Chapter 18

Live Cell Imaging Analysis of Receptor Function

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Abstract

Cell surface receptors are crucial in the regulation of a wide variety of signalling responses to extracellular stimuli such as soluble growth factors or matrix proteins. To respond effectively to rapidly changing environmental cues, many receptors are rapidly endo- or exo-cytosed to either subcellular or membrane compartments or they recruit specific intracellular binding partners. Recent advances in microscopy techniques have made it possible to study receptor behaviour in live cells to gain a better understanding of dynamics, binding partners and sub-cellular localisation. Here we describe several common currently used techniques to study receptor behaviour in living cells.

Key words: Microscopy, receptor, fluorescence, FRAP, FRET, FLIM, TIRF, Green fluorescent protein variants, endocytosis.

1. Introduction

Receptors are key to many fundamental processes in cell biology. Analysis of receptor function using biochemical assays has provided a great deal of information regarding posttranslational modification and binding partners, but does not allow precise determination of subcellular localisation or dynamics. Recent advances in microscopy techniques have enabled direct visualisation of receptor function during processes such as internalisation, dimerisation and intracellular trafficking in response to ligand binding. Such experiments have been made possible by both advancements in imaging technology and protein labelling techniques. In this paper we outline several imaging techniques and strategies that are currently used to analyse receptor function

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in live cells. The advantages, disadvantages and troubleshooting suggestions are discussed for each technique.

1.1. Fluorescent Labelling of Receptors

The most popular method for receptor labelling for live imaging is by generating a genetically encoded fluorescent tag on either the N or C terminus of the protein (**Fig. 18.1a**). Typically this is the green fluorescent protein (GFP) or a variant of it, which can be excited at specific wavelengths to produce a fluorescent signal. Many variants of the original GFP have been produced within the past 15 years and when these are exposed to light of a specific wavelength, they produce characteristic emission (1, 2). Photo-activatable (PA) or photo-convertible variants of the GFP tag have also been used to study localised protein dynamics (3). These can only be viewed when exposed to a burst of light, for example, PA-GFP requires activation with light of a wavelength of 405 nm, only then will the activated PA-GFP be viewable using excitation at a wavelength of 488 nm. These PA-constructs allow activation and tracking of the behaviour of a specific population of molecules (4). Since the generation of the first PA-GFP (5), variants have been developed with similar photo-activation or conversion properties but at different wavelengths of fluorescence (6–8). These fluorophores have been used successfully to study protein behaviour (9) and trafficking of integrin receptors (10). Fluorescent protein tags are relatively large (around 27 kDa) and as such may affect protein folding, function or disrupt interactions with potential binding partners. It is important therefore to rigorously test localisation and biochemical behaviour of the expressed tagged receptor prior to imaging to ensure function is not compromised.

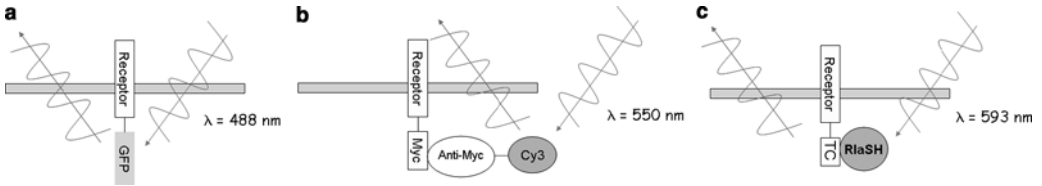

Fig. 18.1. Three most commonly used methods for labelling receptors in live cell imaging studies: (A) receptors labelled with genetically encoded fluorescent tags (e.g. GFP); (B) small molecule tags (e.g. myc) followed by anti-tag antibody detection; or (C) tetracycteine motifs for ReASH detection.

