Multiplexed FRET to Image Multiple Signaling Events in Live Cells

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ABSTRACT We report what to our knowledge is a novel approach for simultaneous imaging of two different Förster resonance energy transfer (FRET) sensors in the same cell with minimal spectral cross talk. Previous methods based on spectral ratiometric imaging of the two FRET sensors have been limited by the availability of suitably bright acceptors for the second FRET pair and the spectral cross talk incurred when measuring in four spectral windows. In contrast to spectral ratiometric imaging, fluorescence lifetime imaging (FLIM) requires measurement of the donor fluorescence only and is independent of emission from the acceptor. By combining FLIM-FRET of the novel red-shifted TagRFP/mPlum FRET pair with spectral ratiometric imaging of an ECFP/Venus pair we were thus able to maximize the spectral separation between our chosen fluorophores while at the same time overcoming the low quantum yield of the far red acceptor mPlum. Using this technique, we could read out a TagRFP/mPlum intermolecular FRET sensor for reporting on small Ras GTP-ase activation in live cells after epidermal growth factor stimulation and an ECFP/Venus Cameleon FRET sensor for monitoring calcium transients within the same cells. The combination of spectral ratiometric imaging of ECFP/Venus and high-speed FLIM-FRET of TagRFP/mPlum can thus increase the spectral bandwidth available and provide robust imaging of multiple FRET sensors within the same cell. Furthermore, since FLIM does not require equal stoichiometries of donor and acceptor, this approach can be used to report on both unimolecular FRET biosensors and protein-protein interactions with the same cell.

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The signaling networks that underlie complex cellular processes such as cell survival, proliferation and differentiation comprise a vast number of different proteins and secondary messenger molecules whose interactions are key in determining cell behavior. Understanding the mechanisms behind different cell responses requires knowledge of how these interactions are correlated in time and space. To this end Förster resonance energy transfer (FRET), the nonradiative transfer of energy from an excited state fluorophore (the donor) to a spatially colocalized chromophore (the acceptor), provides a means to image the spatial and temporal dynamics of protein interactions in live cells (1). To date, most FRET imaging experiments have utilized only a single donor-acceptor pair because the ability to image multiple FRET pairs within a single cell—and thus to correlate multiple signaling events—has been limited by the significant spectral overlap of the commonly used fluorophores, particularly the genetically expressed fluorescent proteins. Previous strategies to overcome this problem have included the use of a single shared acceptor for the two donor species (2), but this approach could only be applied to study of interactions where the binding partner was the same for both donor-labeled species. The recent growth in red and orange fluorescent proteins has addressed this issue to some extent by increasing the spectral bandwidth avail-able (3,4), although some of these longer emitting proteins exhibit relatively low quantum efficiencies. Several of these new proteins have been demonstrated to work well as FRET pairs (4) and the first multiplexed ratiometric measurement of FRET, between mOrange/mCherry and ECFP/EYFP, was recently described (5).

Ratiometric FRET measurements can, however, be compromised by cross talk which can significantly limit the achievable signal/noise ratio. Depending on the wavelength used to excite the mOrange donor and the filter used to detect its emission, it is difficult to avoid an artifact from direct excitation of EYFP. This can be minimized by exciting and detecting at longer wavelengths but only at the expense of increased direct excitation of mCherry and unwanted detection of mCherry emission in the red-shifted donor channel. Together with the inevitable bleed-through of donor fluorescence into the acceptor channel, this cross talk can result in a high noise background against which it may be difficult to resolve a genuine change in intensity ratio. One approach to mitigate this is to use a novel fluorescent protein with a large Stokes shift for the second donor (6). This solves the problem of fluorescence from direct excitation of the first acceptor being detected in the second donor channel, since both donors can now be excited at a wavelength beyond the excitation spectrum of the first acceptor. Nonetheless, sensitized emission from this acceptor may still be detected in the second donor channel.

Here, we report a method for imaging two FRET pairs within the same cell that minimizes these sources of cross talk. Recently, a novel red protein TagRFP was introduced (7) that has a higher brightness than others in the same spectral class (mRFP, mCherry) and whose fluorescence can easily be resolved from that of EYFP. TagRFP can therefore serve as a suitable donor for

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multiplexing with ECFP and EYFP. TagRFP, in turn, requires a further red-shifted protein to serve as acceptor, for which mPlum, having the longest emission spectrum of all currently available fluorescent proteins, is a possible choice. One reason why this pair had not previously been utilized for FRET is that the low quantum yield of mPlum makes it unsuitable for ratiometric measurements. Our approach overcomes this problem by using fluorescence lifetime imaging (FLIM) to monitor FRET between the TagRFP/mPlum pair. Unlike spectral ratiometric measurement, FLIM only requires the donor fluorescence signal to be measured. By combining FLIM of the TagRFP/mPlum pair with spectral ratioing of an ECFP/Venus pair, we were hence able to maximize the spectral separation of our chosen FRET pairs while avoiding the issue of low sensitized emission from the second (long wavelength) acceptor. In addition, since fluorescence lifetime is largely independent of fluorophore concentration, FLIM-FRET does not require equal stoichiometries of donor and acceptor and so can be used to image intermolecular FRET as well as dual labeled biosensors.

