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Review

Structural and biophysical characterisation of G protein-coupled receptor ligand binding using resonance energy transfer and fluorescent labelling techniques☆

Richard J. Ward, Graeme Milligan *

Molecular Pharmacology Group, Institute of Molecular Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK

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ABSTRACT

The interaction between ligands and the G protein-coupled receptors (GPCRs) to which they bind has long been the focus of intensive investigation. The signalling cascades triggered by receptor activation, due in most cases to ligand binding, are of great physiological and medical importance; indeed, GPCRs are targeted by in excess of 30% of small molecule therapeutic medicines. Attempts to identify further pharmacologically useful GPCR ligands, for receptors with known and unknown endogenous ligands, continue apace. In earlier days direct assessment of such interactions was restricted largely to the use of ligands incorporating radioactive isotope labels as this allowed detection of the ligand and monitoring its interaction with the GPCR. This use of such markers has continued with the development of ligands labelled with fluorophores and their application to the study of receptor–ligand interactions using both light microscopy and resonance energy transfer techniques, including homogenous time-resolved fluorescence resonance energy transfer. Details of ligand–receptor interactions via X-ray crystallography are advancing rapidly as methods suitable for routine production of substantial amounts and stabilised forms of GPCRs have been developed and there is hope that this may become as routine as the co-crystallisation of serine/threonine kinases with ligands, an approach that has facilitated widespread use of rapid structure-based ligand design. Conformational changes involved in the activation of GPCRs, widely predicted by biochemical and biophysical means, have inspired the development of intramolecular FRET-based sensor forms of GPCRs designed to investigate the events following ligand binding and resulting in a signal propagation across the cell membrane. Finally, a number of techniques are emerging in which ligand–GPCR binding can be studied in ways that, whilst indirect, are able to monitor its results in an unbiased and integrated manner. This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

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* Corresponding author at: Wolfson Link Building 253, University of Glasgow, Glasgow G12 8QQ, Scotland, UK Tel.: +44 141 330 5557; fax: +44 141 330 5481.
E-mail address: Graeme.Milligan@glasgow.ac.uk (G. Milligan).

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1. Introduction

1.1. Guanine nucleotide binding protein-coupled receptors (GPCRs)

GPCRs are the largest family of transmembrane proteins in the human genome and their encoded genes make up a significant proportion of the genome devoted to cellular signalling. Indeed signalling via GPCRs is one of the most important processes in the regulation of physiological functions. GPCRs are membrane-spanning polypeptides whose most notable structural feature is a serpentine arrangement of seven sequential α-helices all of which cross from one face of the membrane to the other to form transmembrane domains (TMD) with the amino-terminus of the protein outside the membrane, whilst the carboxy-terminus resides within the cytoplasm (Fig. 1). The function of these receptors is to detect external (to the cell) stimuli and pass a signal across the membrane to a cascade of other signalling molecules to elicit a physiological response. A wide range of external stimuli may be involved ranging from photons to odorants, hormones, neurotransmitters and lipids, and these vary in size from ions and small molecules to peptides and proteins. It is the breadth of this range of stimuli which makes GPCRs such important drug targets and, consequently, the focus of much pharmacological research. Investigation of the human

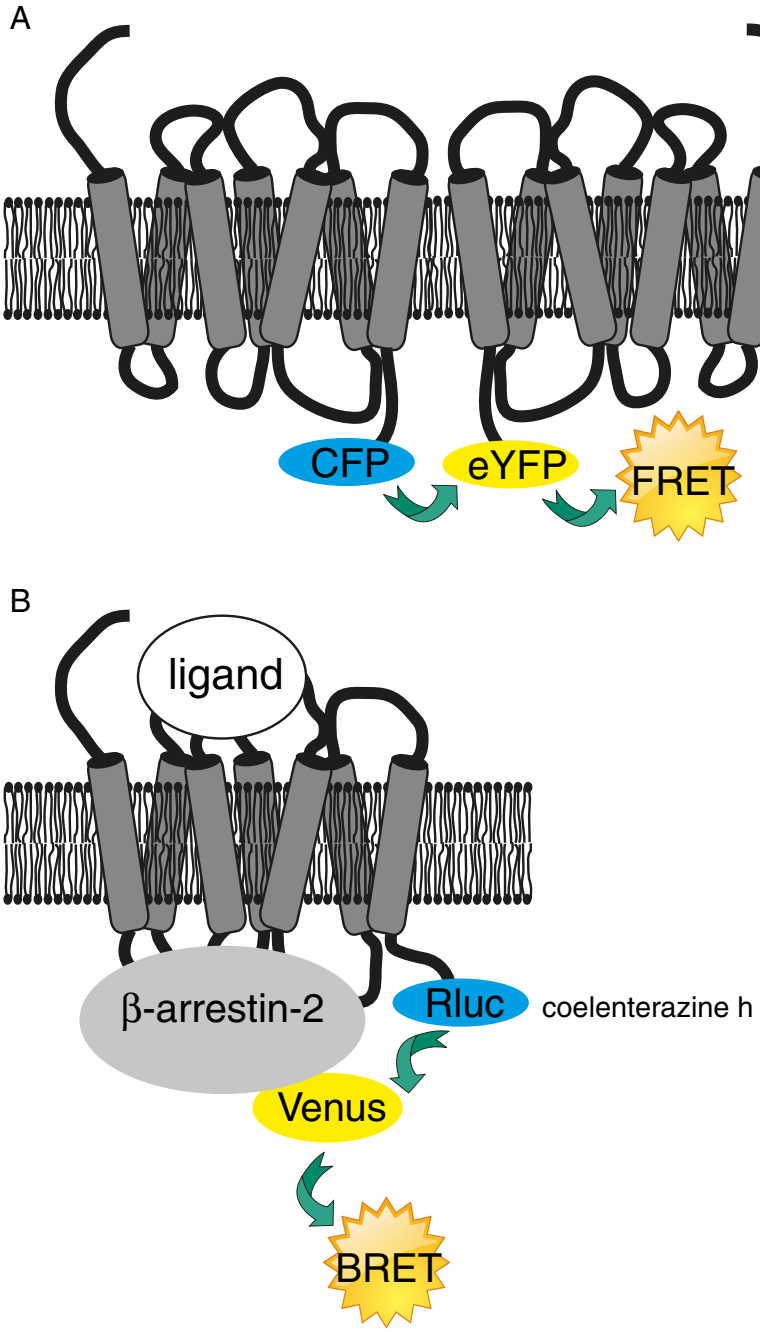

Fig. 1. Comparison of fluorescence resonance energy transfer (FRET) with bioluminescence resonance energy transfer (BRET). A. FRET between fluorescent proteins attached to the carboxy-termini of interacting GPCRs. Upon excitation with light at 430 nm the CFP emits light at 470 nm, which then excites the eYFP, causing the eYFP to emit light at 535 nm, the FRET signal. B. BRET[1] based β-arrestin-2 recruitment assay. The Rluc tagged GPCR is activated by ligand binding, causing β-arrestin-2 linked to the fluorescent protein Venus to be translocated to the membrane to interact with the receptor. This brings Rluc and Venus into close proximity, and when Rluc catalyses the oxidation of coelenterazine h and emits light at 480 nm this excites the Venus fluorescent protein, which subsequently emits light at 530 nm.

genome sequence has revealed that there are in excess of 800 genes that encode GPCRs, around 350 of which are predicted to generate non-olfactory receptors [1]. The physiological function of many of these is, as yet, unknown and they are termed orphan receptors. An important part of GPCR research is the search for natural ligands that regulate these orphan receptors in order to define their physiological function [2].

GPCR research began with observations of physiological responses to ligands in intact organisms or in preparations of organs or cell types. This, together with the use of ligands able to block such responses, resulted in classification of receptors into groups defined by their pharmacology [3]. This phase of GPCR research was followed by one in which biochemical techniques explored the details of signalling processes and downstream pathways, whilst the cDNAs of many GPCRs and other associated proteins were cloned and investigated by a variety of means including visualisation in living cells by the incorporation into their primary sequences of cDNAs encoding fluorescent proteins [4–7]. The determination of GPCR structures by X-ray crystallography, however, was comparatively slow to develop given the great significance of the class of receptors and this reflects the inherent difficulties of firstly purifying in soluble form and then crystallising integral membrane proteins. The first GPCR structure to be solved was that of bovine rhodopsin, a light-activated GPCR, which is the first step in the signalling pathway leading to vision [8]. This was the only GPCR structure available for some years and, as a consequence, was the basis of a great deal of modelling to infer and predict structural information for other GPCRs [9,10]. In recent years, however, GPCR structures which were once thought obdurate have begun to appear regularly in the literature. Examples include the human β_2 adrenergic receptor [11], human A_{2A} adenosine receptor (bound to an antagonist) [12], chemokine receptor CXCR4 (bound to antagonists) [13], and μ, δ and κ opioid receptors (bound to antagonists) [14–16]. There has also been structural definition of a complex between the β_2 adrenergic receptor and its predominant interacting partner, the Gsα subunit [17]. It seems likely that this progress is set to continue, particularly taking into account the development of thermostabilised receptors [18] and various other methodological breakthroughs described in ref. [19]. Most of the known GPCR structures have been determined from crystals which have been grown in the presence of receptor ligands, either agonists and antagonists, to stabilise the receptor in an active or inactive conformation respectively, which in turn has raised questions about conformational changes that underpin such transitions of state. In a number of cases, the unexpected observation has been that there are often rather limited differences between the agonist- and antagonist-bound forms, unless the appropriate G-protein α subunit is present within the system, either in its entirety or as a fragment [19]. This has revealed changes in the structures predicted largely from previous biophysical and mutagenic studies [20] which suggested that GPCR activation would involve an outward movement of trans-membrane domain 6 (TMD6) and possible breakage of an ionic lock generated with amino acids at the bottom of TMD3 [19]. Additional work is required to determine if this type of mechanism is generally applicable to the other families of GPCRs that are unrelated to rhodopsin.

