Published in final edited form as:

Methods Mol Biol. 2011 ; 746: 425–440. doi:10.1007/978-1-61779-126-0_25.

Analysis of GPCR Localization and Trafficking

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Abstract

Localization and trafficking of G protein-coupled receptors (GPCRs) is increasingly recognized to play a fundamental role in receptor-mediated signaling and its regulation. Individual receptors, including closely homologous subtypes with otherwise similar functional properties, can differ considerably in their membrane trafficking properties. In this chapter, we describe several approaches for experimentally assessing the subcellular localization and trafficking of selected GPCRs. Firstly, we describe a flexible method for receptor localization using fluorescence microscopy. We then describe two complementary approaches, using fluorescence flow cytometry and surface biotinylation, for examining receptor internalization and trafficking in the endocytic pathway.

Keywords

Microscopy; Receptor endocytosis; Flow cytometry; Biotinylation; Receptor trafficking

1. Introduction

The subcellular localization of receptors can impact fundamentally on the strength and specificity of cellular signaling. Individual GPCRs can differ considerably in their localization between the plasma membrane and various intracellular compartments, as well as between different domains of the plasma membrane. In many cases, this represents a steady state, reflecting dynamic trafficking pathways that are subject to physiological regulation (1, 2). Clinically relevant drugs influence the number or subcellular distribution of receptors in target tissues and, for some drugs, effects on receptor localization are thought to represent the primary therapeutic mechanism (3). Accordingly, it is of considerable interest to have experimental methods suitable for assessing receptor localization and trafficking.

In this chapter, we discuss several approaches for assessing the subcellular distribution and trafficking of receptors, based on our experience with several members of the GPCR family. We focus on immunochemical methods that require receptor-specific antibodies or the expression of mutant receptors fused to an epitope-tag. These methods complement pharmacological and cell fractionation assays used traditionally to assess receptor distribution, and can be applied both to cell culture and intact tissue preparations. Each of the methods described is highly flexible, and can be modified to accommodate particular experimental questions and constraints. They are also flexible with regard to the deployment

of newer experimental technologies, such as engineered fluorescent proteins and covalent protein tagging.

1.1. Visualization of Subcellular Localization of GPCRs

How does one begin to investigate the localization and trafficking fate of a GPCR? An obvious starting point is to visualize the subcellular distribution of receptors using immunocytochemical staining and microscopy. A major limitation in many cases has been the relative dearth of antibodies that are capable of specifically detecting receptors of interest when expressed at their (typically low) native levels. The widespread availability of recombinant receptors, as well as application of methodologies such as epitope and fluorescent protein tagging (discussed elsewhere in this volume), has greatly facilitated progress in this area. There is increasing application of enzyme-directed covalent modification (e.g., SNAP-tag) as an alternate means of receptor labeling. Of course one must consider, and control for, the possibility that recombinant receptors may not faithfully mimic native receptor properties. Tagged, recombinant receptors are typically amenable to conventional methods of chemical fixation, immunocytochemical staining, and microscopic imaging.

Generally receptor localization is carried out using fluorescence microscopy, after staining tagged receptors with a fluorescent antibody conjugate. Receptors tagged with an intrinsically fluorescent protein [such as green fluorescent protein (GFP)] can be visualized directly, but may lose fluorescence intensity after fixation. Anti-GFP antibodies and conventional fluorescent conjugates are often used to augment detection if this is a problem. Fluorescent staining methods are generally rapid and amenable to imaging the localization of multiple receptors and/or other proteins in the same preparation, taking advantage of the availability of a wide range of spectrally resolved fluorescent conjugates and suitable optics for multicolor acquisition. Conventional fluorescence imaging methods are intrinsically limited in their ability to resolve small structures by Abbe's law, which specifies that the ability of light to resolve objects is directly proportional to the illuminating wavelength (4). Even with the highest quality microscope and objective lens, the resolution limit of epifluorescence or confocal fluorescence microscopy is on the order of ~300 nm, a usable range for many (but not all) questions of receptor localization. Much higher spatial resolution (on the order of a few nanometer) can be achieved using electron microscopy. This is because the effective wavelength of an electron beam is considerably smaller than that of light. Immunoelectron microscopy is similar in principle but involves additional sample preparation and reagents, description of which is beyond the scope of this chapter.

