Video Article

FRET Microscopy for Real-time Monitoring of Signaling Events in Live Cells Using Unimolecular Biosensors

Julia U. Sprenger[1], Ruwan K. Perera[1], Konrad R. Götz[1], Viacheslav O. Nikolaev[1]

[1]Emmy Noether Group of the DFG, Department of Cardiology and Pneumology, European Heart Research Insitute Göttingen, Georg August University Medical Center, Göttingen, Germany

Correspondence to: Viacheslav O. Nikolaev at viacheslav.nikolaev@med.uni-goettingen.de

URL: https://www.jove.com/video/4081
DOI: doi:10.3791/4081

Keywords: Molecular Biology, Issue 66, Medicine, Cellular Biology, FRET, microscope, imaging, software, cAMP, biosensor

Date Published: 8/20/2012

Citation: Sprenger, J.U., Perera, R.K., Götz, K.R., Nikolaev, V.O. FRET Microscopy for Real-time Monitoring of Signaling Events in Live Cells Using Unimolecular Biosensors. *J. Vis. Exp.* (66), e4081, doi:10.3791/4081 (2012).

Abstract

Förster resonance energy transfer (FRET) microscopy continues to gain increasing interest as a technique for real-time monitoring of biochemical and signaling events in live cells and tissues. Compared to classical biochemical methods, this novel technology is characterized by high temporal and spatial resolution. FRET experiments use various genetically-encoded biosensors which can be expressed and imaged over time *in situ* or *in vivo*[1-2]. Typical biosensors can either report protein-protein interactions by measuring FRET between a fluorophore-tagged pair of proteins or conformational changes in a single protein which harbors donor and acceptor fluorophores interconnected with a binding moiety for a molecule of interest[3-4]. Bimolecular biosensors for protein-protein interactions include, for example, constructs designed to monitor G-protein activation in cells[5], while the unimolecular sensors measuring conformational changes are widely used to image second messengers such as calcium[6], cAMP[7-8], inositol phosphates[9] and cGMP[10-11]. Here we describe how to build a customized epifluorescence FRET imaging system from single commercially available components and how to control the whole setup using the Micro-Manager freeware. This simple but powerful instrument is designed for routine or more sophisticated FRET measurements in live cells. Acquired images are processed using self-written plug-ins to visualize changes in FRET ratio in real-time during any experiments before being stored in a graphics format compatible with the build-in ImageJ freeware used for subsequent data analysis. This low-cost system is characterized by high flexibility and can be successfully used to monitor various biochemical events and signaling molecules by a plethora of available FRET biosensors in live cells and tissues. As an example, we demonstrate how to use this imaging system to perform real-time monitoring of cAMP in live 293A cells upon stimulation with a β-adrenergic receptor agonist and blocker.

Video Link

The video component of this article can be found at https://www.jove.com/video/4081/

Protocol

1. Setting up a FRET Imaging Microscope

In principle, any inverted fluorescence microscope which is available in the lab and has a camera port can be adapted for FRET imaging. The final setup should include the following crucial components: a microscope, a light source with or without additional shutter, a beam-splitter for emission light and a CCD-camera (see **Figure 1**). The hardware devices, especially the light source, the shutter and the camera are integrated into and controlled by the imaging software which allows image acquisition and analysis. Below we describe a procedure to assemble a simple FRET system from commercially available components.

