing steps are required. Indeed, only the bound ligand leads to a FRET signal, such that the unbound ligands, though still present in the assay, are not detected; they don't need to be washed away. Also, such a specific FRET signal resulting from the ligand binding to its receptor also avoids the detection of any unspecific binding due to hydrophobicity of the ligand or its interaction with the plastic or any other support used in the assay. These FRET assays are then straightforward and can be miniaturized and are therefore HTS compatible (they can be performed in 384-well, 1,536-well, and even 3,456-well plates). The existing HTRF®-compatible plate readers allow fast reading, and finally no hazardous waste are produced.

One of the most attractive features of this Tag-lite® binding assay is the double specificity brought by the receptor labeling on the one hand and by the fluorescent ligand on the other hand. Therefore, and by contrast to radioactive binding, the nonspecific binding of the fluorescent ligand does not provide any nonspecific FRET signal since the ligand in that case will not be in proximity with the labeled receptor. Thus, the double labeling confers to the method a high signal-to-noise ratio [18–20].

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## 2 Materials

### 2.1 Cells

1. HEK293 cells.

### 2.2 Cell Culture and GPCR Labeling

1. Phosphate buffered saline (PBS): 0.9 % NaCl, 10 mM sodium phosphate pH 7.
2. Trypsin-EDTA solution: 0.05 % Trypsin-EDTA.
3. Dulbecco's Modified Eagle Medium-GlutaMAX™ (DMEM-GlutaMAX™) (*see Note 1*).
4. Opti-MEM® Reduced Serum Medium, GlutaMAX™.
5. Fetal calf serum (FCS).
6. Penicillin/streptomycin.
7. Poly-L-ornithine.
8. Black 96-well plate.
9. Lipofectamine® 2000.
10. Tag-lite® buffer (Cisbio Bioassays).
11. SNAP-Lumi4-Tb and CLIP-Lumi4-Tb (Cisbio Bioassays).
12. Plasmid coding for tagged receptor (*see Note 2*).
13. Unlabeled ligands were usually purchased from Tocris (R&D Systems Europe).
14. Vi-CELL Cell Viability Analyzer (Beckman Coulter).
15. Prism 6 (GraphPad Software).

### 2.3 FRET

1. GPCR ligands derivatized with TR-FRET acceptors (fluorescein, Alexa Fluor® 488, d2, d1, Alexa Fluor® 647, or Cy5) can be found in the literature [21] and can therefore be synthesized (*see Note 3*) and prepared at a concentration of about 200 µM (*see Note 4*).
2. Microplate readers compatible with time-resolved FRET (*see [www.HTRF.com](http://www.HTRF.com) (see Note 5)*).

### 3 Methods

#### 3.1 Expression of G Protein-Coupled Receptor in Cells

The receptor expression in cell lines should be optimized for each G protein-coupled receptor, and the quantity of plasmid used for the transfection to get a correct receptor expression has to be defined.

1. Keep HEK293 cells in culture in an atmosphere of 95 % air and 5 % CO<sub>2</sub> in DMEM-GlutaMAX™ medium supplemented with fetal calf serum (10 %) and penicillin/streptomycin antibiotics (1 %) at 37 °C. Split cells before they reach confluence.
2. Transfect cells using manufacturer Lipofectamine® 2000 transfection protocol. Coat 96-well black plates flat bottom with poly-L-ornithine diluted at 0.1 mg/mL in sterile PBS (50 µL/well) during 30 min at 37 °C.
3. Wash plates with 100 µL sterile PBS per well.
4. Harvest HEK293 cells when they are at 80 % of confluence, count on Vi-CELL, and resuspend cells in Opti-MEM® medium at a density of 500,000–1,000,000 cells/mL and plate (100 µL/well).
5. Dilute Lipofectamine® 2000 Transfection Reagent (0.5 µL/well) in Opti-MEM® (50 µL/well) (5 min at room temperature), and then mix with plasmid coding for GPCR of interest (25–200 ng/well).
6. After 20 min at room temperature, add 50 µL/well of plasmid-Lipofectamine® mix on previously plated cells. Perform the labeling receptor step on cells 24 or 48 h after transfection.

