Protocol

Confocal FLIM of Genetically Encoded FRET Sensors for Quantitative Ca^{2+} Imaging

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Fluorescence lifetime imaging (FLIM) is a powerful imaging mode that can be combined with confocal imaging. Changes in the fluorescence decay time of a donor in an intramolecular Förster resonance energy transfer (FRET)-based biosensor provide intrinsic quantitative data. Here, we describe a protocol using both the Ca^{2+} sensor TN-XL, which uses troponin C, as the Ca^{2+}-sensing unit, and the FLIM technology based on time-correlated single-photon counting.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Antibiotics (penicillin and streptomycin)
Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum
HEK293 cells (or other cell lines)
Ionomycin (5 μM, for Ca^{2+} calibration)
NanoJuice core transfection reagent (Novagen)
NanoJuice transfection booster (Novagen)
Plasmid pcDNA3-TN-XL (or vector encoding any other FRET-based Ca^{2+}-sensor)
Trypsin
Tyrode solution for probing Ca^{2+} signaling <R>

Equipment

Avalanche photodiodes (2) or other photon-counting devices

These are part of the time-correlated single-photon counting (TCSPC) upgrade kit; here we use PDMs (Micro Photon Devices).

Cell culture plate (12 well)

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Confocal microscope with single-beam scan-head (e.g., PCM2000 [Nikon])
Coverslips (20 mm, round, for microscopy)
Dichroic 532 DCLP emission filters (e.g., 475/42 and 532 LP)
Excitation filter (434/17)
Incubator (37°C, humidified CO_2)
Photon-counting device

This is part of the TCSPC upgrade kit; here we use the HydraHarp-400 (PicoQuant GmbH).

Picosecond-pulsed laser in the range of 400–450 nm

Monochromatic pulsed diode lasers are part of the TCSPC upgrade kit; here we use a white-light laser (SC400-6, Fianium).

This protocol necessitates handling a free-space Class 4 laser. Use of such a laser requires strict safety precautions and the experimenter should hold an appropriate qualification. It is essential that investigators meet their national safety regulations.

Software

This is part of the TCSPC upgrade kit. Here we use SymPhoTime (PicoQuant), but others such as SPC-Image (Becker and Hickl) are available.

TCSPC-based upgrade kit for a single-beam laser scanning microscope

This is available from PicoQuant or Becker & Hickl for many models of the major microscope manufacturers (Zeiss, Leica, Nikon, Olympus, Bio-Rad); for a custom-built system, the major parts are listed separately above (see also Step 1).

METHOD

Custom Setups

1. (*For custom-built setups only*) For the TCSPC, ensure you have a pulsed laser (pulse duration in the order of picoseconds ([psec] or shorter) set at a repetition frequency that allows pulse intervals of at least five times the expected fluorescence decay time of the fluorophore (typically below 80 MHz). Note the following:
   - The laser needs to be coupled into a scan head as depicted in Figure 1A, which gives a schematic overview of the setup.
   - This coupling also holds true if implementing TCSPC upgrade kits for an existing setup.
   - Further parts that need to be implemented are photon-counting detectors, which feed signals, together with trigger signals from the laser (pulse trigger) and the scanner (frame- and line clock), into a specialized photon-counting device.

Cell Culture and Transfection

2. Maintain HEK293 cells in DMEM with 5% (v/v) fetal bovine serum and antibiotics in an incubator with 5% CO_2.
3. Ensure that each well of a 12-well plate contains a coverslip and plate 2×10^4 cells in 1 mL of complete growth medium for transfection after ~24 h.
4. For each well, thoroughly mix 0.5 µL of NanoJuice core transfection and 2 µL of NanoJuice transfection booster with 50 mL of DMEM and incubate at room temperature for 5 min.
5. Add 1 µg purified plasmid pcDNA3-TN-XL to the mixture and gently mix.
6. After incubation for 20 min at room temperature, add the entire volume of transfection mixture to the cell culture well and incubate for 48–72 h at 37°C and 5% CO_2.

