Time-resolved fluorescence ligand binding for G protein–coupled receptors

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G protein–coupled receptors (GPCRs) and their ligands are traditionally characterized by radioligand-binding experiments. These experiments yield excellent quantitative data, but have low temporal and spatial resolution. In addition, the use of radioligands presents safety concerns. Here we provide a general procedure for an alternative approach with high temporal and spatial resolution, based on Tb^+-labeled fluorescent receptor ligands and time-resolved fluorescence resonance energy transfer (TR-FRET). This protocol and its design are detailed here for the parathyroid hormone receptor, a class B GPCR, and its fluorescently labeled 34-amino acid peptide ligand, but it can be easily modified for other receptors and their appropriately labeled ligands. We discuss three protocol options that use Tb^+-labeled fluorescent ligands: a time-resolved fluorescence separation option that works on native receptors but requires separation of bound and unbound ligand; a TR-FRET option using SNAP-tag-labeled receptors for high-throughput screening; and a TR-FRET option that uses fluorescently labeled antibodies directed against an epitope engineered into the Flag-labeled receptors' N terminus. These protocol options can be used as standard procedures with very high signal-to-background ratios in order to characterize ligands and their receptors in living cells and in cell membranes via straightforward plate-reader measurements.

INTRODUCTION

GPCRs regulate numerous physiological and pathophysiological processes. Two key properties make them prime targets for drug treatment: their ability to control both cellular and organ functions and the fact that the binding sites for their natural ligands are generally very specific. Consequently, a large fraction of existing drugs, as well as of the current drug developments, are focused on GPCRs[1–3]. On the basis of their structural properties, GPCRs are usually divided into different subfamilies[4,5]. The largest and functionally most important one is represented by the class A or rhodopsin-like receptors, and most existing drug targets belong to this family[3]. The class B or secretin receptor family comprises a small group of receptors characterized by large N termini involved in ligand binding[6], whereas the similarly small class C or metabotropic glutamate receptor family also bears large N-terminal structures, the ligand-binding venus flytrap domains, which act as obligatory GPCR dimers[7].

The traditional technique for characterizing GPCRs and for searching for new ligands is radioligand binding[6,8–11]. These assays usually involve the incubation of receptor-containing cells or cell membranes with a radioactively labeled specific ligand, followed by separation of bound and free radioligand and subsequent quantification of the bound radioactivity. For most GPCRs, these assays are quite reliable and sensitive, and are thus suitable for high-throughput screening. In addition, radioligands not only can be used as simple quantitative assays with membrane preparations, but also can serve to localize receptors in tissues and organs with the help of autoradiography[12–15]. However, radioligand-binding assays have two major drawbacks. First, the use of radioactive compounds entails a series of safety and disposal concerns and precautions. Second, these assays have only limited spatial and temporal resolution. The spatial resolution is limited by the techniques to localize a radioactive emitter and depend both on the type of emission and on the detection method. The temporal resolution is limited by the fact that these assays represent endpoint determinations and that, therefore, each time point requires a separate sample. To overcome these drawbacks, nonradioactive assays using fluorescent methods have gained increasing importance. The availability of labels with good fluorescent properties and novel labeling strategies have greatly advanced the use of fluorescence techniques in GPCR research[16].

A key development in obtaining good signal-to-noise ratios in fluorescence assays was the development of techniques using rare-earth lanthanides together with appropriate organic cages[17]. Their fluorescence emission half-lives are much longer than those of background fluorescence, and by using time-resolved (TR) fluorescence measurements they offer the possibility of detecting fluorescence at time points when background fluorescence has largely disappeared. The lanthanides can serve as donor fluorophores in FRET measurements, in which they transmit their excitation energy to a nearby acceptor via radiation-less Förster energy transfer[18,19]. Compared with conventional FRET techniques, TR-FRET has the advantage of much lower background. This usually allows one to carry out these assays without separation steps, and hence they have been termed homogeneous time-resolved fluorescence (HTRF) assays[18,20,21].

Fluorescent ligands can be used for receptor-binding assays in two major ways. The first approach is analogous to radioligand-binding assays and involves a binding step followed by separation of bound and free fluorescent ligand and subsequent quantification of the bound ligand. The second method is based on FRET, especially on TR-FRET using lanthanide labels, and is homogeneous, i.e., it does not require a separation step (HTRF). For both approaches, the generation of fluorescent ligands and ligand-binding studies have been described for a large number of receptors, including the α_1- and β-adrenergic, angiotensin-II, CXCR1 and 2 chemokine,

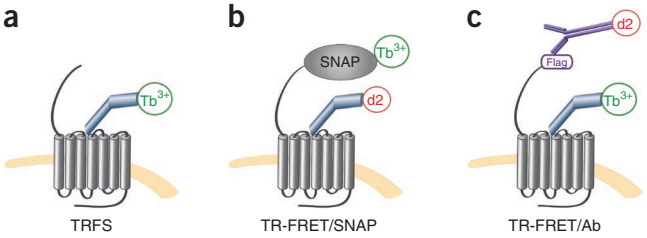

Figure 1 | Illustration of time-resolved ligand-binding protocol formats for PTHRs. (**a**) TRFS protocol. Binding of Tb^{3+}-cryptate-labeled PTH (green and blue) to the PTHR is detected by direct Tb^{3+} fluorescence (gray). (**b**) TR-FRET/SNAP. Tb^{3+}-cryptate-labeled SNAP-PTHR (green and gray) and d2-labeled PTH (red and blue) generate TR-FRET upon ligand binding. (**c**) TR-FRET/Ab. TR-FRET is accomplished by the binding of Tb^{3+}-cryptate-labeled PTH (green and blue) to the PTHR and d2-labeled anti-Flag antibodies (red and magenta) directed to the PTHR.

cholecystokinin, dopamine-D_2, γ-aminobutyrate$_B$, growth hormone secretagogue receptor type 1a, melanin-concentrating hormone, melanocortin-4, neurotensin 1, neurokinin, oxytocin, δ-opioid, and vasopressin V_{1a} receptors, as well as for non-GPCR receptors such as the epidermal growth factor and tumor necrosis factor-α type 1 receptors[20,22–32].

To be useful for receptor characterization, as well as for high-throughput screening, binding assays need to have high signal-to-noise ratios. This is a key advantage of FRET-based assays, because they require the use of and close proximity to two labels, which enhance specificity. HTRF approaches with their particularly high signal-to-noise ratio have been described for a series of different GPCRs and other receptor classes[20,22–28,30–33]. Although FRET- and TR-FRET ligand-binding assays have been described for a large number of GPCRs, there is so far no optimized and standardized protocol for TR-FRET ligand/receptor binding. Therefore, we have set out to develop and describe such a protocol. In this protocol, we describe optimized procedures for three formats of TR fluorescence ligand-binding protocols: (i) the 'traditional' approach that involves separation of bound and free ligand as in radioligand binding, referred to here as the time-resolved fluorescence separation (TRFS) assay (**Fig. 1a**); plus the new options (ii) TR-FRET, based on a SNAP-tag-labeled receptor (**Fig. 1b**); and (iii) TR-FRET, based on an antibody-labeled receptor (**Fig. 1c**). These formats may be used for kinetic, saturation and competition binding assays.

Furthermore, we briefly summarize various strategies to prepare cells for such assays (**Box 1**) and explain a set of general rules regarding the development and use of receptor-binding protocols (**Box 2**). The assays can be carried out with different types of materials, most notably intact cells, but they can also be carried out on frozen cells and cell membranes (**Box 3**).

Development of the method

This protocol is based on three key elements: (i) techniques to measure fluorescence and, in particular, TR-FRET; (ii) techniques to generate and characterize functionally intact fluorescent ligands

Box 1 | Sample preparation

Samples containing the receptor of interest can be obtained by various means. Endogenous material (i.e., from human or animal cells or tissues) can generally only be investigated with the TRFS format (Step 5A; **Fig. 1a**). Optimal specificity and signal-to-noise ratio are achieved by the introduction of a second label in TR-FRET formats, which generally require recombinant, N-terminally modified receptors—either bearing a direct label (Step 5B; **Fig. 1b**) or an epitope for a labeled antibody (Step 5C; **Fig. 1c**). For these TR-FRET formats, recombinant receptors are expressed either transiently or stably in transfected cells.

