Chapter 6

FRET Microscopy for Real-Time Visualization of Second Messengers in Living Cells

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Abstract

Förster Resonance Energy Transfer (FRET) microscopy is a useful tool in molecular biology and medical research to monitor and quantify real-time dynamics of protein-protein interactions and biochemical processes. Using this well-established technique, many novel signaling mechanisms can be investigated in intact cells or tissues and even in various subcellular compartments. Here, we describe how to perform FRET measurements in living cells expressing FRET-based biosensors and how to evaluate these data. This general protocol can be applied for FRET measurements with various fluorescent biosensors.

Key words FRET, Microscopy, Imaging, Fluorescence, Biosensor

1 Introduction

Förster or Fluorescence Resonance Energy Transfer (FRET) microscopy is a powerful tool for real-time monitoring of signaling events in living cells and tissues [1, 2]. Classical biochemical techniques require thousands of cells to analyze a limited number of time points without any spatial resolution at the cellular level. The great advantage of FRET microscopy is the ability to visualize temporal and spatial changes of, for example, second messengers, not only within a single cell but also in subcellular compartments [3]. This technique is widely used for, e.g., pH measurements [4], detection of disease-related molecules [5], and visualization of compartmentation of second messengers such as cyclic nucleotides in cardiomyocytes [6].

FRET microscopy became a well-established method to investigate many novel signaling mechanisms and biochemical processes in living cells. Typical unimolecular fluorescent biosensors consist of a binding domain for the molecule of interest, which is flanked between two fluorescent proteins that act as energy donor and acceptor [2, 7]. The donor protein is excited with a single-wavelength light. The emitted light energy can be partially

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transferred to the neighboring acceptor protein, which also emits fluorescence light without being directly excited. The binding of the molecule of interest to the binding domain leads to a conformational change of the biosensor, resulting in an altered distance between donor and acceptor. With increased distance between the two fluorophores, the emitted light of the donor loses the ability to excite the acceptor, which leads to a reduction of transferred energy [2]. By monitoring the donor/acceptor fluorescence ratio, changes in concentration of the molecule of interest can be recorded and analyzed in real time. Alternatively, bimolecular sensors can consist of two interacting proteins, one fused to the donor and another one to the acceptor fluorophore to monitor changes in protein-protein interaction over time. FRET biosensors can be introduced into living cells by plasmid transfection, viral gene transfer or they can be expressed in transgenic animal models [8].

This chapter describes the method of how to perform FRET microscopy, calculate FRET ratio, and evaluate cellular dynamics of second messengers in living cells.

2 Materials

2.1 FRET Imaging System

1. Inverted fluorescent microscope (Nikon ECLIPSE T*i*-S) equipped with an oil immersion objective (Plan Apo λ 60×/1.40 oil) and a suitable filter cube (including an excitation filter for the donor fluorophore and a longpass dichroic mirror, e.g., ET436/30 and LP455, respectively, if the donor fluorophore is CFP).
2. Fluorescence light source (coolLED *p*E-100) with a wavelength similar to the maximum spectral absorbance of the donor, in our case 440 nm for the cyan fluorescence protein (CFP).
3. Beam-splitter (Photometrics Dual-View DV2) including a filter cube consisting of a dichroic mirror and two emission filters for the donor and acceptor fluorophores. For CFP and yellow fluorescent protein (YFP) as a donor-acceptor pair, the DV2-cube 05-EM is routinely used. It includes a 505dcxr dichroic mirror, D480/30m and D535/40m emission filters.
4. CMOS Camera (QIMAGING optiMOS).
5. Arduino digital input/output board.
6. Microscopy software (Micro-Manager1.4.5 together with ImageJ). Refer to a previously published protocol that describes how to connect all imaging system components and how to set up the software [9].

2.2 Materials for FRET Measurements

1. Immersion oil for microscopy.
2. FRET Buffer: 144 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2 \times 6H_2O$, 1 mM $CaCl_2$, and 10 mM HEPES. Weigh

8.42 g NaCl, 0.40 g KCl, 2.03 g $MgCl_2 \times 6H_2O$, and 2.38 g HEPES. Add 1 ml of a 1 M $CaCl_2$ solution and water up to a volume of 1 L. Adjust pH with 1 M NaOH to 7.4, filter through a 0.22 μm filter, and store at room temperature.

3. Round glass coverslides (25 mm diameter) with adherent cells expressing FRET biosensor.
4. Attofluor cell chamber for microscopy (Invitrogen) or similar.