There are several patterns of subcellular localization typically described for GPCRs, and recognizing these can be a useful first step in inferring the basic membrane trafficking properties of receptors. Many GPCRs are localized primarily in the plasma membrane, particularly in cells not exposed to an agonist. Other GPCRs may be localized primarily in the endoplasmic reticulum (5) or subcompartments of the Golgi apparatus (6), or may be found in endosomes at steady state (7). The addition of agonist for between 5 and 120 min often causes a redistribution of receptors from the plasma membrane to a more punctate distribution within the interior of the cell, reflecting the occurrence of ligand-induced

endocytosis. Co-staining with various markers can help determine these localization patterns. For receptors that undergo ligand-induced internalization, subcellular localization at various times after inducing receptor endocytosis can provide information about the subsequent trafficking fate of receptors in the endocytic pathway.

Receptor localization in the plasma membrane is often obvious in cultured cells, based on a smooth peripheral pattern of receptor immunoreactivity. This can be verified by establishing surface accessibility of receptors, or by colocalization with fluorescent concanavalin A (a lectin that labels surface glycoproteins), or the Na⁺, K⁺ ATPase that is localized primarily in the plasma membrane of most cell types. A number of markers have been established that help the investigator to distinguish different types of internal membrane compartments relevant to GPCR trafficking. These include fluorescent transferrin and transferrin receptor antibodies (these mark early endosomes and the major recycling pathway), EEA1 (concentrated on early endosomes), LAMP1 and 2 (concentrated on late endosomes and lysosomes), BiP and calnexin (localized to endoplasmic reticulum), galactosyl transferase (Golgi apparatus), and TGN38 (trans-Golgi network). Specific antibodies or fluorescent conjugates recognizing these proteins are available commercially, and colocalization relative to GPCRs is practical using the multilabeling flexibility of fluorescence microscopy.

1.2. Use of Flow Cytometry to Determine Cell-Surface Expression Levels of GPCR

Although microscopic visualization offers a quick and generally easy method to estimate the predominant subcellular localization of GPCRs, and to infer basic aspects of receptor trafficking, this method is not easily quantifiable. As such, it is difficult to determine absolute receptor numbers or precisely assess redistribution of receptors. We routinely use fluorescence flow cytometry as a more quantifiable tool, which is particularly useful for assessing receptor internalization and recycling. In the simplest application, a fluorescent antibody conjugate is used to detect surface-accessible receptors before and after the exposure of cells to agonist, and then again after agonist removal. Changes in surface-accessible receptor immunoreactivity are then measured as a function of time. This allows one to use kinetic models to estimate rates of receptor internalization and recycling (8). Flow cytometric assays are similar in principle to cell surface ELISA, but we generally find that flow cytometry has a wider linear range of detection. It is possible to calibrate flow cytometry methods to allow determination of absolute receptor number, although this method is typically used to assess relative changes.

1.3. Use of Biotin-Protection, Degradation Assay to Measure Receptor Stability

Changes in receptor localization and surface receptor immunoreactivity are useful in assessing rapid trafficking processes, but such changes do not provide direct information about generally slower processes such as receptor biosynthesis or proteolytic destruction. A traditional method for inferring receptor biosynthesis and proteolysis is via radioligand binding assay (discussed elsewhere in this volume). Radioligand binding remains an extremely useful technique but is limited to receptors for which suitable ligands (usually high-affinity antagonists) are available with appropriate specificity and in radiolabeled form. Also, as radioligand binding reports changes in net receptor number (e.g., changes in $B_{\max}$ estimated from saturation isotherms), it can be challenging to distinguish the effects of