#### 3.2 Labeling of G Protein-Coupled Receptor Expressed at the Cell Surface

As mentioned above, various tags have been developed to get fluorescent tagged receptors. We describe below the methods to label SNAP-tag® and CLIP-tag® fused receptors, these two being used in routine in our laboratory:

1. Incubate cells expressing SNAP-tag® or CLIP-tag® receptors in the presence of their cognate substrates, i.e., SNAP-Lumi4-Tb or CLIP-Lumi4-Tb (*see Note 6*).
2. Dilute SNAP-Lumi4-Tb or CLIP-Lumi4-Tb substrates in Tag-lite® buffer to get a final concentration of 100 nM and 500 nM, respectively.
3. Remove medium from cells.
4. Dispense 100 µL/well of SNAP-Lumi4-Tb (100 nM) or CLIP-Lumi4-Tb (500 nM) solution.
5. Incubate cells for 2 h at 37 °C (*see Note 7*).
6. Remove the medium and proceed to four washes with Tag-lite® buffer.



### 3.3 Saturation Binding Experiments

Saturation experiments have to be carried out to define the concentration at which the fluorescent ligand (tracer) has to be used. One prerequisite to carry out saturation experiments is that ligands should be in excess with respect to the receptor expression (*see Note 8*).

1. After the four times washing step, add 50  $\mu\text{L}$  Tag-lite<sup>®</sup> per well.
2. Prepare a serial dilution of the fluorescent ligands in Tag-lite<sup>®</sup> buffer. At this point, prepare all the ligands at four times the desired final concentrations.
3. Add 25  $\mu\text{L}$  of fluorescent ligands par well.
4. Add 25  $\mu\text{L}$  of Tag-lite<sup>®</sup> or 25  $\mu\text{L}$  of unlabeled ligand in excess (*see Note 9*) in each well to determine total binding or nonspecific binding, respectively.
5. Measure donor fluorescent signal at 620 nm (fluorescence of the donor), and record FRET signal either at 520 nm (for green acceptor) or at 665 nm (for red acceptor) in a time-resolved mode (*see Note 10*).
6. To determine that equilibrium is reached, measure fluorescent signals at various times until the FRET signal is stable (*see Note 11*) (Fig. 2a).
7. Analyzed data as described in data analysis section.

### 3.4 Competition Binding Experiments

One prerequisite to carry out competition experiments is that ligands (tracer and competitors) should be in excess with respect to the receptor expression (*see Note 8*).

1. Prepare the tracers at concentration four times the  $K_d$  to use them at a final concentration close to  $K_d$ , and make dilutions in Tag-lite<sup>®</sup> buffer.
2. Perform serial dilutions of the competitors in Tag-lite<sup>®</sup> buffer, and prepare ligands at four times the desired final concentration.
3. After the four times washing step of the labeling procedure, dispense 50  $\mu\text{L}$  of Tag-lite<sup>®</sup> per well.
4. Add 25  $\mu\text{L}$  of tracer previously prepared in all the wells.
5. Add 25  $\mu\text{L}$  of one of the various competitor solutions issued from the serial dilution in the well.
6. Include two controls, total binding and nonspecific binding, in the plate. For the total binding or the nonspecific binding, substitute 25  $\mu\text{L}$  of Tag-lite<sup>®</sup> buffer or 25  $\mu\text{L}$  of unlabeled ligand at high concentration (*see Note 9*), respectively, to 25  $\mu\text{L}$  of competitor solution.
7. Measure donor fluorescent signal at 620 nm (fluorescence of the donor), and record FRET signal either at 520 nm (for green acceptor) or at 665 nm (for red acceptor) in a time-resolved mode (*see Note 10*).

8. For the first experiments, read fluorescent signals at different times to define the duration of the incubation required to reach equilibrium (Fig. 2b). If long incubation is required to reach equilibrium, perform overnight incubation at 4 °C.