An alternative strategy is to use antibody labelling for live cell imaging (**Fig. 18.1b**). Kits are available which allow direct labelling of an antibody to the receptor of interest with a fluorescent reporter such as the Cy™ (GE Life Sciences), Alexa Fluor® (Molecular Probes) or DyLight™ Fluor (Pierce) dyes. This technique has been used to image a number of receptors including the $p75^{NTR}$ (11). In some cases this technique may prove more valuable as it permits the use of antibodies engineered to recognise different regions of the receptor or different conformations and

post-translational modifications. The antibodies being used may also have a blocking or inhibitory effect on receptor function; this needs to be considered when choosing an antibody. An additional way to use labelled antibodies is to genetically encode a small peptide tag on to the receptor of interest and then use a fluorescently labelled anti-tag antibody to study receptor behaviour. Examples of this include the use of both HA and myc tags to study nicotinic acetylcholine receptors (nAChR) (12) and flag tags to look at Class 1 metabotropic glutamate receptors (mGluRs) (13). These labelled anti-tag antibodies can be introduced into live cells either by direct microinjection into the cytoplasm or by brief treatment of cells with a mild permeabilising agent (such as saponin) to allow entry of antibody added to the medium. The same principle also applies to some fluorescent dyes such FIAsH-EDT_2 and RIAsH-EDT_2 dyes (Invitrogen; **Fig. 18.1c**). These particular dyes are fluorescent upon binding to the tetracysteine tag, consisting of a Cys-Cys-Pro-Gly-Cys-Cys consensus sequence (14). This has the benefit of being relatively small compared to fluorescent protein tagging and has been used successfully in the study of AMPA receptors (15). In some cases direct labelling of a receptor may not be possible and in these cases, direct labelling of a ligand may be more appropriate. The same labelling strategies discussed for receptors can also be considered for the ligand. The advantage of labelling ligand is that in many cases this can then be produced as a recombinant protein and labelled directly with dyes prior to treating cells. Labelled epidermal growth factor (EGF) is a good example of this with successful imaging being performed with Cy3- or Cy5-tagged EGF (16) or EGF tagged with quantum dots (17). Quantum dots are highly photostable semiconductor crystals that emit light when exposed to UV radiation or visible light depending on the crystal (18). One of the common studies to perform with labelled ligands is pulse-chase experiments. Here, cells are exposed to a dose of labelled ligand (pulse) and then at a later time point, unlabelled ligand (chase). In cases where both ligand and receptor are labelled, it is possible to investigate interactions between the two.

1.2. Overview of Strategies for Imaging Receptors in Living Cells

A number of different imaging techniques can be used to study fluorescently labelled receptors (**Fig. 18.2**). Wide-field (or epi-fluorescence) microscopy allows cells to be imaged with fluorescence and phase contrast, thus the position of a fluorescently tagged receptor can be seen in relation to cell movement. Imaging can be performed on a fluorescent microscope equipped with a charge-coupled device (CCD) camera and the appropriate excitation and emission filter sets to distinguish spectral regions of interest. When using sensitive CCD cameras, this method of imaging is relatively non-toxic to cells due to the low illumination/exposure times that are required to achieve high-quality images (19).