receptor biosynthesis from degradation using this technique. This is a particular concern in transfected cell systems, and for those receptors whose down-regulation is slow, as significant changes in receptor destruction can be effectively masked by the high level of biosynthesis driven by nonendogenous promoters typically used in these systems. Pulse-chase metabolic labeling and immunoprecipitation methods, described in detail elsewhere, are useful for investigating receptor biosynthesis. We have found nonradioactive methods, based on chemical modification of receptors by surface biotinylation, advantageous for selectively assessing endocytic trafficking, and proteolysis. Using a variation in which existing surface receptors are rendered nonreactive, the method can also be used to assess delivery of receptors to the plasma membrane from internal pools (6). Chemically “cleavable” biotinylation reagents can be used to allow selective labeling of internalized receptors, even if this pool is minor quantitatively (9, 10). The particular method described below (in Subheading 3.3) incorporates this principle.

2. Materials

2.1. Cell Fixation Materials

2.1.1. Complete Cell Culture Medium

1. Dulbecco's modified Eagle medium (DMEM).
2. 10% fetal bovine serum.
3. 100 U/mL penicillin and 100 µg/mL streptomycin.
4. 4 mM L-glutamine.
5. 25 mM glucose.

2.1.2. Poly-L-Lysine solution

1. Sterile H₂O.
2. Poly-L-lysine solution (0.1% w/v in H₂O).

Make a 1/100 dilution of the poly-L-lysine solution in sterile H₂O.

2.1.3. Phosphate-Buffered Saline

1. 135 mM NaCl.
2. 15 mM Na₂HPO₄·7H₂O.
3. 2.68 mM KCl.
4. 1.47 mM KH₂PO₄.
5. 0.68 mM CaCl₂.
6. 0.49 mM MgCl₂·6H₂O.

2.1.4. Phosphate-Buffered Saline/EDTA

1. 135 mM NaCl.

2. 15 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$.
3. 2.68 mM KCl.
4. 1.47 mM KH_2PO_4 .
5. 1 mM EDTA.

2.1.5. Fixation Buffer

1. Phosphate-buffered saline (PBS).
2. 4% Formaldehyde (made from paraformaldehyde).

Dissolve 4 g of paraformaldehyde in 100 mL PBS, heating gently (to about 65°C with stirring) in a fume-hood. Be careful because formaldehyde fumes can be dangerous, particularly if inhaled or exposed to the eyes. Add 2–4 drops of 1 M NaOH to help the paraformaldehyde dissolve. Chill on ice, aliquot and store at –20°C.

In some cases, the PBS can be replaced with a PIPES-buffered solution, or supplemented with an osmotic substance such as 300 mM sucrose. These modifications can help in maintaining the morphology of some subcellular compartments.

2.1.6. Permeabilization Buffer

1. PBS.
2. 1% bovine serum albumin.
3. 0.5% Triton X-100 (or Nonidet P40).

2.1.7. Quench/Wash Buffer Tris-Buffered Saline

1. 50 mM Tris base.
 2. 275 mM NaCl.
 3. 6 mM KCl.
 4. 2 mM g/L CaCl_2 .
- Adjust pH to 7.4 with HCl.

2.2. Flow Cytometry Materials

2.2.1. Complete Cell Culture Medium

2.2.2. PBS

2.2.3. Primary/Secondary Antibody Solutions

1. Antibody (appropriate concentration; we typically use M1 anti-Flag at 1 $\mu\text{g/mL}$).
2. PBS.
3. 0.1% BSA.

2.2.4. Fixation Solution

1. PBS.
2. 1% Formaldehyde.

2.3. Biotinylation Materials**2.3.1. Poly-Lysine Solution****2.3.2. PBS****2.3.3. 100× Biotinylation Solution**

1. 30 mg/mL cleavable biotin (EZ-link NHS-SS-biotin, Thermo Scientific).
2. DMSO.

2.3.4. 1× Biotinylation Solution

1. PBS.
2. 100× Biotinylation Solution.

Dilute the 100× biotinylation solution in PBS just prior to addition to cells.

2.3.5. Tris-Buffered Saline

1. 50 mM Tris base.
 2. 275 mM NaCl.
 3. 6 mM KCl.
 4. 2 mM g/L CaCl_2 .
- Adjust pH to 7.4 with HCl.