1.2. Receptor oligomerisation

GPCRs were, until comparatively recently, considered to exist and to function as monomeric polypeptides and, indeed, recent work has demonstrated that they are functional after purification and reconstitution as monomers in high density lipoprotein particles. Examples of this include both the β_2-adrenergic receptor [21] and the μ-opioid receptor [22]. Such experiments notwithstanding, a large body of evidence has built up over recent years to support the concept of receptor oligomerisation, as reviewed in ref. [23–28]. A well characterised example of receptor dimerisation/oligomerisation (which also has implications for trafficking the receptor to the membrane) is that of the type C $GABA_B$ (γ-aminobutyric acid) receptor which exists at the cell membrane as an obligate heterodimer/heterotetramer consisting of $GABA_{B1}$ and $GABA_{B2}$ subunits. The $GABA_{B1}$ subunit is only able to be trafficked to the membrane if interacting with the $GABA_{B2}$ subunit as it contains an endoplasmic reticulum retention sequence in the carboxy-terminal which is masked by the carboxy-terminal sequence of the $GABA_{B2}$ subunit. Additionally the $GABA_B$ subunits have further complementary functions as the $GABA_{B1}$ binds the ligand whilst $GABA_{B2}$ recruits and passes a signal to the G protein [29]. Subsequent work has suggested that dimers of the $GABA_B$ receptor may interact to form tetramers [30] or larger complexes and that this may regulate receptor signalling properties [31]. Despite this many questions regarding receptor oligomerisation remain, not least of which is the relevance of much of the work performed in engineered cell lines to receptor interactions in more physiologically relevant situations.

1.3. Radioactive ligand binding

The use of radioactive ligand binding is central to the study of GPCRs and their interactions with ligands and has been used for many years. This technique provides a convenient and easy, (assuming appropriate ligands are available, which will inherently not be the case for orphan receptors) way to determine receptor expression levels in both whole and broken cell formats and it is also used for many pharmacological analyses including determination of binding affinities. However in recent years moves have been made to reduce the use of radioactive isotopes (typically ^3H and ^{125}I) for reasons of safety and the expense of radioactive waste disposal. Despite this, for many uses, such as the determination of binding kinetic 'on' and 'off' rates, radioactive ligand binding remains an attractive and frequently sole option. Alternative techniques such as that described in Section 4, using an htrFRET approach based upon the use of a donor-labelled SNAP-tagged receptor and a ligand tagged with an htrFRET acceptor are becoming available [32], but still do not yet quite match the versatility of radioactive ligand binding.

1.4. Label free techniques

In recent years a number of "label-free" techniques have been developed to report interactions between ligand and receptor. This has largely been driven by the needs of screening programmes which aim to identify pharmacologically useful compounds from chemical libraries and where there is a desire to capture concurrent information on the totality of signals generated upon receptor activation. Examples of these platforms include CellKey™ (MDS Analytical Technologies) and xCELLigence (Roche) systems, which measure changes in electrical impedance across a cell monolayer as cells alter their shape or move in response to receptor activation, and the Epic® (Corning Incorporated) system which measures changes in 'mass redistribution' in response to similar receptor stimulation [33–35]. An example of the use of the xCELLigence system was described recently by Stallaert and colleagues [36] to dissect the contributions of distinct elements of cellular response to the β_2-adrenergic receptor ligand isoproterenol. This study made use of pharmacological inhibitors to identify G_i, G_s and Gβγ signalling events and to identify the production of cAMP and the ERK1/2 pathways as elements of the impedance response. Similar approaches employing the Epic® system have shown that signals reflecting activation of each of G_q, G_i, G_s and G_{13} can be observed and deconvoluted from a complex overall pattern [37].

1.5. Resonance energy transfer techniques

The last 10–15 years have seen huge growth in the use of biophysical techniques such as Resonance Energy Transfer (RET or Förster

RET) in the analysis of interactions between GPCRs to form oligomers and also interactions between GPCRs and other proteins [38]. The principal advantage of such approaches is that they are able to monitor the close proximity and potential interactions between proteins in living cells and so provide information distinct from conventional biochemical techniques, such as co-immunoprecipitation. The growth in the use of RET techniques has been fuelled by advances in both optical instrumentation and the development, first of variants of the green fluorescent protein (GFP), originally isolated from the crystal jelly *Aequorea victoria* [39], and subsequently other suitable fluorophores. Among the first variants of GFP to be developed were the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) [40], forms which are able to function as an efficient RET donor/acceptor pair, though in many instances these have now been superseded by the further development of a range of fluorescent proteins which have distinct excitation/emission spectra, higher quantal yield and therefore brighter fluorescence and which have been engineered to be strictly monomeric in nature (an A206K mutation for GFP derivatives) [41]. This latter feature is highly desirable as wild type fluorescent proteins often aggregate to form dimeric or tetrameric complexes [42,43]. That the fluorescent proteins should be monomeric is clearly of great importance if they are being used to study protein–protein interactions as a tendency to interact on the part of the fluorescent proteins themselves could result in false positive results. RET involving transfer of energy between fluorescent proteins (such as from CFP, as a donor, to YFP, as an acceptor) is known as fluorescence resonance energy transfer (FRET) (Fig. 1A). An alternative form of RET is Bioluminescence Resonance Energy Transfer (BRET), which has become widely used to examine the interactions between GPCRs and other proteins. A key example of work employing BRET based techniques includes studies of the recruitment of β-arrestins to interact with the inner membrane face of a GPCR upon activation of the GPCR by an agonist ligand, a system widely used in screening assays to identify novel activators of GPCRs [44]. In principle, BRET is similar to FRET, but rather than the energy donor being a fluorescent protein, the energy donor is a luciferase type enzyme usually cloned from the Sea Pansy *Renilla reniformis*. Here the donated energy is derived from the enzymatic oxidation of a substrate by the luciferase which is then emitted as light, rather than the excitation of a fluorescent protein with light, which is then re-emitted as light of a different wavelength (Fig. 1B).

RET, as described above, involves the transfer of energy from a donor fluorophore to an acceptor fluorophore and in order for this to happen there must be of the order of 30% or more overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor [45]. In addition the distance between the donor and acceptor and also their orientation with respect to one another are important if RET is to occur. Considering the issue of distance first, for RET to be observed, the donor and acceptor fluorophores must be within the Förster radius (usually 3-6nm) and because the RET efficiency reduces as a function of the inverse sixth power of the separation [46], then it is only possible to generate a useful RET signal if the donor and acceptor are 1–10 nm apart [46]. It is this level of spatial resolution which is crucial for the interpretation that such experiments are indicative of direct protein–protein interactions between the partner proteins linked to the energy donor and acceptor species. The relative orientation between the RET pair, that is the orientation of emission and absorption dipoles (transitional dipole moments) within the fluorescent protein structure must be considered and must be orientated at an angle other then 90°. If this angle is 90° then the RET signal will be abolished due to self cancelling oscillations. By contrast if the dipole moments are parallel then signals will have the maximum value. A positive RET signal may be assumed to be an indication that two proteins are in close proximity, but is not proof of a direct interaction between them. Similarly, a negative signal does not necessarily mean that there is no direct interaction between the proteins linked to the RET partners but may reflect distance and relative orientation issues described above.

1.5.1. Fluorescence resonance energy transfer (FRET)

FRET is a form of RET in which energy transfer occurs between fluorophores according to the criteria described in the previous section. This is often performed between two fluorophores attached to different, but putatively interacting proteins, known as intermolecular FRET (Fig. 2A). An alternative possibility is intramolecular FRET which occurs between two fluorophores that have been incorporated into a single protein, (Fig. 2B). In the former case the objective is to monitor the interaction between the pair of proteins to which the fluorophores are attached, whilst in the latter, since the fluorophores are "fixed" within the limits of the flexibility of the protein into which they are incorporated, the intention is to monitor changes in the conformation of the protein due to, for example, the binding of a ligand (see Section 3). Clearly, in the case of intramolecular FRET equal expression of the two fluorophores is "built in", whereas for intermolecular FRET additional experiments are required to ensure equivalent expression.

FRET can be monitored in a number of different ways of which the most commonly used are as follows: a) acceptor photobleaching (after bleaching the acceptor with intense light, an increase can be seen in donor emission, as energy is no longer being donated to the acceptor); b) sensitised emission (the emission from the acceptor due to energy transferred from the donor); and c) fluorescent lifetime decay (after excitation with a pulsed laser, donor fluorescence decays more rapidly with increasing FRET efficiency). In all cases appropriate experimental controls are important to prevent artifactual results due to bleed-through between the channels measuring the fluorophore emission or the quenching of donor or acceptor fluorescence [47].

The development of FRET techniques has been rapid and of particular relevance to the study of GPCRs which has been the introduction of homogenous time-resolved FRET (htrFRET). This is based upon the use of lanthanide FRET donors, usually terbium, within a cryptate cage. The advantage of this is that as a FRET donor the lanthanides exhibit long lived fluorescence. This allows FRET to be measured after a time delay during which the background fluorescence, common to all excited biological systems, decays and therefore this greatly increases the signal to background window. This temporal selectivity, together with good spectral compatibility of the donor and acceptor and the lack of polarisation of the lanthanide emission (resulting in only weak dependence of htrFRET upon the relative orientation of donor and acceptor) ensures that htrFRET has the ability to deliver improved signal to noise ratio than alternative FRET techniques [27].

Early experiments using this approach to monitor δ-opioid receptor homodimerisation employed donor and acceptor htrFRET fluorophore-labelled antibodies that bind specifically to receptors tagged with the c-Myc and FLAG peptide epitopes [48]. This basic approach was subsequently adopted to demonstrate homodimerisation of the α_{1A}- [49] and α_{1B}- [50] adrenergic receptors and heterodimerisation between each of the $GABA_{B1}$–$GABA_{B2}$ receptor subunits [51] and chemokine CXCR1–CXCR2 receptors [52]. However, the use of antibodies linked to donor and acceptor fluorophores has potential disadvantages, including their large size which could result in steric hindrance and their bivalent nature which might intrinsically drive or contribute to the observed interactions. The main advantages are based on the specific high affinity interaction between the antibody and epitope tag, which allows the antibody to be used at a relatively low concentration and that the approach can be used directly in native cells, provided appropriate antibodies to the extracellular domains are available, as in the case of many 'cluster of differentiation' (CD) proteins used to define specific cell populations in immunological research [53,54].