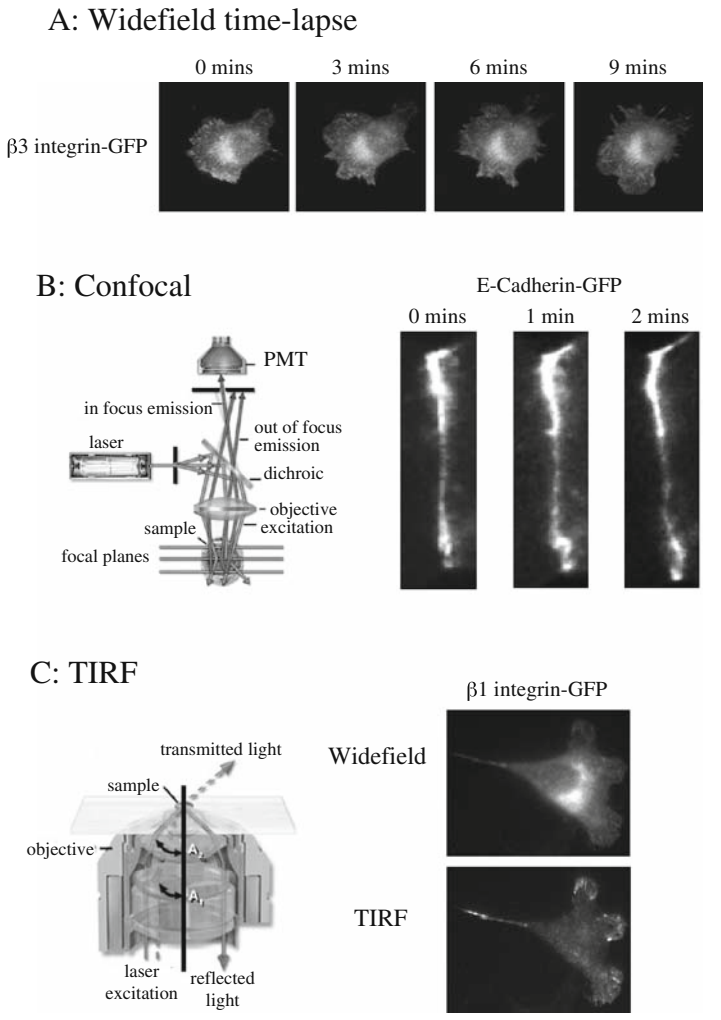

Fig. 18.2. Overview of methods to image receptors in live cells. (A) Examples of images acquired using wide-field fluorescence time-lapse microscopy to image dynamics of β3 integrin-GFP in fibroblasts. (B) Cartoon schematic of basic set-up of a confocal microscope (left panel) and (right panels) example images of E-Cadherin expressed in human epithelial cells imaged over 2 min. (C) Cartoon schematic of the principles of TIF microscopy (left panels) and example comparative images of β1 integrin-GFP in a mouse fibroblast by wide-field microscopy (top panel) and the same cell imaged by TIRF (bottom panel).

An alternative to wide-field microscopy is the highly sensitive confocal microscope. Here, instead of polychromatic light passing through filter sets, lasers are used to excite at specific pre-defined wavelengths. This then excites the tagged receptor and the emitted light from the sample is received by a photo-multiplier tube detector. Using this form of microscopy allows for greater resolution in the *z*-axis than wide-field microscopy as the emitted light is from a narrow focal plane. Depending on the speed of the

system, images can be taken anywhere from 4 to 30 frames/s using a fast resonant scan head. Faster scan speeds are more suited for live imaging and some systems will also allow Z-stack images to be acquired over time. A drawback of using this form of microscopy for live imaging is that the exposure of cells to lasers can result in tag photobleaching and phototoxicity, which can affect cell behaviour thus leading to artefacts.

Another method for viewing cell surface receptors in contact with the substrate (such as integrins) is total internal reflection (TIRF) microscopy. This technique works through production of an evanescent wave, which is achieved when light is totally reflected when passing from a solid phase to a liquid phase (**Fig. 18.2**). The resulting wave decays exponentially and so only penetrates a short distance into the cell, approx 100nm (20). Thus, this makes TIRF an ideal method for viewing events near to the plasma membrane, such as viewing protein recruitment to a receptor or dynamics of a receptor to, from or within the membrane.