2.3.6. Complete Cell Culture Medium

1. DMEM.
2. 10% fetal bovine serum.
3. Penicillin and streptomycin.

2.3.7. Stripping Buffer-1

1. 50 mM Tris-HCl, pH 8.8.
2. 100 mM NaCl.
3. 1 mM EDTA.
4. 1% BSA.
5. 100 mM MESNA.

2.3.8. Stripping Buffer-2

1. 5 mM glutathione.

2. 75 mM NaCl.
3. 10% fetal bovine serum.
4. pH with 7.5 mL of 1 M NaOH.

2.3.9. Quench Buffer

1. 100 mL PBS.
2. 1 g iodoacetamide.
3. 1 g BSA.

2.3.10. Lysis Buffer: 10 mL

1. 250 mM NaCl.
2. 50 mM Tris/HCl, pH 7.4.
3. 5 mM EDTA.
4. 0.5% Triton X-100.
5. 10 g/L iodoacetamide.
6. 1× Complete mini protease inhibitor cocktail tablet (Roche).

Add the iodoacetamide and inhibitor cocktail tablet fresh.

3. Methods

3.1. Cell Fixation and Staining

For the purposes of this chapter, it is assumed that your GPCR of interest is expressed in HEK293 cells; however, the general principles remain the same for whichever cell line/tissue you are using. Stable cell-lines expressing your GPCR should be maintained in complete cell culture medium until the time of experiment. Coverslips should be sterilized and placed into appropriate cell culture dishes; typically we use 12-well plates and 18-mm diameter glass coverslips. The use of poly-L-lysine provides a charged surface on the glass, and allows significantly greater attachment of cells. This becomes important with the large number of washes required and ensures that there will still be cells attached to the coverslips at the end of the protocol.

1. Add 1 mL of poly-L-Lysine solution to the wells to completely cover the coverslip.
2. Leave for 10 min.
3. Aspirate off the poly-L-lysine solution and allow coverslips to air-dry for 1 h.
4. Remove the medium from the cells and lift with PBS/EDTA.

5. Return to complete cell culture medium and replate on to coverslips so they will be ~80% confluent on the day of experimentation, typically 24–48 h after plating.

The conditions of the experiment itself will vary depending on the specific question being asked, but the principles for fixation and staining remain the same as for a simple endocytosis experiment. Cells should be left untreated, others should have agonist added to the culture medium for 10, 30, and 90 min to measure endocytosis. Following endocytosis, the cells may be washed and returned to complete medium in the presence of an antagonist to allow the receptors to recycle.

Following agonist treatments it is important to rapidly cool the cells to prevent further trafficking (which is essentially zero at 4°C) and then fix the cells. A number of different fixatives can be used, depending on the particular antigenic properties of the protein you wish to stain, but the most commonly used is formaldehyde. Formaldehyde is a small membrane permeable fixative that forms methylene crosslinks with various parts of the cellular proteins and maintains a lot of the cellular architecture. Organic solvents, such as ice-cold methanol are sometimes used, particularly if aldehyde fixation negatively impacts antigenicity. Following fixation it is important to quench the fixation reaction to prevent the formaldehyde interfering with the staining steps; this can be achieved by incubation with a primary amine, such as glycine, or by carrying out multiple washes in Tris-buffered saline (TBS).

6. Wash cells twice with ice-cold PBS.
7. Fix cells for 10 min with 1 mL/well fixation buffer.
8. Wash cells three times in TBS.
9. Quench fixation buffer for 20 min with 1 mL/well TBS.

Once fixed, the cells need to be permeabilized to allow antibody access to intracellular compartments. A number of different detergents can be used, commonly Triton or NP-40. Milder reagents, such as saponin and digitonin may also be used. When used at low concentration these selectively extract a subset of lipids, effectively creating holes in membranes with less perturbation of overall membrane structure. Following permeabilization it is important to block nonspecific binding of antibodies by incubating the cells in PBS containing 1% bovine serum albumin (alternative blocking solutions are 1% gelatin or 10% serum from the species that the secondary antibody was raised in), typically, the blocking solution also contains detergent (as do all the antibody solutions).

