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Time-resolved FRET based assays to characterize hetero-oligomer pharmacology

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Abstract

Although G protein-coupled receptor (GPCR) oligomerization is a matter of debate, it has been shown that the nature of the GPCR partners within the oligomers can influence the pharmacological properties of the receptors. Therefore, finding specific ligands for homo- or hetero-oligomers opens new perspectives for drug discovery. However, no efficient experimental strategy to screen for such ligands existed yet. Indeed, conventional binding strategies do not discriminate ligand binding on GPCR monomers, homo- or hetero-oligomers. To address this issue, we recently developed a new assay based on a time-resolved FRET method that is easy to implement and that can focus on ligand binding specifically on the hetero-oligomer.

Key words: Tag-lite[®] screening, G protein-coupled receptor, fluorescent ligand, HTRF[®], time-resolved FRET, lanthanide, terbium, self-labeling enzyme, binding experiment, homo- and hetero-oligomerization

1. Introduction

G protein-coupled receptors (GPCRs) constitute the larger family of membrane proteins and they participate to the regulation of all physiological functions. They are the major targets for drug development in therapeutics with about 30% of the drugs currently on the market [1]. However, several reasons make the screening for new drugs targeting GPCRs still interesting. First, only few receptors are yet the target of drugs; second, because full agonists present side effects, there is a need to screen for biased agonists devoid of any side effects. Finally, the concept of GPCR oligomerization, although still a matter of debate, opens new perspectives. Indeed, GPCRS have the propensity to oligomerize forming homo-oligomers if all receptors are similar or hetero-oligomers if the receptors are different. Various publications have shown that the binding and/or coupling properties of a given receptor can be modified upon oligomer formation with another GPCR. Therefore, finding ligands selective for oligomers opens tremendous perspectives in terms of drug development.

However, until recently, no experimental strategy exists to screen for ligands selective for oligomers. The method [2] described below, derived from the Tag-lite[®] assays based on a time-resolved strategy [3–9], is therefore promising to find oligomer-specific ligands. The method is compatible with high-throughput screening, easy to implement and constitutes an attractive strategy for both academic laboratories and big pharmaceutical companies.

1.1. Principle of the method

The principle of the strategy is based on a Förster (or Fluorescence) resonance energy transfer (FRET) [10] between a receptor tagged with a fluorophore and a fluorescent ligand bound to a second receptor (**Figure 1a**). As for all other FRET-based strategies, the non-radiative transfer occurs between a fluorophore donor and an acceptor. Three parameters are crucial to make the non-radiative transfer efficient: (i) the energy compatibility between the fluorophores. The greater the overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor, the better the FRET; (ii) the relative orientation of the donor and the acceptor (*see Note 1*): the FRET is maximal when the dipole transition moments of the donor and the acceptor are parallel; (iii) the distance between the donor and the acceptor. For every pair of fluorophores, a distance called the Förster distance (R_0) can be determined. It corresponds to the distance that gives half maximal FRET efficiency. Because the efficiency of the transfer varies as a function of the inverse of the distance to the 6th power, a distance between the fluorophores superior to 1.5 times R_0 or inferior to 0.5 R_0 results in the absence of FRET or a maximal efficiency, respectively. Classic pairs of fluorophores usually have a R_0 between 35 Å and 80 Å.

FRET techniques do not usually exhibit a high signal-to-noise ratio because of various contaminating signals due to (i) the direct excitation of the acceptor fluorophore at the excitation wavelength of the donor; (ii) the emission of the donor at the emission wavelength of the acceptor; or (iii) the background fluorescence of the biological samples or medium. Therefore various acquisition protocols have been developed to eliminate this contamination from the FRET signal. They consist of measuring the fluorescence of the donor or the acceptor after photobleaching of the acceptor or the donor, respectively. These methods require complex data analysis such as normalization by the expression of the donor or the acceptor or the subtraction of the background fluorescence. All these steps can again in turn generate artifacts.

An alternative strategy consists in implementing time-resolved FRET, a strategy derived from the HTRF® technology, which displays a higher signal-to-noise ratio due to two properties: the temporal selectivity and the spectral compatibility [11]. The temporal selectivity is provided by the use of cryptates of lanthanide, more specifically cryptate of europium and terbium, as donor (**Figure 1b**). The cryptate moiety (**Figure 1c**) is essential [12]: (i) it allows the linkage of the lanthanide on the molecule of interest (ligand or receptor); (ii) it is an antenna increasing the quantum yield of the donor; (iii) it protects lanthanides from quenching, especially quenching by water. Although lanthanides are luminescent but not fluorescent molecules, their resonance energy transfer on a fluorophore is following the same rules. Lanthanides exhibit a long-lasting emission, greater than 1 ms, i.e. more than 100.000 times greater than classic fluorophores. Therefore, the emitted fluorescence of an acceptor due to a FRET with a lanthanide cryptate will be long-living as well. This special feature allows introducing a time delay (usually 50 μ s) between the excitation of the sample and the measurement of the fluorescence in order to discriminate the real fluorescence signal resulting from a FRET from all contaminations with a short-live fluorescence (**Figure 1b**).

