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Homogeneous Time-Resolved Fluorescence Assay to Probe Folded G Protein-Coupled Receptors

Adam M. Knepp, Thomas P. Sakmar, and Thomas Huber¹

Laboratory of Chemical Biology and Signal Transduction, The Rockefeller University, New York, New York, USA

Abstract

Continued advances in G protein-coupled receptor (GPCR) structural biology and biochemistry depend in part on strategies to stabilize these polytopic membrane proteins in purified systems. New methods to measure properly folded GPCRs are needed to facilitate the identification of suitable conditions and ensure sample quality. Most GPCRs do not contain an intrinsic reporter on their functionality, so probes must be introduced. Here we describe a fluorescence-based approach to quantitatively measure the chemokine receptor CCR5 with labeled antibodies. The assay is exceptionally sensitive and high-throughput. We detail procedures to label antibodies, characterize the system, and process data. We also describe several useful applications, including optimization of incorporation into nanodiscs, measurement of receptor stability, and competition binding assays.

1. Introduction

GPCRs are conformationally flexible and amphipathic, and thus present considerable technical challenges. As methods to recombinantly express GPCRs have improved, difficulties stabilizing receptors have emerged as the largest impediment to studies in reconstituted systems and to the obtainment of high-resolution structures (Tate and Schertler, 2009). Detergents are used to extract GPCRs from the lipid bilayer during purification, and the solubilized receptor is prone to unfolding and aggregation. Great care must be exercised to ensure that the receptor maintains proper folding during these procedures. Subtle disruptions to the native conformation may render the protein nonfunctional.

A variety of tools have been developed to improve GPCR stability. These approaches include truncations of particularly flexible regions, meticulous optimization of detergent/lipid mixtures, combinations of favorable mutations, insertion of domains such as T4 lysozyme, and formation of complexes with small molecules or antibody Fab fragments. The result of these approaches has been an impressive explosion of X-ray crystal structures, including the first snapshot of a GPCR complexed with a heterotrimeric G protein (Rasmussen *et al.*, 2011). Unfortunately, these strategies are nearly impossible to rationalize,

¹Corresponding author: hubert@rockefeller.edu.

and many of them are undesirable for functional assays. Thus, the tedious, systematic search for successful conditions must be carried out for each receptor under study.

Because the number of conditions that must be screened may be quite large, assays to measure foldedness and stability should ideally be high-throughput and require minimal amounts of receptor. Often the parameter examined is thermal stability, which has been shown to predict successful crystallization (Dore *et al.*, 2011; Robertson *et al.*, 2011; Warne *et al.*, 2008). Existing methods to probe thermal stability include surface plasmon resonance, radioligand binding, and changes in cysteine reactivity. These techniques usually require micrograms of receptor and cannot necessarily be automated for high-throughput screening.

Homogeneous time-resolved fluorescence (HTRF) is a special fluorescence resonance energy transfer (FRET) method that exploits several advantages of lanthanoidcryptate reagents (Mathis, 1995). These complexes, such as europium cryptate (EuK), have exceptionally long fluorescence half-lives, permitting measurement on time scales at which transient autofluorescence is not problematic, resulting in improved signal-to-background compared to most fluorescence techniques. EuK also has a large Stokes shift, so emission wavelengths are easily isolated from the excitation light source. Finally, EuK and the acceptor fluorophore used, a modified allophycocyanin protein termed XL665, exhibit an unusually large Förster radius. This property is especially beneficial for reasons described below.

In order to employ the HTRF technology to the problem of GPCR stability, the fluorophores must be conjugated to probes for receptor function or, as a proxy, proper folding. Ligand binding is the most obvious candidate, but GPCR ligands are tremendously variable, and few are amenable to chemical labeling with a fluorophore. Alternatively, antibodies that recognize specific receptor conformations can be used. These reagents bind properly folded receptors but are inactive under denaturing conditions. Highly specific conformationally sensitive antibodies have been developed as therapeutics and as tools for crystallization (Mancia *et al.*, 2007; McKnight *et al.*, 1997; Wu *et al.*, 1997). While they are currently available for a limited number of receptors, a growing appreciation of their utility may spur the development of more.

The assay formulation we employed, with both donor and acceptor fluorophores linked to monoclonal anti-CCR5 antibodies, is shown in Figure 1. The 2D7 mAb binds a conformationally sensitive epitope on the second extracellular (EC2) loop of CCR5. Biotinylated 1D4 mAb binds an engineered linear C-terminal epitope derived from rhodopsin. Streptavidin-conjugated XL665 links to 1D4-biotin. This chapter will describe a general method to efficiently label IgG with EuK, how to characterize the FRET signals, and then several applications of the method.

