Approaches to characterize and quantify oligomerization of GPCRs

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Summary

Fluorescence Resonance Energy Transfer (FRET) is an approach widely used to detect protein-protein interactions in live cells. This approach is based on the sensitization of an "acceptor" molecule by the energy transfer from a "donor" when there is an overlap between the emission spectrum of the "donor" and the excitation spectrum of the "acceptor" and close proximity between the two fluorophore species (in the region of 8 nm). Various methods exist to quantify FRET signals: here we describe the application of homogeneous time-resolved FRET (htrFRET) combined with Tag-lite^{TM} technology and its application to determine not only protein-protein interactions but also the capability of GPCR mutant variants to form homomers compared to the wild type GPCR within the plasma membrane of transfected cells.

1. Introduction

G protein-coupled receptors (GPCRs) are a vast class of trans-plasma membrane proteins that play pivotal roles in cell signalling and in the regulation of a broad range of biological processes. Dysregulation of many of these result in serious pathological conditions and, as such, GPCRs represent a major target class for therapeutic drug development ***(1)***.

GPCRs have been classically described as monomeric units that bind in a stoichiometric 1:1 ratio to both ligand and downstream signalling proteins such as members of the family of heterotrimeric guanine nucleotide binding proteins (G-proteins). Indeed, studies in which monomeric GPCRs were incorporated into nanodiscs demonstrated that each of the $^{2}{}_{2}$-adrenoceptor, rhodopsin, and the μ-opioid receptor can function effectively as monomers ***(2, 3, 4, 5)***. However, in recent years a growing number of studies have supported the hypothesis

that GPCRs may also exist as dimeric or oligomeric complexes ***(6, 7)***. In the case of various members of the class C, metabotropic glutamate receptor family it is clear that formation of either homo- or hetero-complexes define the pharmacology and function of the receptor ***(7)***. However, in the case of the more numerous class A, rhodopsin-like receptors, the situation is more uncertain and both the basic concept of GPCR oligomerization and its functional relevance remain controversial topics ***(6)***.

The first structural evidence supporting organization of a class A GPCR into dimers/oligomers was provided by the application of atomic force microscopy to murine rod outer segment discs that indicated that rhodopsin is arranged as a series of parallel arrays of dimers ***(8)***. Since 2007 innovative crystallographic techniques have resulted in rapid growth in the number of available atomic level GPCR structures ***(9)***. Such structures are consistent with, for example, the μ-opioid and the $^{2}{}_{1}$-adrenoceptor, being able to organize as parallel dimers and/or tetramers, and suggest the existence of multiple dimer interfaces in different GPCR homodimers. Transmembrane domain (TMD) V and TMD VI residues provide the main interface for μ-opioid receptor dimers while TMD I, TMD II and helix 8 residues generate a second, more limited, interface ***(10)***. A TMD I–TMD II–helix 8 interface (with slightly different contact residues) was also found in crystals of the turkey $^{2}{}_{1}$-adrenoceptor ***(11)*** where it constitutes the most prominent interaction interface. An additional interface involving TMD IV and TMD V residues was also observed for this receptor ***(11)***.

Both biochemical and biophysical approaches have been employed to study the basis of GPCRs dimerization/oligomerization ***(12)***. However, traditional biochemical techniques such as immunoblotting, cross-linking and co-immunoprecipitation, have clear limitations in efforts to study interactions involving integral membrane proteins owing to the need to use non-physiological buffers and detergents that may cause aggreration or disruption of native biological interactions.

In the past decade, several biophysical methods based on Resonance Energy Transfer (RET) between two molecules, known as the “donor” and “acceptor”, have been developed to examine the quaternary organization of integral membrane proteins, including GPCRs, in intact cells. These include both Bioluminesence Resonance Energy Transfer (BRET) and Fluorescence Resonance Energy Transfer (FRET). Moreover, when combined with protein complementation assays that allow the reconstitution of a fluorophore from two non-fluorescent polypeptides that are brought into proximity by interactions between the proteins they are attached to, larger scale complexes can, at least potentially, be detected and imaged. Although these techniques overcome a number of the issues with the biochemical approaches noted above, they both generally require genetic manipulation of the proteins, thus limiting *in vivo* analysis without a committed effort to generate transgenic organisms and report proximity between proteins rather than association *per se*.