Cell transfection is performed using standard protocols:

- A *standard transient transfection* procedure for HEK293 cells can be performed with 500,000 cells per 3.5-cm-diameter dish in 2 ml of DMEM/FCS medium, using 0.5 µg of plasmid DNA (e.g., pcDNA3 coding for the respective receptor; it should be endotoxin-free for best expression) and a transfection reagent such as Effectene, used according to the manufacturer's protocols.
- An *accelerated transient transfection* procedure can be done directly in the multiwell plates. For each well of a coated 96-well plate (Step 1 of the PROCEDURE), use 50,000 cells, 50 µl of medium and 200 ng of plasmid DNA as described above.
- *Batchwise transfection* is an alternative for high-throughput applications. Cells are transfected as in the standard protocol, but in 10-cm-diameter or larger dishes, by appropriately adjusting volumes and amounts of reagents. This also allows *batchwise labeling* of receptors in Step 5B(i–iii). To do so, label with 100 nM benzylguanine-Tb^{3+}-cryptate in Tag-lite labeling medium for 1 h at 37 °C under 5% CO_2. Remove the medium and wash the dish four times in Tag-lite medium. Cells may then be split into 384-well culture plates (5,000 cells per well). Cells or membranes may also be stored frozen at this stage for later use. See **Box 3** for use of living cells versus cell membranes.

Samples should usually be measured in triplicate. The following controls should be included:

(A) Non-expressing cells (i.e., mock-transfected cells when recombinant receptors are used, or cells not expressing the receptor of interest when endogenously expressed receptors are studied)

(B) For each concentration of labeled ligand

(i) Total binding (no competing ligand)

(ii) Nonspecific binding (in the presence of a saturating concentration (i.e., >100-fold K_i value) of an unrelated competing ligand).

The values measured for nonspecific binding (ii) should correspond to the values of control samples (A). Specific binding is calculated as total minus nonspecific binding (i–ii), and it should be much higher than the nonspecific binding. Non-expressing cells can also be used to ascertain the efficiency of receptor labeling (Step 5B(iv)); in these cases, the fluorescent signal from transfected cells should be much larger than that from nontransfected cells. Samples should be arranged on microtiter plates such that triplicates of these controls are measured at the beginning and at the end of each plate.

Box 2 | Designing ligand-binding experiments

The design of ligand-binding experiments should follow a strict routine in order to determine the experimental parameters:
• *Incubation time*: Usually it is advisable to achieve equilibrium, which may take from a few minutes to many hours depending on the off-rate of the ligand. Note that the time to reach equilibrium depends on the temperature and the ligand concentration. Start with kinetic experiments and chose assay times well above the attainment of equilibrium.
• *Receptor/ligand concentrations*: Only a small fraction of labeled ligand should be bound to avoid ligand depletion. Ligand depletion means that the free ligand concentration is no longer (roughly) equivalent to the total ligand concentration, because so much is bound, and it results in skewing of saturation or competition curves unless appropriate corrections are made. Change the number of receptors (i.e., cells or protein amounts or expression levels), which should proportionately change the amount of bound ligand. Adjust the ligand and receptor concentrations such that no more than 10–20% of the ligands are bound.
• *Ligand affinity*: By using the incubation times and receptor amounts determined above, generate a receptor saturation curve by using various concentrations of labeled ligand in order to determine its affinity (K_d value). If the affinity turns out to be higher than that expected and, therefore, lower ligand concentrations need to be used, kinetic experiments may need to be repeated to assure that equilibrium is achieved also at lower ligand concentrations.
• *Competition experiments*: Affinities (K_i values) of unlabeled ligands are determined in competition experiments using a labeled ligand concentration well below its K_d value. Competitor concentration intervals should reach 2–3 orders of magnitude below and above the predicted K_i value of the competing ligand using at least half-log dilutions. Note that the competing ligand may take longer to equilibrate than the labeled ligand.

and GPCRs; and (iii) the assays to actually measure the binding of fluorescently labeled ligands to GPCRs, which are either also fluorescently labeled or in their native form. The protocol that we describe here concerns largely the last element.

Techniques for measuring FRET in general have been described in several recent reviews, including the physical background and the details of the methodology[34–36]. In brief, FRET requires two fluorophores, where the emission spectrum of one (the donor) overlaps with the excitation spectrum of the other (the acceptor). If the two are in close proximity (usually <10 nm), excitation of the donor can result in emission by the acceptor. This emission of the acceptor, as well as the reduced emission of the donor, can be measured to give a FRET ratio. FRET also reduces the lifetime of the donor emission, and measurement of this lifetime is another, albeit technically more demanding, way to measure FRET. The techniques for measuring FRET will be described here only as far as is necessary for the protocol itself.

Generation of a fluorescent ligand often requires its *de novo* synthesis[37–40]. In the case of larger ligands containing reactive functional groups, it may also be possible to attach a fluorescent label via specific coupling chemistry[41]. The generation of fluorescent ligands is beyond the scope of this review. It is important to characterize the receptor-binding properties of such fluorescent ligands and to ascertain their affinity and specificity for the receptor of interest[37,39,42]. The use of europium and terbium cryptates as fluorescent labels for TR-FRET studies has allowed the development of TR-FRET systems for the study of GPCRs[43,44]. For FRET studies, one label is usually attached to the ligand, whereas a second label is placed in the receptor, so that FRET occurs only when the ligand and receptor are in close proximity (i.e., upon binding). A variety of techniques exist to fluorescently label receptors. In addition to chemical labeling, which generally requires a purified receptor[45], fusion with fluorescent proteins, insertion of binding sequences for labels such as the fluorescent arsenical hairpin binder FlAsH, insertion of a recognition motif for a fluorescently labeled antibody, or fusion with a protein that can be used for autocatalytic labeling with a fluorophore have all been used with GPCRs[21,46–48]. The necessity of using a good fluorophore for a good signal-to-noise ratio has made fluorescently labeled antibodies and fusions with DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT)[49], commercially available as SNAP-tag, particularly popular. Several reports on a number of GPCRs have used combinations of receptors labeled via an N-terminal SNAP-tag together with labeled ligands to investigate receptor binding[22–24] and oligomerization[48,50].

Box 3 | Binding experiments on living cells versus cell membranes

Binding assays to GPCRs can be done using living cells and cell membranes. Each of the two preparations has advantages and disadvantages:
• *Living cells*:
Advantages: affinities correspond to *in vivo* situation (i.e., agonists often have lower affinities than in cell membranes).
Disadvantages: nonspecific binding may be higher; receptors (and their ligands) may internalize and thereby affect apparent binding; ligand affinities may change over time owing to regulatory processes (e.g., phosphorylation); and living cells cannot be stored well.
• *Cell membranes*:
Advantages: agonist affinities are often higher (owing to the formation of a high-affinity ternary complex of agonist, receptor and G protein in the absence of guanine nucleotides); freezing and storage are usually possible; and batchwise preparation is possible.
Disadvantages: ligand affinities may deviate from physiological situation; ligands and other compounds may exert effects at intracellular sites.

To ensure that these assays truly reflect the respective receptor's binding properties, these assays need to be validated along the lines that were established two to three decades ago for radioligand binding[51–54]. This requires not only the determination of specificity but also the study of binding kinetics, ligand affinity in saturation experiments and competition assays with a series of ligands. In **Box 2**, we present a set of rules to follow in setting up such receptor-binding experiments, and our optimized procedure has been developed along these lines.

The protocol described here can be easily adapted to any other ligand/receptor pair to which fluorescent labels can be attached. We describe it here for one specific ligand/receptor pair, parathyroid hormone (PTH) and its receptor, the PTH-receptor-1 (PTHR), with comments on where to pay attention when a new ligand/receptor pair is addressed.

PTH is an 84–amino acid peptide hormone that acts as a full agonist on PTHR and thereby causes stimulation of phospholipase C-β and adenylyl cyclase, and ultimately affects calcium and phosphate reuptake in the kidney, bone metabolism and calcium homeostasis[55,56]. We use a shorter, 34–amino acid derivative (in full: human [Nle^{8,18}, Y^{34}]PTH(1–34)) that is used by most researchers as a full agonist for this receptor[56,57], and we refer to this analog as PTH′(1–34).

The PTHR is a member of the small GPCR class B, which is characterized by a large extracellular N terminus that participates in ligand binding. Crystal structures of PTH(1–34) alone and bound to the isolated PTHR N terminus[58,59], as well as structure-function studies, indicate sites where PTH can be labeled without compromising its receptor binding; these sites are positions 13 and 35 (i.e., the C terminus)[46,60]. Identification of such 'neutral' sites in a ligand is important in order not to negatively affect the binding of the resulting fluorescent ligand. As PTH(1–34) is a cysteine-free peptide, site-specific labeling can be achieved by replacing amino acid 13 with cysteine or by attaching a cysteine 35 at the C terminus, respectively, in the peptide synthesis. These derivatives containing a single cysteine residue are then labeled with a cysteine-reactive fluorophore.