1. Connect your light source to the microscope. For example, use a single-wavelength light emitting diode (CoolLED *p*E-100, 440 nm) which selectively excites enhanced cyan fluorescent protein (CFP) used as a donor in most FRET biosensors. It can be directly and easily connected to the epifluorescence illumination port of the microscope. There are also multi-wavelength LEDs available which can be connected in a similar way. Instead of LED, other standard light sources such as XBO75 xenon arc lamp (often used for Olympus and Zeiss microscopes) or HBO mercury lamp (usually installed on Nikon microscopes) can be used. In the case of a fluorescent lamp, you should also place a shutter between the lamp and illumination port to enable software-assisted control of the excitation light coming onto your sample. CoolLED does not require any additional shutter since it can be directly switched on and off by the software. The disadvantage of the single-color LED systems is a limited number of excitation wavelengths, while a fluorescent lamp with various filter sets is a more flexible option, especially when working with multiple and bimolecular biosensors. Alternatively, monochromator light sources (*e.g.* Polychrome V, TILLPhotonics) can be used; they usually have an integrated shutter which can be software-controlled via a trigger signal.
2. Place an appropriate filter cube into the microscope. For routine FRET measurements with CFP and enhanced yellow fluorescent protein (YFP) or any of their variants as a FRET pair, we use a simple filter cube containing an ET436/30M excitation filter (which can be omitted when LED is used, but is indispensable when using a xenon or mercury lamp rather than LED) and a DCLP455 dichroic mirror. The

microscope should also be equipped with an objective suitable for a good-resolution fluorescence microscopy, for example with a plan-fluor, plan-neofluar or plan-apochromat 40x, 60x or 100x oil-immersion objective.
3. Switch on the fluorescent light and check whether the light spot is evenly distributed across the field of view. If this is not the case, additional alignment of the LED or the lamp is required to achieve the optimal illumination of the specimen. This can be performed by using the screws which position the diode or the lamp in space.
4. Connect the beam-splitter via a C-mount to one of the microscope's emission ports. For example, use the DV2 DualView (Photometrics) which splits the emission light into two (donor and acceptor) channels which can be simultaneously monitored on a single CCD camera chip. Alternatively, there are other comparable products such as Optosplit (Cairn Research) or a beam splitter integrated into the Hamamatsu ORCA-D2 dual-wavelength camera. For the CFP/YFP FRET pair, we use the 05-EM filter set containing the 505dcxr dichroic mirror plus ET480/30M and ET535/40M emission filters for CFP and YFP, respectively, which is supplied with the DV2. Instead of a beam-splitter, two filter cubes for the donor (containing the ET436/30M excitation filter for CFP, the DCLP455 dichroic mirror and the CFP emission filter) and acceptor (containing the CFP excitation filter, DCLP455 and the YFP emission filter) channels are used in many FRET systems. Alternatively, one filter cube without any emission filter and an automatic emission filter wheel position before the camera can be installed. In this case, a motorized microscope or the filter wheel alternates between the two emission filter positions within ~200-300 msec to perform the ratiometric imaging. This minor delay is acceptable when imaging rather slow intracellular processes, such as cAMP signals, where truly simultaneous acquisition of both channels is not critical.
5. Connect the CCD camera (use for example ORCA-03G or ORCA-R2 from Hamamatsu Photonics) to the beam splitter. Use a FireWire cable to connect the camera to the IEEE1394 computer interface, as described in the manual supplied with the camera. Install camera drivers without switching on the camera.
6. Finally, to establish the communication between the computer and the light source, connect the Arduino I/O board (use for example Arduino Duemilanove or Arduino Uno) to the LED or to the shutter by using a BNC cable which should contain a normal BNC plug on the LED side and two single wires on the other end connected into the pins GND (0) and 8 of the board as shown in **Figure 2**. The assembled board can be directly connected to a USB-port of your computer.

2. Setting up the Imaging Software

To control and synchronize the light source with image capturing by the camera, imaging software should be installed on the computer. There are several commercially available software packages including MetaFluor (Molecular Devices), Slidebook (Intelligent Imaging Innovations), VisiView (Visitron Systems). Here we demonstrate the use of the open-source Micro-Manager freeware which offers a high degree of flexibility for low-cost imaging.