The second parameter responsible for the sensitivity of time-resolved FRET-based strategies is the spectral compatibility (**Figure 1d**). The cryptates of terbium and europium are excited by UV at wavelengths between 330 nm and 350 nm. Both of them display very large pseudo Stoke shifts: the emission of the europium cryptate starts around 600 nm while terbium cryptate exhibits a more complex spectrum with four emission peaks around 490 nm, 550 nm, 585 nm and 620 nm. The consequence of this large pseudo Stoke shift is the absence of a direct excitation of the acceptor at the excitation wavelength of the donor. Moreover the narrowness of the emission peaks makes

terbium cryptate compatible with multiple acceptors such as fluorescein-like or dy647-like fluorophores while limiting the contamination of the FRET signal by the emission of the donor.

Although labeling the receptor with an acceptor and the ligand with the donor to characterize the pharmacology of the receptor hetero-oligomer, is possible, we almost exclusively used the reverse labeling, i.e. the labeling of the receptor with the donor and the ligand with the acceptor. This choice is based on at least two reasons: first, most fluorescent ligands have already been derivatized with classic organic fluorescent acceptors compatible with the cryptates of lanthanide to perform TR-FRET experiments but almost never with cryptates of lanthanides; and second, the signal-to-noise ratio when labeling the receptor with the donor and the ligand with the acceptor is higher than for the reverse labeling.

1.2. Labeling of G protein-coupled receptors

Various strategies can be used to label GPCRs. A first set of strategies is based on a non-covalent labeling of the receptor with fluorescent antibodies against either the receptor itself or a tag fused to the receptor N-terminus. Although positive results have been obtained, it is noteworthy that antibodies are large molecules (150 kDa) and induce steric hindrance [13]. One alternative consists in using fluorescently labeled nanobodies which are much smaller. A second drawback is linked to the non-covalent binding itself, which results in an equilibrium between unlabeled and labeled GPCRs. Since an excess of antibodies is usually used to get the largest fraction of labeled GPCRs, washing steps are necessary to eliminate free antibodies which can be the source of a non-specific FRET signal. The kinetics of dissociation of the antibodies/receptor complex should therefore be slow to keep the receptor labeled after the washing steps.

The second set of strategies is based on the covalent labeling of the receptors. This can be reached by the fusion of a self-labeling protein (also called suicide enzyme) to the N-terminus of the receptor. The self-labeling proteins catalyze the transfer of a chemical group from a substrate to themselves. Providing a fluorescent substrate to the self-labeling protein will therefore result in the transfer of the fluorescent group onto the self-labeling protein/receptor chimera. Interestingly, different self-labeling proteins with each their specific substrates have been developed allowing the labeling of multiple receptors at the same time. One of the most commonly self-labeling protein used is the SNAP-tag[®] (20 kDa) that has been derived from O⁶-alkylguanine-DNA alkyl transferase (AGT) [14–17], a DNA-repair enzyme. Optimization of the structure of the wild type enzyme increased its enzymatic activity and reduced its size. Addition of fluorescent benzylguanine (BG) substrates results in the transfer of the fluorescent benzyl group to the protein. More recently, also other self-labeling proteins such as CLIP-Tag[®] (21 kDa) [18] and Halo-Tag[®] (33 kDa) [19] have been developed to label receptors.

These strategies have two main advantages: (i) because of the high enzymatic activity of these proteins, 100% of the receptors can be labeled [20] and the labeling is not sensitive to washing steps and (ii) the steric hindrance is reduced since these proteins are much smaller than antibodies. The main drawback is that the fusion of the self-labeling protein to the receptor can influence the pharmacological properties of the receptor, although it has not yet been reported.

1.3. Ligand labeling

As mentioned above, various acceptors are compatible with a lanthanide cryptate and particularly with the terbium cryptate because of its multiple emission peaks. These acceptors can be organic fluorophores or fluorescent proteins such as Green Fluorescent Protein (GFP) or its derivatives. The

choice is mainly driven by the easiness to synthesize the fluorescent ligands. Organic fluorophores can often be easily linked to small ligands during their synthesis [21, 22, 13, 23, 4, 24, 9]. By contrast, labeling large ligands such as proteins is more difficult and expensive and fusing them to fluorescent proteins can be a good alternative if the fluorescent partner does not induce a large steric hindrance. Moreover, before starting, you have to be sure that your device is compatible with green and red FRET measurements and thus equipped with the appropriate filters.

However, there are no obvious general rules for ligand labeling. It seems that each ligand is unique. We have observed that the linkage of fluorescein dramatically alters the affinity of some ligands for their cognate receptors while linkage of a d2 acceptor does not, but we also observed the contrary suggesting that all physicochemical parameters (size, polarity, hydrophobicity/hydrophilicity...), the length of the spacer if any, the position of the derivatization on the ligands seems to be important. Although analyses of structure-activity relationship of the ligand-receptor complex are very helpful, our experience shows that the impact of ligand labeling on its affinity is not always predictable.