An alternative to the use of antibodies for the addition of htrFRET donor and acceptor fluorophores is the use of a 'self-labelling' protein that behaves as a 'suicide enzyme'. Such systems include the 'SNAP' tag. This is a 23 kDa protein, based upon the DNA repair enzyme

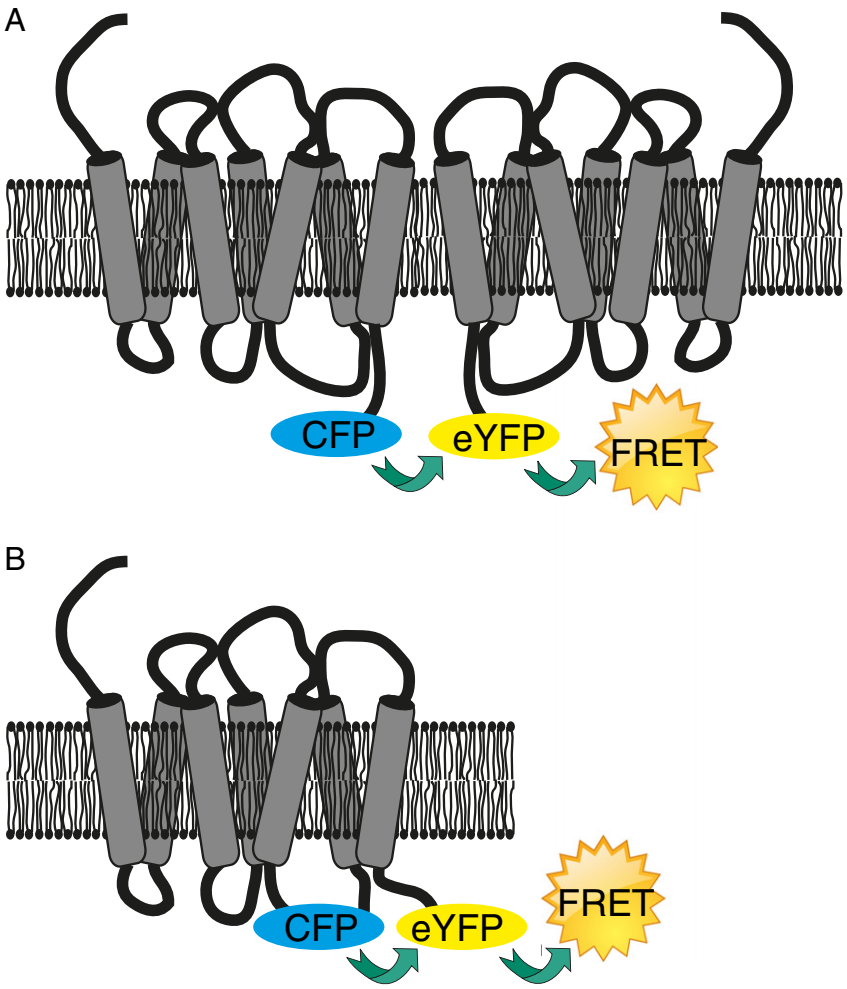

Fig. 2. Principle of intermolecular and intramolecular FRET. A. Two co-expressed GPCRs labelled with carboxy-terminal eYFP and CFP fluorescent protein tags. If these interact, the tags are brought into close proximity and intermolecular FRET can then occur. B. A GPCR tagged with CFP in the third intracellular loop and eYFP at the carboxy-terminal, emits an intramolecular FRET signal, which can then be modified by conformational changes in the receptor in response to ligand binding.

O^6-alkylguanine-DNA alkyl-transferase, which can react with a benzylguanine derivative substrate that incorporates a suitable fluorophore hence transferring the fluorophore-linked benzyl group covalently and irreversibly onto the 'SNAP' protein (Fig. 3A). This, alongside the similar but non cross-reacting 'CLIP' tag (Fig. 3B), provides the basis of the Tag-lite™ system (CisBio Bioassays) which allows cells expressing proteins in which their extracellular domains have been modified to encode the 'SNAP' and/or 'CLIP' tags to be simultaneously labelled with htrFRET donor and acceptor species. This system has been used to study GPCR oligomerisation in a number of examples of both homo- and hetero-configurations [30,55–57].

2. Fluorescently-labelled ligands; applications and uses

GPCR ligands that have been modified to include a fluorophore have been in use for more than 30 years and have been applied in a wide range of analyses designed to examine aspects of GPCR localisation, internalisation, pharmacology and function, as reviewed in [58–61]. Specific examples include the use of the orexin OX_1 receptor agonist oxerin A linked to TAMRA (carboxytetramethylrhodamine) to monitor the internalisation of the receptor and its interaction with β-arrestin-2 linked to GFP [62] and the use of fluorescent adrenoceptor and cannabinoid ligands to examine receptor distribution in small arteries [63]. Fluorescent derivatives have been made for both peptide and small molecule ligands, initially without detailed analysis of the effect that the fluorophore might have on the pharmacology of the ligand at its cognate GPCR. Subsequently the design of fluorescent ligands has become a much more thoughtful process as described in refs. [60,64], with detailed analysis of such behaviours being defined prior to functional application.

A number of applications for fluorescent ligands have recently emerged in which FRET is generated between a receptor-attached fluorophore and a bound fluorescent ligand. Albizu and co-workers used novel fluorescent vasopressin and oxytocin receptor ligands in conjunction with fluorophore-labelled antibodies specific to a receptor N-terminal HA (influenza hemagglutinin) tag to develop a ligand screen. Herein, positive 'targets' competed with the fluorescent ligand and so reduced the FRET signal allowing the identification of novel ligands and the investigation of their pharmacology [65]. Subsequently, similar studies have been performed using receptors with N-terminally fused fluorescent proteins as the second FRET component. Examples include the identification of ligands for the growth hormone secretagogue type 1a [66] and vasopressin V_2 receptors [67]. The latter study also described FRET experiments performed to demonstrate vasopressin V_{1A}–V_2 cell surface heterodimerisation using the selectivity of ligands labelled with FRET donor and acceptor. The mechanism of receptor ligand binding to the muscarinic M_1 acetylcholine receptor has also been investigated using such an approach [68,69].

A potentially important and powerful application of FRET between fluorescent ligands and a labelled receptor was described by Zwier

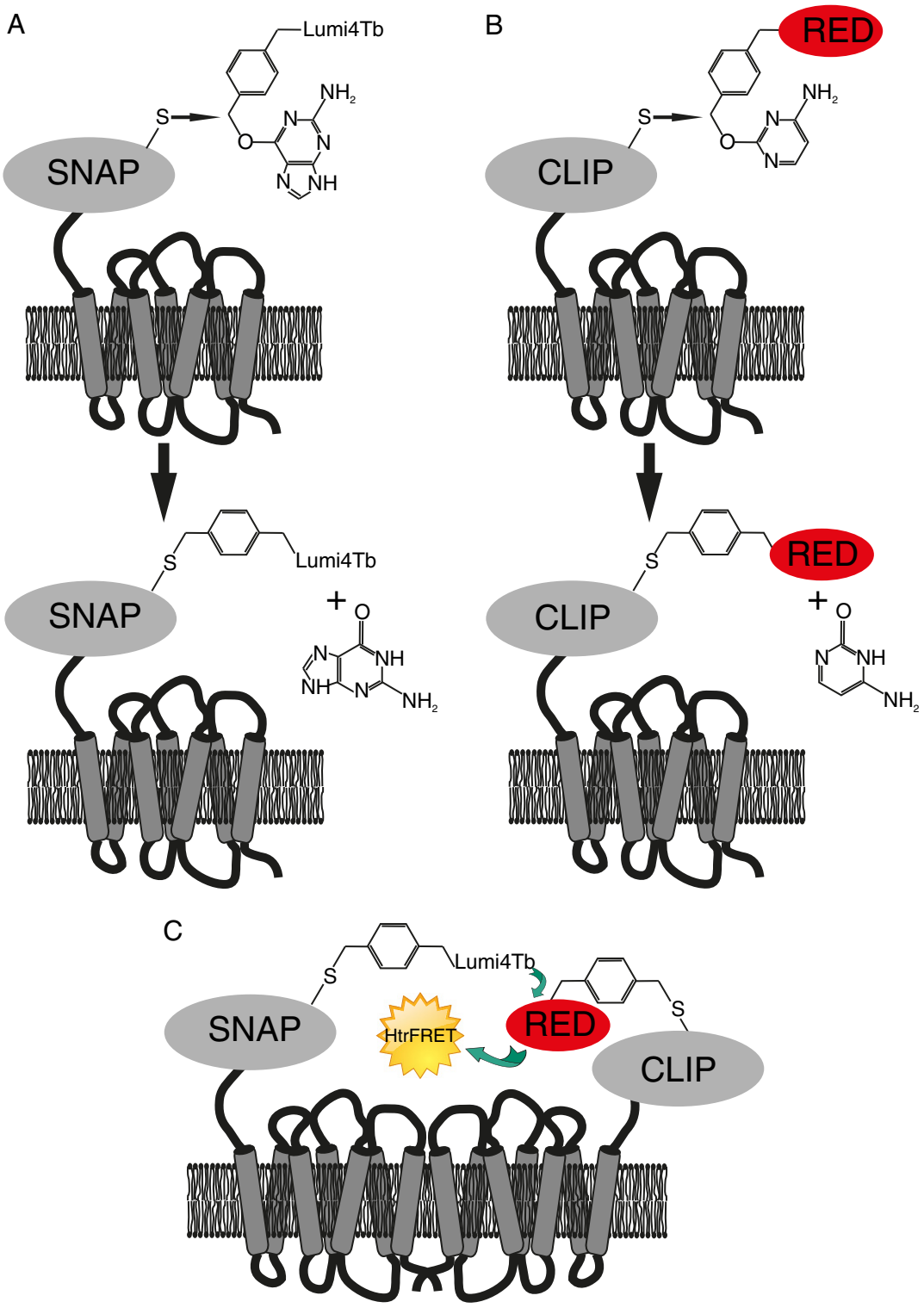

Fig. 3. SNAP and CLIP labelling; the Tag-lite htrFRET system. A. Amino-terminally SNAP tagged GPCR reacts with O^6-benzylguanine-Lumi4Tb, irreversibly labelling the SNAP tag with the Taglite htrFRET donor, Lumi4Tb. B. Simultaneously, an amino-terminally CLIP-tagged GPCR reacts with O^6-benzylcytosine-RED and is hence labelled with a Taglite htrFRET acceptor. C. An htrFRET signal can then be generated between the donor and acceptor, should they be in sufficiently close proximity.