2. Solid-phase labeling of IgG with europium cryptate

To maximize assay sensitivity, antibodies should be directly labeled rather than relying on a secondary antibody. Typically, conjugations require fairly large amounts of antibody and/or fluorophore, which can make in-house labeling of an array of reagents prohibitively

expensive. We therefore developed a scalable procedure for labeling small amounts of IgG with EuK. The method can be generalized to other IgG and amino-derivatized fluorophores.

2.1 Materials

Ni-NTA magnetic agarose beads (QIAGEN)

Magnetic Eppendorf tube separation rack

Sulfo-SMCC (Pierce)

SPDP (Pierce)

Europium trisbipyridine diamino cryptate (EuK, Cisbio)

IgG to label (results shown with anti-CCR5 mAb 2D7)

Labeling buffer: 100 mM phosphate buffer pH 7.5, 150 mM NaCl, 0.0037% *n*-dodecyl- β -D-maltoside (DM)

Elution buffer: Labeling buffer + 200 mM imidazole

SEC buffer: 100 mM phosphate buffer pH 7.0, 150 mM NaCl, 0.5 mg/mL bovine serum albumin (BSA)

0.5 M sodium borate buffer pH 8.2

Sodium fluoride

Tris base

TCEP

N-acetylcysteine

Thiomersal, for IgG storage

Äkta Explorer (GE)

Superdex 10/300 GL gel filtration column

EnVision Multilabel Plate Reader (PerkinElmer)

2.2 Immobilization of IgG and Labeling Strategy

Labeling reactions in solution have two technical drawbacks that are bypassed by solid-phase chemistry. First, the conjugated product must be separated from excess reagents and side products by chromatographic or other means. This can add several additional purification steps to the procedure and decrease yield. Immobilization of the IgG permits simple and thorough washing steps, particularly with magnetic beads. Second, the tethering to beads results in significant concentration of IgG compared to commercial solutions. This

reduction in reaction volume increases the efficiency of the conjugation and eliminates the necessity of a large molar excess of the label. Both of these advantages are particularly noteworthy when the antibodies and/or labels are expensive, which is often the case.

IgG molecules bind magnetic Ni-NTA beads through a conserved histidine-rich region of the Fc stem. 2D7 mAb and EuK were activated separately before combining for the final conjugation: 2D7 with sulfo-SMCC and EuK with SPDP. This strategy was chosen because we found that reduction of SPDP to produce the free thiol resulted in significant breaking of IgG disulfide linkages. When EuK is activated with SPDP, TCEP treatment is carried out in the absence of IgG and antibody integrity is preserved. The overall reaction scheme is shown in Figure 2A. The labeling procedure is detailed below, and results are discussed in the next section.

1. Transfer 200 μ L of a 5% slurry of Ni-NTA magnetic agarose beads to an Eppendorf tube.
2. Wash the beads with 1 mL labeling buffer, spin down, and remove the supernatant with the aid of a magnetic rack. The volume of the beads should be 10 μ L, plus a few μ L of liquid that cannot be completely removed.
3. Add 100 μ g (1.3 nmol) of the IgG to be labeled. (Note: According to the manufacturer's specifications, the binding capacity for 150 kDa IgG should be $\sim$ 375 μ g/200 μ L bead suspension.) Allow binding to proceed for 30 min at room temperature with shaking. The small amount of DM in the labeling buffer facilitates complete mixing in all steps with the beads.
4. After completion of IgG binding, spin and remove the supernatant as before.
5. Activate the IgG with 2 μ L of 3.75 mM sulfo-SMCC (7.5 nmol, 5.6 equiv.). React for 60 min at room temperature with shaking.
6. In a separate reaction tube, mix 3.68 μ L of 0.68 mM EuK (2.5 nmol, 2 equiv. with respect to IgG), 0.67 μ L of 0.5 M sodium borate buffer, 1.32 μ L of 1 M NaF, and 0.67 μ L of 7.58 mM SPDP (5.0 nmol, 2 equiv. with respect to EuK). Shake for 60 min at room temperature.
7. Stop IgG/sulfo-SMCC activation by spinning the beads down and removing the supernatant.
8. Quench EuK/SPDP activation with 0.67 μ L of 7.58 mM Tris, which reacts with remaining SPDP molecules.
9. Cleave the SPDP disulfide by adding 0.67 μ L of 8.27 mM TCEP. React for 15 min at room temperature.
10. Combine activated IgG and EuK. Mix overnight at 4°C.
11. Quench labeling reaction with 1 μ L of 80 mM *N*-acetylcysteine, which reacts with remaining maleimide-activated IgG.
12. Wash beads with at least 2×1 mL labeling buffer to remove excess reagents. Spin beads and remove supernatant after each wash.

