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Förster Resonance Energy Transfer-Based Imaging at the Cell Surface of Live Cells

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Abstract

Understanding the molecular mechanisms of protein–protein interactions at the cell surface of living cells is fundamental to identifying the nature of cellular processes. Here, we discuss how fluorescence-based approaches have been successfully developed to visualize protein–protein interactions in living cells. Förster resonance energy transfer (FRET) is unique in generating fluorescence signals between proteins that are highly spatially sensitive. Furthermore, total internal reflectance fluorescence (TIRF) microscopy combined with FRET is a robust technique used to assay protein/protein interactions and the functionality of proteins assembled at the cell surface membrane.

Keywords

Protein–protein interactions; Cell surface; FRET; TIRF; Live-cell imaging

1 Introduction

Biological systems are composed of basic physiological and chemical processes in which molecular events among thousands can be feasibly studied through optical imaging techniques. Fluorescence microscopy is a powerful tool in the biomedical and biological sciences enabling visualization of the myriad proteins and processes of cellular physiology. Progress has been made in the last decade in developing methods in different modes of epifluorescence microscopy. One method of detecting molecular interactions involves Förster resonance energy transfer (FRET) in which energy is transferred from a “donor” fluorophore in an excited state whose emission spectrum overlaps the absorption spectrum of an “acceptor” fluorophore (1). The FRET efficiency depends on many factors, but the most critical is the donor–acceptor distance, with the efficiency maximal at $\sim 50\text{\AA}$ (r_0), a distance only expected if the donor and acceptor fluorophores are in intimate proximity. What makes FRET such good evidence of intermolecular interactions is that the FRET efficiency declines with the inverse sixth power of the donor–acceptor distance, more precisely $1/(R - r_0)^6$, where R is the distance between donor and acceptor (2). Thus, although the FRET efficiency is dependent on other factors, such as the degree of overlap of the donor emission and acceptor absorption spectra, the relative orientation of the donor absorption and acceptor transition moments, and the refractive index of the medium, the

steep dependence on distance makes this approach powerful in establishing the physical interaction between fluorescent molecules (3).

Traditional FRET experiments on membrane proteins performed under confocal microscopy have often been hampered by contamination of events at the plasma membrane, which are most relevant, by those occurring in the cytoplasm. Thus, a major tool in the field is the use of total internal reflectance fluorescence (TIRF) microscopy, which exploits the behavior of light at the interface between two media of differing refractive index. At such an interface, at an angle greater than a critical angle, the main light beam does not penetrate the second medium, but rather is totally internally reflected back into the first medium. However, a component of the light energy, called the evanescent wave, does penetrate into the second medium at a perpendicular angle and, importantly, decays away exponentially with distance (4, 5). At a glass/water interface typical of living cells in solution, and using a fluorescent microscope objective with numerical aperture (N.A.) of typically 1.45, the evanescent wave, and thus the excitation of the cell, only penetrates to a depth of ~200 nm. Thus, if one is performing FRET experiments under TIRF illumination, only fluorophores located within or close to the plasma membrane are selectively detected within the evanescent field, whereas molecules in the cytoplasm will not be illuminated. This allows for a drastic reduction in the fluorescence background coming from irrelevant proteins in the cytoplasm, and a very high signal-to-background ratio in and around the membrane of living cells. TIRF combined with FRET is especially useful for studying the interactions among plasma membrane proteins such as ion channels, receptors, and their associated signaling molecules, and trafficking events in which the movement of proteins into or away from the membrane can be visualized in real time. Examples of the TIRF/FRET approach include interactions among G-proteins, enzymes, ion channels, scaffolding proteins, and other associated proteins. For example, GTPases were shown to regulate trafficking of $K_{ir}2.1$ channels (6), calmodulin, and A-kinase anchoring protein 79/150 binding to M-Type (KCNQ) K^+ channels (7, 8) and activation of GIRK K^+ channels by $G_{\beta\gamma}$ subunits (9).