[32]. Herein, an N-terminally 'SNAP'-tagged receptor was labelled with a htrFRET donor (see Section 1.5.1) and a FRET acceptor-labelled ligand was then added, generating an htrFRET signal (Fig. 4). This is a highly adaptable technique which can be modified for screening, the investigation of the kinetics of ligand binding and a host of other pharmacological analyses. However it is dependent upon the use of a 'SNAP'-tagged receptor (which can be easily generated) and the availability of an appropriate htrFRET labelled ligand.

The technique of fluorescence anisotropy is also one which makes use of fluorescently-labelled ligands, and is based upon the property that light emitted by a fluorophore does not have the same intensity along all axes of polarisation. The fluorescence anisotropy signal is

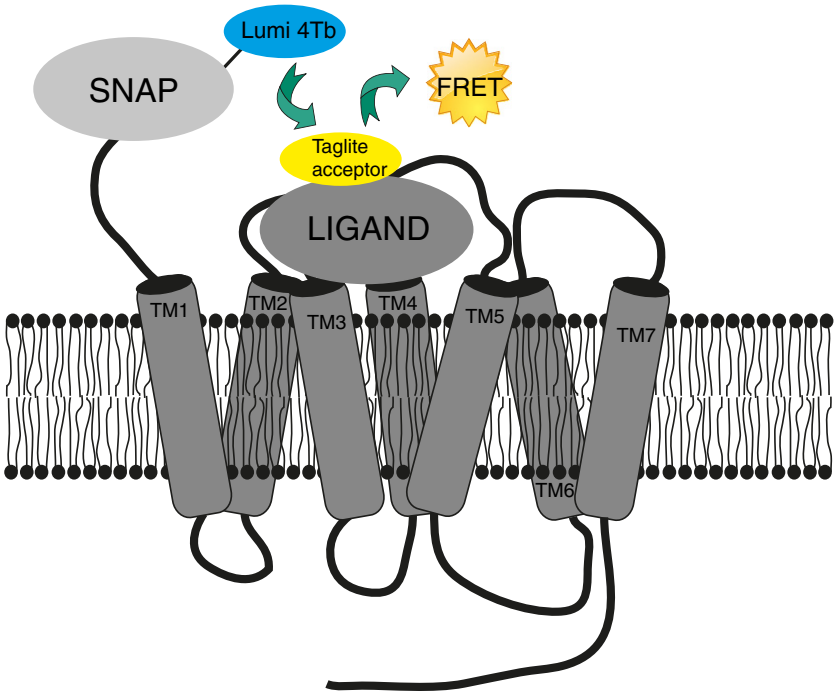

Fig. 4. HtrFRET between an amino-terminal SNAP-tagged receptor and an htrFRET acceptor labelled ligand An amino-terminal SNAP-tagged GPCR labelled with the Taglite htrFRET donor, Lumi4Tb, binds a ligand labelled with a Taglite acceptor. The htrFRET signal can be used in a similar way as radioactive ligand binding to investigate the receptor–ligand interaction.

generated from the difference in degree of rotation possible for a free ligand and one bound to a (much larger) receptor. Thus, when irradiated with plane-polarised light the rapidly rotating free ligand fluorophore emits highly depolarised light, whereas for ligand bound to a receptor (and rotating more slowly) the degree of depolarisation of the emitted light is less. Therefore, by monitoring the polarisation of the emitted light it is possible to assess the level of binding of a fluorescent ligand to a receptor. This technique was recently compared to a traditional radio-ligand binding approach for the muscarinic M_1 receptor by Huwiler and co-workers [70] and found to give similar IC_{50} values. Fluorescence anisotropy has also been used by Woll and colleagues [71] to monitor the binding of fluorescently labelled peptides, as opposed to small molecule ligands.

3. Monitoring GPCR activation in response to ligand: the FRET sensor

The basis of monitoring ligand-induced GPCR activation by intramolecular FRET is that ligand binding induces small conformational changes within the GPCR around the ligand binding site, which are then amplified to become larger movements at the cytoplasmic face of the receptor, in turn transmitting a signal to (usually) a G protein. This movement at the cytoplasmic face of the activating receptor is generally considered to be a change in position of TMD6 in relation to TMD3. As noted above, this view of receptor activation was developed from use of a variety of biophysical techniques [72] and has now been validated by some of the recently reported GPCR crystal structures [19,20]. Consequently the idea behind an intramolecular GPCR FRET sensor is that a FRET signal is generated from two appropriate fluorophores incorporated into the structure of the GPCR in such a way that a change in conformation alters the relative position of the fluorophores and hence alters the FRET signal.

The first experiments of this type were performed by Kobilka and colleagues and involved incorporating fluorescent labels into purified human β_2 adrenergic receptor and then using fluorescence spectroscopy to determine changes in the environment of the fluorescent label [73,74]. It was found that concentration–response curves to a full agonist (isoproterenol) could be generated and that such changes in fluorescence could be blocked by β-adrenergic antagonists. Furthermore, using a series of agonists with different levels of efficacy, a correlation was observed between changes in fluorescence and pharmacological efficacy [73]. Subsequently the β_2 adrenergic receptor was mutated to remove certain cysteine residues (the binding site of the fluorescent probe) and it was found that cysteines in TMD3 and TMD6 were essential for ligand-induced modulation of fluorescence [74]. This approach was refined further by the use of a fluorescence quenching reagent [75], which indicated movement of TMD6, and demonstrated that different agonists produce differences in the kinetics of movement [76].

The use of FRET (as opposed to fluorescence quenching) in experiments of this type was described by Granier [77] and again involved the purified β_2-adrenergic receptor, mutated to include a FlAsH (Fluorescein Arsenical Hairpin binder) binding sequence (CCPGCC) at two alternative positions in the C-terminus. These bound the fluorophore FlAsH, which then acted as a FRET donor. The FRET acceptor was Alexa Fluor 568 maleimide and this reacted with cysteine 265 in intracellular loop 3 (IL3) of the receptor close to the cytoplasmic end of TMD6. The FRET signal from this labelled receptor was used to calculate the distances between the alternative donors and the acceptor and also to demonstrate ligand specific changes in the FRET signal.

Thus, such experiments using purified receptors were able show that receptor activation involves conformational changes such as the movement of TMD6 and that different agonists produce specific conformational changes. The use of purified receptors however is not ideal or indeed always possible. Consequently the focus of such receptor "sensor" experiments has largely moved to the use of live cells expressing receptor constructs which incorporate fluorescent proteins, or a peptide sequence capable of being labelled *in situ*, as part of the primary sequence. This approach was first described by Vilardaga [78] using the parathyroid hormone receptor (PTHR) and α_{2A}-adrenergic receptor. Constructs were generated with CFP and YFP fused into the primary sequence of the third intracellular loop

and C-terminal tail respectively for PTHR and in the reverse order for the α_{2A}-adrenergic receptor (Fig. 5A). These constructs were designated "cameleons" based on previous efforts to generate sensors sensitive to alterations in cellular Ca^{2+} levels. Despite the addition of two approximately 30kDa fluorescent proteins into their sequence, the GPCR variants were found to exhibit typical ligand binding characteristics, albeit with reduced affinity for certain ligands, to be expressed at the cell surface in the same way as the unmodified receptors, and to possess broadly similar signalling characteristics as the wild type protein. The FRET signal obtained after treatment with agonist was reduced (compared to the untreated) by 15% for the PTHR sensor and by 5% for the α_{2A}-adrenergic receptor sensor. In both cases, the rapid kinetics that would be expected from the physiological response to the activation of these receptors was confirmed. That the kinetic response to parathyroid hormone was found to be slower (1 s for PTHR as opposed to 40 ms for the α_{2A}-adrenergic receptor) may indicate a more complex binding process of the peptide hormone than of the small molecule agonist noradrenaline.

An important aspect of these studies was the demonstration that agonist-induced FRET change represented a valid reflection of receptor activation. This was characterised in a number of ways. For example, to preclude the possibility that the FRET change was due to interaction with a downstream signalling molecule such as a G protein or a β-arrestin, FRET experiments were performed using membrane preparations from cells expressing the FRET sensor (to prevent interaction with cytosolic proteins) and the sensor was