10. Permeabilize cells for 10 min with 1 mL/well permeabilization buffer.
11. Incubate the cells for 30 min in blocking buffer.

12. Dilute primary antibodies to appropriate concentration in blocking buffer (see Note 1).
Any number of antibodies can be added at one time, provided that there is enough specificity for secondary antibodies. Typically, two are used at any time, normally raised in mouse and rabbit; however, two mouse antibodies can be used if they are monoclonal subtypes (e.g., IgG1 and IgG2).
13. Incubate cells for 1 h in primary antibody solution.
14. Wash cells three times in TBS (see Note 2).
15. Leave in TBS for 30 min in TBS.
16. Dilute fluorophore-conjugated secondary antibodies to appropriate concentration in permeabilization buffer.
The secondary antibodies should have the required immunological specificity (e.g., anti-mouse vs. anti-rabbit IgG, or anti-IgG1 vs. anti-IgG2). There are several excellent commercial sources for these reagents, and specific advice regarding their selection and use, such as at Jackson ImmunoResearch (Malvern PA, USA). For double-labeling, one would typically choose a green fluorescent conjugate (such as fluorescein or Alexa488) for one secondary antibody and a red conjugate (such as TexasRed or Alexa594) for the other. The specific fluorochromes used should be chosen according to the ability of the available imaging equipment to spectrally resolve them (see Note 3).
17. Stain cells for 1 h in secondary antibody solution.
18. Wash cells twice in TBS.
19. Leave cells for 10 min in TBS.
20. Remove TBS.
21. Repeat steps 19 and 20, three times (see Note 2).

¹In our experience the most critical determinant of success or failure in immunocytochemical staining is the primary antibody. In all cases, one must find the optimal antibody concentration and incubation time. For monoclonal antibodies and epitope-tagged receptors in dissociated cells, the staining is typically highly robust over a range of times and concentrations. For anti-sera, as well as for all types of staining applied to tissue sections, these conditions are more critical. An important control for nonspecific staining for tagged receptors is to apply the same conditions to cells not expressing the tagged receptor, or expressing the same receptor tagged with an irrelevant epitope. For endogenous proteins the nonspecific control is often limited to blocking peptide or immunogen. This is an important control, but is less definitive because cross-reactive, but irrelevant antigens can also be blocked. In this case the ideal control is to stain cells/tissue from the relevant knockout animals, if available.

²The second most important variable for successful staining is adequate washing after antibody incubations. This must be determined for each situation, but, in general, one must wash extensively and make sure that complete solution changes occur in each wash. The same principle applies to flow cytometric assays, although this method can be more forgiving, because the passage of cells through the saline solution used in the internal plumbing of the instrument provides additional washing before analysis.

³In any multilabel experiment, one should verify specific detection of each antigen. In our experience, the two most common causes of trouble are immunochemical cross-reactivity between primary and secondary reagents (i.e., one of the secondary antibodies has significant affinity for the “wrong” primary antibody) and spectral “bleed-through” (i.e., the imaging system does not adequately separate the fluorochromes used to label the secondary antibodies). One can test for immunochemical cross-reactivity in control specimens using single labeling for the “wrong” combination of primary and secondary antibodies. One can test for spectral bleed-through in control specimens using single labeling for the “right” individual combination of primary and secondary antibodies, and imaging in the “wrong” channel on the microscope. To be valid, these controls must be carried out in parallel specimens and using the same microscope acquisition settings.

- Lastly the samples need to be mounted on to microscope slides before imaging.
22. Place two drops of fluoromount onto a microscope slide.
 23. Lift coverslips out of the wells using a needle and forceps.
 24. Wash in ddH₂O.
 25. Place coverslip cell side down onto the drop of fluoromount.
 26. Seal coverslip with nail polish to prevent drying and movement under microscope.
 27. Store in the dark at 4°C. It is generally advisable to visualize specimens soon (within a few hours) after preparation. Depending on the dissociation rate of the antibodies used in staining, however, it is sometimes possible to store specimens in the cold for several days.