The methods and equipment discussed in this chapter utilize enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP), which is the most commonly used fluorescent protein FRET pair, and we will focus on this set of donor and acceptor fluorescent proteins (FPs). Given that TIRF works best at a glass/water interface and that we are usually most interested in dynamic events happening in living cells, our TIRF/FRET experience is solely using live cells, as opposed to fixed cells in which the typical mounting medium and flattened cell architecture are both not very suitable. Cultured cells are most convenient since multiple FP-tagged proteins can be transfected simultaneously and also these cells adhere well to a glass substrate. Furthermore, these cells can be feasibly imaged and the FRET efficiency quantified using a regular epifluorescence microscope. Such FRET efficiency is often measured by either quantifying the increase in emission of the acceptor fluorophore upon energy transfer from the donor (sensitized emission) or by comparing the emission of the donor before and after photobleaching of the acceptor (acceptor photobleaching, donor dequenching). The former method is best when dynamic FRET is sought, since neither fluorophore is destroyed during a sequence of images, but necessitates a careful series of control images. On the other hand, the latter method is most efficient when a static FRET measurement is the goal, and is also relatively

simple to quantify accurately. This chapter describes how to perform TIRF/FRET measurements using the acceptor photobleaching method on heterologously transfected cultured cells. We describe the setup as currently sold by Nikon Instruments, the vendor who sold us our TIRF/FRET setup (still in use) some 10 years ago.

2 Materials

2.1 Equipment

1. Inverted Eclipse Ti Microscope with through-the-lens TIRF imaging (Nikon Instruments).
2. Vibration isolation system (“air table”) to minimize drift and noise on which the microscope is situated (i.e., Technical Manufacturing Corp., Peabody, MA, USA).
3. Laser light excitation is controlled by a computer-controlled acoustic optical tunable filter (AOTF) that controls output by computer. The lasers should include at least solid-state diode lasers with 442 and 514 nm lines. Although it should be noted that it is also possible to use an Argon (40 mW) laser outputting 488 and 514 nm lines for excitation of GFP and YFP, respectively, and a 442 solid-state diode laser for excitation of CFP, the development of economical, highly durable solid-state lasers has largely eclipsed the use of gas lasers.
4. Microscope optics equipped with an Apo TIRF 60× oil immersion high-resolution (1.45 N.A.) objective.
5. It is most helpful to have the microscope contain the following filter cubes: wide-field CFP and YFP, TIRF CFP and YFP, and a cube for using the dual-view chip-splitter containing only a dichroic mirror, as described below.
6. High-resolution charge-coupled device (EMCCD) camera (e.g., Photometrics Cool SNAP HQ2).
7. Dual-view chip splitter (Optical Insights, Photometrics, Tucson, AZ, USA).

2.2 Software

1. NIS-Elements for image acquisition and data analysis. The software controls laser light delivery, microscope, and the EMCCD camera (Nikon Instruments).
2. Microsoft Excel (used in conjunction with NIS-Elements for formula-based data analysis).

2.3 Epifluorescence Imaging

1. Chinese Hamster Ovary (CHO) or other suitable cell line expressing a membrane targeted tandem construct of eCFP and eYFP (Rho-pYC) to act as a positive control for strong FRET efficiency. It consists of the C-terminal prenylation site of Rho (RQKKRRGCLLL) appended to the C-terminus of a YFP-CFP fusion protein (10) (see Note 1).

2. CHO (or equivalent) cells expressing the CFP-tagged protein to be tested and eYFP-M (membrane bound). These act as a negative control for FRET efficiency, since they are known to be too far apart in the membrane to exhibit FRET, and make a good control for “spurious” or incidental FRET.
3. Tissue-culture cells expressing the eCFP-tagged and eYFP-membrane bound proteins that you wish to assay for molecular interactions.
4. Cell imaging solution: 160 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, pH 7.4 with NaOH. Other suitable saline solutions are equally suitable.
5. Glass-bottom culture dishes with No. 1.5 coverglass (0.16–0.19 mm) on the bottom, 35 mm (Mat-tek) (see Note 2).
6. Objective immersion oil.