13. Elute IgG with $2 \times 50 \mu\text{L}$ elution buffer. Incubate each elution with beads for 30 min at room temperature.

2.3 Purification of EuK-labeled IgG and Characterization

The labeled IgG was first analyzed by non-reducing SDS-PAGE (Figure 2B). The gel shows that the chosen labeling strategy results in minimal fragmentation. In fact, the labeled product is actually cleaner than the initial 2D7 sample, which appears to contain some impurities.

Next, the product was supplemented with 0.5 mg/mL BSA and loaded onto a Superdex 200 10/300 GL gel filtration column equilibrated with SEC buffer. We found that using BSA as a carrier protein significantly improved recovery of the microgram quantities of sample. This column is suitable for samples of this molecular weight. Absorbance was monitored at 280 and 305 nm for protein and EuK (Figure 2B). These absorbance peaks showed an approximate labeling ratio of 1.3 EuK/IgG. They also revealed some sample heterogeneity. The main peak is monomeric 2D7-EuK, and the earlier and later peaks are presumed to be dimeric and fragmented IgG, respectively.

To further confirm the presence of EuK, SEC fractions were collected and loaded into a microplate, and fluorescence was measured. The peak profile corresponds very well to UV absorbance off of the column (Figure 2C). The fluorescence points were fit to three Gaussian peaks to determine the fractions most likely to contain monomer. We estimated that 30 μg of labeled 2D7 monomer were ultimately pooled. The sample was supplemented with 0.01% thiomersal (an antimicrobial) and stored at 4°C.

3. HTRF assay standards and signal analysis

3.1 Materials

EuK-labeled IgG (e.g. anti-DNP-EuK and 2D7-EuK)

Biotin-BSA (Pierce)

DNP-NHS (Cisbio)

Biotin-1D4 mAb

XL665-Streptavidin (XL665-SA; Cisbio)

Competition binding reagents: DNP-NHS, 2D7, 1D4, 1D5 peptide (TETSQVAPA)

Heterologously expressed CCR5 with 1D4 epitope (as described (Knepp *et al.*, 2011))

Appropriate buffer with 50 mM NaF and 1 mg/mL BSA

Black bottom 384-well microplate

PerkinElmer EnVision multilabel plate reader

3.2 Standard experiment

Signal detection was first optimized in a system similar to the immunosandwich in Figure 1. The standard experiment used commercially available reagents: anti-DNP-EuK (Cisbio), DNP-NHS (Cisbio), biotin-BSA (Pierce) and XL665-SA (Cisbio). Biotin-BSA-DNP was prepared by reacting DNP-NHS with biotin-BSA according to the manufacturer's protocol. The predicted distance from donor to acceptor in this scheme is shorter, making signal detection easier. Competition with DNP-NHS, which does not bind XL665-SA, was used to demonstrate the specificity of the observed HTRF signal.

The assay volume used is 50 μ L/well, so total volumes to be mixed should be calculated accordingly. All HTRF buffers should contain the additives 50 mM NaF, which is necessary for optimal EuK fluorescence, and 1 mg/mL BSA, which prevents low concentration proteins from sticking to the plate wells.

1. Combine biotin-BSA-DNP (5 nM) and XL665-SA (10 nM) in PBS pH 7.0 in a 1:1 ratio (v/v) and mixed for 15 min on ice (mixture 1).
2. From the mixture in step 1, make a 10-fold diluted solution in the same buffer (mixture 2).
3. Prepare anti-DNP-EuK at concentrations of 2.5 nM and 0.25 nM.
4. Make a 4-fold serial dilution of DNP-NHS with concentrations from 5.12 μ M to 1.22 pM, plus a negative control with no DNP-NHS. Preparing the serial dilution across the row of a 96-well microplate makes the mixing in step 5 easier.
5. Mix both the 2.5 nM and 0.25 nM anti-DNP-EuK solutions with each of the DNP-NHS concentrations for 15 min at a 1:1 ratio (preparing series 1 and 2, respectively).
6. Combine mixture 1 with series 1 (25 μ L each/well), and mixture 2 with series 2 (25 μ L each/well) in a 384-well microplate. In the experiment shown, four replicates were prepared for each condition.
7. Incubate the plate at 4°C for at least 1 h.
8. Measure plate fluorescence with excitation at 320 nm and emission collection in 10 μ s windows at 615 and 665 nm. Averaging over 5000 flashes per well yielded an acceptable compromise between measurement time and signal-to-background. Sum emission counts over windows 2–10; the first window is excluded to minimize contributions of autofluorescence. The ratio F_{665}/F_{615} gives a normalized measure of sensitized emission. The negative control for this sample is the mixture with a saturating concentration of DNP-NHS, which prevents donor and acceptor from being linked. From this, the signal enhancement ΔF can be calculated:

$$\Delta F = \left(\frac{F_{665, sample}}{F_{615, sample}} - \frac{F_{665, negative}}{F_{615, negative}} \right) \div \frac{F_{665, negative}}{F_{615, negative}}$$