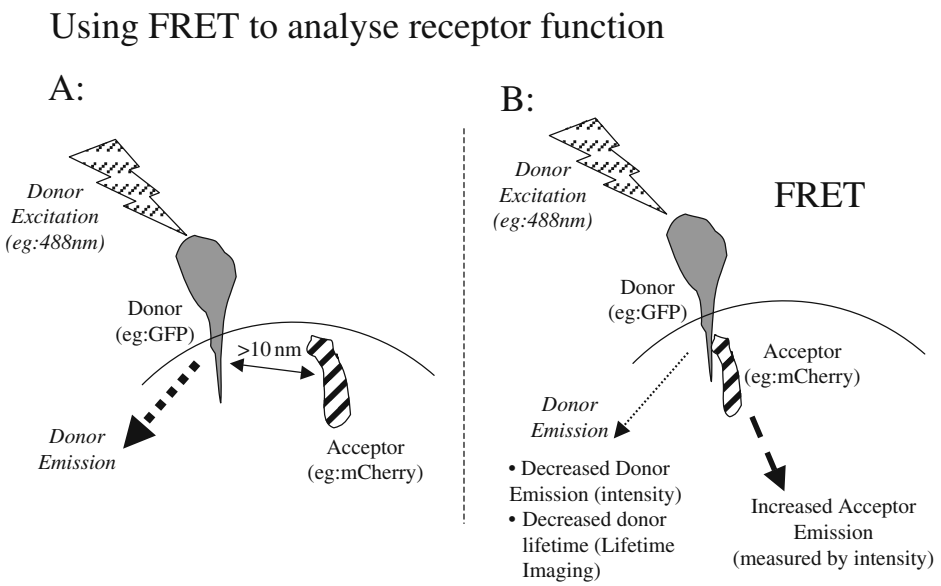

Fig. 18.3. Using FRET to analyse receptor function. (A) Donor and acceptor molecules are more than 10 nm apart, therefore excitation of the donor does not result in transfer of energy to acceptor. (B) When acceptor fluorophore is less than 10 nm from donor fluorophore, excitation of donor can result in both increased acceptor emission and decreased donor emission. FRET efficiencies can be calculated by measuring these parameters.

Fluorescence resonance energy transfer (FRET) is a technique used to measure the interaction between two fluorescently tagged proteins, when the donor (a protein tagged with a shorter wavelength tag) comes in close proximity with an acceptor located on the binding partner (**Fig. 18.3**). Non-radiative resonance energy transfer takes place from the donor to the acceptor, resulting in increased fluorescence of the acceptor, decreased fluorescence of

the donor and a reduction in fluorescence lifetime of the donor. Any of these three occurrences can be measured to ascertain the levels of FRET between the two interacting proteins. FRET is a powerful technique to study both receptor homo- and hetero-dimerisation and receptor association with intracellular binding partners in live cells (21).

Fluorescence recovery after photobleaching (FRAP) is an excellent method to study the dynamics of receptors and to quantify the speed of mobilisation within certain compartments of a cell (**Fig. 18.4**). Using a confocal microscope, regions of interest are defined and bleached until a total loss of fluorescent signal in that area is achieved. The rate of recovery of fluorescent signal within the region of interest is then recorded over time. The resulting data can be used to calculate the speed of recovery of intensity: the faster the time the faster the dynamics of your receptor. It can also be used to calculate the mobile and immobile fractions of the receptor, with the percentage recovery seen being the mobile fraction and the percentage recovery not seen being the immobile fraction (22). Fluorescence loss in photobleaching (FLIP) uses the same principle as FRAP apart from instead of measuring recovery, loss of intensity is recorded. The chosen region of interest is bleached repeatedly while images of the whole cell are taken. If fluorescence is lost outside the bleached region, then that indicates that your tagged protein has moved from, or through, this area (23).

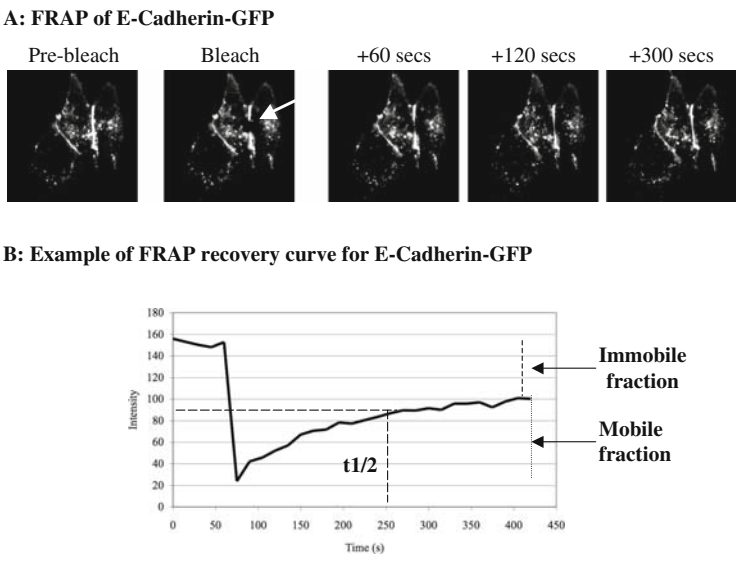

Fig. 18.4. FRAP analysis of receptor dynamics. (A) Sample images of a time course of images acquired on a confocal microscope to analyse dynamics of E-Cadherin-GFP by FRAP. Panels show a single pre-bleach image followed by an image acquired immediately post-bleach (bleached region of interest box highlighted in white) and subsequent recovery images. (B) An example of a recovery curve obtained from the FRAP data set.