In this chapter we describe the application of homogenous time-resolved FRET (htrFRET) using SNAP-Tag-lite™ technology combined with alanine scanning mutagenesis to characterize and quantify oligomerization of GPCRs and mutant variants of interest at the surface of suitably transfected and engineered cells. The procedure involves the generation of a construct in which the GPCR of interest is linked to the SNAP-tag ***(13, 14, 15)***, in such a way that the SNAP-tag is located on the extracellular face of the plasma membrane when the GPCR is correctly expressed and displayed. Subsequently incubation of the cells with a combination of an appropriate energy “donor” and ”acceptor” pair allows detection of energy transfer between the two fluorophores if the protein forms a homo-oligomeric complex (**Figure 1**).

The SNAP-tag is a modified version of the DNA repair protein O_6-alkylguanine-DNA alkyltransferase (AGT) that displays faster reaction kinetics with O_6-benzylguanine (BG) substrates and no longer interacts with DNA. BG substrates can be conjugated with

fluorophores including Europium cryptate (or a Terbium cryptate), which can act as long-lived fluorescence energy donors, as well as molecules such as the Cy5-like dye (d2), which can act a suitable energy "acceptor" in a FRET assay ***(13)***. Due to the particular properties of such rare-earth cryptates, which generate fluorescence emission with lifetime in the millisecond range, compared to the nanosecond range for standard fluorophores, it is possible to record the FRET signal between donor to acceptor in a 'time resolved' manner (htrFRET). Suitable time gating of the signal allows autofluorescence to rapidly decay, producing a decrease in background signal. Moreover, a further consequence of the properties of the selected fluorophores is that they produce a large increase in the signal to noise ratio when compared to classical FRET methods ***(13)***.

2. Materials

2.1. Molecular biology

1. Mammalian expression plasmid containing the cDNA sequence coding for the SNAP-tag modified-GPCR of interest (such as PSEM1-26 m plasmid supplied by Covalys Biosciences AG/New England Biolabs).

2. Mammalian expression plasmid not containing the cDNA sequence coding for the GPCR of interest to use as a negative control in htrFRET assay.

3. Enzymes suitable for cloning (such as restriction enzymes and DNA ligase)

4. Primers .

5. Pfu DNA polymerase .6. dNTP mix, 10 mM each .7. DpnI.

8. XL1-Blue competent cells.

9. Luria-Bertani (LB) Broth dissolved in distilled water according to the manufacture's protocol. Autoclave at 121°C, 15 min and store at room temperature.

10. LB-agar plates: dissolve LB-agar in distilled water according to the manufacture's protocol. Autoclave at 121°C, 15 min, allow to cool to 55°C and add the appropriate amount of the desired antibiotic for selection. Pour LB-agar into Petri dishes and allow to set. Invert the plates and store at 4°C.

11. Miniprep kit.

2.2. Cell Culture

1. Suitable cells for transfection (e.g. Human Embryonic Kidney 293 cells, HEK293T cells).

2. Suitable growth medium, such as Dulbecco´s Modified Eagle´s medium containing 0.292 g/L L-glutamine, 1% penicillin/streptomycin mixture and 10% heat-inactivated fetal bovine serum.

3. Cell culture dishes 60 mm x 15 mm style.

4. Transfection reagent such as polyethylenimine (PEI).

5. Sodium chloride: 150mM NaCl. Autoclave at 121°C, 15 min and store at room temperature.

6. Poly-D-lysine: 0.1mM poly-D-lysine dissolved in sterile tissue culture grade water. Store at 4°C.

7. Trypsin.

2.3. HtrFRET assay

1. Dimethyl sulfoxide (DMSO).

2. Phosphate buffered saline (PBS, 10X): 1370mM NaCl, 27mM KCl, 100mM Na_2HPO_4, 20mM KH_2PO_4 dissolved in distilled water. Store at room temperature. To obtain the working solution, PBS 1X, dilute PBS 10X in distilled water in a ratio 1:9 and assess the pH of the solution that must be 7.4.