### 3.5 Analysis of Saturation and Competition Curves

Two parameters are classically used to measure the FRET signal:

1. The FRET signal measured at 520 nm or at 665 nm for green or red acceptor, respectively. These parameters are the simplest ones but did not take into account the donor intensity variation from one well to another due to experimental conditions. By contrast, the 520/620 or 665/620 ratio for green or for red acceptor considered potential variation of the donor intensity (*see Note 12*).

2. Saturation curves.

FRET signal can be represented as a function of time. It allows determining the minimum time to reach equilibrium (Fig. 2a). FRET signal can also be plotted as a function of tracer concentration to get saturation curve. Total signal can be fitted by a saturation curve. Specific FRET signal is obtained after subtracting nonspecific signal to total FRET signal. Specific FRET signal should present a plateau proving that saturation has been reached.

The following equation is used to fit the data:

$$F = F_{\max} \cdot [\text{tracer}] / (K_d + [\text{tracer}])$$

in which  $F$  is the FRET signal,  $F_{\max}$  the maximal FRET signal,  $[\text{Tracer}]$  the concentration of tracer, and  $K_d$  the constant of dissociation of the tracer for the receptor. It is noteworthy that for some receptors, more complex equations considering two binding sites or the Hill equation have to be considered to get good fits of the experimental data.

3. Competition curves.

As for saturation curves, FRET signal is acquired at different times, and FRET signal can be plotted as a function of time. Equilibrium is reached when the signal is stable (Fig. 2b). It is noteworthy that the time to reach equilibrium can be different from the one determined in saturation experiments since it depends on the binding of both tracer and competitor.

FRET signal can be plotted as a function of competitor concentration, and the curve can be fitted with the following equation:

$$F = F_{\min} + \left( F_{\max} - F_{\min} / \left( 1 + 10^{(\text{Log}([\text{competitor}]) - \text{Log}(\text{IC}_{50}))} \right) \right)$$

in which  $F$  is the FRET signal,  $F_{\max}$  the maximal FRET signal (obtained in the absence of competitor),  $F_{\min}$  the minimal FRET signal (obtained in the nonspecific conditions),  $[\text{competitor}]$  the concentration of competitor, and  $\text{IC}_{50}$  the concentration of competitor leading to the half-maximal FRET signal. The inhibition constant can be deduced from the  $\text{IC}_{50}$  value when using the Cheng-Prusoff equation:

$$K_i = \text{IC}_{50} / (1 + [\text{tracer}] / K_d)$$

in which  $K_i$  is the inhibition constant of the competitor,  $\text{IC}_{50}$  the competitor concentration leading to a half-maximal FRET signal, and  $K_d$  the dissociation constant of the tracer.

As for saturation experiments, more complex models such as two binding sites model, for example, have to be considered to get good fits.

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## 4 Notes

1. DMEM-GlutaMAX™ offers a greater stability than classic DMEM with glutamine; however, the latter can be substituted to the former for most of the G protein-coupled receptors except glutamate receptors.
2. Various tags have been developed. They are either self-labeling proteins (also called suicide enzymes) such as SNAP-tag®, CLIP-tag®, or HaloTag® or substrates for enzyme (ACP-tag®). For all self-labeling proteins, specific substrates have been developed. The plasmids can be homemade plasmid or purchased from different manufacturers. A large collection of these plasmids are now commercially available from Cisbio Bioassays (Cisbio Bioassays, Codolet, France) (see [www.HTRF.com](http://www.HTRF.com)). Tags are generally fused to the N-terminus of the receptor, and the fusion has been shown not to impact receptor functioning. However, it has to be checked for all receptors. Insertion of SNAP-tag® or CLIP-tag® inside extracellular loops generally affects receptor conformation and modifies receptor binding and functioning properties. By contrast, ACP-tag® is much smaller, and its insertion in extracellular loops is generally better tolerated.
3. A large collection of these ligands are now commercially available from Cisbio Bioassays since they are used in Tag-lite® binding assays (see [www.HTRF.com](http://www.HTRF.com)).
4. Ligands are dissolved in 10 % DMSO in case of peptide or protein ligand or in 100 % DMSO for organic ligand. The concentration of the stock solution is determined by using the Beer-Lambert relationship,  $A = \epsilon l C$ , in which  $A$  is the absorbance,

$\epsilon$  is the molar extinction coefficient,  $l$  is the width of the cuvette, and  $C$  is the concentration of the solution.