2. Materials

1. Substrates designed for live cell imaging, such as glass coverslips (e.g. Menzel-Gläser) glass-bottom minidishes (MatTek, Ashland, MA) or LabTek® 2- or 8-well chamber slides (Nunc, Rochester, NY).
2. Extracellular matrix protein, e.g. fibronectin or vitronectin (*see* **Note 1**).
3. Phenol-red free medium (*see* **Note 2**).
4. Buffering solution such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (*see* **Note 3**).
5. Cells to be analysed – normal or transfected with tagged receptor.
6. Labelled antibodies or fluorescent ligands. These must be prepared in advance of imaging and diluted in an appropriate buffer. Labelled proteins should be filtered using a 0.22-mm syringe filter (Millipore, Bedford, MA) prior to use to avoid interference from aggregates of unbound fluorescent dye.
7. Proper filter sets (GFP, filters labels).
8. Immersion oil (e.g. from Cargille Laboratories, NJ, or Leica Microsystems, Wetzlar, Germany).

3. Methods

3.1. Wide-field Imaging of Fluorescently Tagged Receptors

1. Plate cells of interest into imaging dishes (*see* **Note 1**). Time of plating prior to imaging depends upon the experimental aims.
2. Prepare microscope. If an environmental chamber is used (surrounding microscope) ensure the chamber is pre-heated to 37°C and temperature is stable before use to avoid fluctuations in focus during imaging. Switch on fluorescence illuminator 20 min before use to allow stabilisation.
3. For pulse-chase experiments using labelled ligand, add ligand to cells on ice, allow binding to occur, wash off excess unbound ligand and replace with fresh media.
4. Place the dish onto the stage ensuring both the stage insert and dish are securely held in place. Allow around 10 min for stabilisation of temperature (this will reduce focal drift) and settling of the dish/plate.

5. If fluorescently labelled ligand or exogenous proteins are to be used, add these to the media at this stage to allow binding prior to imaging.
6. Identify a labelled/transfected cell of interest. This can be done either using illumination down the eyepieces or using direct visualisation onto the camera (*see* **Note 4**).
7. Set up acquisition software (this will vary – refer to manufacturers guidelines) and acquire an image of the cell in both GFP and phase-contrast channels.
8. Once the desired cell is in focus and stable, begin time-lapse acquisition (*see* **Note 5**).
9. Once acquisition is complete, the acquired images can be saved as a movie file (e.g. avi or quicktime) or saved as individual files (e.g. tiffs) and be presented as a montage as in **Fig. 18.2a**, where adhesions containing GFP-tagged β3-integrin can be seen. The change in localisation of these adhesions can be seen in relation to the cell's movement. Also, the assembly and disassembly of these sites can be quantified over the movie time period.

3.2. Confocal Microscopy Analysis of Receptor Localisation

1. Prepare cells and confocal microscope as in **Section 3.1**.
2. Once dishes containing cells are secure on the microscope stage, use the wide-field illuminator to find cells of interest down the eyepieces (*see* **Note 6**).
3. Once cells are located, switch to confocal PMT detector mode. Select the correct laser and filter set configuration.
4. Acquire a single-scan image of cells of interest and ensure focal plane/laser gain levels are correct and the resulting image is not saturated.
5. Set the time-lapse protocol window to acquire a single-scan image every 10–15 s over 5 min. Modify as necessary as in **Section 3.1**.
6. Z-stacks can also be acquired throughout the entire cell at every time point to enable collection of three-dimensional data sets over time. This enables a 3D reconstruction of the cell to be generated post-acquisition to analyse receptor behaviour within the entire cell rather than at a single focal plane. An example of this is shown in **Fig. 18.2b** where Z-stacks of cells expressing E-Cadherin-GFP have been taken (*see* **Note 7**).
7. In the analysis of dynamics of photo-activatable variants of GFP (PA-GFP) or photo-switchable fluorescent proteins, avoid exposure of cells to UV light prior to experimental photo-activation, as this can lead to low-level GFP activation.