1.4. Overview of the assay implementation

The development of an assay to investigate hetero-oligomer binding properties requires various steps:

1. A full characterization of the pharmacological properties of the tracer, the fluorescent ligand, should be performed on each receptor expressed alone (**Figure 2a,b**) to determine the affinity of the tracer for each receptor (**Figure 2c,d**). The tracer should display a high affinity and selectivity for one of the two receptors. Competition experiments can be carried out to validate the method with the tracer if it exhibits high affinity for the receptor (**Figure 2e**).
2. The implementation of the assays on hetero-oligomers (**Figure 3**): the expression of both receptors should be optimized (**Figure 3a**) in order to get the amount of hetero-oligomers compatible with the sensitivity of the technique (**Figure 3b**). Saturation experiments should be carried out while labeling receptor 1 or receptor 2 (**Figure 3c**). Finally, competition experiments can be implemented with reference compounds for each receptor (**Figure 3d**).

The affinity of the tracer and of the competitors for each receptor can be influenced by the receptor hetero-oligomerization because of cross cooperativity between the two protomers. In this assay, the cooperativity phenomena can be investigated by comparing the binding data obtained on receptors expressed alone or co-expressed.

2. Materials

2.1. Cells

1. HEK293 or CHO cells (see **Note 2**)

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's Medium (DMEM).
4. MEM Non-essential amino acids (Gibco®, Thermo scientific, Waltham, USA)
5. OptiMEM® Reduced Serum Medium (Life Technologies, Gibco, Saint Aubin, France)

6. Fetal calf serum (FCS) (Lonza, Verviers, Belgium).
7. Penicillin/streptomycin.
8. Poly-L-Ornithine (Sigma Aldrich, Saint Quentin Fallavier, France)
9. Black- or white 96-well plate (e.g. Greiner Cell Star 96-well plate, Dominique Dutscher, Brumath, France).
10. Lipofectamine™ 2000 (Invitrogen, Cergy Pontoise, France) or jetPEI DNA Transfection Reagent (Ozyme, Saint Quentin en Yvelines, France)
11. Tag-lite® labeling medium (Ref#LABMED Cisbio, Codolet, France).
12. SNAP-Lumi4®-Tb and HALO-Lumi4®-Tb (Cisbio, Codolet, France).
13. Plasmid coding for tagged receptor (see **Note 3**)
14. Unlabeled ligands are usually purchased from Tocris (R & D Systems Europe Ltd, Lille, France)
15. Automatic Cell Counter Eve (NanoEntek, Pleasanton, USA).
16. Prism-7 (GraphPad Software).

2.3. FRET

1. GPCR ligands derivatized with TR-FRET acceptors (fluorescein, AlexaFluor 488, d2, d1, AlexaFluor 647 or Cy5 can be found in the literature and can therefore be synthesized (see **Note 4**) and prepared ideally at a stock concentration of about 300 µM. (see **Note 5**).
2. Microplate readers compatible with Time-resolved FRET (see <https://www.cisbio.com/drug-discovery/htrf-microplate-readers>) (see **Note 6**).

3. Methods

3.1. Expression of G protein-coupled receptors in HEK293 cells

The receptor expression in cell lines should be optimized for each G protein-coupled receptor when co-expressed or expressed alone.

1. HEK293 cells are grown in culture in an atmosphere of 95% air and 5% CO₂ at 37°C in DMEM medium supplemented with Fetal Calf Serum (10%) and penicillin/streptomycin antibiotics (1%). Split cells before they reach confluence.
2. Transfect cells using manufacturer's Lipofectamine 2000® Transfection protocol. Coat black- or white-96-well 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. Dilute Lipofectamine® 2000 Transfection Reagent (0.5 µL/well) in Opti-MEM (25 µL /well) (5 min at room temperature), and then add to Opti-MEM mixture containing plasmid coding for the GPCR of interest (25 to 200 ng/well in 25 µL Opti-MEM per well). Incubate the mixture 20 minutes at room temperature. Add 50 µL of the DNA:lipofectamine mixture to each well.
5. Harvest HEK293 cells when they are at 80% of confluence, count cells on Automatic Cell Counter Eve, resuspend cells in Opti-MEM® medium at a density of 250 000 or 500 000 cells/mL to follow 24h or 48h after transfection respectively. Plate 100 µL per well on top of the DNA mixture.
6. Binding experiments can be carried out 24h or 48H after transfection depending on the level of expression of the receptors.