2.3 Materials for Calculation of the Spectral Bleedthrough Correction Factor

1. 6-well plate with adherent cells (HEK293 cell line) plated on autoclaved glass coverslides in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal calf serum, L-glutamine, and antibiotics.
2. Donor fluorescence protein expression plasmid, e.g., pECFP-N1.
3. Lipofectamine 2000 transfection reagent.

3 Methods

3.1 FRET Measurements in Living Cells

1. Place a coverslide with adherent cells into a cell chamber for microscopy.
2. Wash the cells with 400 μL of FRET buffer to remove nonadherent and dead cells (*see* **Note 1**).
3. Add 400 μL of fresh FRET buffer.
4. Put a small drop of immersion oil onto the objective and place the measuring chamber onto the microscope.
5. Focus on the cell layer using transillumination light and search a cell that is nicely attached to the coverslide (*see* **Note 2**).
6. Switch to the fluorescent light and check whether the biosensor is homogeneously expressed in the cell. If not the case, search for another cell.
7. Start imaging software and adjust the settings to reach a good signal-to-noise ratio of the cell (*see* **Note 3**).
8. Select the time interval between the individual images (e.g., 5–10 s) (*see* **Note 4**).
9. Start data acquisition.
10. Run a plugin that splits the image into donor and acceptor channels and calculates the ratio for each pixel (*see* **Note 5**).
11. Mark the region of interest and run the plugin that calculates the acceptor to donor ratio over time and displays a graph (*see* **Note 5**).
12. Wait until a stabile baseline is reached (*see* Fig. 1). We suggest waiting at least for 15 frames. Make sure that the starting FRET ratio values of each individual experiment are in a similar

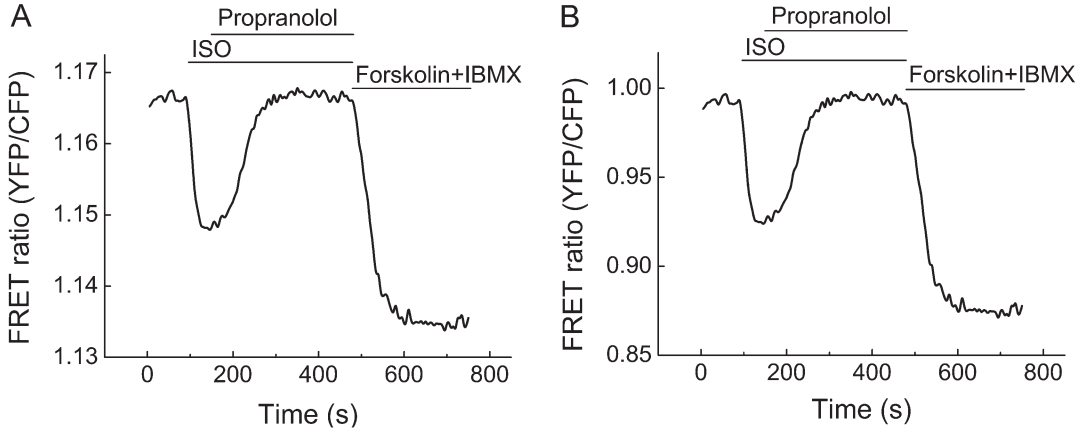

Fig. 1 Representative real-time FRET measurement of an adult mouse cardiomyocyte expressing a FRET biosensor for 3′,5′-cyclic adenosine monosphosphate (cAMP). The cell was sequentially stimulated with the β-adrenergic agonist isoprenaline (ISO, 100 nM, 90 s), the β-blocker propranolol (10 μM, 170 s), and the adenylyl cyclase activator forskolin (10 μM) plus the phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthin (IBMX, 100 μM, 470 s). An uncorrected FRET ratio (YFP/CFP) is presented in (**a**), while (**b**) shows a corrected and normalized ratio trace obtained as described in Subheading 3.2. Decrease of FRET ratio corresponds to an increase of intracellular cAMP

range; otherwise variable spectral bleedthrough correction factors are needed.

13. Add the pharmacological compound of interest by carefully pipetting 400 μL of solution to the measuring chamber. This step can be repeated with further compounds as often as necessary (*see* Fig. 1).
14. Stop the experiment and save the images for offline data evaluation. Clean both the cell chamber and the objective of the microscope and start a new experiment.