Our multiplexed FRET instrument is built around an inverted Olympus microscope into which are coupled a continuous wave diode pumped solid state laser for blue excitation of ECFP and a spectrally filtered ultrafast supercontinuum source for excitation of the second donor, TagRFP (Fig. 1). The ECFP and Venus fluorescence emission are resolved from TagRFP emission by a 560LP dichroic and split into two channels using a dual channel imager (DualView) from Optical Insights. These two channels are imaged onto the same charge coupled device chip and ratioed to provide a FRET image, which in turn can be mapped to changes in calcium concentration throughout the cell. FLIM images of the TagRFP donor are acquired using a high speed wide-field time-gated method (8).

For the work reported here, live COS-7 cells were stimulated with epidermal growth factor and spectral ratiometric imaging was used to monitor changes in calcium flux using an ECFP/Venus Cameleon sensor (YCAM 3.6). FLIM meanwhile was used to observe FRET between a TagRFP labeled Ras Binding Domain from C-Raf-Kinase (Raf-Ras Binding Domain) and mPlum labeled H-Ras (Fig. 2). Binding of Raf-RBD to Ras will only occur after activation of Ras, hence measurement of FRET between the TagRFP/mPlum pair provides a sensitive means for monitoring Ras activity. FRET between the TagRFP donor and mPlum acceptor is indicated by a decrease in TagRFP fluorescence lifetime. Fig. 2 *B* shows the spectral bands used to detect the fluorophores.

Fig. 3 shows ratiometric images of the Cameleon sensor at intervals before and after stimulation, together with fluorescence

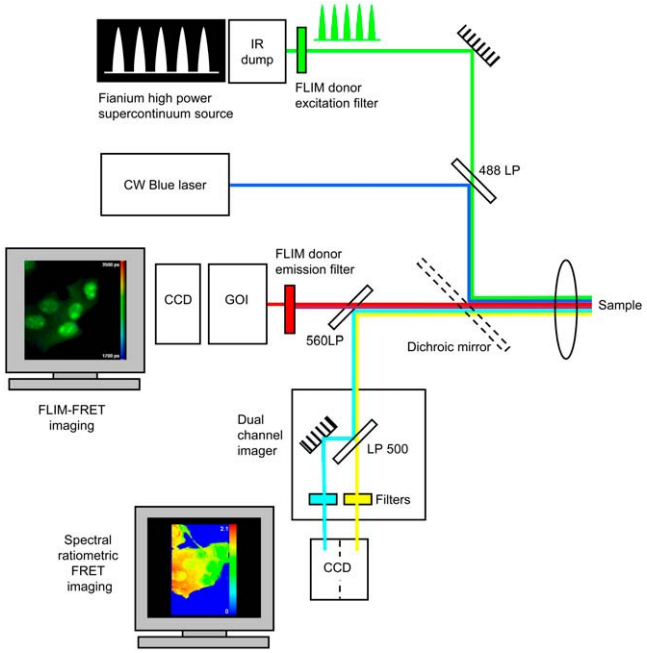

FIGURE 1 Multiplexed FRET microscope. Spectral ratiometric images of Cameleon were acquired by exciting ECFP with a continuous wave blue laser and resolving the fluorescence into two spectral channels. For FLIM measurements an ultrafast supercontinuum excitation source (Fianium, UK) was spectrally filtered for TagRFP excitation. TagRFP fluorescence was imaged onto a GOI (gated optical intensifier (Kentech Instruments, model HRI) and time-gated fluorescence images were recorded on a Hamamatsu ORCA-ER charge coupled device camera.