3. Black 96 well plate (Greiner Bio-One).

4. SNAP-Lumi4Tb (Cisbio).

5. SNAP-Red (Cisbio).

6. Labeling medium 5X (Cisbio).

2.4 Equipment

1. Standard cell culture facility including biological safety cabinet and 37°C incubator with 5% CO_2.

2. Thermal cycler.

3. Microplate reader (such as Pherastar BMG Labtech).

4. GraphPad Prism 5.2 software for data analysis.

3. Methods

3.1. Preparation of expression plasmids

1. Modify PSEM1-26 m plasmid, containing the SNAP-tag coding sequence by the addition of a small linker region encoding the metabotropic glutamate receptor 5 signal sequence (MVLLLILSVLLLKEDVRGSAQS) between the *Cla1* and *EcoR1* sites of the multiple cloning site and upstream of the SNAP-tag (MCS1). PCR amplify the cDNA coding the

GPCR of interest using primers designed to add *BamH1* and *Not1* consensus sequences to the fragment termini and then ligate into the multiple cloning site downstream of the SNAP-tag (MCS2) of the modified plasmid.

3.2. Generation of GPCR mutant variants by alanine substitution mutagenesis

1. Design mutagenic primers: both the forward and the reverse primers must contain the desired mutation (preferably in the centre of the sequence) and anneal to the same sequence on opposite strands of the plasmid. Primers should have a minimum GC content of 40%, be between 25 and 45 bases in length, with a melting temperature (Tm) of e 78°C and terminate in one or more C's or G's.

2. Generate the SNAP-tag-GPCR mutant variants using a modification of the QuikChange method (Stratagene, Agilent Technologies, Santa Clara, CA). To amplify the SNAP-tag-GPCR construct and introduce the desired mutation combine in a sterile nuclease-free microcentrifuge tube the PCR reaction components in a total volume of 50 µL (**Table 1**). Prepare also a PCR control reaction without including the Pfu DNA polymerase.

3. Place the PCR reactions in a thermal cycler and after the initial denaturation step at 95°C for 1 min start the 18-20 cycles program consisting of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and DNA synthesis at 72°C for 2 min for every 1 kb DNA to be amplified. Final DNA extension at 72°C for 10 min and after that the tube is maintained at 4°C (*see* **Note 1**).

4. Add 1 µL (equivalent to 10 U) of DpnI to the PCR reaction tube to digest the methylated non-mutated DNA and incubate at 37°C for at least 60 min.

5. Add 1-10 µL of the digest reaction to 50 µL of XL1-Blue competent cells, incubate 15 min on ice, heat-shock at 42°C for 90 sec and then incubate 2 min on ice. Add 450 µL of LB-

Broth and incubate with shaking at 37°C for 60 min. Spread the cell suspension on LB-agar plates containing the correct antibiotic for selection and incubate overnight at 37°C.

6. Select 3 colonies randomly and grow them overnight in 5 mL LB-Broth. The next day isolate the plasmids using miniprep kit (follow manufacturer`s protocol) (*see* **Note 2**).

7. Check the presence of mutation in the isolated plasmids by sequencing.

3.3. Transient expression in mammalian cells

1. Plate mammalian cells onto 60 mm cell culture dishes and grow to 70-80% confluency overnight at 37 °C with 5% CO_2.

2. Transfect the cells with the plasmid of interest following the PEI protocol ***(16)***. Combine the DNA with PEI (ratio 1:6) in 250 µL of 150 mM NaCl solution. Mix thoroughly the DNA-PEI mixture and incubate for 10 min at room temperature. Change medium to the cell plates and add the DNA-PEI mixture to the cell medium in a drop-wise manner. The following transfections are needed:

a. Empty plasmid not containing the GPCR of interest (*see* **Note 3**).

b. Expression plasmid containing the GPCR of interest (wild type or mutant variant of the GPCR).