1. Download this software at http://valelab.ucsf.edu/~MM/MMwiki/index.php/Micro-Manager_Version_Archive. We recommend installing the 1.4.5 release which is easily configurable in our hands.
2. Connect the Arduino board to a USB port of your computer. Download the software to control the Arduino board from http://www.arduino.cc/en/Main/software. Follow the instructions found on this website and run this software just one time prior to starting Micro-Manager. Upload a code for use of the board with Micro-Manager software. The code can be downloaded at http://valelab.ucsf.edu/~MM/MMwiki/index.php/Arduino.
3. Switch on the LED and the camera. Start the software and configure Micro-Manager communication with the camera and LED (other light source and shutter) by selecting Tools>Hardware Configuration Wizard (see **Figure 3**). Add the required components including your camera (*i.e. Hamamatsu_ DCAM*) and Arduino Board (add *Arduino-Switch*, *Arduino-Hub* and *Arduino-Shutter* devices). During the next steps, use the default settings suggested by the wizard. Save the new system configuration when prompted by the wizard.
4. Use the main menu to open Tools>Device Property Browser. Scroll down to "Arduino Switch State" and select "1". Close the dialog. Make sure that the "auto-shutter" box is ticked in the main software dialog. Press File>Save System State to save the software configuration which should be opened anytime after starting the software. This will establish the communication between the board and the software needed to perform image acquisition.
5. Press "Live" button to monitor the signal coming from the camera. Make sure that fluorescent light goes on any time "Live" or "Snap" function is selected. Before starting the first measurements, follow the instructions supplied with your beam splitter to perform the optical alignment of both channels.

3. Cell Culture and Transfections

1. Prepare 6-well plates with autoclaved round 24 mm glass coverslides (1 coverslide per well). Alternatively, glass-bottomed cell-culture dishes can be used.
2. Plate 293A cells onto the plates or dishes in D-MEM medium (supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin solution) so that the cells reach 50-70% confluency after one day.
3. 24 hr after plating, transfect the cells in a laminar flow bench with a FRET sensor plasmid using the calcium phosphate transfection method (see 3.5). cAMP biosensor plasmids can be obtained from our group upon request. The reader is also referred to the comprehensive reviews[7,8] describing other available cAMP biosensors.
4. Before transfecting cells for the first time, prepare transfection reagents. Make up a 2.5 M $CaCl_2$ and 2xBBS solutions (the latter containing 1.5 mM Na_2HPO_4, 50 mM BES, 280 mM NaCl, adjust to pH 6.95 with NaOH) in deionized water. Sterile filtrate the solutions using a regular 0.2 µm filter.
5. To transfect a 6-well plate or 6 glass-bottomed dishes, mix 10 µg of FRET sensor plasmid, 50 µl of 2.5M $CaCl_2$ solution and sterile water up to 500 µl. Mix well.
6. Add 500 µl of 2x BBS. Mix well and incubate the mixture for 10 min at room temperature.
7. Pipette 165 µl of the transfection mix dropwise onto each well or dish. Gently stir the plate and put it back into the incubator. Cells are usually ready for FRET measurements 24 hr after transfection. Make sure that cells have not reached confluency at this point, as this might influence the activity of several cell-surface receptors.

4. FRET Measurements in Live Cells

1. Before measuring for the first time, prepare the FRET buffer containing 144 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES in deionized water and adjust the pH to 7.3 with NaOH. Dilute the compounds to be used in your imaging experiments with the FRET buffer.
2. Start the imaging software. Load previously defined system configuration. Select File>Load System State to choose the previously configured system state.
3. Mount a coverslide with adherent transfected 293A cells in the imaging chamber (*e.g.* Attofluor cell chamber). Wash the cells once with FRET buffer and add 400 µl of FRET buffer. When using glass-bottomed cell-culture dishes wash the adherent cells and add 2 ml of the buffer per dish. We perform all measurements at room temperature in the FRET buffer containing HEPES, so that no CO_2 control is necessary.
4. Put some immersion oil onto the objective and transfer the imaging chamber onto the microscope. Focus on the cell layer using transillumination light.
5. Switch on the fluorescent light by pressing the "Live" button, and select a cell for the experiment. Choose a cell with an optimal sensor expression, meaning that it should be not too bright and not too dim. After finding an appropriate cell, switch off the fluorescent light immediately to avoid photobleaching of the FRET sensor.
6. Adjust the exposure time under the "Camera settings" (usually 10-50 msec) in a way that leads to a good signal-to-noise ratio of the acquired image after pressing the "Snap" button. Too long excitation times might result in photobleaching, while too short times result in low image quality.
7. Press the "Multi-D Acq." button and set the number of time points and the time interval for image acquisition. For our cAMP-FRET sensor, we acquire one image every 5 s.
8. Start the measurements by pressing the "Acquire" button.
9. During any measurement, one can use the "FRET online" plug-in (available in the **Online Supplement**, all plug-ins must be copied into the "plugins" folder of your Micro-Manager software before starting it) to monitor FRET ratio changes online. Run this plug-in and select a region of interest in the FRET ratio image using the "Freehand selections" tool. Add the region into the ROI manager and press the "Get average" button in the "Time series analyzer" window. The FRET ratio trace will be displayed. To update the trace during the measurement, run the "FRETratioOnline2" plug-in and press the "GetAverage" button.
10. As soon as the FRET ratio has reached a stable baseline apply the desired compound by accurately pipetting it into the dish/chamber. To treat cells with pharmacological compounds, a perfusion system instead of simple pipetting can be used at this stage.
11. After finishing the experiment, save the time-lapse stack of images. Remove the measurement chamber from the microscope and clean the objective using an objective tissue.
12. Go back to step 4.3 to repeat the measurement with a new sample.