3.2 Data Evaluation

1. Open the recorded images using the "Open Micromanager File" function and run a plugin that splits the acquired images into donor and acceptor channels.
2. Select the region of interest for which the average intensity should be calculated. Determine the individual values for donor and acceptor intensities for all frames.
3. Calculate the FRET Ratio: $\mathrm{FRET} = \dfrac{\text{average acceptor intensity}}{\text{average donor intensity}}$.
4. To correct for the spectral bleedthrough effect, subtract the correction factor b (*see* Subheading 3.3) from the FRET ratio resulting in the corrected FRET ratio.

$$\mathrm{FRET}_{\mathrm{corr}} = \mathrm{FRET} - b$$

5. Normalize the corrected FRET ratios to the average value of the baseline.

3.3 Calculating Spectral Bleedthrough Factor

The emission spectrum of the donor and acceptor overlap in the region of the maximum emission of the acceptor, which leads to a detection of the donor fluorescence in the acceptor channel [9]. It is necessary to calculate a spectral bleedthrough factor (*b*) to account for this phenomenon. The bleedthrough of the acceptor into the donor channel is usually negligible. For the determination of this correction factor, HEK293 cells are transfected with a plasmid that encodes only for the donor fluorophore.

1. Take 300 µL of DMEM without additives.
2. Add 3 µg of the donor fluorophore plasmid DNA. Mix.
3. Add 7 µL of Lipofectamine 2000. Mix well.
4. Incubate for 20 min (up to 40 min is possible).
5. Add 50 µL of transfection mix per well to the cells.
6. Incubate the cells for at least 24 h before measuring.
7. Perform FRET measurements as described in Subheading 3.1. Finish the experiment when a stable baseline is reached. Save the images and stop the experiment (*see* **Note 6**).
8. For determination of the correction factor proceed **steps 1–3** of Subheading 3.2. The calculated FRET ratio corresponds to the correction factor *b*.
9. Calculate the mean of at least ten cells to obtain a reliable correction factor.

4 Notes

1. Make sure to remove most of the dead and not adherent cells before starting the measurements since those could overlay the measured cells, making an experiment unusable. If necessary, wash several times.
2. By gently tapping with the fingers on the side of the microscope, it can be checked whether the cell is attached nicely to the coverslide.
3. To reach a good signal-to-noise ratio, the intensity of the LED and the exposure time can be varied. By increasing the light intensity and exposure time, better pictures are obtained but the propensity for photobleaching is increased as well. If photobleaching occurs (recognized by a rundown of the measured FRET ratio), the intensity of the LED and (or) the exposure time should be reduced. If these steps are not helpful, pick another cell.
4. Shorter time steps between the images lead to a more detailed trace but can also result in cell damage and photobleaching. Consider also that FRET measurements require a large amount

of image data storage (one experiment with 300 images requires approximately 300 MB).

5. ImageJ plugins are available online and easily customizable. Several tutorials on how to modify those plugins are available. *See* also our published plugins [9].
6. The exposure time and LED intensity should be chosen in a similar way as in real FRET experiments.

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References

1. Herbst KJ, Ni Q, Zhang J (2009) Dynamic visualization of signal transduction in living cells: from second messengers to kinases. IUBMB Life 61:902–908
2. Zaccolo M (2004) Use of chimeric fluorescent proteins and fluorescence resonance energy transfer to monitor cellular responses. Circ Res 94:866–873
3. Sprenger JU, Nikolaev VO (2013) Biophysical techniques for detection of cAMP and cGMP in living cells. Int J Mol Sci 14:8025–8046
4. Chan YH, Wu C, Ye F et al (2011) Development of ultrabright semiconducting polymer dots for ratiometric pH sensing. Anal Chem 83:1448–1455
5. Busch C, Schroter T, Grabolle M et al (2012) An in vivo spectral multiplexing approach for the cooperative imaging of different disease-related biomarkers with near-infrared fluorescent forster resonance energy transfer probes. J Nucl Med 53:638–646
6. Froese A, Nikolaev VO (2015) Imaging alterations of cardiomyocyte cAMP microdomains in disease. Front Pharmacol 6:172
7. Börner S, Schwede F, Schlipp A et al (2011) FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. Nat Protoc 6:427–438
8. Sprenger JU, Perera RK, Steinbrecher JH et al (2015) In vivo model with targeted cAMP biosensor reveals changes in receptor-microdomain communication in cardiac disease. Nat Commun 6:6965
9. Sprenger JU, Perera RK, Gotz KR et al (2012) FRET microscopy for real-time monitoring of signaling events in live cells using unimolecular biosensors. J Vis Exp:e4081