Figure 3 shows the results of the standard experiment. A maximum F of approximately 5 is observed in the experiment with higher concentrations of EuK and XL665. The competition curves demonstrate the specificity of the HTRF signal, and its robustness suggests that the fluorescence acquisition parameters are suitable. After establishing these conditions, the more complex immunosandwich with expressed CCR5 was characterized.

3.3 GPCR immunosandwich assay

Figure 1 shows the components used in the GPCR assay, with CCR5 as a model receptor. 2D7-EuK obtained from the labeling procedure in the previous section binds a conformationally sensitive split epitope QKEGL-TL on the EC2 loop of the receptor. Biotinylated 1D4 mAb (prepared according to a published procedure (Knepp *et al.*, 2011)) binds the engineered C-terminal epitope TETSQVAPA to complete the immunosandwich. Given suitably labeled antibodies, the assay can be generalized to other GPCRs and membrane proteins. The acceptor fluorophore is XL665, which is conjugated to streptavidin. This FRET pair has a Förster radius of approximately 95 Å, making signal detection feasible even though the fluorophores are probably separated by at minimum the distance between the intracellular and extracellular faces of the receptor.

Before proceeding to the applications in the next section, it is important to first characterize the parameters described here: donor/acceptor fluorescence, time-dependence, dynamic range, and specificity. These quality control checks are necessary to ensure that the fluorescence signal accurately reflects the quantity of folded target receptor. The basic procedure is the same in all experiments; slight changes depending on the condition being tested are noted. The assay was performed in 40 µL/well, so total volumes should be calculated accordingly.

1. Express CCR5 with the C-terminal 1D4 tag in HEK-293T cells as described (Knepp *et al.*, 2011).
2. Solubilize CCR5 in detergent. The following optimized (Navratilova *et al.*, 2005) buffer (Buffer N) was used in all experiments containing solubilized CCR5, unless otherwise noted: 20 mM Tris-HCl (pH 7.0), 0.1 M (NH₄)₂SO₄, 10% (v/v) glycerol, 0.07% cholesteryl hemisuccinate, 0.018% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 0.008% 1,2-dioleoyl, *sn*-glycero-3-phospho-L-serine, 0.33% DM, and 0.33% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate. 1 mL of this buffer was used to solubilize 5×10^6 cells.
3. Mix 1D4-biotin and XL665-SA at a final concentration of 128 nM each for 15 min on ice.
4. To the mixture from step 3, add an equal volume of 8 nM 2D7-EuK.
5. Transfer 20 µL of the mixed components from step 4 to a 384-well microplate. Add 20 µL of detergent-solubilized CCR5. The final concentrations of assay components are: 2 nM 2D7-EuK, 32 nM XL665-SA, 32 nM 1D4-biotin. In competition experiments, CCR5 is first preincubated with the appropriate concentration of 2D7 mAb, 1D4 mAb, or TETSQVAPA ("1D5") peptide.

6. Incubate the plate at 4°C overnight, unless a signal time course is desired.
7. Measure plate fluorescence and analyze signal as described in the previous section. The negative control for this assay is a mixture of donor and acceptor components in the absence of CCR5.

As controls, the fluorescence at 615 and 665 nm was measured in the presence and absence of 1D4-biotin, XL665-SA, 2D7-EuK, and CCR5 (Figure 4A). The bars show that 1D4-biotin and XL665-SA in the absence of the EuK donor results in minimal counts at 615 nm and some emission at 665 nm. 2D7-EuK without acceptor gives a strong signal at 615 nm and some bleed-through at 665 nm. When these three components are combined in the absence of CCR5, the 615 nm signal is close to that of 2D7-EuK alone, and the 665 nm is only slightly greater than of the acceptor alone. This suggests that minimal FRET occurs without the receptor to bring the donor and acceptor near each other. In the presence of CCR5, sensitized emission is observed, with a decrease in 615 nm fluorescence and increase at 665 nm. This results in an increased F_{665}/F_{615} ratio.

The time course of the CCR5-dependent signal was measured (Figure 4B). The signal increases significantly during the first few hours of incubation at 4°C as the system equilibrates, and is then stable overnight. For maximum signal-to-background and assay reproducibility, plates were incubated for 12–16 hours before fluorescence measurement.