5. Offline Data Analysis

FRET imaging data can be analyzed offline at any time after the experiment using ImageJ software. As a supplement to this protocol, we provide the "FREToffline" plug-in used in our laboratory to split the acquired images into donor and acceptor channels and to measure fluorescent intensities in multiple regions of interest. These intensities can further be copy-pasted into an Excel or Origin spreadsheet to calculate the corrected FRET ratio. To visualize the FRET changes of unimolecular biosensors, simple ratiometry is often used. In this case, only the donor fluorophore (CFP) is excited, and two images are taken at CFP and YFP emission peaks. The calculated YFP/CFP ratio (sometimes also referred to as FRET/CFP ratio) represents the degree of FRET between the two fluorophores. In unimolecular biosensors, the numbers of CFP and YFP moieties are equal, so that the simple ratiometry is sufficient to represent the FRET efficiency[12].

1. Use ImageJ software to open the experiment file by selecting Plugins>Micro-Manager>Open Micro-Manager File.
2. Run the "FREToffline" plug-in which splits the time-lapse stack into individual CFP and YFP channels.
3. If background correction is required, this can be performed using ImageJ software.
4. Click on the YFP stack of images and select one or several regions of interest using the "Freehand selections" tool and add them to the "MultiMeasure" plug-in window by pressing the "Add" button.
5. Select the regions of interests in the "MultiMeasure" window and press "Multi" to obtain a table with mean grey values for each frame and each region. Copy the data into clipboard by selecting all using Ctrl+A and by pressing Ctrl+C. Open an Excel or Origin spreadsheet and paste the data by pressing Ctrl+V.
   The measurements performed by the program can be configured under Analyze>Set Measurements where you can define the parameters to be measured. We usually select only "Mean grey value" in this dialog.
6. Click onto the CFP stack of images. Perform the same as described in 5.5. Paste the CFP intensity data into the same Excel or Origin spreadsheet.
7. Using Excel or Origin software, calculate the corrected FRET ratio. When simple single-chain unimolecular biosensors are imaged, we correct only for the bleedthrough of the donor fluorescence into the acceptor channel. In this case, the corrected acceptor/donor ratio is:
   Ratio = (YFP - B x CFP) / CFP
   Where B is the correction factor which can be determined by transfecting cells with CFP plasmid and measuring a percentage of the donor fluorescence in the YFP channel (B =YFP/CFP). Omitting this correction for unimolecular biosensors is possible, since it would only affect the overall amplitude of the FRET response without any qualitative effect on the shape of the curve.