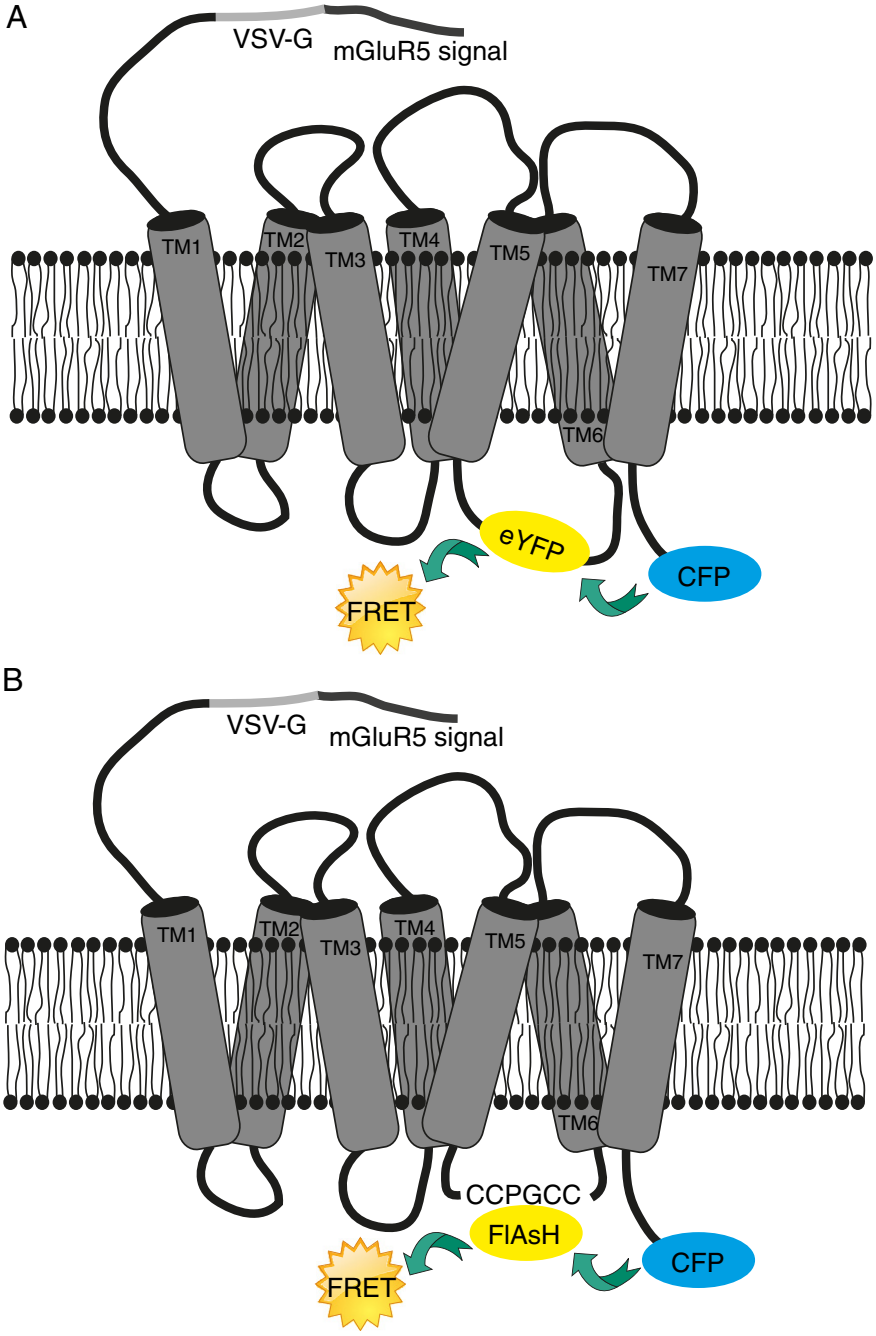

Fig. 5. FRET sensor. A. A FRET sensor consisting of a GPCR labelled at the carboxy-terminal with CFP and in the third intracellular loop with eYFP. B. An alternative GPCR FRET sensor labelled at the carboxy-terminal with CFP and with the Fluorescein Arsenical Hairpin binder (FlAsH) binding sequence inserted into the third intracellular loop. This binding sequence can then be labelled with a biarsenical fluorophore and provides a smaller and less structurally and functionally challenging FRET acceptor then the eYFP in A.

found to respond to PTH in a similar way as in intact cells, even after peripheral membrane proteins were removed or denatured by treatment with urea. Additionally, treatment of cells expressing the α_{2A}-adrenergic receptor sensor with pertussis toxin (which prevents receptor interactions with G_i/G_o subunits by catalysing an ADP-ribosylation reaction) was without effect upon the agonist-dependent change in FRET signal. To show that the FRET change did indeed require agonist binding Vilardaga and co-workers used an antagonist to the PTHR which was unable to induce any change in the FRET signal [78]. Thus antagonist ligands were able to bind, but not activate the receptor and caused no modulation of the FRET signal when added alone. Finally, because a bound G protein is known to promote the active, agonist bound state of the receptor, experiments were carried out in which purified Goα subunit was added to membranes expressing the α_{2A}-adrenergic receptor sensor, which were then treated with a sub-saturating concentration of the agonist UK14,304. This resulted in a change in FRET due to promotion of the active state by $G_o\alpha$ binding and this was significantly reduced by the addition of the poorly hydrolysed GTP analogue, GTPγS which results in G protein activation. Thus the change in FRET signal produced by these constructs upon agonist treatment appeared to mirror effects known to be produced by the receptor moving from an inactive to an active state.

Since these early experiments a number of other sensors of this type have been constructed for class A GPCRs, examples of which include bradykinin B_2 [79], β_1-adrenergic [80], β_2-adrenergic [81] and a distinct parathyroid hormone receptor sensor made using the fluorescent proteins Cerulean (FRET donor) and Citrine (FRET acceptor) [82]. A perhaps surprising aspect of many of these constructs was the similarity of their behaviour to the unmodified receptors. Given that third intracellular loop of many receptors plays a key role in G-protein interactions it is perhaps surprising that more variation in signalling behaviour has not been seen routinely. In order to identify and potentially limit the effects of the insertion of a fluorescent protein into the third intracellular loop of a receptor [83], comparisons were undertaken using FRET sensors constructed from the human A_{2A} adenosine receptor with CFP and YFP inserted into the third intracellular loop and carboxy-terminus respectively, with one in which CFP was inserted into the carboxy-terminal and the much smaller FlAsH sequence (CCPGCC, as described above) in the third intracellular loop (Fig. 5B). Herein CFP acted as FRET donor and FlAsH-labelled CCPGCC as the FRET acceptor. It was found that the CFP–YFP based sensor was able to generate a FRET signal corresponding to receptor activation, but was unable to couple to the downstream effector adenylyl cyclase. By contrast, however, the CFP–FlAsH version of the sensor was able to change the agonist-activated FRET signal by 5-fold, whilst retaining comparable kinetics and downstream signalling as the CFP-YFP sensor [83]. Hoffmann and co-workers also found that this was true for the mouse α_{2A}-adrenergic receptor [83]. It appears, therefore, that utilising the FlAsH sequence and labelling can be advantageous in that it minimises possible disruption to the receptor structure and function, probably because the tetracysteine–biarsenical tag is much smaller than a fluorescent protein (the use and optimisation of the FlAsH binding sequence and its associated fluorophore (commercially available as TC-FlAsH, previously known as Lumio Green) is described in [84–87] together with other similar but alternatively coloured reagents).

A number of other FRET biosensors incorporating the CFP-FlAsH FRET pair were subsequently described for a variety of receptors including the β_2-adrenergic receptor [88], muscarinic M_1, M_3 and M_5 receptors [89], the muscarinic M_2 receptor [90] and the orexin OX_1 and OX_2 receptors [91]. The development of all of these sensors was a more complex process than simply inserting the FlAsH sequence into the third intracellular loop of the receptor at random and fusing a CFP variant to the C-terminal tail. In most cases considerable optimisation of the positioning of the FlAsH sequence was required to maximise the subsequent alteration in FRET signal upon addition of an agonist and to ensure that the sensor was expressed effectively at the cell membrane. This is well illustrated by reference to the work of Xu and colleagues [91] describing the development of FRET sensors for the orexin OX_1 and OX_2 receptors. To generate the OX_1 sensor, the CFP FRET donor was added to the full length carboxy-terminal, or to versions of the receptor with truncated carboxy-terminal sequences (from wild-type length, 65 amino acids to 40 or 30 amino acids). The sequence FLNCCPGCCMEP, (containing the minimal FlAsH binding sequence CCPGCC), was introduced into the third intracellular loop, as either a replacement of 12 wild-type amino acids to maintain the loop length or to replace 34 residues to shorten the loop from 59 to 36 amino acids. This replacement occurred at the "centre" of the loop, however constructs were also made in which the 34 amino acids were replaced at either end of the loop sequence, that is close to either TMD5 or TMD6. Finally, the amino-terminus of the receptor was modified with addition of an mGluR5 signal sequence (MVLLLILSVLLLKEDVRGSA, [92]) to ensure effective delivery to the plasma membrane. Stable, antibiotic-controlled inducible cell lines were generated for these constructs and it was found that the greatest change in FRET upon addition of the peptide agonist orexin A was produced by cells harbouring a sensor with a full length carboxy-terminal tail and the FlAsH sequence placed in the middle of a shortened third intracellular loop. This sensor displayed a reduction in the FRET signal upon agonist treatment (consistent with the activation process involving an increase in the distance between the carboxy-terminal and the third intracellular loop), which was dependent on the concentration of agonist applied (Fig. 6A) and blocked in the presence of antagonist ligands (Fig. 6B). The change in FRET was comparatively slow, (something also noted for the PTH receptor sensor described above) and it may well be that this again reflects a more complex, multi-step binding process between a receptor and a peptide

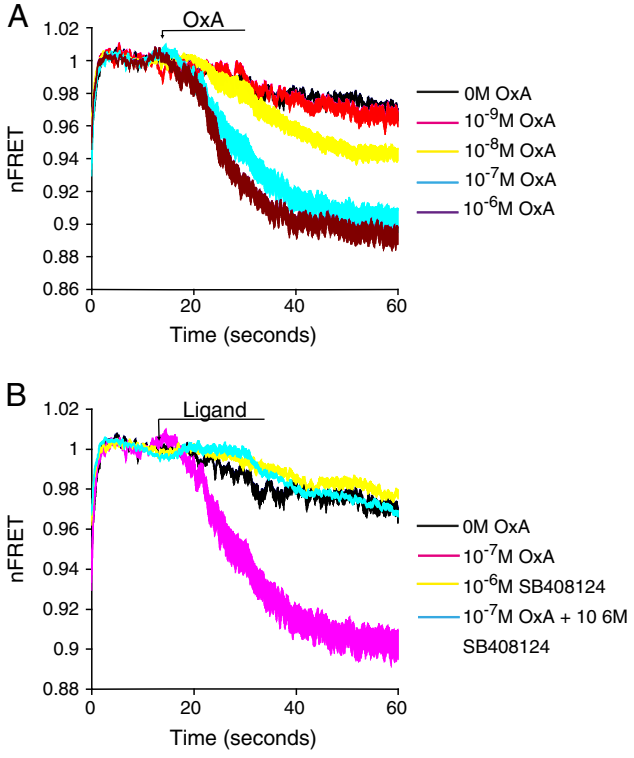

Fig. 6. OX_1 receptor FRET sensor response to ligands. The OX_1 FRET sensor displays a reduction in FRET signal upon treatment with the endogenously produced peptide agonist orexin A (OxA), suggesting that agonist binding causes an increase in the separation between the FRET donor and acceptor. A. This effect is dependent upon the concentration of OxA added and B. sensitive to the presence of the OX_1 receptor antagonist SB408124.
Modified from [85].

ligand, than would be involved in the binding of a small molecule ligand. The orexin OX_2 sensor was generated in a similar manner to that of the OX_1, thus the CFP donor was fused to the full length carboxy-terminal (78 amino acids) or a truncated version (60 amino acids) and the FlAsH sequence introduced into the third intracellular loop, replacing 12 amino acids to maintain the length of the loop at 57 or replacing 28 amino acids to shorten the loop to 40. It was found that the greatest alteration in FRET signal was produced by a sensor with both the truncated carboxy-terminal and the shortened third intracellular loop. Consequently it can be seen that considerable trial and error is involved in the development of a successful FRET sensor and it should be noted that, at least in the case of the orexin receptor constructs, much of the screening had to be carried out after production of inducible stable cell lines, rather than via transient transfection, in order for the sensors to be efficiently expressed at the cell membrane. It should also be noted that Xu and co-workers [91] performed a wide range of studies to ensure that the pharmacological and signalling characteristics of the FRET sensors were very similar to those of the wild-type orexin receptors.

