Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors

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Fluorescence resonance energy transfer (FRET) with fluorescent proteins is a powerful method for detection of protein-protein interactions, enzyme activities and small molecules in the intracellular milieu. Aided by a new violet-excitable yellow-fluorescing variant of *Aequorea victoria* GFP, we developed dual FRET–based caspase-3 biosensors. Owing to their distinct excitation profiles, each FRET biosensor can be ratiometrically imaged in the presence of the other.

Consideration of fluorescence emission maxima alone disguises an inconvenient characteristic of fluorescent proteins: their excitation and emission profiles typically approach or exceed 100 nm in spectral width (considering >10% of maximum intensity). It is for this reason that, in spite of the plethora of color variants reported, imaging of 4 or more fluorescent proteins with filter-based widefield fluorescence microscopy is generally complicated by spectral 'bleed-through'[1]. The broad spectral profiles of available fluorescent proteins have also hampered the development of spectrally compatible FRET pairs for ratiometric imaging of two FRET-based biosensors in a single cell. For example, blue fluorescent protein (BFP) coupled with GFP, or cyan fluorescent protein (CFP) coupled with yellow fluorescent protein (YFP), are FRET pairs that each have good overlap between the donor emission and acceptor absorbance, acceptable levels of excitation cross-talk and strong sensitized emission[2]. However, owing to the substantial excitation and emission profile overlaps between these variants, it is not feasible to image the intensity ratios of both FRET pairs when they are present at the same intracellular locale.

Previously reported strategies for imaging of two FRET pairs have involved the 'sharing' of one fluorescent protein between the two pairs. In one example that involved a triple fluorescent protein fusion, a single variant was used as both the acceptor in one FRET pair and a donor in another[3]. In other examples, the same fluorescent protein has been used as the acceptor in two separate FRET pairs and the lifetime[4] or intensity[5] of the donor fluorescent proteins had been imaged. These prior studies demonstrate that sharing of one fluorescent protein between two FRET pairs can be useful in certain applications, but it is impractical for quantitative ratiometric imaging of two FRET pairs in a single cell. To broaden the versatility of dual FRET pair imaging, we engineered two spectrally distinct FRET pairs that can each be ratiometrically imaged in the presence of the other.

We recently described the engineering of monomeric teal fluorescent protein (mTFP1)[6]. Owing to its narrower and red-shifted absorbance peak, mTFP1 compares favorably to CFPs such as enhanced CFP (ECFP) or Cerulean, when used as a FRET donor to a YFP such as mCitrine. We recognized that the weak excitation of mTFP1 at wavelengths below 400 nm presented an opportunity to combine its use with a violet-excitable fluorescent protein such as BFP or a long Stokes shift Sapphire-type variant[7].

During the development of the violet-excitable blue-fluorescing *A. victoria* GFP variant mKalama1 (ref. 8), we fortuitously discovered a long-Stokes-shift violet-excitable yellow-fluorescing variant. We subjected this new fluorescent protein to aggressive directed protein evolution and eventually produced a brightly fluorescent variant (extinction coefficient (ε) = 44,800 $M^{-1}cm^{-1}$, quantum yield (Φ) = 0.58) with 20 mutations relative to GFP (**Table 1**, **Supplementary Methods** and **Supplementary Note 1** online). The final variant, designated as mAmetrine (sequence deposed in GenBank with accession number EU024649), retained the property of violet excitation (excitation wavelength, λ_{ex} = 406 nm) owing to a protonated ground state, and yellow emission (emission wavelength, λ_{em} = 526 nm) resulting from excited state proton transfer and π-π stacking interactions with the chromophore. The photostability of mAmetrine is modest relative to that of popular fluorescent protein variants, with a half-time for

Table 1 | Properties of fluorescent proteins

Protein	λ_{ex} (nm)	λ_{em} (nm)	ε (10^3 M^{-1} cm^{-1})	ϕ	Brightness[a] (mM^{-1} cm^{-1})	pK_a[b]	Photostability[c] (s) Widefield	Photostability[c] (s) Confocal
mAmetrine	406	526	45	0.58	26	6.0	2.8	281
mTFP1	462	492	64	0.85	54	4.3	82	2,721
mCitrine	516	529	77	0.76	59	5.7	6.6	349
tdTomato	554	581	138	0.69	95	4.7	159	419
mT-Sapphire	399	511	44	0.60	26	4.9	12	397

[a]Product of ε and ϕ. [b]pH at which the fluorescence intensity is 50% of its maximum value. [c]Time to photobleach from 1,000 to 500 photons s^{-1} $molecule^{-1}$ under widefield and confocal (50 μW) illumination, respectively[1].