Experimental design

To illustrate the whole spectrum of options of the protocol, we generated four variants of labeled PTH′(1–34) by using two attachment sites in PTH′(1–34), cysteine 15 or cysteine 35, and two fluorophores, Tb^{3+}-cryptate (Lumi4-Tb, Cisbio Bioassays) or d2 (a near-infrared proprietary fluorophore; Cisbio Bioassays)[61]. Tb^{3+}-cryptate (donor) is usually excited at 340 nm and has multiple emission peaks, notably at 490 nm, 545 nm (maximum), 583 nm and 620 nm (ref. 62). In FRET, terbium or europium serve as donors, owing to their extended emission times, and several of the emission peaks may be used. This enables the use of multiple acceptor fluorophores. In the TR-FRET protocol described here, the three emission peaks at 545, 583 and 620 nm transfer energy to the d2 acceptor, which shows an emission peak at 655 nm. Ratiometric determination of the 620-nm donor emission and the 655-nm acceptor emission can then be used to measure FRET (other donor emission peaks may similarly be used as internal controls, but may suffer from more different absorbance by the sample compared with the acceptor emission). The PTH variants containing the respective cysteines were generated by peptide synthesis, and the labels were attached to these cysteines by the use of maleimide-activated

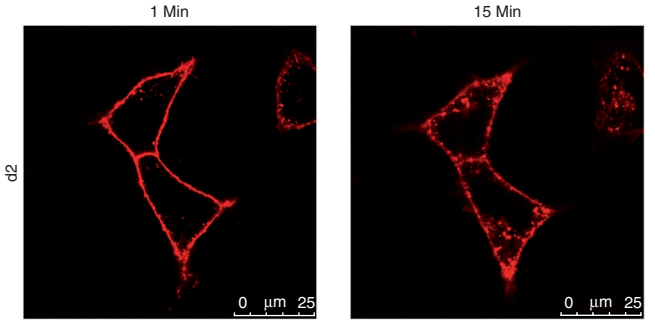

Figure 2 | Confocal imaging of [C^{13}-d2]PTH′(1–34) on living cells. HEK293A cells were transiently transfected with PTHR according to TRFS protocols. Cells were plated 24 h after transfection on 24-mm coverslips and grown for 48 h. A concentration of 100 nM [C^{13}-d2]PTH′(1–34) was diluted in assay buffer containing 0.2% (wt/vol) nonfat milk. Cells were incubated with 100 nM [C^{13}-d2]PTH′(1–34) for the indicated time intervals. Confocal images were taken with a Leica SP5 laser-scanning confocal microscope.

Tb^{3+}-cryptate or d2, respectively. As an alternative, Alexa Fluor 488 has also been used as an acceptor for TR-FRET experiments together with terbium chelate[21].

To verify that the fluorescent labels did not impair the interaction with the PTHR, all these ligands were characterized by competition binding (to determine their affinity) and cAMP signaling (to determine their efficacy, i.e., their ability to activate the receptors) assays. They were all found to have preserved affinities and to bind normally to the PTHR (see below), as well as to induce full receptor activation as measured by generation of cAMP (**Supplementary Fig. 1a**) and receptor internalization (**Fig. 2**). Although complete preservation of agonist activity is not a prerequisite for the use of a fluorescently labeled ligand in this protocol, maintained receptor binding is important to be sure that the relevant receptor is indeed investigated.

The first format of our protocol, the TRFS procedure (**Fig. 1a**), relies on fluorescently labeled ligand alone. Even though any fluorescent label can, in principle, be used for direct binding assays, much better signal-to-noise ratios can be obtained with TR fluorescence measurements based on Tb^{3+}-cryptate-labeled ligands. In classic ligand-binding assays, less than 10(−20)% of the added ligand should be bound to the receptors in order to avoid artifacts caused by ligand depletion (**Box 2**). Regardless of the type of label used, the remaining unbound ligand causes a substantial background signal, which means that it usually needs to be removed from the sample by filtration, washout or similar procedures. In general, washing procedures require slow dissociation rates (k_{off}) relative to washing time, so that little specifically bound ligand is lost. In the case of PTH, the dissociation from the receptor is known to be very slow, and, consequently, washing steps do not need to be rapid (**Supplementary Fig. 1b**)[63,64]. Therefore, we used simple washing of adherent living cells in the TRFS procedure. To be close to a physiological setting, we further chose cell-based assays for our ligand-binding protocol, but the assays also work with cell membranes (**Box 3**). GPCRs are known to have often quite different affinities in membranes compared with intact cells[65]. To avoid the separation step and to be able to perform the assays in a homogeneous format, the unbound ligand should not produce a signal. This can be achieved by using FRET between the ligand and the receptor, because then only the bound ligand produces a

signal. Again, the best signal-to-noise ratio can be expected using TR-FRET. In principle, TR-FRET can be measured with the donor label attached to the receptor and the acceptor label on the ligand, or vice versa.

Two strategies are described to label the receptors with Tb^{3+}-cryptate. The first uses an N-terminal AGT fusion (SNAP-tag, Tag-lite Technology, Cisbio Bioassays; **Fig. 1b**), whereas the second uses a short epitope (usually a Flag or hemagglutinin (HA) epitope) engineered into the receptor's N terminus plus a Tb^{3+}-cryptate-labeled antibody against this epitope (**Fig. 1c**). We describe the option here for a d2-labeled antibody and a Tb^{3+}-labeled PTH. A reverse arrangement of the labels is also possible, but it might result in different FRET efficacies. Both approaches, SNAP-tag and the antibody tag, thus require recombinant receptors, which carry either a small (epitope) or a larger (SNAP-tag) modification. They also both require a labeling step, which can be carried out directly before each experiment or batchwise; in the latter case, when you are using the SNAP-tag modification, the labeled receptors can be stored frozen and used later.

Specifically, it has been shown for the PTHR that fusion with large fluorescent proteins at the extracellular N terminus does not substantially impair ligand-receptor binding[46]. A SNAP-tag can therefore be fused to the very N terminus of the PTHR for labeling with O^6-benzylguanine-Tb^{3+}-cryptate, which serves as the donor for FRET. The ligand can be labeled with the acceptor d2 (we used [C^{13}-d2]PTH′(1–34) or [C^{35}-d2]PTH′(1–34)). TR-FRET between the donor Tb^{3+}-cryptate and the acceptor d2 then occurs upon ligand binding and obviates the need for a separation step to remove unbound ligand.

Alternatively, a Flag-epitope can be inserted into the exon E2, which is known to be dispensable for ligand binding[66]. d2-labeled antibodies directed against this Flag epitope then served as the acceptor fluorophore, whereas PTH′(1–34) labeled with Tb^{3+}-cryptate (designated as Tb^{3+}) at C^{13} or C^{35} was the donor, again in a separation-free TR-FRET (HTRF) format.

TR-FRET is determined by ratiometric measurements of emissions at 620 and 655 nm (see above). Ratiometric measurements allow for compensation of well-to-well variability and variability of receptor density, as well as quenching of the fluorescence by assay components. Levels of donor (Tb^{3+}-cryptate) and acceptor (e.g., [C^{13}-d2]PTH′(1–34)) fluorophores can be controlled by direct excitation in separate control measurements. The necessary corrections of fluorescence signals, as well as the caveats of ratiometric FRET assays, have been recently described[36].

Applications and comparison with alternative approaches

We show here—using the PTHR as an example—that these protocols allow for a full characterization of a receptor, i.e., that they can be used for kinetic, as well as equilibrium-saturation and competition binding assays. Data for competition binding curves are shown here for several well-studied peptide ligands, i.e., PTH(1–34), PTH′(3–34) amide and PTHrP(7–34); however, they can obviously be used for any competing compound.

These TR-FRET protocols can be applied to any modified GPCR that is recombinantly expressed in cells or, for example, transgenic animals. Provided that the modification (i.e., the N-terminal fusion with an SNAP-tag or with an antibody epitope) does not affect the receptors' properties, the TR-FRET formats allow suitable signal-to-noise ratios together with the convenience of a homogenous, separation-free assay, which is clearly superior for high-throughput screening. In contrast, TRFS protocols are less-suited for high-throughput screening because of the need to separate bound and unbound ligands. However, the major advantage of TRFS protocols is the possible application to native GPCRs in cells and tissues.

In general, fluorescently labeled ligands for GPCRs provide an array of options for use besides those described here (reviewed by Daly and McGrath[42] and McGrath *et al.*[67]). Specific GPCR fluorescent ligands have been synthesized for direct binding assays (e.g., refs. 37,38), as well as for imaging approaches of GPCR localization[68]. The fluorescence of the Tb^{3+}-cryptate fluorophores can also be used in imaging experiments, but the low excitation wavelengths interfere with glass optics of imaging setups and time-gating is necessary to make full use of the long-lived emission. Furthermore, confocal imaging using two-photon laser excitation of [Tb^{3+}-cryptate]-labeled ligands is limited by their relatively weak two-photon excitation at 740 nm. Time-resolved wide-field microscopy offers lower resolution and similar background as confocal microscopy with organic fluorophores. Thus, ligands labeled with d2 as described in this protocol or with other conventional fluorophores are generally preferable for imaging (**Fig. 2**). However, it should be noted that [Tb^{3+}-cryptate]-labeled ligands can be used for microscopic imaging, and this may be advantageous if the same ligand is to be used in imaging and plate-reader experiments.