3.2. Expression of G protein-coupled receptors in CHO cells

1. CHO cells are grown in an atmosphere of 95% air and 5% CO₂ at 37°C in DMEM medium supplemented with Fetal Calf Serum (10%), penicillin/streptomycin antibiotics (1%) and non-essential amino acids (1%). Split cells before they reach confluence.
2. Transfect cells using manufacturer's jetPEI transfection protocol.
3. The day before the transfection, harvest CHO cells when they are at 80% of confluence, count on Automatic cell counter EVE, resuspend cells in DMEM at a density of 100 000 to 170 000 cells/mL and plate (100 µL/well).
4. The day after, dilute jetPEI Transfection Reagent (0.5 µL/well) in 25 µl NaCl (150 mM)
5. Prepare DNA mix (plasmids coding for the receptors of interest and non-coding DNA) in NaCl (150 mM) (200 ng DNA/25µL NaCl/well) in a separate tube (see **Note 7**). Vortex gently.
6. Add the jetPEI solution to the DNA mix and vortex immediately.
7. Keep the jetPEI/DNA mix at room temperature for 30 min.
8. During that time, remove the 100 µL DMEM medium from the plate with cells and add 50 µL of fresh DMEM medium per well.
9. After 30 min incubation, add 50 µl of the jetPEI/DNA mix per well.
10. Binding experiments can be carried out 24h or 48H after transfection depending on the level of expression of the receptors.

1.2 Labeling of G protein-coupled receptors expressed at the cell surface

As mentioned above, various tags have been developed to fluorescently label receptors. We describe below the methods to label SNAP-tag® and Halo®-tag fused receptors with non-permeable substrates, being routinely used in our laboratory. Whatever the tag used, it is important to check that the substrates do not cross-react with the other tags (see **Note 9**).

1. Incubate cells expressing SNAP-tag® or Halo-Tag® receptors in the presence of their cognate substrates, i.e. SNAP-Lumi4®-Tb or Halo-Lumi4®-Tb.
2. Dilute SNAP-Lumi4®-Tb or Halo-Lumi4®-Tb substrates in Tag-lite® labeling medium to get a final concentration of 100 nM (see **Note 8**).
3. Remove medium from cells.
4. Wash cells once with 100 µl Tag-lite® labeling medium.
5. Dispense 100 µL/well of SNAP-Lumi4®-Tb (100 nM) and/or HALO-Lumi4®-Tb (100nM) solution (see **Note 9**).
6. Incubate cells for 1H to 2H at 37°C.
7. Remove the medium and proceed to 4 washes with Tag-lite® labeling medium.

1.3 Optimization of the condition to detect receptor hetero-oligomerization

It is important to define experimental conditions which optimize receptor hetero-oligomerization. Transfections should be carried out with various ratios of plasmids coding for the two receptors containing a different tag (in this example SNAP for receptor 1 and Halo for receptor 2). For each transfection, we suggested to label homo- and hetero-oligomers (**Figure 3**). For the labeling of homo-oligomers, both substrates should be mixed before and then added to the cells in order to get the

same proportion of receptor labeled with the donor and with the acceptor. This is not crucial for hetero-oligomer labeling since the substrates do not cross react with the other self-labeling protein.

To this end:

1. Prepare mixes of substrates as follows :
 - SNAP-Lumi4[®]-Tb (100 nM) and SNAP-red (300 nM) or SNAP-green (100 nM) (receptor 1 homo-oligomer);
 - Halo-Lumi4[®]-Tb (100 nM) and Halo-red (300 nM) (receptor 2 homo-oligomer);
 - SNAP-Lumi4[®]-Tb (100 nM) and Halo-red (300 nM) (receptor 1/receptor 2 hetero-oligomer);
 - Halo-Lumi4[®]-Tb (100 nM) and SNAP-red (300 nM) or SNAP-green (300 nM) (receptor 1/receptor 2 hetero-oligomer) (see **Note 10**)
2. Remove medium from cells.
3. Wash cells once with 100 μ L with Tag-lite[®] labeling medium.
4. Dispense 100 μ L/well of the different mixes prepared in step 1.
5. Incubate cells for 1H to 2H at 37°C.
6. Remove the medium and proceed to 4 washes with Tag-lite[®] labeling medium.
7. Measure Donor fluorescent signal at 620 nm (fluorescence of the donor) and FRET signal either at 520 nm (for green acceptor) or at 665 nm (for red acceptor) in a time-resolved mode. (see **Note 11** and **Note 12**)

1.4 Saturation Binding experiments

As mentioned above, saturation experiments have to be performed on each receptor expressed alone and on receptors co-expressed. These experiments should allow determining the concentration of the fluorescent ligand that should be used for the competition experiments. One prerequisite to carry out saturation experiments is that ligands should be in excess with respect to the receptor expression (see **Note 13**).

1. After the washing steps from 1.2, add 50 μ l Tag-lite[®] labeling medium per well.
2. Prepare a serial dilution of the fluorescent ligands in Tag-lite[®] labeling medium. At this point, prepare all the ligands at four times the desired final concentrations.
3. Add 25 μ L of fluorescent ligands per well.
4. Add 25 μ L of Tag-lite[®] labeling medium or 25 μ L of unlabeled ligand in excess in each well to determine total binding or non-specific binding, respectively (see **Note 14**).
5. Measure the fluorescent signal of the donor at 620 nm and FRET signal either at 520 nm (for green acceptor) or at 665 nm (for red acceptor) in a time-resolved mode.
6. The dissociation constant can only be determined when equilibrium is reached. Because the assay is performed in homogeneous conditions, meaning the fluorescent ligand is not washed away, the evolution of the TR-FRET signal can be followed over time. When the signal reaches a plateau for all the concentrations of the tracer, one can consider that the equilibrium is reached (see **Note 15**).
7. Analyze data as described in the data analysis section.