Image and focus cells initially using brightfield illumination and a single scan using phase contrast/differential interference contrast (DIC) settings on the confocal scan head acquired.

8. Set up the time-lapse acquisition protocol to undergo a "bleach/activation" cycle (at 405 nm for PA-GFP) followed by acquisition of the desired number of images every 10–15 s using the 488-nm laser (to detect the activated GFP).
9. Highlight the area of interest to be photo-activated (e.g. plasma membrane) with a region of interest box using the bleaching function protocol. Activate the 405-nm laser at 100% power and illuminate the cell for a set number of iterations within the defined region of interest.
10. Once activation is complete, visualise and track the GFP over time within the cell.

3.3. TIRF Analysis of Receptor Behaviour

TIRF is only suitable for imaging receptors that are presented at the interface between the cell and coverslip. Correct alignment and calibration of TIRF illumination angle (both for wide-field and laser-based systems) must be checked prior to imaging to ensure a good TIRF signal is achievable.

1. Cell preparation as in **Section 3.1** (*see* **Note 8**).
2. Place imaging chamber securely onto microscope stage ensuring that the sample is completely flat on the stage.
3. Acquire starting images of cells under wide-field and TIRF illumination. Optimise focal plane and adjust TIRF laser angle if necessary. If an automatic focussing device is available on the microscope, activate this to ensure correct focal plane is maintained throughout the acquisition period.
4. Set up time-lapse software to acquire an image every × seconds over × number of repeats. Start experiment.
5. The images obtained again can be saved as in **Section 3.1**, step 9. Sample images can be seen in **Fig. 18.2c** where a fibroblast expressing GFP-tagged β1 integrin is seen in wide-field and in TIRF. The TIRF image shows β1 integrin containing adhesions which are in contact with the coverslip.

3.4. FRET Analysis of Receptor Dimerisation or Binding Partners

The common method used to measure FRET in live samples is using confocal acquisition of intensity-based measurements. Other techniques such as fluorescence lifetime imaging (FLIM) are used but tend to be less common, and due to lengthy imaging periods required for photon counting are often not best placed for live cell imaging studies. The following is an example of how to set up a FRET experiment for analysis by fluorescence intensity ratiometric method.

1. Co-transfect cells with donor- and acceptor-labelled molecules of choice. Common fluorophore pairs used are CFP/YFP and GFP/mRFP (or mCherry) (*see* **Note 9**).
2. Plate and mount cells on microscope stage as in **Section 3.1**.
3. Locate expressing cells and acquire a single-confocal scan to optimise focal plane and laser settings. Use a pseudocolour look up table (LUT) to ensure images are not saturated at any point.
4. Set up the time-lapse and bleach control windows to acquire a single-scan pre-bleach, followed by a bleach at a defined region of interest, followed by a single-scan post-bleach with no delay between acquisition scans. Set the bleaching laser to 100% output (see **Note 10**).
5. Set up region(s) of interest for bleaching. Run protocol and save data. If bleaching is incomplete, repeat on a different sample using increased bleach time.
6. Many software packages provide FRET analysis tools for post-acquisition purposes. Use these plug-ins where possible. If software is not installed on imaging system, the programme ImageJ (available from: http://rsb.info.nih.gov/ij/) can be used. Numerous plug-ins are available to download that allow you to analyse FRET data.
7. When analysing data consider the following: (a) corrections for fading during image acquisition (changes in intensity across the whole cell before and after bleaching); (b) corrections for differences in starting intensity of donor and acceptor fluorophores (this is essential when analysing ratiometric FRET data); (c) crosstalk detected between different fluorophores (i.e. wavelength "bleedthrough"), which can be tested by acquiring each channel as a single line scan individually with the other laser line inactivated.
8. Images should be smoothed using a 3×3 box mean filter (this can be done in ImageJ or photoshop), background subtracted and post-bleach images fade compensated. A FRET efficiency ratio map over the whole cell is calculated using the following formula: $(\text{donor}_{\text{postbleach}} - \text{donor}_{\text{prebleach}})/\text{donor}_{\text{postbleach}}$. Ratio values are extracted from pixels falling inside the bleach region as well as an equally sized region outside of the bleach region and the mean ratio determined for each region and plotted on a histogram. The non-bleach ratio is then subtracted from the bleach region ratio to give a final value for the FRET efficiency ratio. Data from images must be used only if acceptor bleaching efficiency is greater than 70%.