However, compared with conventional fluorophores, lanthanides present high fluorescence intensities, no orientation bias and minimized autofluorescence background because of TR emissions, making them excellent for assays using plate readers. Furthermore, lanthanide cryptates are very stable. Examples include Tris-bipyridine Eu^{3+}-cryptate or Tb^{3+}-cryptate (Lumi4-Tb^{3+}), which are convenient for TR-FRET assays. These coordination complexes improve the spectral properties of lanthanides and enable coupling reactions for labeling of peptides[43,44]. Moreover, lanthanide complexes possess large Stokes shifts and multiple emission peaks. For the lanthanide terbium (Tb), these multiple emission peaks allow direct measurement of fluorescence emission at 620 nm and via TR-FRET measurement of red or green acceptor emissions.

As an alternative to the protocol described here, fluorescent ligands have also been developed for other types of ligand-binding assays. In fluorescence polarization assays, the rotational speed of ligand-bound fluorophores is reduced upon binding to the receptor, resulting in more polarized emission after a short excitation pulse, which can be measured[39,69,70]. This technique works best with tagged ligands that are small compared to the receptors (i.e., up to 5 kDa), it usually requires good expression of the receptors (above 1 pmol mg^{-1} (ref. 71)), and it is sensitive to nonspecific binding and fluorescent background[70]. Fluorescence correlation spectroscopy is similarly based on the higher mobility of unbound versus bound ligand, and it has been used for several GPCRs[72]; it has also been used successfully for binding studies with lipophilic ligands[73]. The analysis of lifetime changes occurring in FRET (i.e., the shortening of the donor fluorescence lifetime) has also recently been adapted to a multiwell high-throughput format[74]. Finally, fluorescent ligands of sufficient specificity and low nonspecific binding can also be used in flow cytometry[40,75]. Compared with these techniques, TR-FRET assays are usually easier to use and thus have been established for a large number of GPCRs and other receptors as enumerated above[20,22–28,30–33].

MATERIALS

REAGENTS

Plasmids and labeling reagents

- Mammalian expression plasmid encoding Flag-PTHR1 (i.e., full-length human PTHR1 (PTHR) with the Flag epitope DYKDDDDA replacing the endogenous sequence 94–101 (a site often used for insertion of a HA-epitope YPYDVPDYA to allow receptor identification with antibodies[76])), as well as wild-type and HA-tagged receptors, are available from our laboratory upon request. These receptors are used here as model GPCRs, and the protocol can, in principle, be used for any other analogous GPCR construct
- Plasmid encoding human PTHR1 with an N-terminal AGT-tag (SNAP-PTHR) is available from Cisbio Bioassays (product code PSNAPPTH1); again, the protocol can be equally used with other similarly labeled GPCRs
- EndoFree plasmid maxi kit (Qiagen, cat. no. 12362)
- O^6-benzylguanine-Tb^{3+}-cryptate (Tag-lite SNAP-Lumi4-Tb, product reference: SSNPTBC), anti-Flag antibodies labeled with the red fluorophore d2 and Tag-lite labeling medium (Product reference: LABMED) are available from Cisbio Bioassays ▲ **CRITICAL** Cisbio Bioassays is the only commercial supplier of Lumi4-Tb and Tag-lite reagents.

Chemicals and reagents

- NaCl (Applichem, cat. no. A4256)
- $MgCl_2$ hexahydrate (Applichem, cat. no. A4425)
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Applichem, cat. no. A3724)
- NP-40 (nonylphenoxypolyethoxyethanol; Applichem, cat. no. A1694)
- Na-deoxycholate (Applichem, cat. no. A1531)
- SDS (Applichem, cat. no. A1502)
- DMSO (Sigma-Aldrich, cat. no. D8418) ! **CAUTION** DMSO is irritating to the eyes, respiratory system and skin; wear gloves and eye/skin protection.

Cells, cell culture media and reagents

- Human embryonic kidney cells (HEK293A cells, Invitrogen, cat. no. R705-07) Human embryonic kidney cells (HEK293T cells, Invitrogen, cat. no. R700-07)
- DMEM (Gibco/Invitrogen, cat. no. 10313-021)
- Penicillin/streptomycin (100× solution; Gibco/Invitrogen, cat. no. 15140-122)
- L-Glutamine, 200 mM (Gibco/Invitrogen, cat. no. 25030-081)
- FCS (Gibco/Invitrogen, cat. no. 16000-044)
- Trypsin-EDTA solution (Gibco/Invitrogen, cat. no. R-001-100)
- Transfection reagent, Effectene (Qiagen, cat. no. 301425)
- Transfection reagent, Lipofectamine (Invitrogen, cat. no. 11668-019)
- Poly-D-lysine (Sigma-Aldrich, cat. no. P0296)

Ligands (PTH variants)

- PTH variants human [$Nle^{8,18}$, Y^{34}]PTH′(1–34), subsequently termed PTH(1–34), and its variants for cysteine-directed labeling (i.e., human [$Nle^{8,18}$, C^{13}, Y^{34}]PTH(1–34), which is shortened to [C^{13}]PTH′(1–34), and human [$Nle^{8,18}$, Y^{34}, C^{35}]PTH(1–34), which is shortened to [C^{35}]PTH′(1–34)) were custom-synthesized by Peptide Specialty Laboratories. Again, for other GPCRs, similarly modified ligands will have to be synthesized in order to generate fluorescently labeled ligands
- Fluorescently labeled ligands were generated from the above-mentioned Cys-derivatized PTH variants by reacting with maleimide-based cysteine-reactive dyes: Tb^{3+}-cryptate-labeled or d2-labeled [C^{13}]PTH′(1–34) or [C^{35}]PTH′(1–34), respectively. These ligands, namely [C^{13}-Tb^{3+}]PTH′(1–34), [C^{35}-Tb^{3+}]PTH′(1–34), [C^{13}-d2]PTH′(1–34) and [C^{35}-d2]PTH′(1–34), are available upon request from Cisbio Bioassays. Analogous labeled ligands may be generated for other GPCRs
- Human PTH(1–34), wild-type (PTH(1–34)wt) (Tocris Bioscience, cat. no. 3011)
- Bovine [$Nle^{8,18}$, Y^{34}]PTH(3–34) amide (PTH′(3–34) amide), and human PTHrP(7–34) amide (Polypeptide Laboratories, cat. no. SC888). Again, for other GPCRs, these ligands will be substituted by appropriate specific ligands

EQUIPMENT

- EnVision Xcite multilabel reader (PerkinElmer, cat. no. 2104-0020); other plate readers capable of measuring TR-FRET may be used as alternatives
- Excitation filter UV2 (TRF), 340 nm (PerkinElmer, cat. no. 2100-5010, barcode 101)
- Emission filters (PerkinElmer): Terbium, 545 nm (cat. no. 2100-5070); APC, 665 nm (cat. no. 2100-5110, bar code 205); and Cy5, 620 nm (cat. no. 2100-5240, bar code 118)
- Mirror module D400 (PerkinElmer, cat. no. 2100-4170, bar code 412)
- Black-bottom culture plates, 96 wells (PerkinElmer, cat. no. 6005660)
- Six-well culture plates (Nunc, cat. no. 140675)

REAGENT SETUP

Cell culture medium DMEM should be supplemented with 10% (vol/vol) FCS, 2 mM (1% (vol/vol)) L-glutamine and 0.1 mg ml^{-1} (1% (vol/vol)) penicillin/streptomycin solutions before use. The medium can be stored for up to 4 weeks in a refrigerator (at 4 °C).

Assay buffer All assay formats can be conducted by using Tag-lite labeling medium. Store the Tag-lite labeling medium at 4 °C until reconstitution. Dilute Tag-lite labeling medium 1:5 with distilled water before use under a laminar air flow bench in order to prevent contamination. As an alternative to Tag-lite labeling medium, an assay buffer containing 20 mM HEPES, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2% (wt/vol) nonfat milk (pH 7.5) can be used. This nonfat milk–containing assay buffer should be freshly prepared each day.

Lysis buffer Prepare lysis buffer containing the following reagent concentrations: 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% (vol/vol) NP-40, 0.5% (wt/vol) Na-deoxycholate and 0.1% (wt/vol) SDS (pH 7.5). To do so, add NaCl, Tris and EDTA to double-distilled water; adjust the pH and then add NP-40 and Na-deoxycholate to the pH-adjusted solution. The buffer should be stored at 4 °C and can be used for up to 4 months.