Imaging is typically carried out using a high-quality fluorescence microscope. Resolution of subcellular structures typically requires use of water or oil immersion objective lenses that provide a relatively high numerical aperture (NA). Optical resolution is inversely proportional to NA, and objectives used for examining subcellular localization of receptors generally have NA³ 1. Oil immersion lenses offer the highest possible NA, and thus attainable spatial resolution. Water immersion objectives are often preferred for thicker specimens because of spherical aberration that becomes problematic when imaging deeper into aqueous medium. Standard epifluorescence microscopy can be useful for cultured cells that are relatively flat. Confocal fluorescence microscopy is often preferred, however, especially for thicker specimens. Confocal microscopes reject a fraction of the emission or scattered light originating from out of the focal plane, allowing one to localize receptors in “optical sections” with reduced background. Many excellent instruments are available commercially or in multiuser facilities. By way of example, we typically use a shared Zeiss LSM510 confocal microscope fitted with a 63 × 1.4 NA oil-immersion objective lens for imaging relatively flat tissue culture cells. However, there are many other excellent instruments and options. There are also a variety of newer developments in fluorescence imaging methodology, which may be preferred for particular questions or applications (see Note 4).

⁴We typically use conventional epifluorescence or laser-scanning confocal microscopy for fixed specimens. One major area of rapid advance in the analysis of GPCR trafficking is the imaging of receptor localization and redistribution in living specimens. Such approaches can provide important insights into regulatory events occurring acutely in response to physiological or pharmacological stimuli. Fluorescent antibody conjugates can be useful for live-cell imaging, but the popularity of this approach has exploded with the availability of intrinsically fluorescent protein tags, such as GFP and its many variants. Live imaging involves many of the same principles and techniques described above, with some adaptations. Additional fluorescence imaging methods, such as spinning disk confocal microscopy and two-photon microscopy are useful for determining three-dimensional localization of receptors in cells and tissue preparations. Total internal reflection fluorescence (TIRF) microscopy is gaining popularity for imaging receptor redistributions among microdomains of the plasma membrane (11, 12). Another area of current advance is toward achieving higher spatial resolution than the theoretical diffraction “limit” of optical microscopy. This has the exciting potential to reveal, potentially even in live preparations, fine details of receptor localization that were previously accessible only in fixed specimens using electron microscopy. These so-called super-resolution methods of fluorescence imaging involve the merging of conventional optical techniques with additional physical principles. One approach, called photoactivated localization microscopy (PALM), is based on the principle of single-particle tracking and adapted to the availability of improved photo-convertible fluorescent dyes (13). Another promising approach, called stimulated emission decay (STED) microscopy, is a variation of laser-scanning confocal microscopy that increases the effective resolving power of the laser excitation (14). A number of recent studies have carried out live-imaging of GPCR

3.2. Flow Cytometry

1. Plate cells into 12-well plates so that each time point/drug treatment can be measured in triplicate.
2. Treat cells.
3. Wash cells twice with ice-cold PBS.
4. Add 0.5 mL of PBS to each well and lift cells by mechanical trituration and place in flow cytometry tubes.
5. Dilute primary antibodies to appropriate concentration in staining buffer (see Note 1).
6. Stain cells for 1 h in primary antibody solution.
7. Centrifuge tubes at $500 \times g$ for 5 min.
8. Resuspend cells in 3 mL of PBS.
9. Repeat steps 7 and 8, two times (see Note 2).
If the primary antibody is directly conjugated to a fluorophore then skip ahead to step 15.
10. Leave in PBS for 30 min.
11. Dilute fluorophore-conjugated secondary antibodies to appropriate concentration in permeabilization buffer.
12. Stain cells for 1 h in secondary antibody solution.
13. Wash cells twice by centrifugation.
14. Leave cells for 10 min in PBS.
15. Add 0.5 mL of fixation buffer.
16. Analyze surface fluorescence using a flow cytometer.