6. Representative Results

Figure 1 shows an example of a fully assembled FRET imaging setup consisting of a Nikon inverted microscope, CoolLED, DV2 DualView and the Hamamatsu ORCA-03G CCD camera. To establish the communication between the hardware components and the computer, the I/O Arduino board is connected to the computer and to the CoolLED as shown in **Figure 2**. To control the light source and image capturing by the camera in a synchronized fashion, Micro-Manager software has to be installed and properly configured (see **Figure 3**). This freeware can be easily adapted to individual experimental needs by adding necessary plug-ins. **Figure 4A** shows a representative raw FRET ratio trace from

a measurement using the cAMP sensor Epac1-camps[13] expressed in 293A cells to monitor the effects of β-adrenergic agonist isoproterenol applied at time point 150 sec and the β-blocker propranolol added at time point 300 sec (performed as described in 4.3-4.10). These data can be analyzed offline and corrected for the bleedthrough of CFP into the YFP channel as described in 5.1-5.7 to obtain the corrected FRET ratio trace shown in **Figure 4B**. This representative experiment shows a slow decrease in the monitored FRET ratio upon isoproterenol treatment which indicates an increase of intracellular cAMP. Propranolol as a β-blocker reverses the isoproterenol signal, leading to a decrease of cAMP to basal levels. These changes in FRET signal can be monitored online during any experiments (as described in 4.9). Such experiments can be performed with a variety of commonly used biosensors designed to monitor different second messengers or biochemical processes.

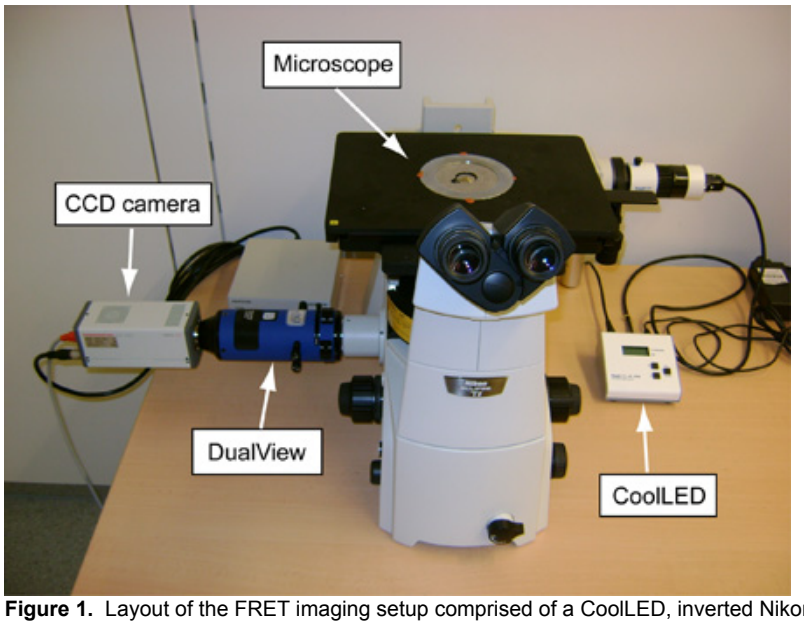


Figure 1. Layout of the FRET imaging setup comprised of a CoolLED, inverted Nikon microscope, DV2 DualView, and ORCA-03G CCD camera.