In this chapter we are not describing protocols (and therefore not listing any materials required) for cell culture and transfection as these can be found elsewhere (i.e., Chapters 2, 4, and 5 of this book).

3 Methods

3.1 TIRF/FRET Experiments on Cultured Cells Using Epifluorescence Microscopy

It is most convenient to image eCFP and eYFP emission simultaneously, which can be easily performed using the dual-view chip splitter, which is put between the side-camera port and the CCD camera. This device is equipped with a filter cube containing a dichroic mirror, and emission filters of your choice. For concurrent eCFP/eYFP imaging, we use an HQ470 nm/30 m and HQ550 nm/30 m emission filters for eCFP and eYFP and a 505 nm dichroic mirror for separation of emission wavelengths. In this configuration, the microscope cube can contain only a dual-band TIRF dichroic mirror to selectively send excitation light to the sample, and emission light to the detector. The approximate TIRF angle is adjusted by eye to give the signature TIRF illumination to the experimental chamber. Any fine TIRF angle adjustment can be made through the *NIS-Elements* software. Fluorescence images are collected and processed with a 16-bit, cooled charge-coupled device camera (e.g., Photometrics Cool SNAP HQ2) interfaced to the *NIS-Elements* software. This camera uses a front-illuminated EMCCD with on-chip multiplication gain. Images are collected (100–600 ms exposure time) immediately before and after photobleaching eYFP. Images are not binned or filtered, with pixel size corresponding to a square of 122 × 122 nm.

3.2 Imaging of Cells

1. Just prior to fluorescence imaging, replace the cell culture medium with an appropriate cell imaging solution (see Subheading 2.3, step 4).

¹Cells can typically be kept in culture for imaging for 3 days post-transfection, but often appear to be unhealthy and do not provide reliable data on the fourth and subsequent days post-transfection.

²The coverglass with bottom thickness No. 1.5 is preferable to accommodate the short working distance of most high-power objectives.

2. Place the chamber on the stage on top of the oil immersion 60× objective (1.45 N.A.).
3. Using bright field transmitted light, focus on an isolated cell. Note that there will not be contrast optics in the light path, so focusing on the cells will be hard. We often “find” the cells under epifluorescence, without using transmitted light (see Note 3).
4. Using the 200 W metal-halide lamp (which gives similar spectral output to more traditional mercury vapor lamps) and alternating CFP and YFP filter cubes, pick a cell with robust expression of both fluorophores (see Note 4).
5. Under TIRF illumination and either the TIRF CFP or YFP filter cubes, the focal plane is adjusted immediately before each image acquisition to obtain a “sharp” TIRF image. The membrane proteins should appear punctate if the cell is focused correctly (see Note 5).
6. Take the “before” photobleaching images of the cell using the 442 and 514 nm laser lines (see Note 6).
7. Photobleach the eYFP fluorophores by using the metal-halide lamp and the wide-field YFP filter cube for a minimum of 7–10 min, which should be sufficient to achieve >80% photobleaching of the eYFP. Photobleaching is done through the wide-field metal-halide lamp since the molecules in live cells are diffusible in the membrane and are likely to move. Thus, wide-field illumination allows for the eYFP fluorophores in the entire cell to be photobleached and eliminates the problem of limited area laser photobleaching under TIRF illumination, which would be insufficient for donor dequenching (see Note 7).
8. After photobleaching, take the “after” photobleaching images of the cell using the same 442 and 514 nm laser lines.
9. Using the CFP “before” image, use the *NIS-Elements* analysis package draw tool to trace the cell perimeter. Export the averaged intensity to *Excel* using the log data function.
10. Using the draw tool in *NIS-Elements*, draw a second circle in the blank region of the CFP “before” image to estimate the background level. Export the averaged intensity to *Excel* using the log data function.