3.4. Optimization of htrFRET conditions and determination of the cell surface GPCR oligomeric interaction

1. Trasfect HEK293T cells with 2 µg of plasmid containing the SNAP-GPCR construct of interest (for details see step 2 in section 3.3) (*see* **Note 3**). This step is performed in day 1.

2. Plate 30000 cells per well (100 µL per well) onto black 96 well plates previously pre-treated with poly-D-lysine and incubate overnight at 37 °C with 5% CO_2 (*see* **Note 4**). This step is performed in day 2.

3. Prepare labeling medium 1X solution diluting the labeling medium 5X with distilled water in a ratio1:4 and keep the solution at room temperature. This step is performed in day 3

4. Reconstitute 5nmol SNAP-Lumi4Tb adding 50 µL of DMSO to get a 100 µM SNAP-Lumi4Tb stock solution, make 5 µL aliquots and store at -80°C. To obtain the 10 µM SNAP-Lumi4Tb working solution dilute SNAP-Lumi4Tb stock solution with DMSO in 1:9 ratio (*see* **Note 5**). This step is performed in day 3.

5. Reconstitute 20nmol SNAP-Red adding 200 µL of DMSO to get a 100 µM SNAP-Red working solution, make 5 µL aliquots and store at -80°C (*see* **Note 5**). This step is performed in day 3.

6. Prepare different solutions with a fixed concentration (10nM) of the energy donor SNAP-Lumi4Tb and different concentrations of the energy acceptor SNAP-Red (0-1000 nM) in labeling medium 1X. (*see* **Note 5**). This step is performed in day 3.

7. Aspirate the cell growth medium and replace it, adding to each well 50 µL of one of each solution made in the step 4 (*see* **Note 5**). This step is performed in day 3.

8. Incubate the plates for 1 h at 37°C in a humidified atmosphere (5% CO_2), (*see* **Note 4**). This step is performed in day 3.

9. Aspirate the SNAP-Lumi4Tb and SNAP-Red containing medium and wash the plate 4 times with 100 µL PBS 1X. This step is performed in day 3.

10. Add 100 µL of labeling medium 1X. This step is performed in day 3.

11. Measure the fluorescence emission using a microplate reader (Pherastar). Excite the donor SNAP-Lumi4Tb with light of wavelength 337 nm and detect simultaneously the emission light of donor SNAP-Lumi4Tb at 620 nm and the emission light of acceptor SNAP-Red at 665 nm. This step is performed in day 3.

12. Analyse the data using GraphPad Prism 5.2 (or similar). Plot the htrFRET signal at 665 nm on the Y axis and the logarithm of molar concentration of SNAP-Red on the X axis and apply the Gaussian equation of the nonlinear regression curve fit analysis (**Figure 2**) **3.5. Studying the effect of mutation of the GPCR on oligomeric organization**

1. Transfect HEK293T cells with increasing amounts of plasmid containing SNAP-GPCR wild type or SNAP-GPCR mutant variant (of interest) sequence (for details see step 2 in section 3.3) (*see* **Note 3**). This step is performed in day 1.

2. Plate 30000 cells/well (100 µL per well) from each transfection plate onto black 96 well plates and incubate overnight at 37°C with 5% CO_2 (*see* **Note 4**). This step is performed in day 2.

3. Prepare a solution in labeling medium 1X containing a fixed concentration (10 nM) of energy donor SNAP-Lumi4Tb and a concentration of energy acceptor SNAP-Red as determined in section 3.4. (*see* **Note 5**). This step is performed in day 3.

4. Incubate for 1 h at 37°C in a humidified atmosphere (5% CO_2) (*see* **Note 5**). This step is performed in day 3.

5. Aspirate the SNAP-Lumi4Tb and SNAP-Red mixture and wash the plate 4 times with 100 µL PBS 1X. This step is performed in day 3.