Tag-lite SNAP-Lumi4-Tb (Tb^{3+}-cryptate) Before use, store the SNAP-tag substrates at −20 °C. Add the appropriate volume of DMSO to the vial containing Tag-lite SNAP-Lumi4-Tb (Tb^{3+}-cryptate) in order to obtain a 100 μM stock solution (see also the product information supplied with the kit). Carefully shake the vial until the compound is completely dissolved. ! **CAUTION** The compound is irritating to the eyes, respiratory system and skin; wear gloves and eye and skin protection. Stock solutions are stable for 6 months at −80 °C (avoid repeated freeze-and-thaw cycles). The stock solutions may be frozen and thawed once. Prepare a 100-nM Tag-lite SNAP-Lumi4-Tb (Tb^{3+}-cryptate) solution from the 100 μM stock solution by a 1:1,000 dilution in Tag-lite labeling medium.

EQUIPMENT SETUP

TR-FRET detection instrumentation An important characteristic of this protocol is that it does not require sophisticated instrumentation and can essentially be done with standard laboratory equipment. A plate reader or analogous instrument capable of TR-FRET detection is required for the readout of the protocol. Many such devices are available, and several have been certified for use with Tag-lite reagents. For TR-FRET detection in a strict high-throughput context, it is advisable to measure emissions of donor and acceptor simultaneously with an Envision HTS multilabel reader (PerkinElmer, cat. no. 2104-0010) and an optical module for dual emission (for laser excitation: PerkinElmer, cat. no. 2103-4290; for flash-lamp excitation: PerkinElmer, cat. no. 2100-4160). However, for the protocols presented here, a successive detection of the emissions at 620 and 655 nm using the Envision Xcite multilabel reader is sufficient.

PROCEDURE

Cell preparation ● TIMING 1 h

1| Coat a black-bottom 96-well culture plate with poly-D-lysine at 37 °C for 30 min, aspirate the poly-D-lysine solution and dry the wells under a laminar air flow bench. If you are unfamiliar with handling the cell line being used, prepare a clear-bottom 96-well plate as mentioned above in parallel in order to visually monitor cell confluence and viability.

2| Plate ~50,000 cells in 100 µl of medium per well of a coated black-bottom 96-well culture plate by using careful trypsin-EDTA digestion and fresh medium. Cells expressing the receptor of interest can be obtained by various means; in particular, cells may be transfected with receptor constructs that enable TR-FRET assay formats; these transfected cells can be used for the procedure 24 h after transfection (**Box 1**). Grow the cells in these plates for 24 h. As best expression of GPCRs is generally achieved 48 h after transfection, cells are usually left to grow for 24 h after plating, before harvesting or measurement.

Assay solutions ● TIMING 0.5–1 h

3| Prepare a 5× solution of the d2- or Tb^{3+}-cryptate-labeled ligand in assay buffer (see Step 5A–C for choice of labeled ligand). The final ligand concentration should usually be below the ligand's K_d value. The final ligand concentration is varied in saturation experiments. In all assays described here (with the exception of saturation assays), the final concentration of d2- or Tb^{3+}-cryptate-labeled PTH is 5 nM (i.e., the 5× solution is 25 nM). For details see **Box 2**.

4| Prepare 5× solutions of unlabeled competing ligands in assay buffer. The concentration of competing ligands is varied in competition experiments. It should span at least three orders of magnitude around the (presumed) K_I value of the ligands. To determine nonspecific binding, use a (final) concentration of competing ligand that is 2–3 orders of magnitude above its K_I value. To determine nonspecific binding of labeled PTH ligands, use 40 µM unlabeled PTH or, preferably, another unrelated ligand at saturating concentrations. For details see **Box 2**.

Binding assay ● TIMING 2–4 h

5| Three different options can be used for the binding assay proper (Experimental design): the classic reference assay involving separation of free and bound ligand, here referred to as TRFS, using only fluorescently labeled ligand and no second label (option A; **Fig. 1a**); TR-FRET, based on SNAP-tag-labeled receptor (TR-FRET/SNAP) (option B; **Fig. 1b**); and TR-FRET, based on antibody-labeled receptor (TR-FRET/Ab) (option C; **Fig. 1c**). The latter two options involve a fluorescent receptor label and a fluorescently labeled ligand, and they measure FRET between these two labels. These options represent separation-free, more-modern assay options. General considerations on how to develop a binding assay are described in **Box 2**.

(A) TRFS assay

(i) At 24 h after plating, carefully discard the culture medium either by simply decanting the 96-well plate and gently tapping it on paper tissue or by gentle aspiration.

(ii) Quickly after removing the medium, add 50 µl of assay buffer to each well by using a multichannel pipette.
▲ **CRITICAL STEP** To prevent cell loss, pipette the assay buffer gently into the wells.

(iii) Add 20 µl of 5× solutions of competing ligands prepared in Step 4 (for competition assays or nonspecific binding) or 20 µl of assay buffer.

(iv) Add 10 µl of 10× solutions of any other component that you might want to investigate or 10 µl of assay buffer (if no such additions are desired, simply increase the assay buffer in Step 5A(ii) to 60 µl).

(v) Add 20 µl of 5× solution of the labeled ligand prepared in Step 3 and incubate it under gentle shaking. The standard final concentration of Tb^{3+}-cryptate-labeled PTH ligands is 5 nM, and the standard incubation time is 4 h at 20 °C.

(vi) Terminate incubation by performing a separation step. Wash each well with 100 µl of assay buffer at room temperature (20 °C), and discard the supernatant by decanting and tapping the well plate on paper tissue. Repeat this step three times.
▲ **CRITICAL STEP** Be careful to avoid cell loss during the washing steps. Cell loss decreases the precision of TRFS measurements (**Fig. 3**).

(vii) Finally, add 50 µl of lysis buffer to each well and incubate the wells for at least 10 min at 20 °C before taking measurements.
■ **PAUSE POINT** Lysates may be stored overnight at 4 °C.

(viii) To measure the bound fluorescence signal, use the EnVision reader (PerkinElmer) or comparable equipment. An appropriate filter setup for TRFS consists of the excitation filter UV2 (TRF), 340 nm; the emission filter Terbium, 545 nm; and the mirror module D400. Choose scan mode (6 × 6 points per well) with an 0.8-mm point distance in order to improve accuracy of the measurements. Use one well to optimize measurement parameters (height, delay and integration time) for your equipment setup (e.g., by the optimization wizard of the EnVision Software). For TRFS using flashlight EnVision readers, the optimization of the measurement parameters may result in typical settings of 1.9-mm height, 500-µs delay and 1,400-µs integration time. For excitation with flashlight lamps, use 100 flashes and 100% excitation light. Compared with flashlight lamps, laser-equipped setups usually yield superior signal-to-noise ratios. For laser excitation, use 20 flashes per well.
? TROUBLESHOOTING