A useful application for FRET sensors of the type described above has been the study of allosteric modulators of GPCR ligand activation. These are ligands, ranging from small molecules to peptide and protein toxins, which bind to sites distinct from the normal (endogenous) ligand binding site (orthosteric site) and modulate (either positively or negatively) the effect of agonist or inverse agonist ligands. This concept has been reviewed extensively in refs. [93–96]. The use of a FRET sensor in the investigation of allosteric modulators of the muscarinic M_2 receptor was described by Maier-Peuschel in ref. [90] and involved the use of a receptor with CFP fused to the carboxy-terminal and a FlAsH binding sequence in the third intracellular loop. This FRET sensor displayed a reduced FRET signal on treatment with orthosteric agonists, which was restored by removal of the agonist or addition of antagonist. The allosteric ligands studied (gallamine and dimethyl-W84), which caused no sensor response when administered alone, were able to modulate markedly the effect of agonist and to do so with kinetics much faster than the response to an orthosteric antagonist, suggesting that they act by inducing a receptor conformation which has reduced affinity for the agonist.

4. FRET between fluorescent ligands and the detection of endogenously expressed receptor complexes

The concept of receptor dimerisation or oligomerisation is one for which extensive evidence has been generated in studies performed in cell systems heterologously expressing GPCRs (see Section 1.2). Numerous examples of modified receptor pharmacology in cells expressing pairs of receptors have been described and interpreted as reflecting their heterodimerisation. However, much less data is available in native cells and tissues. This is a substantial challenge as the primary sequence modifying receptor labels and tags essential to the techniques described above are not available or that such modified receptors must be expressed transgenically. One potentially attractive approach is the use of fluorescently-labelled antibodies as they can provide specificity and be of high affinity. However, developing high quality antibodies to GPCRs has generally been challenging. In addition, the size of antibodies and their bivalent nature raises questions regarding both the effects of steric hindrance and the possibility of dimerisation actually being driven by the addition of antibody, rather than monitored by it. A recently described novel approach is that of measuring FRET between receptor ligands labelled with FRET-competent fluorophores. This was first demonstrated by using fluorescently labelled ligands of the vasopressin V_{1A}, V_2 and dopamine D_2 receptors, in a heterologous expression system [97,98]. The fluorophores used were designed for htr-FRET measurements. Within these studies the authors demonstrated, using htrFRET competent fluorophore-labelled oxytocin receptor ligands, that oxytocin receptor multimers can be detected in prepared membranes prepared from and intact patches of native lactating rat mammary gland tissue. This approach offers broad applicability, assuming that suitable ligands can be prepared. It must be noted, however, that these experiments were performed using tissue expressing levels of the oxytocin receptor, (1–3 pmol·mg protein^{-1}, [97]), comparable to those used in many heterologous expression system studies and a challenge in moving forward will be to both define and then lower the limits of detectability of such signals to allow receptor complexes expressed at much lower levels to be identified.

5. Conclusions

The identification and analysis of novel (and pharmacologically useful) GPCR ligands, together with the further analysis of those which are already known, are of great importance and, as such, the focus of much effort. Many drugs that are in present use act via GPCRs and its reasonable to assume that the effort to deorphanise those GPCRs whose endogenous ligands are as yet unknown will result in further potential drugs targets. The fluorescence based techniques described here to study the interactions between ligands and GPCRs will be an important part of this. Clearly the screening of compound libraries against GPCRs, as part of the search for new drugs, requires readouts which are amenable to “high throughput” screening and as such, FRET and fluorescent labelling techniques provide assay platforms which are homogeneous and which can be scaled down in volume for economy both financial and in terms of the number of assays which can be carried out “per plate”.

Once new GPCR ligands are identified then fluorescent labelling and htrFRET can be used in place of the traditional radio-labelling approach for the study of their binding and its effects upon the interactions between the GPCR and other ligands. Subsequently, the transduction of a ligand induced signal by a GPCR can be analysed using a receptor modified as a FRET sensor.

References

[1] R. Fredriksson, M.C. Lagerström, L.-G. Lundin, H.B. Schiöth, The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups and fingerprints, Mol. Pharm. 63 (2003) 1256–1272.
[2] O. Civelli, R.K. Reinscheid, Y. Zhang, Z. Wang, R. Fredriksson, H.B. Schiöth, G protein-coupled receptor deorphanisations, Annu. Rev. Pharmacol. Toxicol. 53 (2013) 127–146.
[3] J. Black, A personal view of pharmacology, Annu. Rev. Pharmacol. Toxicol. 36 (1996) 1–33.
[4] G. Milligan, E. Kostenis, Heterotrimeric G-proteins: a short history, Br. J. Pharmacol. 147 (Suppl. 1) (2006) S46–S55.
[5] L.S. Barak, J. Zhang, S.S. Ferguson, S.A. Laporte, M.G. Caron, Signalling desensitisation, and trafficking of G-protein coupled receptors revealed by green fluorescent protein conjugates, Methods Enzymol. 302 (1999) 153–171.
[6] A.J. McLean, N. Bevan, S. Rees, G. Milligan, Visualizing differences in ligand regulation of wild-type and constitutively active mutant β_2-adrenoceptor-green fluorescent protein fusion proteins, Mol. Pharm. 56 (1999) 1182–1191.
[7] G. Milligan, Exploring the dynamics of regulation of G protein-coupled receptors using green fluorescent protein, Br. J. Pharmacol. 128 (1999) 501–510.
[8] K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, Crystal structure of rhodopsin: a G protein coupled receptor, Science 289 (2000) 739–745.
[9] J.A. Ballesteros, L. Shi, J.A. Javitch, Structural mimicry in G protein coupled receptors: implications of the high-resolution structure of rhodopsin for structure–function analysis of rhodopsin-like receptors, Mol. Pharm. 60 (2001) 1–19.
[10] E. Archer, B. Maigret, C. Escrieut, L. Pradayrol, D. Fourmy, Rhodopsin crystal: new template yielding realistic models of G-protein-coupled receptors? Trends Pharmacol. Sci. 24 (2003) 36–40.
[11] S.G. Rasmussen, H.-J. Choi, D.M. Rosenbaum, T.S. Kobilka, F.S. Thian, P.C. Edwards, M. Burghammer, V.R.P. Ratnala, R. Sanishvili, R.F. Fischetti, G.F.X. Schertler, W.I. Weis, B.K. Kobilka, Crystal structure of the human β2 adrenergic G-protein coupled receptor, Nature 450 (2007) 383–388.
[12] V.-P. Jaakola, M.T. Griffith, M.A. Hanson, V. Cherezov, E.Y.T. Chien, J.L. Lane, A.P. Ijzerman, R.C. Stevens, The 2.6 Å crystal structure of a human A_{2A} adenosine receptor bound to an antagonist, Science 322 (2008) 1211–1217.
[13] B. Wu, E.Y.T. Chien, C.D. Mol, G. Fenalti, W. Liu, V. Katritch, R. Abagyan, A. Brooun, P. Wells, F.C. Bi, D.J. Hamel, P. Kuhn, T.M. Handel, V. Cherezov, R.C. Stevens, Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists, Science 330 (2010) 1066–1071.

[14] A. Manglik, A.C. Kruse, T.S. Kobilka, F.S. Thian, J.M. Mathiesen, R.K. Sunahara, L. Pardo, W.I. Weis, B.K. Kobilka, S. Granier, Crystal structure of the μ-opioid receptor bound to a morphinan antagonist, Nature 485 (2012) 321–327.

[15] H. Wu, D. Wacker, M. Mileni, V. Katritch, G.W. Han, E. Vardy, W. Lui, A.A. Thompson, X.-P. Huang, F.I. Carroll, S.W. Mascarella, R.B. Westkaemper, P.D. Mosier, B.L. Roth, V. Cherezov, R.C. Stevens, Structure of the human κ-opioid receptor in complex with JDTic, Nature 485 (2012) 327–332.

[16] S. Granier, A. Manglik, A.C. Kruse, T.S. Kobilka, F.S. Thian, W.I. Weis, B.K. Kobilka, Structure of the δ-opioid receptor to naltrindole, Nature 485 (2012) 400–405.

[17] S.G. Rasmussen, B.T. DeVree, Y. Zou, A.C. Kruse, K.Y. Chung, T.S. Kobilka, F.S. Thian, P.S. Chae, E. Pardon, D. Calinski, J.M. Mathiesen, S.T.A. Shah, J.A. Lyons, M. Caffrey, S.H. Gellman, J. Steyaert, G. Skiniotis, W.I. Weis, R.K. Sunahara, B.K. Kobilka, Crystal structure of the β2 adrenergic receptor–Gs protein complex, Nature 477 (2011) 549–555.

[18] C.G. Tate, A crystal clear solution for determining G-protein-coupled receptor structures, Trends Biochem. Sci. 37 (2012) 343–351.

[19] M. Audet, M. Bouvier, Restructuring G-protein-coupled receptor activation, Cell 151 (2012) 14–23.

[20] B.K. Kobilka, G protein coupled receptor structure and activation, Biochem. Biophys. Acta 1768 (2007) 794–807.