FLIM Data Acquisition

7. Transfer the coverslip with cells expressing the Ca^{2+} sensor to the measuring chamber containing Tyrode solution.

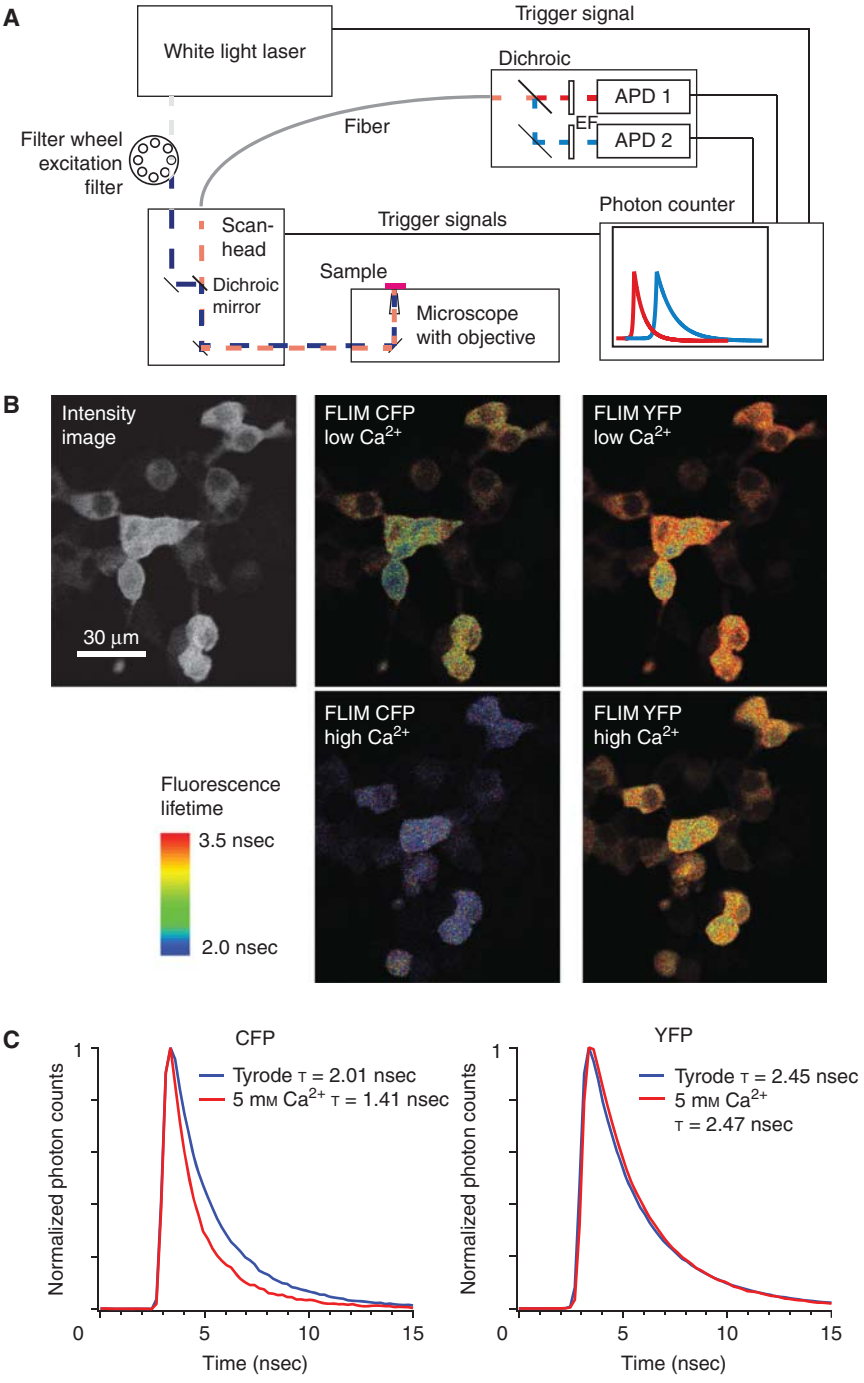

FIGURE 1. Confocal FLIM measurements of genetically encoded Ca^{2+} sensors. (*A*) Cartoon illustrating the experimental setup. A picosecond-pulsed white-light laser ensures flexibility of wavelength. An excitation filter in a filter wheel picks the appropriate wavelength band. A confocal scan-head with fiber adapters performs the confocal scanning, and avalanche photodiodes detect single photons that are counted in a photon counter. (*B*) Images of HEK293 cells transfected with the Ca^{2+} sensor TN-XL and incubated with the Ca^{2+} ionophore ionomycin (5 µM). *Left*: Intensity image; *middle* row: fast FLIM image of the CFP (cyan) channel at low (native) Ca^{2+} concentration (*upper* image) and high (saturated, 5 mM) Ca^{2+} concentration (*lower* image); *right* row: same as *middle* row, but for the YFP (yellow) channel. (*C*) Diagrams of the time-resolved photon counting that can be used to extract the fluorescence lifetime (exponential fit of the decay).

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8. To minimize noise, perform the measurements in a dark room.