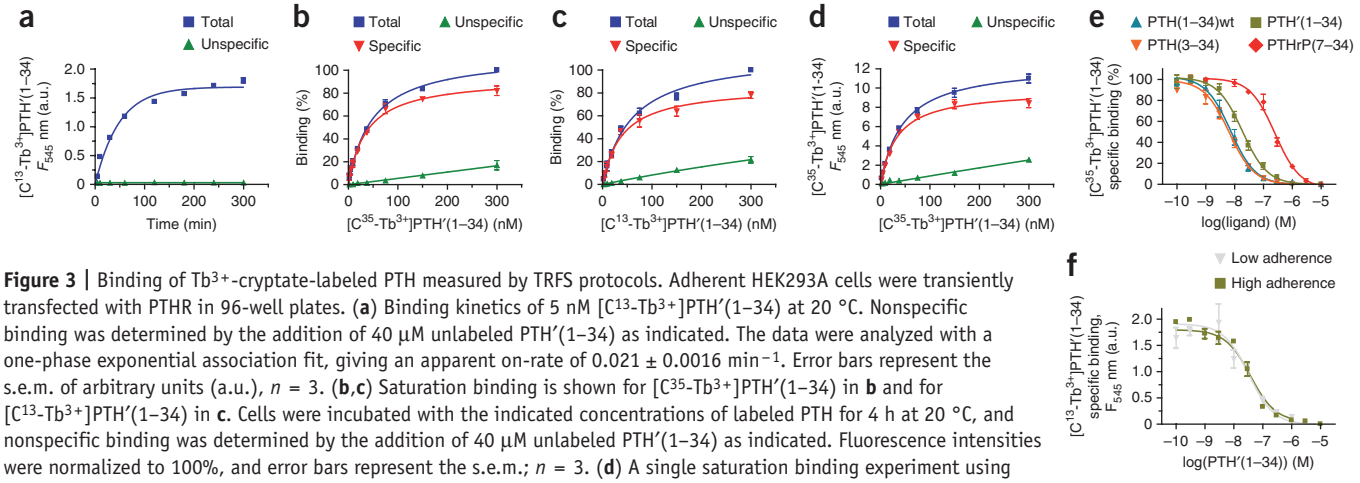

Figure 3 | Binding of Tb^{3+}-cryptate-labeled PTH measured by TRFS protocols. Adherent HEK293A cells were transiently transfected with PTHR in 96-well plates. (**a**) Binding kinetics of 5 nM [C^{13}-Tb^{3+}]PTH'(1–34) at 20 °C. Nonspecific binding was determined by the addition of 40 µM unlabeled PTH'(1–34) as indicated. The data were analyzed with a one-phase exponential association fit, giving an apparent on-rate of 0.021 ± 0.0016 min^{-1}. Error bars represent the s.e.m. of arbitrary units (a.u.), n = 3. (**b**,**c**) Saturation binding is shown for [C^{35}-Tb^{3+}]PTH'(1–34) in **b** and for [C^{13}-Tb^{3+}]PTH'(1–34) in **c**. Cells were incubated with the indicated concentrations of labeled PTH for 4 h at 20 °C, and nonspecific binding was determined by the addition of 40 µM unlabeled PTH'(1–34) as indicated. Fluorescence intensities were normalized to 100%, and error bars represent the s.e.m.; n = 3. (**d**) A single saturation binding experiment using [C^{35}-Tb^{3+}]PTH'(1–34) is shown to illustrate the intra-assay variability. Experimental procedure was conducted according to **c**. Fluorescence intensity is expressed as a.u. and error bars represent the s.e.m. of triplicates. (**e**) Competition binding experiments were carried out with 5 nM [C^{35}-Tb^{3+}]PTH'(1–34) and unlabeled PTH derivatives: PTH(1–34)wt, PTH'(3–34) amide and PTH'(1–34) or PTHrP(7–34) at the indicated concentrations. Cells were incubated for 4 h at 20 °C. Fluorescence intensities were normalized to 100% and error bars represent s.e.m.; n = 3–6. (**f**) Competition binding using PTH'(1–34) and 5 nM [C^{13}-Tb^{3+}]PTH'(1–34) illustrates intra-assay variability of experiments that achieved high cell adherence compared with low cell adherence. Experimental procedure was carried out according to **e**. Error bars represent s.e.m. of triplicates as a.u.

(B) TR-FRET assay using SNAP-tags (TR-FRET/SNAP)

(i) To label SNAP-tag-fused GPCRs (here: PTHR), remove the cell culture medium from 96-well plates at 48 h after transfection by decanting the 96-well plate and gently tapping it on paper tissue. These assays are usually robust enough to permit accelerated transfection protocols (**Box 1**) with measurements obtained as soon as 24 h after transfection.

(ii) Add 100 µl of Tag-lite labeling medium containing 100 nM benzylguanine-Tb^{3+}-cryptate per well. Incubate the mixture for 1 h at 37 °C under 5% CO_2.

▲ **CRITICAL STEP** To prevent cell loss, pipette the medium gently into the wells.

(iii) Remove excess Tb^{3+}-cryptate by removing the medium as described above and washing each well with 100 µl of Tag-lite labeling medium. Repeat this step four times. At the end, add 50 µl of assay buffer to each well with a multichannel pipette.

▲ **CRITICAL STEP** To prevent cell loss, pipette the medium gently into the wells.

(iv) Successful labeling may be verified by simultaneous labeling protocols using nontransfected control cells. Perform direct measurement of Tb^{3+}-cryptate by using the filter setups of TRFS protocol (see Step 5A(vii,viii)). Labeling efficiency can be estimated by the difference of fluorescence signal of samples using transfected and control cells (**Box 1**).

(v) Add 20 µl of 5× solutions of competing ligands prepared in Step 4 (for competition assays or nonspecific binding) or 20 µl of assay buffer.

(vi) Add 10 µl of 10× solutions of any other component that you might want to investigate or 10 µl of assay buffer (if no such additions are wanted, simply increase the assay buffer in Step 5B(iv) to 60 µl).

(vii) Add 20 µl of 5× solution of d2-labeled ligand (prepared in Step 3) and incubate it under gentle shaking. The standard final concentration of d2-labeled PTH ligands is 5 nM, and the standard incubation time is 4 h at 20 °C.

(viii) To measure the bound fluorescence signal by determining TR-FRET between the Tb^{3+}-cryptate-labeled receptor and the d2-labeled ligand, use the EnVision reader (PerkinElmer) equipped with a TR-FRET optical module, or comparable equipment. An appropriate filter setup for TR-FRET consists of the following: excitation filter UV2 (TRF), 340 nm; emission filters Cy5, 620 nm (donor), and APC, 665 nm (acceptor); and mirror module D400. Use one well to optimize measurement parameters (height, delay and window) for your equipment setup (e.g., by the optimization wizard). For TR-FRET/SNAP using laser-equipped EnVision readers, 50-µs delay, 400-µs integration time, 6.5-mm measurement height and 20 flashes per well represent an optimized set of measurement parameters. One point measurement or scan mode may be chosen.

? TROUBLESHOOTING

(C) TR-FRET assay using antibodies (TR-FRET/Ab)

(i) Prepare 10× solutions of d2-labeled anti-Flag antibodies. The amount of antibody must exceed the amount of expressed receptors (epitopes); a final antibody concentration of 1 nM should be largely sufficient to achieve saturation of receptors.

(ii) Remove the cell culture medium from 96-well plates 24–48 h after transfection by decanting the 96-well plate and gently tapping it on paper tissue. These assays are usually robust enough to permit accelerated transfection protocols (**Box 1**) with measurements as soon as 24 h after transfection.

(iii) Quickly after removing the medium, add 50 µl of assay buffer to each well by using a multichannel pipette.
▲ **CRITICAL STEP** To prevent cell loss, pipette the assay buffer gently into the wells.

(iv) Add 10 µl of the 10× d2-labeled antibody solution (prepared in Step 5C(i)).
▲ **CRITICAL STEP** Antibody binding to the receptors (epitopes) may be slower than ligand binding. The on-rate of antibody binding is directly proportional to the antibody concentration, and low concentrations may lead to long equilibration times. Carefully determine assay kinetics to check that equilibrium is achieved. Allow for sufficient incubation time in the binding assay or carry out a preincubation step with the antibody alone if equilibrium is not achieved.

(v) Add 20 µl of 5× solutions of competing ligands prepared in Step 4 (for competition assays or nonspecific binding) or 20 µl of assay buffer.

(vi) Add 20 µl of 5× solution of Tb^{3+}-cryptate-labeled ligand (prepared in Step 3) and incubate it under gentle shaking. The standard final concentration of Tb^{3+}-cryptate-labeled PTH ligands is 5 nM, and the standard incubation time is 4 h at 20 °C.

(vii) To measure the bound fluorescence signal by determining TR-FRET between the d2-antibody-labeled receptor and the Tb^{3+}-cryptate-labeled ligand, proceed exactly as described in option 5B(viii).
? TROUBLESHOOTING

Data analysis ● TIMING 1–2 h

6| The analysis of all binding data (kinetic, saturation and competition) should be done by nonlinear curve fitting. Older procedures, such as Scatchard analysis of saturation curves or Cheng-and-Prusoff correction for competition experiments are approximations that are inferior to nonlinear curve fitting. Several commercial programs are available for this purpose. A particularly popular and accurate program is PRISM[77,78]. An alternative but more basic program, SCTFIT[79,80], is available from the authors upon request.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5A(viii), 5B(viii), 5C(vii)	Experiments give low signal intensities and high scatter (**Fig. 3f**)	Cells may become confluent, affecting GPCR expression and cell adherence (Step 2) Cell viability may be impaired (Step 2 and **Box 1**)	Split a small aliquot of the cells to a clear-bottom 96-well plate in parallel to the black-bottom plate to visually monitor cell numbers and viability (Steps 1 and 2) Avoid confluency Check receptor expression with alternative methods (radioligand binding; expression of GFP-tagged receptor constructs)
		Cell loss during pipetting and/or washing steps (Step 5A(i–vi))	Optimize pipetting and washing steps (Step 5A(vi), e.g., decentered pipetting with truncated pipette tips)
		Cell transfection may be inefficient (Step 2 and **Box 1**)	Optimize transfection (e.g., reduce the amount of plated cells, change amounts of plasmid and transfection reagent; **Box 1**)
5A(viii), 5B(viii), 5C(vii), 6	Fluorescence intensity of labeled ligand is unexpectedly low; inhibition constants of competing ligands are unexpectedly high or variable	Unspecific binding of labeled ligand or competitors to plastic tubes (Steps 3 and 4)	Prepare plastic tubes by coating with Sigmacote (Sigma-Aldrich, cat. no. SL2) before use. Alternatively, use low-binding plastic tubes for Steps 3 and 4