3.5. FRAP/FLIP Analysis of Receptor Kinetics and Recruitment

1. Set cells up as in **Section 3.1**.
2. Set up cells/imaging chambers on microscope stage securely.
3. Find cell(s) of interest and focal plane as in **Section 3.2**. Acquire a single scan at each of the required wavelengths to ensure good signal in each channel.
4. Set up time-lapse and bleach acquisition control panels. Define the region(s) of interest for photobleaching as in **Section 3.4**, and the laser for bleaching (e.g. for bleaching GFP, use 488 nm) set to 100% for bleach cycle (*see* **Note 11**).
5. Set up time-lapse acquisition software to acquire a minimum of four scans before bleaching (to ascertain pre-bleach intensity and fluorophore fading) followed by a single bleach scan, followed by a single scan every x seconds over x cycles (depending upon kinetics of receptor to be imaged).
6. Run imaging experiment.
7. Once the time-lapse protocol has finished, analyse data for recovery (or loss of signal for FLIP) kinetics. Plot intensity over time for the region of interest.
8. The final data set must be corrected for fading of the fluorophore over time (due to illumination). To achieve this, apply the slope of the curve of the pre-bleach image intensities plotted as a function of time, or a background region of interest for the entire time-lapse sequence.
9. Re-plot corrected recovery values as percentage recovery over time.

4. Notes

1. If cells do not adhere, coverslips or imaging chambers can be pre-coated with an extracellular matrix protein such as fibronectin or vitronectin.
2. Phenol red dye is auto-fluorescent and can result in high background levels during imaging. Media such as OptiMEM® can be used. If cells are unhealthy, the media can be supplemented with 1–10% fetal bovine serum (FBS).
3. Buffering solutions are only required if cells are to be imaged in an environment where the CO_2 levels are not regulated.

4. Avoid excessive exposure of cells to fluorescent light to preserve fluorescent signal and protect cells.
5. The interval period and number of images acquired will depend upon the dynamics of the receptor being studied. A good starting point is to acquire an image every 10–15 s for 5 min in both fluorescence and phase contrast channels. This will provide an initial idea of speed of movement of the receptor/ligand and the acquisition protocol can be subsequently modified if necessary.
6. Using Wide-field as opposed to confocal will avoid unnecessary exposure to lasers and help prevent photobleaching and phototoxicity.
7. Two potential caveats exist with acquiring Z-stacks over time. First, depending upon the final resolution of the image obtained and the optical depth of the samples, the acquisition of a full Z-stack can be rather time-consuming. In this case, it may be necessary to reduce either the resolution (from 1024×1024 pixels to 512 or 256 pixels) or the number of Z-stacks to speed up acquisition times. Second, the increased exposure to laser illumination during acquisition of Z-stacks may cause considerable fading of the fluorophore and/or prove toxic to some cells.
8. For TIRF analysis, cells must be plated on glass coverslips and mounted in aqueous medium, e.g. growth media or PBS (phosphate-buffered saline).
9. Ideally, each fluorophore-tagged protein should be expressed at similar levels in each cell to be analysed.
10. The number of iterations (or bleach scan passes) should be optimised for each fluorophore depending upon the intensity of the signal to be bleached and the output power of the laser line used.
11. For FRAP, the bleach region will be a relatively small, defined area of interest such as a membrane compartment or vesicle. For FLIP analysis, the protocol set-up is essentially the same, but the bleach region is much larger to allow calculation of loss of fluorescence in the remaining un-bleached region of interest.

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