For quantification applications, the dynamic range of the signal must be determined (Figure 4C). This was done by incubating labeled components with a serial dilution of detergent-solubilized CCR5. A linear range precedes signal saturation near $F = 2$. The signal was calibrated to an absolute concentration by incubating receptor with a high-affinity fluorescent antagonist FL-maraviroc, as described (Knepp *et al.*, 2011). This shows that an estimated 0.02–1.0 nM CCR5 can be effectively quantified, which corresponds to 0.8–40 fmol in a volume of 40 μ L. The assay is therefore remarkably sensitive.

The specificity of the HTRF signal was determined in competition experiments (Figure 4D–F). Signal enhancement was normalized to the end points of 0% and 100% inhibition. Preincubation with unlabeled components results in decreased signal, and the IC_{50} values of the curves are reasonable.

4. Applications of GPCR HTRF assay

Once the HTRF assay has been characterized for the system of interest, a variety of applications are possible. The assay requires very small quantities of receptor, which is an especially beneficial quality for GPCRs. Additionally, the microplate format enables high-throughput measurements, which is critical when a number of conditions must be tested. An HTRF immunosandwich approach is not limited to the methods discussed below; it could very well be useful in other situations that require a sensitive receptor probe. However, the applications described here should give the reader an idea of the assay's utility. In all sections, CCR5 is presented as a model GPCR.

4.1 Microscale incorporation of a GPCR into nanoscale apolipoprotein bound bilayers (NABBs)

NABBs are discoidal nanoparticles with a lipid bilayer encapsulated by amphipathic belt proteins. The lipid environment better mimics native conditions than detergents, so these particles are useful for membrane protein reconstitution and functional study (Banerjee *et al.*, 2008; Whorton *et al.*, 2007). A microscale approach to incorporate receptors into NABBs is desirable because most GPCRs cannot be purified from natural sources in large quantities. With a method to determine whether GPCR-NABBs retain structural integrity, conditions for the purification strategy can be optimized.

4.1.1 Materials—Heterologously expressed CCR5 with 1D4 epitope (as described (Knepp *et al.*, 2011))

CCR5 solubilization buffer (Buffer N)

1D4-Sepharose resin (as described (Knepp *et al.*, 2011))

1D5 peptide

Micro-Spin columns (Pierce)

Zebrafish apolipoprotein A-I protein (zap1, prepared as described (Banerjee *et al.*, 2008))

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)

Lissamine rhodamine B-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (LRB-DOPE)

NABB buffer: 15 mM Tris-HCl (pH 7.0), 75 mM (NH₄)₂SO₄, 7.5% (v/v) glycerol, 0.33% DM, 1.5% sodium cholate

Buffer S: 20 mM Tris-HCl (pH 7.0), 100 mM (NH₄)₂SO₄, 10% (v/v) glycerol

Pierce Detergent Removal Resin

Äkta Explorer (GE)

Superose 6 10/300 gel filtration column

Dynabeads Protein G (Invitrogen)

Western blot reagents, including 1D4 mAb, anti-His₆ mAb, and HRP-anti-mouse

HTRF reagents and equipment described above

4.1.2 Microscale incorporation method and analysis—Because the emphasis of this chapter is the HTRF assay and its applications, discussion of the procedure to incorporate CCR5 into NABBs is fairly brief. More detailed information on conditions tested and observations has been reported previously (Knepp *et al.*, 2011).

1. Solubilize CCR5 from 2×10 cm plates in Buffer N and add to 50 μ L of packed 1D4-Sepharose resin. After 16 h incubation at 4°C, transfer the resin to a Micro-Spin column and wash with Buffer N. Elute CCR5 by incubating the beads with 2×50 μ L Buffer N containing 400 μ M 1D5 peptide.
2. Mix combined CCR5 elutions with 3.75 nmol purified zap1, 280 nmol POPC, and 22.7 nmol cholesterol in a total volume of 200 μ L containing NABB buffer. For visualization of lipid elution, use a POPC solution containing 0.5% (mol/mol) LRB-DOPE. Incubate the mixture for 30 min on ice.
3. Add the NABB mixture from step 2 to 1 mL of Detergent Removal Resin pre-equilibrated with at least one column volume of Buffer S. Elute at 4°C by gravity flow, adding Buffer S and collecting 200 μ L fractions.
4. Measure 280-nm absorbance of fractions and combine those containing protein.
5. Purify sample by loading onto a Superose 6 10/300 gel filtration column. Monitor absorbance at 280 nm and, if LRB-DOPE was used, 570 nm. Combine fractions containing CCR5-NABBs.
6. To determine 2D7-binding capability, mix CCR5-NABBs with Dynabeads Protein G with or without 2D7 according to the manufacturer's protocol and analyze supernatant fractions by 1D4 Western blot.
7. For HTRF quantification of folded CCR5 in NABBs, prepare and mix the HTRF components as described in section 3. Use PBS pH 7.0 containing 50 mM NaF and 1 mg/mL BSA for this assay. Add CCR5-NABBs (20 μ L), incubate overnight at 4°C, and read plate fluorescence.