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
5A(viii), 5B(viii), 5C(vii), 6	Signal-to-background and signal-to-noise ratios are low	Binding of fluorescently labeled ligand may be low due to affinity loss upon fluorescent modification	Determine affinity or activity of fluorescently labeled ligand in independent experiments (radioligand binding; second messenger assays)
		Insufficient labeling of receptors via SNAP-tag (Step 5B(ii)) or antibodies (Step 5C(i and vi)) Low receptor expression (Step 2 and **Box 1**)	Verify receptor labeling in control experiments with nontransfected control (**Box 1**) and in SNAP-receptor-transfected cells and measure Tb^{3+}-cryptate labeling in cells using the filter setups of the TRFS protocol (Step 5A(viii)) Visualize labeled antibodies microscopically Check receptor expression with alternative methods as above
	Signal-to-background and signal-to-noise ratios are low	Cells or cell lysate and fluorophores are not in optimal focus (Steps 5A(viii), 5B(viii), 5C(vii)) Time-resolved fluorescence peak is not in the measurement window (as above)	Use the optimization wizard of EnVision to adjust measurement height Use the optimization wizard of EnVision to adjust delay and integration window
6	Equilibrium is not achieved	Some ligands have slow on- and off-rates (Steps 5A(v), 5B(vii), 5C(vi))	Use overnight incubation and/or higher temperatures for binding reaction
		Antibody binding (Step 5C: TR-FRET/Ab) may be slow (Step C(vi))	Use more extended preincubation of antibodies with receptors (cells) and/or try higher antibody concentrations

● TIMING

Steps 1 and 2, cell preparation: 1 h
Steps 3 and 4, assay solutions: 0.5–1 h
Step 5, binding assay: 2–4 h
Step 6, data analysis: 1–2 h

ANTICIPATED RESULTS

A requirement for TR ligand binding assays is the availability or development of suitably labeled ligands and their characterization. Here we used four distinct fluorescently labeled PTH variants as ligands, labeled in the center (C^{13}) or at the C terminus (C^{35}) with either Tb^{3+}-cryptate or the red fluorophore d2. The functionality of these labeled ligands was assessed along several lines: (i) their ability to stimulate the second messenger signal cAMP was determined by measuring cAMP levels in HEK293A cells expressing the PTHR, and the stimulation of cAMP levels was found to be comparable to that of wild-type PTH (i.e., about 25 ± 2-fold at 0.1 nM, at a submaximal level, at which differences would be most visible (**Supplementary Fig. 1a**)); (ii) their affinity was determined in TR-fluorescence saturation experiments (**Fig. 3**); (iii) binding of the labeled PTH ligands to the PTHR on intact cells was monitored visually by confocal fluorescence microscopy using [C^{13}-d2]PTH′(1–34) (**Fig. 2**); these assays showed not only the ability of the fluorescently labeled PTH to bind to receptor-expressing cells much better than to nontransfected control cells, but also the ability of the ligand to induce internalization of the receptors, as evidenced by internalization of the ligand (**Fig. 2**).

In binding assays, all options of this protocol, the classic separation-based and the new FRET-based ones, yielded typical kinetic, saturation and competition data with good signal-to-noise and signal-to-background ratios. Results obtained with the ligand/receptor pair PTH/PTHR are shown in **Figures 3–5**.

Results of binding experiments using TRFS (Step 5A), as a reference, are presented in **Figure 3**. Experiments with a new ligand/receptor pair should start with kinetic experiments to assure that equilibrium is achieved in subsequent experiments. At a ligand concentration of 5 nM [C^{35}-Tb^{3+}]PTH′(1–34), kinetic experiments revealed a time constant of k_{ob} 0.021 ± 0.0016 min^{-1}; equilibrium was essentially achieved after 4 h (**Fig. 3a**). This result is in agreement with literature data indicating slow binding kinetics of PTH[46,60]. The dissociation rate of PTH from PTHR was determined to verify that washing steps do not

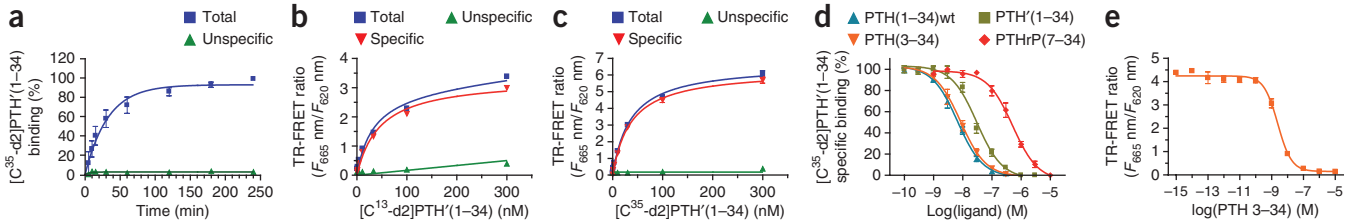

Figure 4 | TR-FRET/SNAP. Adherent HEK293T cells transiently expressing SNAP-PTHR were labeled with benzylguanine-Tb^{3+}-cryptate in 96-well plates. (**a**) Binding kinetics of 5 nM [C^{35}-d2]PTH′(1–34) at 20 °C. Addition of 40 μM unlabeled PTH′(1–34) indicates nonspecific binding. Binding data were analyzed by one-phase exponential association fits, giving an apparent on-rate of 0.031 ± 0.0045 min^{-1}. Error bars represent s.e.m. of arbitrary units (a.u.), n = 4. (**b**,**c**) For saturation binding, cells were incubated with [C^{13}-d2]PTH′(1–34) (**b**) or [C^{35}-d2]PTH′(1–34) (**c**) at indicated concentrations for 4 h at 20 °C. Determination of nonspecific binding was accomplished in the presence of 40 μM unlabeled PTH′(3–34) amide. Error bars represent s.e.m.; n = 3. (**d**) Competition binding using adherent HEK293T cells on 96-well plates. Cells were incubated with 5 nM [C^{35}-d2]PTH′(1–34) and unlabeled PTH derivates, PTH(1–34)wt, PTH′(3–34) amide, PTH′(1–34) or PTHrP(7–34), at indicated concentrations for 4 h at 20 °C. TR-FRET ratios were normalized to 100%, and error bars represent s.e.m.; n = 3–10. (**e**) Representative competition experiment using [C^{35}-d2]PTH′(1–34) and PTH′(3–34) amide is shown to illustrate the intra-assay variability and signal-to-noise ratio. Error bars represent s.e.m. of triplicates.

interfere with the binding assays by causing dissociation. After three washing steps, the dissociation rates were measured continuously by TR-FRET/SNAP assays (**Supplementary Fig. 1b**). A dissociation rate of k_{off} 0.034 ± 0.0032 min^{-1} for labeled PTH in assay buffer and a dissociation rate of k_{off} 0.018 ± 0.0029 min^{-1} for labeled PTH competing with 1 μM PTH′(1–34) was determined. Saturation experiments with the two Tb^{3+}-cryptate-labeled PTH ligands in concentrations ranging up to 300 nM are shown in **Figure 3b–d**. They show that the assay works well even at very high ligand concentrations with good signal-to-noise ratios. Analysis of the saturation curves yielded dissociation constants of 39.5 ± 5.2 and 37.1 ± 5.9 nM for [C^{13}-Tb^{3+}]PTH′(1–34) and [C^{35}-Tb^{3+}]PTH′(1–34), respectively (**Table 2**). Similar affinities have been described for ^{125}I-PTH binding to the receptor (28 ± 3.4 nM)[76]. Competition experiments using this protocol yielded typical sigmoidal inhibition curve binding and affinities that are similar to those derived from radioligand binding experiments reported in the literature[76,81,82] (**Table 3** and **Fig. 3e**). Good cell viability and adherence reduce the noise of TRFS assays, as demonstrated by competition binding experiments obtained by using cells with high or low adherence (**Fig. 3f**). A second requirement of the TRFS format (Step 5A) is the need to lyse the cells before data collection, which increases the precision of the assay by yielding more homogeneous samples (but at the same time decreases the signal intensity). Taken together, the TRFS assay format is similar to classic radioligand competition binding.

Figure 4 shows analogous experiments based on homogeneous (separation-free) assays on TR-FRET between a labeled SNAP-tag in the receptor and a labeled ligand. Again, kinetic experiments showed the slow binding of PTH to its receptor. An on-rate of 0.031 ± 0.0045 min^{-1} was determined (**Fig. 4a**). TR-FRET/SNAP assays can monitor binding kinetics continuously in a single well. Insertion of the SNAP-tag into the receptor did not appear to affect the ligand binding properties of the receptor, as the affinities of labeled and unlabeled ligands were similar to those determined in TRFS experiments (compare **Figs. 4b–e** and **Tables 2** and **3**). They were also similar to the values found in the literature.