[21] M.R. Whorton, M.P. Bokoch, S.G.F. Rasmussen, B. Huang, R.N. Zare, B. Kobilka, R.K. Sunahara, 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 (2007) 7682–7687.

[22] A.J. Kuzak, S. Pitchiaya, J.P. Anand, H.I. Mosberg, N.G. Walter, R.K. Sunahara, Purification and functional reconstitution of monomeric μ-opioid receptors, J. Biol. Chem. 284 (2009) 26732–26741.

[23] G. Milligan, G protein coupled receptor dimerisation: molecular basis and relevance to function, Biochim. Biophys. Acta 1768 (2007) 825–835.

[24] G. Milligan, A day in the life of a G-protein coupled receptor: the contribution to function of G-protein coupled receptor dimerisation, Br. J. Pharmacol. 153 (Suppl. 1) (2008) S216–S229.

[25] G. Milligan, G-protein coupled receptor hetero-dimerisation: contribution to pharmacology and function, Br. J. Pharmacol. 158 (2009) 5–14.

[26] G. Milligan, The role of dimerisation in the cellular trafficking of G-protein coupled receptors, Curr. Opin. Pharmacol. 10 (2010) 23–29.

[27] M. Cottet, O. Faklaris, D. Maurel, P. Scholler, E. Doumazane, E. Trinquet, J.-P. Pin, T. Durroux, BRET and time-resolved FRET strategy to study GPCR oligomerisation: from cell lines toward native tissues, Front. Endocrinol. 3 (2012) 1–14.

[28] D. Pétrin, T.E. Hébert, The functional size of GPCRs — monomers, dimers or tetramers? Subcell. Biochem. 63 (2012) 67–81.

[29] T. Galvez, B. Duthey, J. Kniazeff, J. Blahos, G. Rovelli, B. Bettler, L. Prézeau, J.-P. Pin, Allosteric interactions between GB1 and GB2 subunits are required for optimal $GABA_B$ receptor function, EMBO J. 20 (2001) 2152–2159.

[30] D. Maurel, L. Comps-Agrar, C. Brock, M.-L. Rives, E. Bourrier, M.A. Ayoub, H. Bazin, N. Tinel, T. Durroux, L. Prézeau, J.-P. Pin, Cell-surface protein–protein interaction analysis with time-resolved FRET and SNAP-tag technologies: application to GPCR oligomerisation, Nat. Methods 5 (2008) 561–567.

[31] L. Comps-Agrar, J. Kniazeff, L. Nørskov-Lauritsen, D. Maurel, M. Gassmann, N. Gregor, L. Prézeau, B. Bettler, T. Durroux, E. Trinquet, J.-P. Pin, The oligomeric state sets the GABAB receptor signalling efficacy, EMBO J. 30 (2011) 2336–2349.

[32] J.M. Zwier, T. Roux, M. Cottet, T. Durroux, S. Douzon, S. Bdioui, N. Gregor, E. Bourrier, N. Oueslati, L. Nicolas, N. Tinel, C. Boisseau, P. Yverneau, F. Charrier-Savournin, M. Fink, E. Trinquet, A fluorescent ligand binding alternative using Tag-lite® technology, J. Biomol. Screen. 15 (2010) 1248–1259.

[33] P.H. Lee, A. Gao, C. van Staden, J. Ly, J. Salon, A. Xu, Y. Fang, R. Verkleeren, Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed G protein-coupled receptors, Assay Drug Dev. Technol. 6 (2008) 83–94.

[34] M.F. Peters, F. Vaillancourt, M. Heroux, M. Valiquette, C.W. Scott, Comparing label-free biosensors for pharmacological screening with cell based functional assays, Assay Drug Dev. Technol. 8 (2010) 219–227.

[35] E. Alvarez-Curto, R.J. Ward, J.D.G. Milligan, Novel assay technologies for the discovery of G protein-coupled receptor drugs, in: C.W. Stevens (Ed.), Methods for the discovery and characterization of G protein-coupled receptors, Neuromethods, 60, Springer Science+Business Media, 2011, pp. 231–253.

[36] W. Stallaert, J.F. Dorn, E. van der Westhuizen, M. Audet, M. Bouier, Impedance responses reveal β_2-adrenergic receptor signalling pluridimensionality and allow classification of ligands with distinct signalling profiles, PLoS One 7 (2012) 1–14.

[37] R. Schroder, J. Schmidt, S. Blattermann, L. Peters, N. Janssen, M. Grundmann, W. Seemann, D. Kaufel, N. Merten, C. Drewke, J. Gomeza, G. Milligan, K. Mohr, E. Kostenis, Applying label-free dynamic mass redistribution technology to frame signaling of G protein-coupled receptors non-invasively in living cells, Nat. Protoc. 6 (2011) 1748–1760.

[38] S.S. Vogel, C. Thaler, S.V. Koushik, Fanciful FRET, Sci STKE re2, 2006. 1–8.

[39] D.C. Prasher, V.K. Eckenrode, W.W. Ward, F.G. Prendergast, M.J. Cormier, Primary structure of the *Aequorea victoria* green fluorescent protein, Gene 111 (1992) 229–333.

[40] R. Heim, R.Y. Tsien, Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer, Curr. Biol. 6 (1996) 178–182.

[41] N.C. Shaner, P.A. Steinbach, R.Y. Tsien, A guide to choosing fluorescent proteins, Nat. Methods 2 (2005) 905–909.

[42] J. Zhang, R.E. Campbell, A.Y. Ting, R.Y. Tsien, Creating new fluorescent probes for cell biology, Nat. Rev. Mol. Cell Biol. 3 (2002) 906–918.

[43] N.C. Shaner, R.E. Campbell, P.A. Steinbach, B.N. Giepmans, A.E. Palmer, R.Y. Tsien, Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein, Nat. Biotechnol. 22 (2004) 1567–1572.

[44] K.D.G. Pfleger, M.B. Dalrymple, J.R. Dromey, K.A. Eidne, Monitoring interactions between G-protein-coupled receptors and β-arrestins, Biochem. Soc. Trans. 35 (2007) 764–766.

[45] B.A. Pollock, R. Heim, Using GFP in FRET-based applications, Trends Cell Biol. 9 (1999) 57–60.

[46] T. Förster, Zwischenmolekulare Energiewanderung und Fluoreszenz, Ann. Phys. 2 (1948) 55–75.

[47] E.A. Jares-Erijman, T.M. Jovin, FRET imaging, Nat. Biotechnol. 21 (2003) 1387–1395.

[48] M. McVey, D. Ramsay, E. Kellet, S. Rees, S. Wilson, A.J. Pope, G. Milligan, Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta-opioid receptor displays constitutive oligomerisation at the cell surface which is not regulated by receptor occupancy, J. Biol. Chem. 276 (2001) 14092–14099.

[49] D. Ramsay, I.C. Carr, J. Pediani, J.F. Lopez-Gimenez, R. Thurlow, M. Fidock, G. Milligan, High affinity interactions between human α_{1A}-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the α_{1L}-adrenoceptor, Mol. Pharm. 66 (2004) 228–239.

[50] J.J. Carrillo, J.F. Lopez-Gimenez, G. Milligan, Multiple interactions between transmembrane helices generate the oligomeric α_{1B}-adrenoceptor, Mol. Pharm. 66 (2004) 1123–1137.

[51] D. Maurel, J. Kniazeff, G. Mathis, E. Trinquet, J.-P. Pin, H. Ansanay, Cell surface detection of membrane protein interaction with homogenious time-resolved fluorescence resonance energy transfer technology, Anal. Biochem. 329 (2004) 253–262.

[52] S. Wilson, G. Wilkinson, G. Milligan, The CXCR1 and CXCR2 receptors form constitutive homo- and heterodimers selectively and with equal apparent affinities, J. Biol. Chem. 280 (2005) 28663–28674.

[53] M. Amiot, A. Bernard, B. Raynal, W. Knapp, C. Deschildre, L. Boumsell, Heterogeneity of the first cluster of differentiation: characterization and epitopic mapping of three CD1 molecules on normal human thymus cells, J. Immunol. 136 (1986) 1752–1758.

[54] L. Belov, O. de la Vega, C.G. dos Remedios, S.P. Mulligan, R.I. Christopherson, Immunophenotyping of leukemias using a cluster of differentiation antibody microarray, Cancer Res. 61 (2001) 4486–4489.

[55] E. Alvarez-Curto, R.J. Ward, J.D. Pediani, G. Milligan, Ligand regulation of the quaternary organisation of cell surface M_3 muscarinic acetylcholine receptors analysed by fluorescence resonance energy transfer (FRET) imaging and homogeneous time resolved FRET, J. Biol. Chem. 285 (2010) 23318–23330.

[56] R.J. Ward, J.D. Pediani, G. Milligan, Heteromultimerisation of cannabinoid CB_1 and orexin OX_1 receptor generates a unique complex in which both protomers are regulated by orexin A, J. Biol. Chem. 286 (2011) 37414–37428.

[57] C. Pou, C. Mannoury la Cour, L.A. Stoddart, M.J. Millan, G. Milligan, Functional homomers and heteromers of the dopamine D_{2L} and D_3 receptors co-exist at the cell surface, J. Biol. Chem. 287 (2012) 8864–8878.

[58] C.J. Daly, J.C. Mcgrath, Fluorescent ligands, antibodies, and proteins for the study of receptors, Pharmacol. Ther. 100 (2003) 101–118.

[59] R.J. Middleton, B. Kellam, Fluorophore-tagged GPCR ligands, Curr. Opin. Chem. Biol. 9 (2005) 517–525.

[60] S.J. Briddon, B. Kellam, S.J. Hill, Design and use of fluorescent ligands to study ligand–receptor interactions in single living cells, in: G.B. Willars, R.A.J. Challis (Eds.), Receptor signal transduction protocols, third edition, Methods in Molecular Biology, 746, 2011, pp. 211–236.

[61] L.A. Stoddart, A.J. Vernall, J.L. Denman, S.J. Briddon, B. Kellam, S.J. Hill, Fragment screening at adenosine-A(3) receptors in living cells using a fluorescence-based binding assay, Chem. Biol. 19 (2012) 1105–1115.