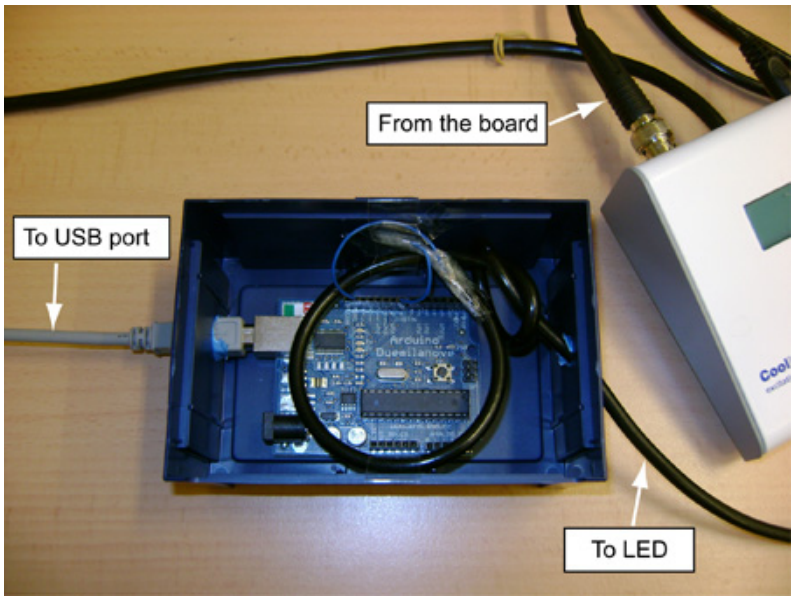


Figure 2. Arduino I/O board and its connections. The board is positioned in a self-mounted plastic box. A standard BNC cable connects the LED to the pins 8 and GND (0) of the board.

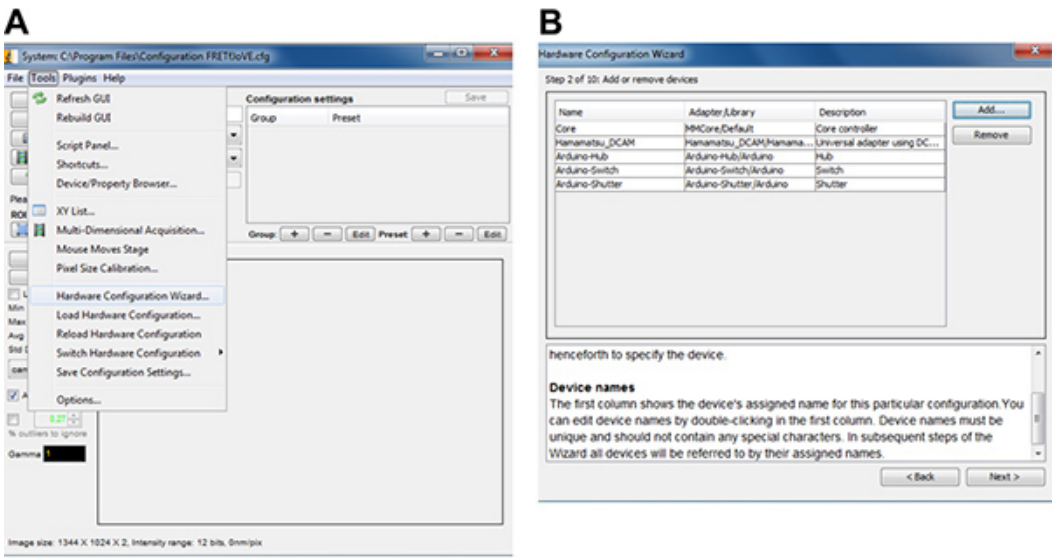
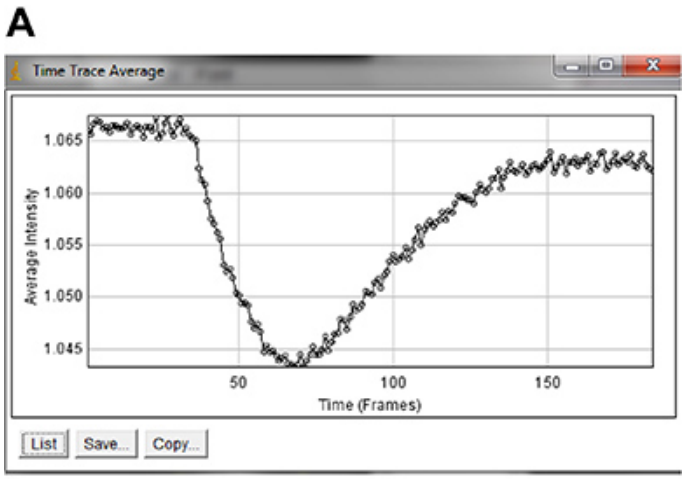


Figure 3. Screenshots demonstrating integration of the system components using Hardware Configuration Wizard. **A**) Start the Hardware Configuration Wizard. **B**) Add the required devices as mentioned in 2.3. Click here to view larger figure.