The details of flow cytometric analysis methods are beyond the present scope, but many institutions have multiuser facilities with suitable instrumentation and expertise. We routinely use a BD FACSCalibur instrument (Becton Dickinson Biosciences, San Jose, CA), and collect data from >10,000 cells per sample. Changes in cell-surface receptor immunoreactivity are typically estimated by determining changes in the mean or geometric mean calculated from the cell population.

3.3. Surface Biotinylation Assay

This assay is a very simple method for analyzing the stability of specific GPCRs in the presence of prolonged agonist treatments; however, it contains a large number of washing steps. Although tissue culture dishes are already treated to allow cell-lines to grow, we treat

trafficking in both cultured cells and tissues. Single-particle tracking has been applied to GPCRs for some time (15), and it is likely that GPCR localization using newer super-resolution methodologies will be forthcoming soon.

the dishes with poly-L-lysine to encourage attachment of the cells to the plates and prevent cell loss during the many washes and manipulations required in this assay. The size of the tissue culture plate required will depend on the receptor expression level and the amount of endocytosis the receptor undergoes. For a cell-line expressing receptors that undergo substantial endocytosis, 60-mm dishes will be sufficient, but can be scaled up to 100-mm dishes if required.

1. Add enough poly-L-lysine solution to cover the bottom of 6 × 60-mm dishes (see ^{Note 5}).
2. Leave for 10 min at room temperature.
3. Aspirate off the poly-L-lysine solution and leave to air dry for 1 h.
4. Plate cells so they are 80% confluent on the day of the experiment (see ^{Note 6}).

On the day of the experiment label the plates 100%, “strip,” NT, agonist 30 min, agonist 90 min and agonist 180 min (lengths of agonist treatments can be altered depending on the stability of any particular GPCR), and place the plates on ice (all manipulations should be carried out on ice to prevent endocytosis or trafficking taking place when not wanted). The first step is to label all of the cell-surface receptors with biotin. The biotinylation reagent used includes a reactive *N*-hydroxysuccinimide (NHS) ester group, which forms a stable amide bond with free amine groups in their nonprotonated form (see ^{Note 7}). The biotin used here is the so-called cleavable sulfo-NHS-SS-biotin, indicating that it contains a disulphide bond that can be broken by thiol reducing agents, thereby removing it from the labeled protein.

1. Wash all plates 2× with ice-cold PBS.
2. Aspirate off the PBS.
3. Carefully add 3 mL of 1× biotinylation solution to each plate (made fresh from 100× biotinylation solution, see ^{Note 8}).
4. Leave plates at 4°C for 30 min.
5. Quench the biotinylation reaction by washing 2× in ice-cold TBS (the amine groups in the Tris–HCl will “quench” unreacted biotin).
6. Leave the 100% strip plates in TBS and place at 4°C.

⁵It is important to include controls to make sure that the biotinylated signal detected represents the labeled receptor and not a nonspecific signal, at least until the experiment is standardized and the characteristic protein mobilities established. Typically this involves analyzing, in parallel, cells not expressing the tagged receptor or expressing an alternately tagged version.

⁶Optimal plating density and cell health is essential to keep cells from lifting off the dish through the extensive series of incubations and washes. This is particularly true for less adherent lines, such as HEK293 cells.

⁷The major reactive groups are the *N*-terminal α-amine (if not blocked) and the ε-amine present in lysine side-chains, both of which are basic, and thus the efficiency of cell-surface biotinylation can be quite low when conducted in PBS (pH ~7.4). If weak signals are obtained, alkalize the biotinylation reagent slightly (note, it is advisable not to exceed pH ~8.5 due to the tendency of alkaline conditions to produce cytotoxicity). Owing to the generally lower *pK_a* of the α-amine compared to ε-amine group (~9 vs. ~10.5), manipulating the reaction pH can also be used to adjust the degree to which lysine side chains are modified relative to the *N*-terminal α-amine.