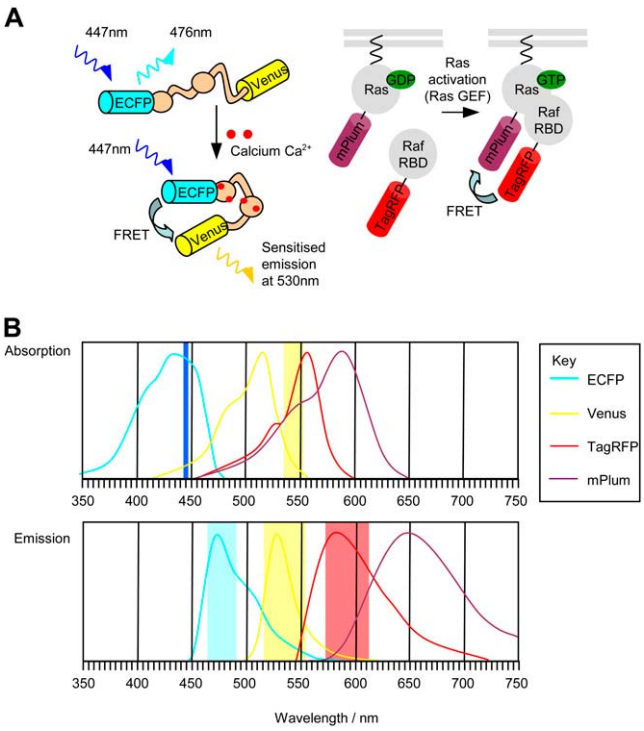

FIGURE 2 FRET constructs and spectral channels used. (*A*) Binding of calcium to calmodulin domain of the YCAM 3.6 Cameleon results in a conformational change and FRET from ECFP to Venus. Activation of mPlum labeled H-Ras (exchange of GDP for GTP catalyzed by guanonucleotide exchange factor (GEF)) results in recruitment of Tag-RFP labeled Raf-Ras Binding Domain to the membrane and FRET between TagRFP/mPlum. (*B*) Absorption and emission spectra of the 4 fluorophores. Shaded areas indicate excitation and emission bands used for imaging multiplexed FRET. Filters are given in the Supplementary Material, Data S1.

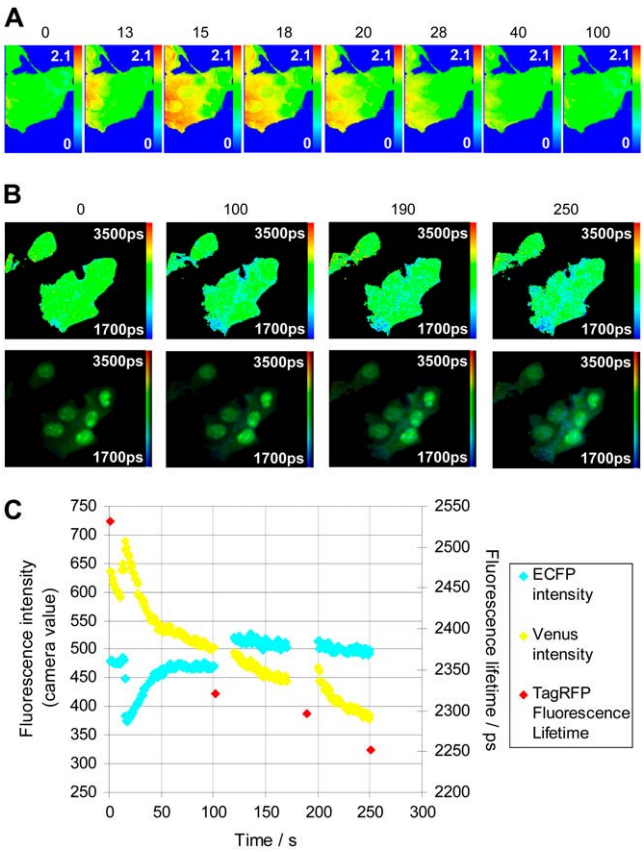

FIGURE 3 Multiplexed FRET imaging of calcium flux and Ras activation. (*A*) Spectral ratiometric images of Cameleon at time points shown (times in seconds). Epidermal growth factor was added 10s from the start. (*B*) Lifetime maps of TagRFP at time points shown (*top row*) and lifetime merged with intensity (*bottom row*). (*C*) Intensity traces in the Venus and ECFP spectral channels from a region of interest in the image and mean lifetime of TagRFP from a region in the membrane. Details of cell culture and plasmid construction are given in the Supplementary Material, Data S1.

lifetime images of the second donor TagRFP. After stimulation with epidermal growth factor, we observed a transient rise in calcium concentration, followed by a sustained Ras activation at the plasma membrane, as evidenced by the shorter fluorescence lifetime around the cell peripheries. Fig. 3 *C* shows mean intensities in the CFP and Venus channels from a region in the cytosol together with mean fluorescence lifetimes at the cell membrane at different times. The Venus and ECFP fluorescence intensities and TagRFP lifetime remained constant in cells not stimulated by EGF.

One limitation of this method is that TagRFP was found to exhibit relatively low photostability. This reduced the number of FLIM images that could be acquired before the fluorophore bleached. This meant that we were able to obtain fewer time points in the time lapse sequence of Ras activation than were obtained for the calcium sensor. At the time of writing, a TagRFP mutant with greater photostability has just become available (9) which should permit a greater number of exposures. We note that, although these experiments were performed using a wide field microscope, it would also be possible to implement optical sectioning using a Nipkow disc confocal microscope, and so achieve optically sectioned FLIM-FRET at high frame rates (10).

In conclusion, we have developed a method for imaging multiple FRET pairs with high signal/noise ratios. This in turn has allowed us to report on spatio-temporal aspects of Ras activation and calcium flux in live cells. Such a system holds promise for elucidating the role of calcium signaling in Ras activation and how the spatial and temporal modulation of calcium concentration give rise to different cellular outputs. As the number of FRET sensors available to biologists continues to expand, we envisage that using this approach it will become possible to explore the interplay and interdependency of a host of cellular parameters.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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