1.5 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 13**).

1. Prepare the tracers at concentration 4 times the K_d to use them at a final concentration close to K_d and make dilutions in Tag-lite[®] labeling medium.

2. Perform serial dilutions of the competitors in Tag-lite® labeling medium and prepare ligands at 4 times the desired final concentration.
3. After the washing steps of the labeling procedure, dispense 50 µL of Tag-lite® labeling medium per well.
4. Add 25 µL of tracer prepared in step 1 to all the wells and 25 µL per well of one of the various competitor solutions from the serial dilution prepared in step 2.
5. Two controls should be included in the experiment: the “total binding” and “non-specific binding” conditions. Substitute the 25 µL of competitor solution with 25 µL of Tag-lite® labeling medium for the total binding and with 25 µL of unlabeled ligand at high concentration for the non-specific binding (see **Note 14**).
6. Measure the fluorescent signal of the donor at 620 nm and the FRET signal either at 520 nm (for green acceptor) or at 665 nm (for red acceptor) in a time-resolved mode.
7. For the first experiments, read fluorescent signals at different times to define the incubation time required to reach equilibrium (Figure 2b). If long incubation is required to reach equilibrium, perform overnight incubation at 4°C.

1.6 Analysis of saturation and competition curves

1. Two methods are generally considered to analyze the FRET signals: (i) the first method takes only in account the raw FRET signal measured at the emission wavelength of the acceptor, 520 nm or at 665 nm for green or red acceptor, respectively. This method is the simplest one but is relevant only if the expression of the receptor is kept constant; (ii) the second method normalizes the FRET signal by the receptor expression. For experiments performed on a single receptor, receptor expression is generally estimated by the intensity of the signal at 620 nm although this peak is not the most intense. Therefore, depending on the acceptor, the ratios 520/620 or 665/620 can be used (see <https://www.cisbio.com/drug-discovery/htrf-ratio-and-data-reduction>). This normalization can be performed well by well. In case of the hetero-oligomer, normalization is more complicated since the donor complex density is dependent on the expression of both receptors but only one receptor can be labeled with the donor at a time. Therefore, the double normalization cannot be performed well by well. The expression of the second receptor can only be estimated by a mean value after the labeling of the second receptor in separate wells. This second normalization is required if one wants to compare experiments performed in various receptor densities (see **Note 16**).
2. Saturation curves
When equilibrium is reached, i.e. the TR-FRET signal is stable at all the ligand concentrations tested, the TR-FRET signal or the 520/620 or 665/620 ratio can be plotted as a function of the tracer concentration to get a saturation curve. The specific TR-FRET signal is obtained after subtracting the non-specific signal from the total TR-FRET signal. The specific TR-FRET signal should reach a plateau proving that equilibrium has been reached. It is noteworthy that the non-specific signal is no longer linear at the highest concentrations of the tracer because of a dynamic FRET between free labeled ligand and the labeled receptor.

The following equation is used to fit the data corresponding to the saturation curve:

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

In which F is the TR-FRET signal, F_{\max} the maximal TR-FRET signal (at the plateau of the saturation curve), $[tracer]$ 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 or more binding sites or the Hill equation have to be considered to get good fits for the experimental data.

3. Competition curves

When equilibrium is reached, the TR-FRET signal can be plotted as a function of the concentration of the competitor. It is worthy to note that the time needed to reach equilibrium is not necessarily the same as for the saturation experiments because it depends on the binding kinetics of the tracer and the competitors of the different receptors. The curve can be fitted with the following equation:

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

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

$$K_i = IC_{50} / (1 + [Tracer] / K_d)$$

In which K_i is the inhibition constant of the competitor, IC_{50} the competitor concentration leading to a half-maximum TR-FRET signal and K_d the dissociation constant of the tracer.

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

2. Notes

1. The relative orientation of the donor and acceptor is a crucial parameter for classic FRET- or BRET-based experiments but it is less important in Time-resolved FRET based because of the use of a cryptate of lanthanides [11].
2. Experiments can in theory be performed in any cell line that can be efficiently transfected. We chose HEK293 and CHO cells since these models are often used in literature. CHO cells adhere very well and hence no poly-D-ornithine is needed, while HEK293 cells are easily washed away, so coating of the 96-well plate is crucial.
3. 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 an enzyme (ACP-tag[®]). For all self-labeling proteins, specific substrates have been developed. The plasmids can be homemade plasmids or purchased from different manufacturers. A large collection of these plasmids is now commercially available from Cisbio (Cisbio, Codolet, France) ([see https://www.cisbio.com/drug-discovery/receptor-binding-assays](https://www.cisbio.com/drug-discovery/receptor-binding-assays)). Tags are generally fused to the N-terminus of the receptor and the fusion has been shown not to impact receptor

functioning. However this needs to be verified for all receptors. Insertion of a SNAP-tag® or a CLIP-tag® inside extracellular loops is not recommended since it generally affects receptor conformation and modifies receptor binding and functioning properties. By contrast, ACP-tag® or Halo-tag® insertions in extracellular loops are generally more tolerated.