Regarding red acceptor-derivatized ligands, the following values for the molar extinction coefficient ( $\epsilon$ ) (L/mol/cm) at 649 nm were used: d1: 250,000; d2: 225,000; and BODIPY: 80,000. Ratio of absorptions measured at 649 and 604 nm was systematically defined. It should be around 3.3. A lower value can reflect a degradation of the ligand or some difficulties to dissolve it.

Regarding green acceptor-derivatized ligands, aliquots for measuring absorption were generally diluted in a 100 mM carbonate buffer at pH 9, and the value of the molar extinction coefficient is 75,000 (68,000 at pH 7.4) at 495 nm.

5. We read TR-FRET signal on PHERAstar reader (BMG LABTECH) and on Tecan Infinite F500 Microplate Reader (Tecan).
6. Since fluorescent ligands are generally derivatized with fluorescent green or red acceptors, tagged receptors have to be labeled with fluorescent donor-derivatized substrates, either SNAP-Lumi4-Tb or CLIP-Lumi4-Tb, to generate a FRET signal. These substrates are non-permeating, and therefore, intracellular tagged receptors cannot be labeled.
7. The duration of the incubation and the concentration of the substrate solution are defined in such a way to get almost 100 % of the receptor labeled. However, these parameters can be modified to get a faster labeling (shorter incubation and higher substrate concentration) or to use less substrates (longer incubation and smaller substrate concentration).
8. It is generally admitted that free ligand quantity should be at least ten times greater than the amount of bound fraction. Because the assays are generally performed in small volumes (100  $\mu$ L in 96-well plates as described here, but smaller volumes may be involved when using 384-well plates), the experimenter has to be sure that ligands are in excess. One possibility is to remove the medium containing the fluorescent ligand after the equilibrium is reached and to compare the fluorescence remaining in the medium to the fluorescence bound to the cell.
9. Unlabeled ligand is added in excess to determine nonspecific binding. This ligand can be the unlabeled homologue of the tracer, but it can also be a well-characterized ligand for the GPCR of interest. It should be used in such a way that the probability of binding of the unlabeled ligand is at least 100 times greater than for the tracer.
10. In the time-resolved mode, the FRET signal measurement is delayed with respect to the excitation. The parameters have been optimized on the various plate readers. The delay and the

time window for the FRET measurement are usually 50  $\mu$ s and 400  $\mu$ s on PHERAstar device and 150  $\mu$ s and 500  $\mu$ s on Infinite F500 device, respectively. The wavelength of excitation is 337 nm on PHERAstar device and 340 nm on Infinite F500 device.

11. Depending of the fluorescent ligands, great variations in the duration of the incubation can be observed. Equilibrium can be reached within 1 h or by contrast after 8 h. For long incubation, overnight incubation can be performed at 4 °C. Moreover, when ligands are or are presumed to be agonists, incubation can be done at a temperature lower than 16 °C to prevent receptor internalization and recycling.
12. It is noteworthy that closed attention should be given in the 520/620 or 665/620 ratio calculation. If variations of the signal at 620 nm are small (<5 %), calculation of the ratios can be carried out. By contrast larger decrease or increase (>50 %) can be observed in saturation or competition experiments, respectively, when tracer or competitor concentration increases. These variations are probably due to a high FRET efficiency between donor and acceptor. In such conditions, two alternative strategies can then be used to calculate the 520/620 or 665/620 ratio: The first and the most relevant strategy consists in the determination of the signal at 620 nm before adding the fluorescent tracer. The second method consists in considering an average value of the signal at 620 nm determined only from nonspecific binding conditions. With the latter method, potential variations in cell density or receptor expression between wells will not be considered.

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