⁸The biotinylation reagent can react also with water. This reagent must be prepared fresh in DMSO, and the powder should be kept in a desiccator to avoid inactivation during storage.

7. Add 5 mL of prewarmed complete medium to the other four dishes and return to the incubator.
8. Let cells equilibrate in the incubator for 30 min before addition of agonist to the three agonist plates (leave the NT plate in the incubator without agonist).

At this point the agonist plates will need to be removed at different times; however, the protocol is the same for all the plates. The NT plate should be left in the incubator for the same length of time as the longest agonist-treated plate. All plates (with the exception of the 100% plate) need to be “stripped” – treated with a reducing agent to remove any noninternalized biotin (see ^{Note 9}).

1. Wash the plates with 2× ice-cold PBS.
2. Add 3 mL of stripping buffer to each plate (except the 100% plate, which remains in PBS).
3. Leave for 10 min at 4°C, rocking gently.
4. Aspirate the first strip and repeat with another 3 mL of stripping buffer.
5. Leave for 10 min at 4°C, rocking gently.
6. Aspirate the stripping buffer.
7. Add 5 mL of quench buffer to each plate (including the 100% plate).
8. Leave at 4°C for 20 min.

The last step in the protocol is the recovery of biotinylated proteins and the detection of receptors. This is achieved by a modified version of an immunoprecipitation assay. Firstly, the cells are lysed and the nuclei and insoluble cytoskeletal components are removed by centrifugation. Samples are then mixed with streptavidin-conjugated agarose beads to recover the biotinylated proteins. Recovered proteins are then separated by size and charge by polyacrylamide gel electrophoresis, and the amount of receptor detected by an anti-epitope (typically Flag or HA) antibody and enhanced chemiluminescence.

1. Aspirate off the quench buffer.
2. Add 1 mL of lysis buffer (ensuring no reducing agents).
3. Scrape cells off of the plate and place in a 1.5-mL microcentrifuge tube.
4. Clear the supernatant by centrifugation at $20,000 \times g$ for 10 min at 4°C.
5. Transfer supernatant to a new set of microcentrifuge tubes.
6. Add 35 µL of streptavidin-conjugated agarose to each tube.
7. Incubate on a rocker at 4°C for 3–16 h.

⁹The reducing agent breaks the disulphide bridge of any biotin still on the surface of the cell. We provide recipes for two different stripping buffers, both of which are effective, but some cells may tolerate one buffer better than the other.

8. Centrifuge at $6,000 \times g$ for 1 min (streptavidin-conjugated agarose beads should sediment rapidly), discard the supernatant, and resuspend in 1 mL of lysis buffer. Repeat three times, rotating for 5 min between each wash and removing as much liquid as possible using a vacuum aspirator attached to a 22- to 25-gauge needle (to prevent aspiration of beads).
9. Repeat step 28, but resuspend in 1 mL of 20 mM Tris/HCl, pH 7.4.
10. Add 10 mL of PNGase-F solution.
11. Incubate at 37°C for 1 h.
12. Add 10 μ L of sample buffer.
13. Incubate at room temperature for 30 min. We do not boil samples, because we find this promotes the formation of aberrant high molecular weight receptor aggregates.
14. Centrifuge $14,000 \times g$ for 10 min at room temperature and load supernatant on SDS-polyacrylamide gels suitable for resolving the candidate GPCR.
15. Transfer to nitrocellulose or PVDF membranes using standard methods and immunoblot for GPCR using anti-epitope antibody (typically HA-11 or M2 Flag).

The different plates should give different amounts of immunoreactivity. The 100% plate is the total amount of receptor present at the cell-surface, the “strip” is a control plate to determine the efficiency of the protocol, and this lane should be essentially empty. The NT lane shows the agonist-independent endo-cytosis occurring during the time-course of the experiments. The agonist for 30 min is the pool of endocytosed receptor. The other agonist plates will demonstrate the stability of the receptor. If these plates are the same or more than the 30-min time point then the receptor is fairly stable and likely to recycle, if they are less than the 30-min point then the endocytosed pool has undergone rapid proteolysis.

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