4. A large collection of these ligands are now commercially available from Cisbio since they are used in Tag-lite® binding assays (see <https://www.cisbio.com/drug-discovery/receptor-binding-assays>).
5. Ligands are dissolved in 10% DMSO in case of a peptide or protein ligand or in 100% DMSO for an 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, ϵ is the molar extinction coefficient, l the width of the cuvette and C the concentration of the solution.
Regarding red acceptor-derivatized ligands, the following values for the molar extinction coefficient (ϵ) (L/mol/cm) at 649 nm were used: d1: 250 000; d2: 225 000; Bodipy: 80 000. The ratio of the absorption measured at 649 nm and 604 nm was systematically defined and 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, pH 9 and the value of the molar extinction coefficient is 75 000 (68 000 at pH 7.4) at 495 nm.
6. We read the TR-FRET signal on a Pherastar reader (BMG Labtechnologies) and on a Tecan Infinite F500 microplate reader (Tecan) but numerous readers are compatible with TR-FRET based strategies.
7. This step can need optimization. First, the final concentration of DNA is dependent on the method used for the transfection. Second, the plasmid including the coding sequences for the receptors of interest should generally be mixed with non-coding plasmid. The ratio of each plasmid should be optimized to get receptor expressions compatible with binding. A too low expression will prevent a reliable measure of the FRET signal; a too high expression will induce ligand depletion.
8. The concentration of substrates is dependent on the self-labeling protein and the substrate itself. 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). The concentrations are indicated in the Table below.

Tag	Name of the substrate	Final concentration (nM)
SNAP-tag®	SNAP-Lumi4®-Tb	100
	SNAP-green	300
	SNAP-red	300
CLIP-tag®	CLIP-Lumi4®-Tb	400
	CLIP-green	300
	CLIP-red	100
HALOTag®	HALO-Lumi4®-Tb	100
	HALO-red	300

9. To perform simultaneous labeling of receptors, ensure that the substrates are specific for one self-labeling protein and cannot cross react with a second one. It is noteworthy that CLIP-substrates can react on the SNAP-tag. Therefore, it is important to add an excess of unlabeled SNAP-substrate to label a CLIP-tag in the presence of a SNAP-tagged protein.
10. For the labeling of homo-oligomers, a mix of both substrates should be prepared in advance and then added to the cells in order to get the same proportion of receptor labeled with the donor and with the acceptor. This is not crucial for hetero-oligomer labeling since the substrates do not cross react with the other self-labeling protein.
11. 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 μ s and 400 μ s on the Pherastar device and 150 μ s and 500 μ s on Infinite 500 device. The wavelength of excitation is 337 nm on the Pherastar device and 340 nm on the Infinite 500 device.
12. Amplitude of the signals obtained with the different mixes should not be compared since the FRET signal will depend on the number of oligomers but also on the distance between the donor and the acceptor and, to a lesser extent, on their relative orientation. The distance and orientation can vary from one complex to another. By contrast, for a given complex, the amplitude of the TR-FRET ratio resulting from different transfections can be compared.
13. In general, the amount of free ligand should be at least 10 times greater than the amount of the bound fraction. Because the assays are generally performed in small volumes (100 μ 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 method to verify this 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.
14. Unlabeled ligand is added in excess to determine the non-specific 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. This ligand needs to bind on the same site as the tracer. 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. Importantly, the non-specific signal depends on the non-specific binding of the ligand but also on the dynamic FRET resulting from the random collisions of the free fluorescent ligand molecule and the tagged receptor. The dynamic FRET is often negligible when fluorescent ligands are used at concentration inferior to 10 nM but can be very important for concentrations superior to 100 nM. This dynamic FRET does not increase linearly in function of the tracer concentration and could not easily be extrapolated. Therefore, it is important to determine the non-specific signal for each tracer concentration.
15. Depending on the fluorescent ligands, great variations in the incubation time can be observed. Equilibrium can be reached within one hour, but sometimes only after 8 hours. For long incubation times, 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.
16. Close attention should be given when calculating the 520/620 or 665/620 ratios. 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 ratios: 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 non-specific binding conditions. With the latter method, potential variations in cell density or receptor expression between wells will not be considered.

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Legends to figure

Figure 1: Principle of the time-resolved FRET technique with HTRF[®] technology. **a**, Principle of the assay to characterize hetero-oligomer pharmacology. **b**, Temporal selectivity in HTRF[®] technology: TR-FRET signal is measured 50 μ s after the excitation, in a time window of 400 to 500 μ s. During the delay, all short-lived fluorescence is decreased to zero and only long-lived time fluorescence FRET and free donor fluorescence can be measured. Because the fluorescence emission of the donor is weak at 665 nm (emission wavelength of the acceptor), the contamination caused by the free donor fluorescence is often negligible. **c**, Spectral compatibility with HTRF[®] donor and acceptors: 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. **d**, Structure of the Lumi4[®]-Tb. SLP = self-labeling protein.