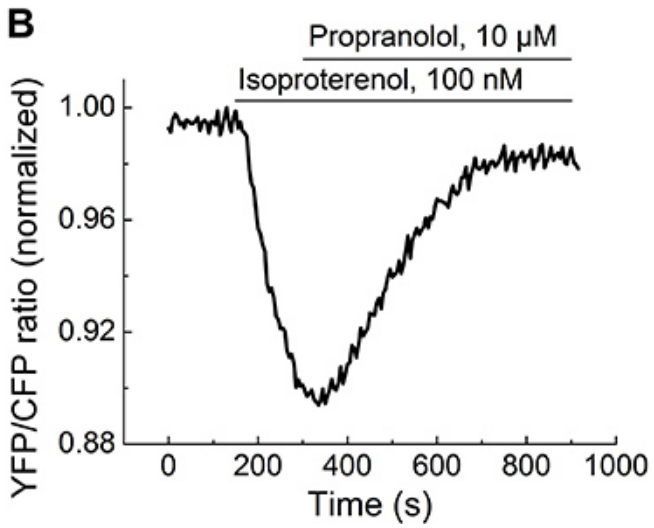


Figure 4. A representative FRET experiment which measures cAMP levels in 293A cells transfected with Epac1-camps. First, cells were stimulated with the β-adrenergic agonist isoproterenol (100 nM, at frame 30 or at 150 sec) to increase cAMP (observed as a decrease in YFP/CFP FRET ratio). Cells were subsequently treated with the β-blocker propranolol (10 µM, at frame 60 or at 300 sec) which leads to an increase

of FRET ratio, reflecting a decrease in cAMP. **A**) Raw online FRET ratio trace from one region of interest corresponding to a single cell monitored during the experiment. **B**) Corrected ratio trace after offline data analysis performed as described in 5.1-5.7.

Discussion

in this protocol, we demonstrate how to build a simple low-cost but powerful FRET imaging system for routine applications with a variety of available biosensors. The system presented here is designed for CFP and YFP, or similar types of fluorescent proteins, as the donor-acceptor pair. Meanwhile, other individual biosensors become available which use for example green and red fluorescent proteins[14]. To adapt the described system for other colors, appropriate light sources and/or filter sets should be selected. In the case of LED, another single LED line, for example 490 nm to excite green fluorescent protein can be used. Single-wavelength LEDs can be easily (within seconds) dismounted and exchanged. Alternatively, there are LED arrays available which contain several lines to excite various fluorescent proteins (*e.g.* pE-2 CoolLED). To enable measurements with alternative FRET pairs, other fluorescent filter cubes can be placed into the microscope, and eventually the emission beam-splitter filters should be exchanged. Photometrics offers additional emission filter sliders for DV2 DualView. Some recently developed applications use two or more biosensors simultaneously to monitor multiple processes at the same time, for example cAMP and cGMP together in one cell[15]. In this case, a QuadView (Photometrics) containing four emission channels can be used, the ImageJ plug-in for image splitting and analysis can be then adapted to be used with four channels and to calculate two FRET ratios. ImageJ software is very flexible in terms of image analysis and online representation of the results. Simple text-edited plug-ins allow adaptation of the software algorithm to the needs of any individual imaging system and experiment. This is sometimes very helpful and offers rapid solutions to the technical problems, which can require long times when they have to be implemented into any commercial software package.

When doing FRET experiments, it is crucial to avoid photobleaching which appears when the excitation times are too long or when the images are taken too frequently. In this case, a photon-induced chemical damage or covalent modifications of fluorophores may occur and decrease FRET efficiency. To avoid photobleaching, one can reduce the exposure time and the frequency of image acquisition. There are also established protocols[12,16] to correct for this phenomenon. During data analysis, it is possible to correct for the cross-talk between donor and acceptor channels (bleedthrough). When using unimolecular FRET biosensors (in which case donor and acceptor fluorophores are always expressed at the same level) for simple ratiometric measurements, it can be enough to correct just for the bleedthrough of the donor into the acceptor channel or even omit this correction altogether because it does not qualitatively affect the shape of the FRET ratio curve. The bleedthrough of the acceptor into the donor channel is usually negligible. When bimolecular biosensors comprised of two different proteins are used, these may be expressed at various levels. In this case, the bleedthrough correction and an additional correction for the direct YFP excitation by the 440 nm light are also advisable. Please, refer to the published protocol[12] where all correction procedures are described in more detail. Additional comprehensive information about the development of biosensors, FRET microscopy, possible pitfalls of the technique and about the data analysis are available in previously published protocols[17-18]. In conclusion, the simple and powerful imaging system described here provides a flexible platform to monitor various biochemical events and signaling molecules with high temporal and spatial resolution in live cells.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors would like to thank Anke Rüttgeroth and Karina Zimmermann for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (grant NI 1301/1-1 to V.O.N) and University of Göttingen Medical Center ("pro futura" grant to V.O.N.).