The large signal-to-noise ratio of 30 (**Table 4**) combined with the homogenous assay procedure requiring no separation step highlight the convenience of the TR-FRET/SNAP approach (Step 5B). Furthermore, homogenous TRF competition assays can be performed with suspension cells in 384-well plates and thus offer time- and material-saving formats that are suitable

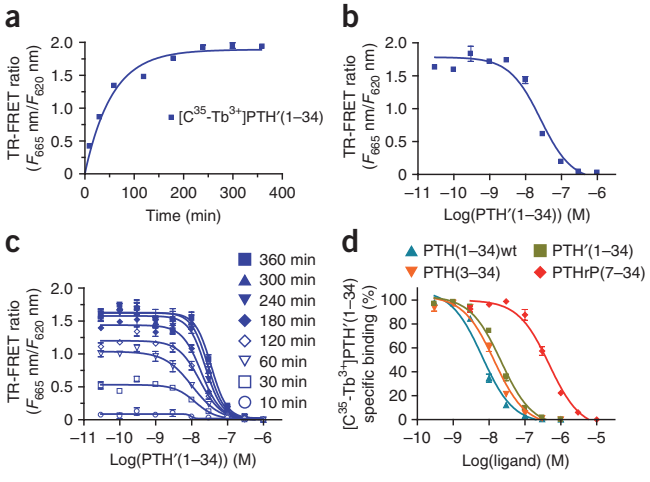

Figure 5 | TR-FRET/Ab. Adherent HEK293A cells were transiently transfected with cDNA coding for human Flag-PTHR and were grown on 96-well plates. (**a**) Binding kinetics of 5 nM d2-Flag antibodies and 5 nM [C^{35}-Tb^{3+}]PTH′(1–34) at 20 °C. TR-FRET ratios were measured after the indicated time intervals. The data were analyzed with a one-phase exponential association fit, giving an apparent on-rate of 0.018 ± 0.0013 min^{-1}. Error bars represent s.e.m.; n = 3. (**b**) Competition binding experiments were carried out using indicated concentrations of PTH′(1–34), 5 nM d2-Flag antibodies and 5 nM [C^{35}-Tb^{3+}]PTH′(1–34) at 20 °C for 4 h. A representative experiment illustrates the intra-assay variability of antibody-TR-FRET. Error bars represent s.e.m. of triplicates. (**c**) Competition binding was carried out according to **b**, and TR-FRET ratios were monitored online at the indicated time intervals. Error bars represent s.e.m.; n = 3. (**d**) Cells were incubated with 5 nM d2-Flag antibodies, 5 nM [C^{35}-Tb^{3+}] PTH′(1–34) and unlabeled PTH derivates, PTH(1–34)wt, PTH′(3–34) amide, PTH′(1–34) or PTHrP(7–34), at the indicated concentrations for 4 h at 20 °C. TR-FRET ratios were normalized to 100%, and error bars represent s.e.m.; n = 4.

TABLE 2 | Affinities of labeled PTH obtained in saturation experiments with TRFS (Step 5A) or TR-FRET/SNAP protocols (Step 5B).

TRFS		TR-FRET/SNAP	
Step 5A		Step 5B	
Compound	K_d ± s.e.m. (nM)	Compound	K_d ± s.e.m. (nM)
[C^{13}-Tb^{3+}]PTH′(1–34)	39.5 ± 5.2	[C^{13}-d2]PTH′(1–34)	46.3 ± 9.1
[C^{35}-Tb^{3+}]PTH′(1–34)	37.1 ± 5.9	[C^{35}-d2]PTH′(1–34)	35.4 ± 4.7

Values represent mean values and s.e.m. for n =3 independent experiments.

for high-throughput screenings (**Box 1**). TR-FRET/SNAP protocols that were conducted with 5 nM tracer concentration obtained signal-to-noise ratios of 30 with adequate standard errors (**Fig. 4e** and **Table 4**).

Binding data using TR-FRET/Ab (Step 5C) are shown in **Figure 5**. Here a fluorescently labeled antibody against an N-terminal epitope engineered into the receptor was used. The experiments shown here used a d2-labeled antibody and a Tb^{3+}-labeled PTH. A reverse arrangement of the labels is also possible, but it may result in different FRET efficacies. Overall, these assays gave results that were similar to those obtained with the other options—in kinetic (**Fig. 5a**) and competition (**Fig. 5b–d** and **Table 3**) experiments. The rate constant of kinetic experiments (on-rate 0.018 ± 0.0013 min^{-1}) was similar to that of ligands in the other formats, indicating that under the conditions chosen here the rate-determining step is dominated by ligand binding also in the presence of antibodies, and that as long as attainment of equilibrium is assured, all assay components can be added simultaneously.

Compared with the protocol using SNAP-tags (option B), the TR-FRET/Ab approach (Step 5C) offers the advantage of a much smaller modification of the receptors (i.e., just the insertion of an epitope), but the signal-to-noise ratio was lower than that in the other approaches (**Table 4**). An explanation may be that the antibodies are larger than the SNAP-tag and thus result in larger distances between donor and acceptor fluorophores and, consequently, lower energy transfers. In addition, it should be noted that if the ligand is labeled with the donor Tb^{3+} this precludes saturation studies with the ligand, because the high concentrations of the donor obscure the acceptor emission signal. Instead, for such experiments, the donor label should be on the receptor (**Fig. 4b**,**c**).

A clear advantage of the homogeneous assay formats (i.e., Steps 5B, 5C) is that the TR-FRET signal can be observed continuously and, thus, the reactions can be monitored online. This is illustrated for the antibody-based approach (Step 5C) in competition binding experiments analyzed at different time points (**Fig. 5c**). These figures show how equilibrium is achieved over time and how the resultant inhibition constants can be reliably read only at equilibrium. Thus, these homogenous assays allow a determination of kinetic and affinity parameters in a single assay—a time- and material-saving approach that is far superior to traditional assays, either radioligand binding or other formats that require separation of bound and free ligand at each time point.

Taken together, the protocol described here provides simple and robust options for characterizing receptor/ligand interactions with TR fluorescence. In particular, the TR-FRET-based assays have a simple and convenient format; they can be recorded online and thus combine kinetic and affinity measurements. Furthermore, batch labeling and freezing of cells allows miniaturization to 384-well plates and high-throughput screening. These assays are demonstrated here in detail for a single ligand/receptor pair. However, they have been used for many other receptors and ligands[20,22–25] and should thus,

TABLE 3 | Affinities of PTH ligands obtained with TRFS (Step 5A), TR-FRET/SNAP (Step 5B) or TR-FRET/Ab (Step 5C) protocols.

	TRFS	TR-FRET/SNAP	TR-FRET/Ab
	Step 5A	Step 5B	Step 5C
Compound	K_I (CI_{95}) (nM)		
PTH(1–34)wt	6.4 (4.7–8.8) (n = 6)	5.8 (4.7–7.1) (n = 9)	5.7 (4.4–7.3) (n = 4)
PTH′(3–34) amide	5.7 (4.0–8.1) (n = 3)	7.3 (5.7 to 9.4) (n = 7)	12.2 (9.3 to 16.1) (n = 4)
PTH′(1–34)	16.4 (12.2–21.9) (n = 6)	26.4 (19.7–35.5) (n = 10)	18.9 (15.5–22.9) (n = 4)
PTHrP(7–34)	221.0 (165.7–294.8) (n = 3)	446.3 (335.3 to 594.0) (n = 7)	413.5 (317.4–538.8) (n = 4)

Mean values and 95% confidence intervals (CI_{95}) are listed for inhibition constants (K_I) determined from the indicated number of independent experiments.

TABLE 4 | Protocol formats with respect to signal-to-background ratio, time efficiency and degree of receptor modification.

Protocol format	Signal-to-background	High throughput	Assay duration[a] (days)	Hands-on time (h)	Receptor modification
TRFS	14	No	3	5	Label-free
TR-FRET/SNAP	30	Yes	2–3	4–5	SNAP-tag
TR-FRET/Ab	9	Yes	2–3	4–5	Antibody epitope

Signal-to-background ratios were obtained at 5 nM tracer concentrations by dividing total by nonspecific signals.
[a]Including cell transfection (**Box 1**).

in principle, be applicable to any receptor for which a fluorescently labeled ligand is available or can be synthesized. Therefore, we believe that these assays can replace more traditional binding assays in order to characterize receptor/ligand interactions and to search for new receptor ligands in high-throughput screening approaches.

Note: Supplementary information is available in the online version of the paper.

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