[62] S. Milasta, N.A. Evans, L. Ormiston, S. Wilson, R.J. Lefkowitz, G. Milligan, The sustainability of interactions between the orexin-1 receptor and β-arrestin-2 is defined by a single C-terminal cluster of hydroxyl amino acids and modulates the kinetics of REK MAPK regulation, Biochem. J. 387 (2005) 573–584.

[63] C.J. Daly, R.A. Ross, C.M. Henstridge, A.J. Irving, J.C. McGrath, Fluorescent ligand binding reveals heterogeneous distribution of adrenoceptors and 'cannabinoid-like' receptors in small arteries, Br. J. Pharmacol. 159 (2010) 787–796.

[64] E. Kozma, P.S. Jayasekara, L. Squarcialupi, S. Paoletta, S. Moro, S. Federico, G. Spalluto, K.A. Jacobson, Fluorescent ligands for adenosine receptors, Bioorg. Med. Chem. Lett. 23 (2013) 26–36.

[65] L. Albizu, G. Teppaz, R. Seyer, H. Bazin, H. Ansanay, M. Manning, Towards efficient drug screening by homogeneous assays based on the development of new fluorescent vasopressin and oxytocin receptor ligands, J. Med. Chem. 50 (2007) 4976–4985.

[66] J.P. Leyris, T. Roux, E. Trinquet, P. Verdié, J.-A. Fehrentz, N. Oueslati, S. Douzon, E. Bourrier, L. Lamarque, D. Gague, J.-C. Galleyrand, C. M'Kadmi, J. Martinez, S. Mary, J.-L. Banerès, J. Marie, Homogeneous time-resolved fluorescence-based assay to screen for ligands targeting the growth hormone secretagogue receptor 1a, Anal. Biochem. 408 (2011) 253–262.

[67] S. Loison, M. Cottet, H. Orcel, H. Adihou, R. Rahmeh, L. Lamarque, E. Trinquet, E. Kellenberger, M. Hibert, T. Durroux, B. Mouillac, D. Bonnet, Selective fluorescent nonpeptidic antagonists for vasopressin V_2 GPCR: application to ligand screening and oligomerisation assays, J. Med. Chem. 55 (2012) 8588–8602.

[68] B. Ilien, C. Franchet, P. Bernard, S. Morisset, C.O. Weill, J.-J. Bourguignon, M. Hibert, J.-L. Galzi, Fluorescence resonance energy transfer to probe human M_1 muscarinic receptor structure and drug binding properties, J. Neurochem. 85 (2003) 768–778.

[69] S.B. Daval, C. Valant, D. Bonnet, E. Kellenberger, M. Hibert, J.-L. Galzi, B. Ilien, Fluorescent derivatives of AC-42 to probe bitopic orthosteric/allosteric binding mechanisms on muscarinic M_1 receptors, J. Med. Chem. 55 (2012) 2125–2143.

[70] K.G. Huwiler, T. De Rosier, B. Hanson, K.W. Vogel, A fluorescence aniosotropy assay for the muscarinic M_1 G-protein-coupled receptor, Assay Drug Dev. Technol. 8 (2010) 351–361.
[71] M.P. Woll, D.A. De Cotiis, M.C. Bewley, D.M. Tacelosky, R. Levenson, J.M. Flanagan, Interaction between the D_2 dopamine receptor and neuronal calcium sensor-1 analysed by fluorescence aniosotropy, Biochemistry 50 (2011) 8780–8791.
[72] M.J. Lohse, S. Nuber, C. Hoffmann, Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein coupled receptor activation and signalling, Pharmacol. Rev. 64 (2012) 299–336.
[73] U. Gether, S. Lin, B.K. Kobilka, Fluorescent labelling of purified β_2 adrenergic receptor, J. Biol. Chem. 270 (1995) 28268–28275.
[74] U. Gether, S. Lin, P. Ghanouni, J.A. Ballesteros, H. Weinstein, B.K. Kobilka, Agonists induce conformational changes in transmembrane domains III and VI of the β_2 adrenoceptor, EMBO J. 16 (1997) 6737–6747.
[75] P. Ghanouni, J.J. Steenhuis, D.L. Farrens, B.K. Kobilka, Agonist-induced conformational changes in the G-protein-coupling domain of the β_2 adrenergic receptor, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 5997–6002.
[76] G. Swarminath, X. Deupi, T.W. Lee, W. Zhu, F.S. Thain, T.S. Kobilka, B. Kobilka, Probing the β_2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists, J. Biol. Chem. 280 (2005) 22165–22171.
[77] S. Granier, S. Kim, A.M. Shafer, V.R.P. Ratnala, J.J. Fung, R.N. Zare, B. Kobilka, Structure and conformational changes in the C-terminal domain of the β_2 adrenoceptor, J. Biol. Chem. 282 (2006) 13895–13905.
[78] J.-P. Vilardaga, M. Bünemann, C. Krasel, M. Castro, M.J. Lohse, Measurement of the millisecond activation switch of the G protein-coupled receptors in living cells, Nat. Biotechnol. 21 (2003) 807–812.
[79] M. Chachisvillis, Y.-L. Zhang, J.A. Frangos, G protein-coupled receptors sense fluid shear stress in endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 15463–15468.
[80] F. Rochais, J.-P. Vilardaga, V.O. Nikolaev, M. Bünemann, M.J. Lohse, S. Engelhardt, Real-time optical recording of the β_1-adrenergic receptor activation reveals super-sensitivity of the Arg389 variant to carvedilol, J. Clin. Invest. 117 (2007) 229–235.
[81] S. Reiner, M. Ambrosio, C. Hoffmann, M.J. Lohse, Differential signalling of the endogenous agonists at the β_2-adrenergic receptor, J. Biol. Chem. 285 (2010) 36188–36198.
[82] Y.-L. Zhang, J.A. Frangos, M. Chachisvillis, Mechanical stimulus alters conformation of type 1 parathyroid hormone receptor in bone cells, Am. J. Physiol. Cell Physiol. 296 (2009) C1391–C1399.
[83] C. Hoffmann, G. Gaietta, M. Bünemann, S.R. Adams, S. Oberdorf-Maass, B. Behr, J.-P. Vilardaga, R.Y. Tsien, M.H. Ellisman, M.J. Lohse, A FlAsH-based FRET approach to determining G-protein-coupled receptor activation in living cells, Nat. Methods 2 (2005) 171–176.
[84] S.R. Adams, R.E. Campbell, L.A. Gross, B.R. Martin, G.K. Walkup, Y. Yao, J. Llopis, R.Y. Tsien, New biarsenical ligands and tetracysteine motifs for protein labelling *in vitro* and *in vivo*: synthesis and biological applications, J. Am. Chem. Soc. 124 (2002) 6063–6076.
[85] B.R. Martin, B.N.G. Giepmans, S.R. Adams, R.Y. Tsien, Mammalian cell-based optimisation of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity, Nat. Biotechnol. 23 (2005) 1308–1314.
[86] B.N.G. Giepmans, S.R. Adams, M.H. Ellisman, R.Y. Tsien, The fluorescent toolbox for assessing protein location and function, Science 312 (2006) 217–224.
[87] S.R. Adams, R.Y. Tsien, Preparation of the membrane-permeant biarsenicals, FlAsH-EDT_2 and ReAsH-EDT_2 for fluorescent labelling of tetracysteine-tagged proteins, Nat. Protoc. 3 (2008) 1527–1534.
[88] J. Nakanishi, T. Takarada, S. Yunoki, Y. Kikuchi, M. Maeda, FRET-based monitoring of conformational change of the β2-adrenergic receptor in living cells, Biochem. Biophys. Res. Commun. 343 (2006) 1191–1196.
[89] N. Ziegler, J. Bätz, U. Zabel, M.J. Lohse, C. Hoffmann, FRET based sensors for the human M_1-, M_3- and M_5-acetylcholine receptors, Bioorg. Med. Chem. 19 (2011) 1048–1054.
[90] M. Maier-Peuschel, N. Frölich, C. Dees, L.G. Hommers, C. Hoffmann, V.O. Nikolaev, M.J. Lohse, A fluorescence resonance energy transfer based M_2 muscarinic receptor sensor reveals rapid kinetics of allosteric modulation, J. Biol. Chem. 285 (2010) 8793–8800.
[91] T.-R. Xu, R.J. Ward, J.D. Pediani, G. Milligan, Intramolecular fluorescence resonance energy transfer (FRET) sensors of the orexin OX_1 and OX_2 receptors identify slow kinetics of agonist activation, J. Biol. Chem. 287 (2012) 14937–14949.
[92] T. Abe, H. Sugihara, H. Nawa, R. Shigemoto, N. Mizuno, S. Nakanish, Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction, J. Biol. Chem. 267 (1992) 13361–13368.
[93] A. Christopoulos, T. Kenakin, G Protein-coupled receptor allosterism and complexing, Pharmacol. Rev. 54 (2002) 323–374.
[94] G. Milligan, N.J. Smith, Allosteric modulation of heterodimeric G-protein coupled receptors, Trends Pharmacol. Sci. 28 (2007) 615–620.
[95] N.J. Smith, G. Milligan, Allostery at G protein-coupled receptor homo and heteromers: uncharted pharmacological landscapes, Pharmacol. Rev. 62 (2010) 701–725.
[96] T. Kenakin, L.J. Miller, Seven transmembrane receptors as shapeshifting proteins: The impact of allosteric modulation and functional selectivity on new drug discovery, Pharmacol. Rev. 62 (2010) 265–304.
[97] L. Albizu, M. Cottet, M. Kralikova, S. Stove, R. Seyer, I. Brabet, T. Roux, H. Bazin, E. Bourrier, L. lamarque, C. Breton, M.-L. Rives, A. Newman, J. Javitch, E. Trinquet, M. Manning, J.-P. Pin, B. Mouillac, T. Durroux, Time-resolved FRET between GPCR ligands reveals oligomers in native tissues, Nat. Chem. Biol. 6 (2010) 587–594.
[98] M. Cottet, L. Albizu, L. Comps-Agrar, E. Trinquet, J.-P. Pin, B. Mouillac, T. Durroux, Time resolved FRET strategy with fluorescent ligands to analyse receptor interactions in native tissues: application to GPCR oligomerisation, Methods Mol. Biol. 746 (2011) 373–387.