4.1.3 GPCR-NABBs results—1D4-Sepharose purification results in a CCR5 yield of approximately 50%. The NABB mixture is prepared by mixing zap1 and lipids at a molar ratio of 1:75, which has been shown to yield discs with 10–12 nm diameter (Banerjee *et al.*, 2008). Adding CCR5 to the zap1/lipid mixture and removing detergent results in efficient formation of NABBs containing receptor, as shown in Figure 5A. The Western blots demonstrate that the SEC peak shoulder centered at ~16.1 mL contains the majority of CCR5. The larger peak at ~17 mL consists of mostly empty NABBs. In this experiment, the molar ratio of CCR5 to NABBs is kept low, at approximately 1:100, in order to reduce the likelihood of incorporating more than one receptor per NABB. The initial check on receptor quality, immunoprecipitation with 2D7, shows that nearly all of the CCR5 present in NABBs is properly folded (Figure 5B).

The HTRF assay was used to measure the yield of folded CCR5 in NABBs following purification, which was useful to optimize preparation conditions. The assay is more quantitative than Western blots and reports on the quality of the sample, not just the amount present. The dynamic range in Figure 5C shows that the immunosandwich assay is effective at quantifying CCR5-NABBs. The signal is specific, as shown by the reduction of signal with a high concentration of unlabeled 1D4 mAb (Figure 5D). Compared to the solubilized starting material, the overall yield of correctly folded CCR5 under these conditions is estimated to be ~15%.

4.2 Temporal and thermal stability measurements

As described in the introduction, stabilizing GPCRs during purification and reconstitution can be a daunting challenge. Methods to probe receptor integrity are necessary when intrinsic reporters, such as the covalently bound chromophore of rhodopsin, are not available. Available approaches can often be prohibitively time-consuming or resource-intensive. The HTRF assay is a sensitive and high-throughput solution, and is based on the principle that receptor denaturation results in loss of the 2D7 epitope. Methods to measure the temporal and thermal stability of CCR5 in detergent and NABBs are described below. The significant stabilizing effects of high-affinity anti-HIV small molecules are also demonstrated.

4.2.1 Materials—Heterologously expressed CCR5 with 1D4 epitope (as described (Knepp *et al.*, 2011))

CCR5 solubilization buffer (Buffer N)

HTRF reagents and equipment described above

Small molecule (e.g. TAK-779)

Gradient thermal cycler

4.2.2 Stability measurement method

1. To measure temporal stability of CCR5, and compare the effects of buffer components, it is simply necessary to thaw a series of solubilized aliquots at suitable time points prior to fluorescence measurement.
2. To measure thermal stability, a thermal cycler is employed. Add detergent-solubilized CCR5 or CCR5-NABBs (11 μ L, at a concentration within the assay dynamic range) to PCR tubes. If the effects of small molecules are to be tested, preincubate the receptor with 8 μ M ligand for 1 h on ice first.
3. Set the thermal cycler to apply a gradient of temperatures across a row of the heating block for 30 min, and then cool to 4°C.
4. Dilute samples by adding an equal volume of the appropriate buffer and add 20 μ L to each well of a 384-well microplate containing the HTRF components. Measure fluorescence.

4.2.3 Analysis of stability data—A comparison of CCR5 stability over time in buffer containing just 1% DM and in the more complex lipid-detergent mixture (Buffer N) is shown in Figure 6A. Aliquots were stored at 4°C for the time indicated prior to measurement. Time points shorter than the time necessary to equilibrate CCR5 with the labeled antibodies are not accessible, but the longer-term trends are observable. Whereas CCR5 in DM denatures over the course of several days, CCR5 in buffer N is remarkably stable. This shows additional buffer components may be necessary to achieve optimal GPCR stability, motivating high-throughput screening techniques such as that described here.