   Because the TCSPC devices are designed as add-ons for confocal scanners, the photon-counting software and the scanning process need to be started separately in most cases. For samples containing several chromophores, it is useful to characterize first the different entities separately. Genetically encoded biosensors, such as FRET-based Ca^{2+}-sensors, should initially be calibrated using the Ca^{2+} ionophore ionomycin, as exemplified for two Ca^{2+} concentrations in Figure 1B,C.

   See Troubleshooting.

9. Deconvolve the data from the so-called instrument response function (IRF), describing the nonideal device characteristic owing to the length of the laser pulses and electronic jitter of the device. There are three approaches for determining the IRF:

   - The IRF can be estimated from an algorithmically derived function implemented in the TCSPC software.
   - A better approach is to measure the IRF, which can be achieved by placing solutions of fluorescent dyes with a very short fluorescence lifetime, such as rose bengal (~0.5 nsec in methanol) (Szabelski et al. 2009) or LDS789 (24 psec in aqueous solution) (Luchowski et al. 2009), in the measurement chamber.
   - Alternatively, the IRF can be measured by replacing the sample with a mirror, but this procedure requires some direct laser light to be detected, for example, by removing the emission filter.

   See Troubleshooting.

10. Analyze the measured data (see Discussion).

TROUBLESHOOTING

Problem (Step 8): A second peak or other phantom signals appear in the lifetime traces.

Solution: The causes of such phantom signals are generally unwanted reflections of the laser pulse leading to a second excitation. Often, such reflections can be avoided by using angled fiber couplers (AFCs) instead of plane fiber couplers (FCs). If such a change is not possible, the synchronization of the laser pulse with the reflection by adapting the fiber length (~3 nsec/m) might be an alternative.

Problem (Steps 8 and 9): Inconsistency of the fluorescence lifetime measurements.

Solution: The most common reason might be so called "pile-up" effects. The TCSPC relies on the assumption that between two laser pulses, a maximum of one photon reaches the detector. If this assumption is not true, the detection gets systematically wrong toward shorter fluorescence lifetimes. To compensate for this, the laser power has to be decreased until the fluorescence lifetime starts to be independent of the laser intensity.

DISCUSSION

Analyzing FLIM data can be challenging. A general consideration, as in any other imaging modality, requires a judgment to be made in terms of spatiotemporal fluorescence lifetime information. For example, does the investigator need a decay time in every pixel, in an entire cell or in a region of interest? Because *n* exponential fits, with a desired accuracy, require a certain number of photons, this information contributes to the determination of the optimized acquisition parameters.

Acquisition software such as the SymPhoTime or SPC-Image provides versatile analysis tools. Alternatively, data can be exported and analyzed externally by software packages such as IGOR Pro or MathLab. FLIM and its analysis is a scientific task that can fill entire books (Valeur 2002; Periasamy and Clegg 2009; Becker 2010). In brief, there are two principle approaches: (i) the fluorescence

lifetime of the identities is determined beforehand—for example, the Ca^{2+}-bound and the Ca^{2+}-free conformation of a small-molecule dye. During the analysis of a double exponential decay, these lifetimes are fed into the fit and the amplitude ratio of the lifetimes correlates with the Ca^{2+} concentration; (ii) a single exponential fit provides an average lifetime, which (in the upper example) also correlates with the Ca^{2+} concentration. For FRET-based sensors, such as TN-XL, the parameter of interest is the (monoexponential fitted) lifetime of the FRET donor as the lifetime of the acceptor does not change (Fig. 1C).

The presented data (Fig. 1B,C) on TN-XL (Mank et al. 2006) are just an example of numerous possible genetically encoded calcium sensors (Gensch and Kaschuba 2012). FLIM allows quantitative measurements on a single spectral channel of the FRET donor. For certain cell types, such as red blood cells, FLIM might be the only option to acquire quantitative Ca^{2+} data (Kaestner et al. 2006). In the case of TCSPC as described in this protocol, the acquisition speed is limited and faster processes might need to be imaged in the linescan mode. However, depending on the particular technology behind the FLIM recordings, sequences have been acquired at a frame rate of 100 Hz (Agronskaia et al. 2003). Finally, aside from the measurement of Ca^{2+} per se, FLIM can also be used to characterize the photophysical properties of genetically encoded biosensors (Tian et al. 2011).

RECIPE

Tyrode Solution for Probing Ca^{2+} Signaling

Component	Final concentration (mM)
NaCl	135
KCl	5.4
$MgCl_2$	1.0
Glucose	10
$CaCl_2$	2
HEPES	10

Prepare fresh and adjust to pH 7.35 with NaOH.

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