³If one is comparing groups of cells, it is imperative to select cells at a random basis that express differing fluorescence emission levels, as long as both eCFP and eYFP fluorophores are robustly expressed. Choosing cells of mostly the same expression will lead to bias of the data.

⁴For the donor-dequenching method of FRET quantification, it is best if there are not more CFP fluorophores than YFP fluorophores.

⁵It is imperative to be sure that the cell is under TIRF illumination. A quick check can be done by moving the TIRF angle in and out of the critical angle. Non-TIRF illumination will show high fluorescence in the cytoplasmic area of the cell. The nucleus should not be visible under TIRF.

⁶During imaging, it is important to be sure that the excitation and fluorescence detection parameters are set to include peak emission wavelengths and such that saturation levels are not reached for any pixel.

⁷It is also very important to note that when searching for a cell, a long period of exposure using the metal-halide lamp light source has the possibility of damaging the cell as well as photobleaching the FPs.

11. Subtract the background averaged intensity from the averaged intensity of the cell measured above. When the CFP image is used, this yields the CFP intensity; when the YFP image is used, this yields the YFP intensity.
12. Repeat steps 10 and 11 for each of the four images (i.e., “before” and “after” photobleaching for both CFP and YFP emission images).

3.3 Quantification of FRET Efficiency in TIRF Illumination

FRET efficiency using the acceptor photobleaching (donor dequenching) paradigm is calculated as the percentage of increased CFP emission after YFP photobleaching. The % FRET efficiency is calculated by drawing the entire area of the cell in the CFP TIRF image and subtracting the background in a cell-free region for each image (see formula below). In addition, % photobleaching efficiency is calculated by drawing the entire perimeter of the cell in the YFP TIRF image and subtracting the background in a cell-free region for each image.

$$\% \text{FRET efficiency} = \left(\frac{(\text{eCFP}_{\text{post}} - \text{eCFP}_{\text{pre}})}{\text{eCFP}_{\text{pre}}} \right) \times 100,$$

where CFP_{pre} and $\text{eCFP}_{\text{post}}$ are the CFP emissions before and after YFP photobleaching, respectively.

Using the protocol detailed in this chapter, Fig. 1 shows representative images of cells transfected with CFP and YFP before and after YFP photobleach. Specifically, Chinese hamster ovary cells were transfected with CFP-tagged angiotensin II AT₁ receptors and YFP-tagged KCNQ3 K⁺ channels. Here, FRET was measured under TIRF illumination by the donor dequenching method and CFP emission was significantly stronger after YFP photobleach, indicating robust FRET.

Positive controls are used as a measure of the highest percentage of FRET possible. Rho-pYC is a CFP and YFP tandem protein that is anchored to the membrane and thus gives an estimate for the maximal energy transferable between two interacting proteins. Conversely, a good negative control pair should give an upper limit for “spurious FRET” between two proteins in the membrane that do not interact, but might be accidentally close. We commonly use a CFP-tagged membrane protein (such as an ion channel) paired with membrane-localized YFP as such a good negative control. Thus, the controls provide the range of levels of FRET for data comparison, both for estimating the strength of FRET for interacting proteins, compared to the maximal possible, and for distinguishing two truly interacting proteins from those that are incidentally close, but do not interact.

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Fig. 1.

Shown are images of Chinese hamster ovary cells transfected with CFP-tagged angiotensin II receptors (AT₁R) and YFP-tagged KCNQ3 K⁺ channels under TIRF illumination, using 442 nm or 514 nm laser lines, respectively. Images of eCFP (*left, in “rainbow pseudocolor”*) and eYFP (*right, in yellow pseudocolor*) emissions are shown before or after YFP photobleach, as labeled. Note the significantly brighter eCFP emission (*warmer colors*) after eYFP photobleach and the profoundly dimmer eYFP emission after 7 min photobleach under wide-field illumination with the YFP filter cube