Thermal denaturation of unliganded CCR5 and CCR5-NABBs was modeled as a first-order process from a folded to an unfolded state. Melting of CCR5-ligand complexes was modeled as a two-step sequence of first-order reactions, with two distinct ligand binding states preceding unfolding. These experiments probe only the kinetics of receptor denaturation, not the equilibrium distribution. Curves were fit using Origin, and the apparent melting temperature, T_M , was derived by determining the point at which 50% of the receptor is unfolded. The models have been described in detail previously (Knepp *et al.*, 2011)

CCR5 thermal stability data are shown in Figure 6B–D. CCR5 solubilized in buffer N has a T_M of ~47 °C. (Figure 6B) Preincubation with TAK-779 results in a significant rightward shift of the melting curve, with the receptor-ligand complex denaturing at ~64°C (Figure 6C). This is comparable to what we previously reported for other CCR5 antagonists (Knepp *et al.*, 2011). The NABBs also confer improved thermal stability (T_M of ~55°C) compared to receptor in detergent, but the difference in curve shapes suggests that denaturation proceeds via a distinct pathway (Figure 6D). Two additional interesting features of the small molecule data are the rise in signal prior to melting, and the ligand-induced reduction in signal. The latter is likely due to an allosteric relationship between the small molecules and the 2D7 mAb. This is explored in more detail in the following section.

4.3 Competition binding between small molecules and antibodies

CCR5 is a primary HIV-1 coreceptor, and this role has prompted development of a number of therapeutics aiming to block viral entry (Horuk, 2009). These include small molecule antagonists (among them, those mentioned in the thermal stability analysis) and monoclonal antibodies. Determining the allosteric relationship between these reagents, if any, can hint at potential synergistic effects. It can also shed light on coupling between different regions of the receptor. Antibodies against multiple extracellular loops and N-terminus have been developed, whereas small molecules generally bind a more buried transmembrane site.

Competition experiments with varied concentrations of antibody and small molecule provide this type of information. This is typically done using radiolabeled reagents, but the fluorescence approach described in this chapter can be more sensitive and high-throughput. This section presents data for 2D7 mAb and TAK-779.

4.3.1 Materials—Heterologously expressed CCR5 with 1D4 epitope (as described (Knepp *et al.*, 2011))

CCR5 solubilization buffer (Buffer N)

HTRF reagents and equipment described above

TAK-779

4.3.2 Competition binding method

1. Preincubate CCR5 solubilized in buffer N with a serial dilution of TAK-779 spanning several orders of magnitude of concentration for 1 h on ice.

2. Prepare HTRF labeled components as before, except vary the concentration of 2D7-EuK. In the experiment presented, the concentrations were 0.5, 1.0, and 2.0 nM. The concentration of 1D4-biotin and XL665-SA is held at 32 nM.
3. Add CCR5 bound to TAK-779 to microplate wells containing HTRF reagents and incubate overnight at 4°C. Measure fluorescence.

4.3.3 Competition binding results and allosteric relationship—The results of the competition experiments are shown in Figure 7. As expected, the signal increases with higher concentrations of 2D7-EuK. The TAK-779 causes a decrease in signal, which could be due to a reduction in the number of 2D7 binding sites or a change in the antibody epitope conformation such that the EuK donor fluorophore is farther from the XL665 acceptor. Bulk competition experiments such as these cannot distinguish between these possibilities. However, the IC₅₀ values of the three curves are very similar, which suggests that TAK-779 exerts a noncompetitive antagonistic effect on 2D7. This aligns with what has been previously reported for CCR5 small molecules, natural ligands, and antibodies (Ji *et al.*, 2007).

5. Conclusion

We report a novel method to quantify folded GPCRs in solution that is sensitive, robust, and high-throughput. The assay relies on the binding of two antibodies against distinct receptor epitopes, one conjugated to a FRET donor fluorophore and the other to a FRET acceptor. A solid-phase technique to efficiently label small quantities of the desired antibodies enabled this strategy. Sub-nanogram quantities of receptor in a single microplate well can be effectively detected, and we describe several applications of the method that demonstrate its versatility. This approach is currently limited by the availability of suitable antibody probes, but new methods to rapidly develop these reagents could expand the number of target receptors in the future.

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FIGURE 1.

HTRF sandwich immunoassay schematic. A hypothetical model of CCR5 based on the crystal structure of rhodopsin (PDB ID: 1U19) is shown. 2D7-EuK recognizes a conformation-sensitive split epitope on the extracellular side of CCR5. Biotinylated 1D4 (1D4-biot) binds an engineered nine-residue C-terminal epitope and is linked to streptavidin-conjugated XL665. FRET is observed between EuK and XL665 when 2D7 binds properly folded CCR5. The Förster radius for this donor-acceptor pair is approximately 95 Å.

FIGURE 2.