References

1. Zaccolo, M. Use of chimeric fluorescent proteins and fluorescence resonance energy transfer to monitor cellular responses. *Circ. Res.* **94**, 866-873 (2004).
2. Mehta, S. & Zhang, J. Reporting from the field: genetically encoded fluorescent reporters uncover signaling dynamics in living biological systems. *Annu. Rev. Biochem.* **80**, 375-401 (2011).
3. Zhang, J., Campbell, R.E., Ting, A.Y., & Tsien, R.Y. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* **3**, 906-918 (2002).
4. Miyawaki, A. Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev. Cell.* **4**, 295-305 (2003).
5. Bunemann, M., Frank, M., & Lohse, M.J. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 16077-16082 (2003).
6. Kotlikoff, M.I. Genetically encoded Ca2+ indicators: using genetics and molecular design to understand complex physiology. *J. Physiol.* **578**, 55-67 (2007).
7. Willoughby, D. & Cooper, D.M. Live-cell imaging of cAMP dynamics. *Nat. Methods.* **5**, 29-36 (2008).
8. Nikolaev, V.O. & Lohse, M.J. Monitoring of cAMP synthesis and degradation in living cells. *Physiology (Bethesda).* **21**, 86-92 (2006).
9. Tanimura, A., *et al.* Use of Fluorescence Resonance Energy Transfer-based Biosensors for the Quantitative Analysis of Inositol 1,4,5-Trisphosphate Dynamics in Calcium Oscillations. *J. Biol. Chem.* **284**, 8910-8917 (2009).
10. Nikolaev, V.O. & Lohse, M.J. Novel techniques for real-time monitoring of cGMP in living cells. *Handb. Exp. Pharmacol.*, 229-243 (2009).
11. Nausch, L.W., Ledoux, J., Bonev, A.D., Nelson, M.T., & Dostmann, W.R. Differential patterning of cGMP in vascular smooth muscle cells revealed by single GFP-linked biosensors. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 365-370 (2008).
12. Borner, S., *et al.* FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nat. Protoc.* **6**, 427-438 (2011).

13. Nikolaev, V.O., Bunemann, M., Hein, L., Hannawacker, A., & Lohse, M.J. Novel single chain cAMP sensors for receptor-induced signal propagation. *J. Biol. Chem.* **279**, 37215-37218 (2004).
14. Hong, K.P., Spitzer, N.C., & Nicol, X. Improved molecular toolkit for cAMP studies in live cells. *BMC Res. Notes.* **4**, 241 (2011).
15. Niino, Y., Hotta, K., & Oka, K. Simultaneous live cell imaging using dual FRET sensors with a single excitation light. *PLoS One.* **4**, e6036 (2009).
16. Palmer, A.E. & Tsien, R.Y. Measuring calcium signaling using genetically targetable fluorescent indicators. *Nat. Protoc.* **1**, 1057-1065 (2006).
17. Brumbaugh, J., Schleifenbaum, A., Stier, G., Sattler, M., & Schultz, C. Single- and dual-parameter FRET kinase probes based on pleckstrin. *Nat. Protoc.* **1**, 1044-1055 (2006).
18. Aoki, K. & Matsuda, M. Visualization of small GTPase activity with fluorescence resonance energy transfer-based biosensors. *Nat. Protoc.* **4**, 1623-1631 (2009).