6. Add 100 µL of labeling medium 1X. This step is performed in day 3.

7. Measure the fluorescence emission using a microplate reader (Pherastar) as described in step 11 section 3.4. This step is performed in day 3.

8. Analyse the data using GraphPad Prism 5.2 (or similar) software, plot the fluorescence emission at 620 nm on the X axis and the htrFRET signal at 665 nm on the Y axis and apply linear regression curve fit analysis to each dataset (**Figure 3**). **4. Notes**

1. The PCR conditions for the synthesis of the mutant constructs are based on the annealing temperature of the primers and may require some modifications of the conditions described here. If the reaction fails, sometimes lowering the primer concentration, increasing the template concentration, or decreasing the annealing temperature can help.

2. If after transformation of the competent cells with the PCR reaction no (or very few) colonies are observed then increase the volume of the PCR reaction used in the transformation protocol.

3. Always perform the htrFRET assay by including cells transfected with an empty vector to establish the background signal.

4. Perform the htrFRET assays in triplicate.

5. In the htrFRET assay always protect the energy donor and the energy acceptor fluorophores from external light.

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References

1. Schöneberg T., Schulz A., Biebermann, H. et al. (2004) Mutant G-protein-coupled receptors as a cause of human diseases. *Pharmacol Ther*. **104**, 173-206
2. Bayburt T.H., Leitz A.J., Xie G. et al. (2007) Transducin activation by nanoscale lipid bilayers containing one and two rhodopsins. *J Biol Chem* **282**, 14875-14881
3. Whorton M.R., Bokoch M.P., Rasmussen S.G. et al. (2007) A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A* **104**, 7682-7687
4. Whorton M.R., Jastrzebska B., Park P.S. et al. (2008) Efficient coupling of transducin to monomeric rhodopsin in a phospholipid bilayer. *J Biol Chem* **283**, 4387-4394
5. Kuszak A.J., Pitchiaya S., Anand, J.P. et al. (2009) Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2. *J Biol Chem* **284**, 26732-26741
6. Milligan G. (2013) The prevalence, maintenance, and relevance of G protein-coupled receptor oligomerization. *Mol Pharmacol* **84**, 158-169
7. Ferré S., Casadó V., Devi L.A. et al. (2014) G protein-coupled recpetor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol Rev* **66**, 413-434
8. Fotiadis D., Liang Y., Filipek S. et al. (2003) Atomic-force microscopy: Rhodopsin dimers in native disc membranes. *Nature* **421**, 127-128
9. Venkatakrishnan A.J., Deupi X., Lebon,G. et al. (2013) Molecular signatures of G-protein-coupled receptors. *Nature* **494**, 185-194
10. Manglik A., Kruse A.C., Kobilka T.S. et al. (2012) Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature* **485**, 321-326
11. Huang J., Chen S., Zhang J.J. et al. (2013) Crystal structure of oligomeric ^{2} 1-adrenergic G protein-coupled receptors in ligand-free basal state. *Nat Struct Mol Biol* **20**, 419-425

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12. Kaczor A.A., Selent J. (2011) Oligomerization of G protein-coupled receptors: biochemical and biophysical methods. *Curr Med Chem* **18**, 4606-4634
13. Maurel D., Comps-Agrar L., Brock C. et al. (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. *Nat Methods* **5**, 561-567
14. Alvarez-Curto E., Ward R.J., Pediani J.D. et al. (2010) Ligand regulation of the quaternary organization of cell surface M3 muscarinic acetylcholine receptors analyzed by fluorescence resonance energy transfer (FRET) imaging and homogeneous time-resolved FRET. *J Biol Chem* **285**, 23318-23330
15. Ward R.J., Pediani J.D., Milligan G. (2011) Heteromultimerization of cannabinoid CB_1 receptor and orexin OX_1 receptor generates a unique complex in which both protomers are regulated by orexin A. *J Biol Chem* **286**, 37414–37428
16. van Rijn R.M., van Marle,A., Chazot P.L. et al. (2008) Cloning and characterisation of dominant negative splice variants of the human histamine H4 receptor. *Biochem J* **414**, 121–131
17. Ballesteros J.A. and Weinstein H. (1995) Integrated methods for modelling G-protein coupled receptors. *Methods Neurosci* 366-428