(A) Scheme depicting strategy to synthesize EuK-labeled 2D7 mAb (**9**). EuK (**1**) is activated by addition of a sulfhydryl by reaction with SPDP (**2**) and reduction by TCEP (top). Separately, 2D7 mAb (**6**) is immobilized on Ni-NTA resin and reacted with sulfo-SMCC (**7**) to generate a maleimide derivative (**8**). A crystal structure of IgG (PDB ID: 1IGY) is shown as a model. The two activated reagents are combined and labeled 2D7 is eluted from the resin with imidazole. (B) Left: Size-exclusion chromatography with monitoring of absorbance at 280 nm (blue; protein) and 305 nm (red; EuK) to determine yield and labeling ratio. Right: Coomassie blue-stained non-reducing SDS polyacrylamide gel electrophoresis showing a single band of the intact 2D7-EuK after labeling. Note the impurities of the initial 2D7 sample have been largely removed by the procedure. (C) Fluorescence of purified, EuK-labeled antibodies. SEC fractions (400 μ L) were collected from 8–19 mL elution and EuK emission was measured at 615 nm.

FIGURE 3.

HTRF standard experiment with donor anti-DNP-EuK and acceptor XL665-SA. The fluorophores are brought in proximity by biotin-BSA-DNP. Two different concentrations of donor and acceptor were used: 0.625 nM anti-DNP-EuK/2.5 nM XL665-SA/1.25 nM biotin-BSA-DNP (triangles) and 0.0625 nM anti-DNP-EuK/0.25 nM XL665-SA/0.125 nM biotin-BSA-DNP (squares). IC_{50} curves depict the competition of biotin-BSA-DNP with DNP-NHS, which does not link to XL665-SA.

FIGURE 4.

(A) Assay controls with fluorescence counts at 615 nm (black) and 665 nm (gray) after excitation at 320 nm. CCR5-specific signal is seen as a signal increase at 665 nm and decrease at 615 nm. (B) Time course of HTRF signal with detergent-solubilized CCR5. After loading samples into a 384-well microplate, fluorescence was measured at the indicated time points. The plate was stored at 4°C between readings. (C) A serial dilution of CCR5 shows the dynamic range of the assay. The signal saturates at ~200% enhancement over background. F is defined as:

$$\Delta F = \left(\frac{F_{665, sample}}{F_{615, sample}} - \frac{F_{665, negative}}{F_{615, negative}} \right) \div \frac{F_{665, negative}}{F_{615, negative}}$$

(D–F) Competition experiments with the 1D5 nonapeptide (D), 1D4 mAb (E), and 2D7 mAb (F) demonstrate signal specificity.

FIGURE 5.

(A) Chromatogram showing co-elution of protein (solid; 280 nm) and fluorescent lipids (dashed; 570 nm). The 1D4 immunoblot below shows that CCR5-NABBs elute in the peak centered at 15.6 mL, exhibiting larger hydrodynamic radius than the majority of the NABBs at 16.1 and 17.0 mL. The anti-His₆ blot detects His-tagged zap1 belt protein. Free zap1 elutes at 18.6 mL (inset). (B) CCR5-NABBs were incubated with Protein G beads and 2D7, and the supernatant fraction was probed with a 1D4 immunoblot. The majority of CCR5 in NABBs is immunoprecipitated by 2D7, indicating properly folded receptor. (C) HTRF signal from a serial dilution of CCR5-NABBs, demonstrating the ability to quantify properly folded CCR5 in NABBs. (D) The HTRF signal is efficiently competed with 1 μ M 1D4, showing signal specificity.

FIGURE 6.

The HTRF assay was applied to make high-throughput temporal and thermal stability measurements with femtomole quantities of CCR5. (A) Temporal stability of CCR5 solubilized in 1% DM (triangles) and a buffer containing a complex mixture of lipids and detergents, as well as 10% (v/v) glycerol (squares). Aliquots were thawed and stored at 4 $^{\circ}\text{C}$ for the indicated time prior to measurement. (B–C) Melting curves of unliganded CCR5 (B) and CCR5 preincubated with the small molecule antagonist TAK-779 (C). TAK-779 shifts the T_M of detergent-solubilized CCR5 from 47 $^{\circ}\text{C}$ to 64 $^{\circ}\text{C}$. (D) Melting curve of CCR5-NABBs. The assembly denatures at 55 $^{\circ}\text{C}$. CCR5-NABBs melt over a much broader range than CCR5 in detergent solution, suggesting some sample heterogeneity or a distinct denaturation pathway.

FIGURE 7.

Competition curves between TAK-779 and 2D7-EuK. CCR5 was preincubated with the indicated concentration of TAK-779, and then the fluorescently labeled assay components were added with 0.5 (triangles), 1.0 (circles) or 2.0 nM (squares) 2D7-EuK.