Figure 2: Time-resolved FRET based binding assay with HTRF[®] technology (**a** and **b**). Saturation (**c** and **d**) and competition (**e**) experiments can be performed. Two steps are required to develop a time-resolved FRET based assay. The tracer should exhibit a high affinity and a high selectivity for one of the receptors.

Figure 3: Time-resolved FRET based binding assay when two receptors are co-expressed with HTRF[®] technology. Estimation of the expression of each receptor (**a**) and of the formation of both homo- or hetero-oligomers (**b**), saturation (**c**) and competition (**d**) curve.

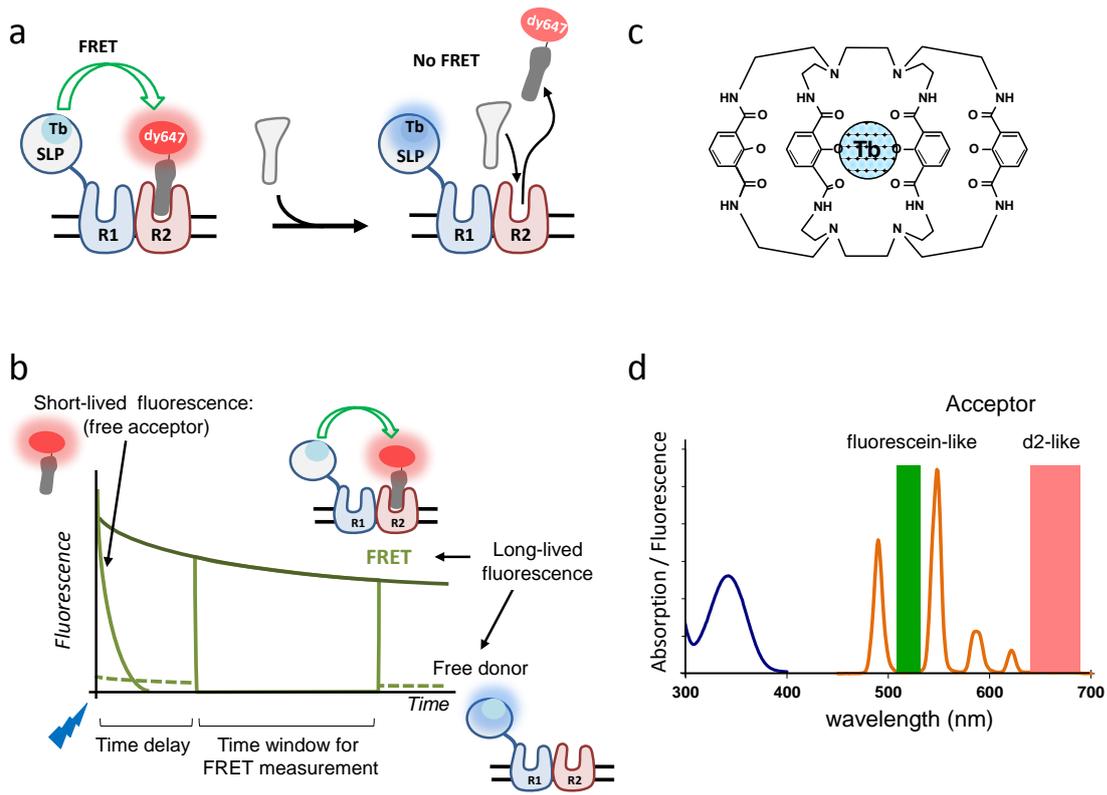


Figure 1

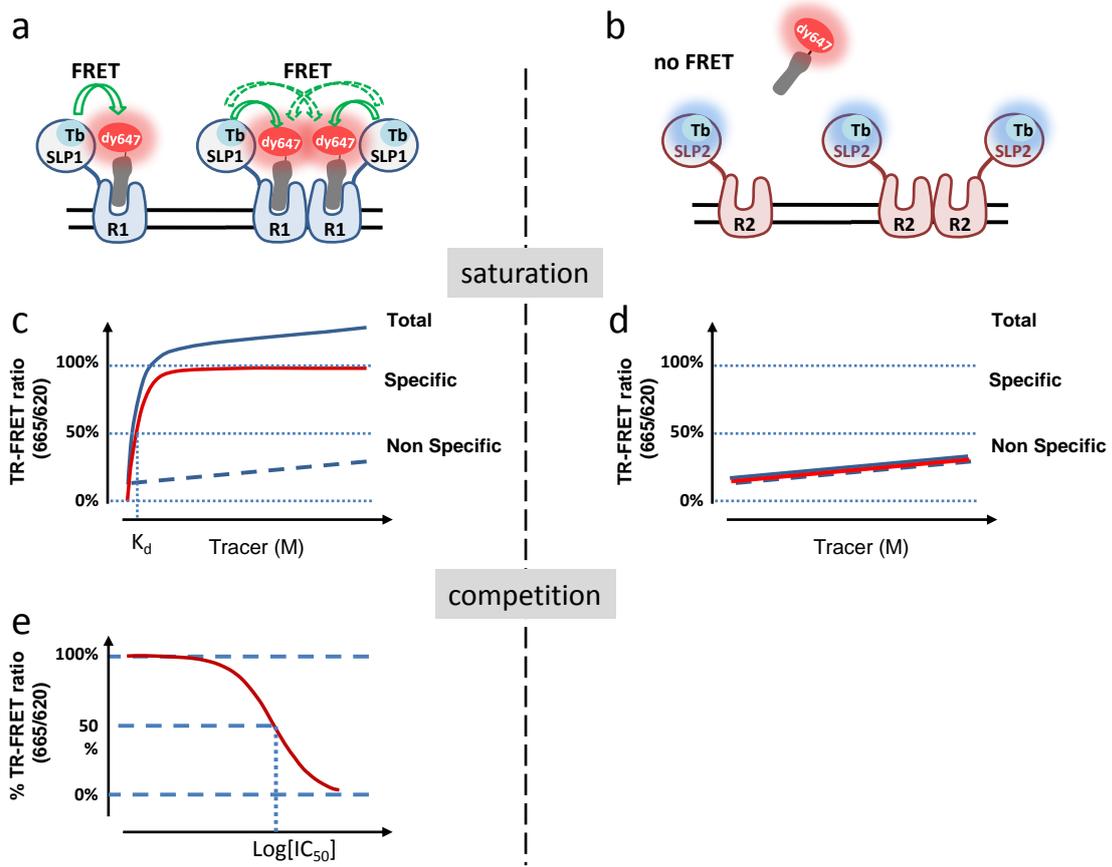


Figure 2

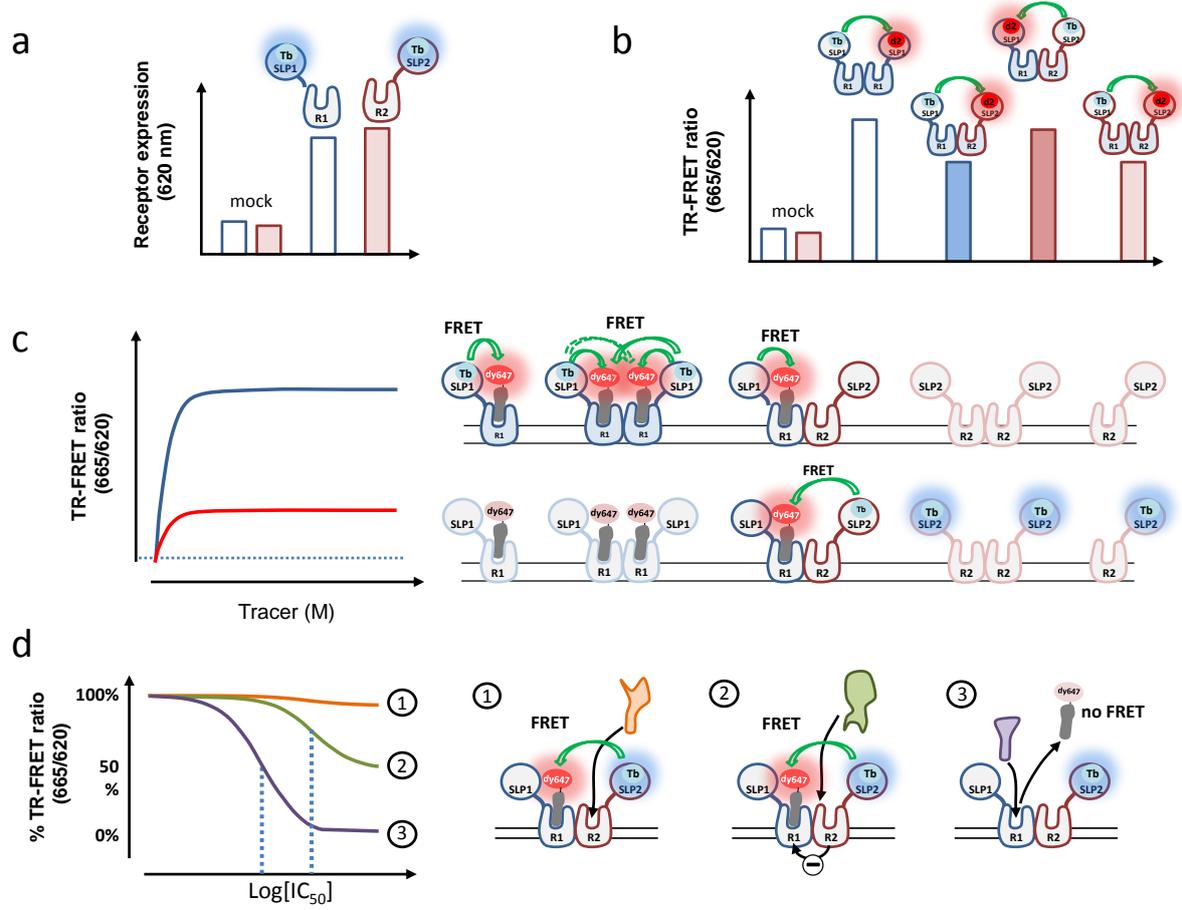


Figure 3