Figure legends

Figure 1. Schematic representation of the procedure described in this book chapter. The procedure involves (1) the generation of the plasmid constructs containing the sequence coding for the SNAP-GPCR of interest or the receptor mutant variant generated by site direct mutagenesis, (2) the expression of those constructs in a suitable mammalian cell system and (3) the seeding of 30000 cells per well of a 96 well plate. Following an htrFRET assay that

requires (4) incubation of the cells with a combination of energy “donor” and “acceptor”, and (5) detection of the signal at 620 nm and 665 nm after excitation at 337 nm (6) the data are analysed

Figure 2. Cell surface oligomeric interactions of VSV-SNAP-hM_3R were defined by htrFRET. Optimal concentrations of the SNAP-tag htrFRET energy donor and acceptor were established by incubating cells with a fixed concentration of energy donor (SNAP-Lumi4Tb, 10 nM) and various energy acceptor (SNAP-Red) concentrations. htrFRET measured at 665 nm after excitation at 337 nm reflects proximity between labelled copies of VSV-SNAP-hM_3R at the cell surface. At low energy acceptor concentrations the htrFRET signal is low and this increases with increasing SNAP-Red concentration, reaching a maximum that represent the optimum ratio of energy donor and acceptor (10 and 100 nM, respectively in this example shown) to detect cell surface oligomers. Further increases of SNAP-Red concentration results in a decrease in htrFRET signal due to the binding competition of SNAP-Lumi4Tb and SNAP-Red for the same tag on the GPCR construct.

Figure 3. HEK293T cells were transfected with increasing amounts (0-2.5 μg of plasmid) of either VSV-SNAP-hM_3R (**circles**) or of a mutant receptor variant Leu80Ala, Ile83Ala, Ile84Ala, Ile87Ala VSV-SNAP-hM_3R (**triangles**) containing four point mutations in which each of residues Leu80, Ile83, Ile84, and Ile87 in TMD I were substituted by alanines, for 24 h and then incubated with the optimal combination and ratio of SNAP-Lumi4Tb (10 nM) and SNAP-Red (100 nM) as estimated insection 3.4. The htrFRET signal determined as fluorescent emission at 665 nm was then plotted as a function of cell surface SNAP-tagged

receptor expression measured by fluorescent emission at 620 nm. A linear dependence between the fluorescence emission at 620 nm and the htrFRET signal at 665 nm is observed in both forms of the receptor. The lower extension of the line for Leu80Ala, Ile83Ala, Ile84Ala, Ile87Ala VSV-SNAP-hM_3R demonstrates that this variant was less effectively delivered to the cell surface than the wild type receptor whilst the reduction in the slope of the line 665nm/620 nm for this mutant indicates an alteration in oligomeric organization consistent with the energy acceptor and donor species being further apart in this variant. The illustration above the data section of the Figure records the amino acid sequence of TMD I of hM_3R. Amino acids in **bold** are those mutated in Leu80Ala, Ile83Ala, Ile84Ala, Ile87Ala VSV-SNAP-hM_3R. The most conserved residue (N) in TMD I across the entire family of class A GPCRs is denoted as residue 1:50 using the Ballesteros and Weinstein equivalent residue positioning system (***17***).

Tables

Table 1. PCR reaction components

Pfu DNA polymerase 10x buffer with Mg_2SO_4	5 µL
dNTP mix, 10 mM each	1 µL
Forward primer	5-50 pmol
Reverse primer	5-50 pmol
DNA template	<0.5 µg
Pfu DNA polymerase (2-3 U/µl)	1.25 U
Nuclease-free water to a final volume of	50 µL