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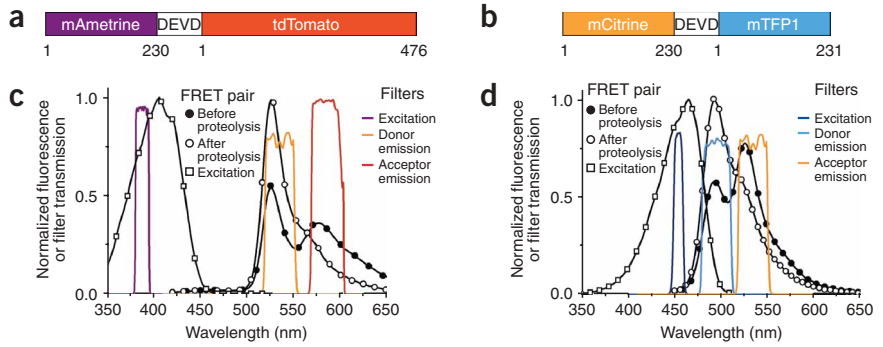

Figure 1 | Caspase-3 biosensors based on dual FRET pairs. (**a**,**b**) Schematics of caspase-3 biosensors. "DEVD" represents the sequence LGGTGSGSGDEVDG. Numbers indicate first and last residue of each fluorescent protein. (**c**) The emission spectrum of mAmetrine-DEVD-tdTomato before and after proteolysis, the excitation spectrum of mAmetrine, and the transmission profiles of excitation and emission filters used for FRET imaging. (**d**) The emission spectrum of mCitrine-DEVD-mTFP1 before and after proteolysis, the excitation spectrum of mTFP1, and the profiles of the excitation and emission filters.

photobleaching that is 42% that of mCitrine and 23% that of mT-Sapphire (**Table 1** and **Supplementary Note 1**), but it is sufficient for certain prolonged imaging experiments as discussed below. Although the fluorescence emission profiles of mAmetrine and mCitrine ($\lambda_{ex} = 516$ nm, $\lambda_{em} = 529$ nm) are practically identical, the dramatically different excitation profiles allow each variant to be specifically excited in the presence of the other.

mAmetrine is potentially a near-ideal donor for pairing with an orange or red fluorescent protein (OFP or RFP) to produce a new FRET pair that would be spectrally compatible with the mTFP1-mCitrine pair for ratiometric imaging of dual FRET–based biosensors. To find an appropriate acceptor for mAmetrine, we systematically tested a variety of non-oligomerizing OFPs and RFPs as acceptors to identify the one with strongest sensitized emission. We found that regardless of the donor (that is, a GFP, a YFP, mT-Sapphire or mAmetrine) the currently available 'true' monomeric OFPs and RFPs[9] give poor sensitized emission and are thus not suitable for use as FRET acceptors for ratiometric imaging (**Supplementary Fig. 1** online). The non-oligomerizing RFP that gives the strongest sensitized emission is tdTomato ($\varepsilon =$ 138,000 M^{-1} cm^{-1}, $\Phi = 0.69$), an exceptionally bright variant of DsRed that consists of concatenated genes encoding the dimeric dTomato[9]. For the combination of mTFP1, mCitrine, mAmetrine and tdTomato to be useful in dual FRET experiments, bleed-through between the four different fluorescence channels must be negligible. Indeed, we found that these four variants permitted facile four-color widefield fluorescence imaging in combination with commercially available bandpass filters (**Supplementary Fig. 2** and **Supplementary Table 1** online).

To test the ability of mTFP1-mCitrine and mAmetrine-tdTomato to function as spectrally compatible FRET pairs for ratiometric imaging, we constructed biosensors of caspase-3 activity[10] by fusing the effector caspase substrate Asp-Glu-Val-Asp (DEVD) between the two fluorescent proteins of the respective FRET pairs (**Fig. 1a**,**b**). *In vitro* characterization of the resulting biosensors, mCitrine-DEVD-mTFP1 and mAmetrine-DEVD-tdTomato, revealed FRET efficiencies of 42% and 45%, respectively. Proteolysis of these constructs resulted in a change in acceptor over donor emission peak intensity ratio from 1.35 to 0.55 and 0.67 to 0.22, respectively (**Fig. 1c**,**d**).

We then co-transfected HeLa cells with plasmids encoding nucleus-targeted mCitrine-DEVD-mTFP1 and nucleus-excluded mAmetrine-DEVD-tdTomato. Then we treated the cells with staurosporine for 100 min to induce apoptosis and imaged them with appropriate filter sets (**Supplementary Movies 1–3** online). In cells that showed no morphological signs of apoptosis, approximately equal exposure times provided similar fluorescence intensities for both the donor and sensitized acceptor emission channels. Within 60 min of the start of imaging, the ratio of sensitized tdTomato (acceptor) to mAmetrine (donor) fluorescence intensity in the cytoplasm of individual cells underwent a stochastic and robust decrease (**Fig. 2**), whereas the emission ratio of a control noncleavable construct (Ser-Ala-Ser-Gly (SASG) linker; **Fig. 3a**,**b**) did not change. After a delay of 2.6 ± 1.6 min (average time difference between midpoints of ratio versus time plots for 17 individual cells), the ratio of sensitized mCitrine (acceptor) to mTFP1 (donor) fluorescence intensity in the nucleus underwent a similar decrease (**Fig. 2** and **Supplementary Table 2** online). When non-targeted mAmetrine-DEVD-tdTomato (that is, freely diffusing between cytoplasm and nucleus) and nucleus-targeted mCitrine-DEVD-mTFP1 were co-expressed, the delay between the onset of caspase-3 activity in the cytoplasm and nucleus was

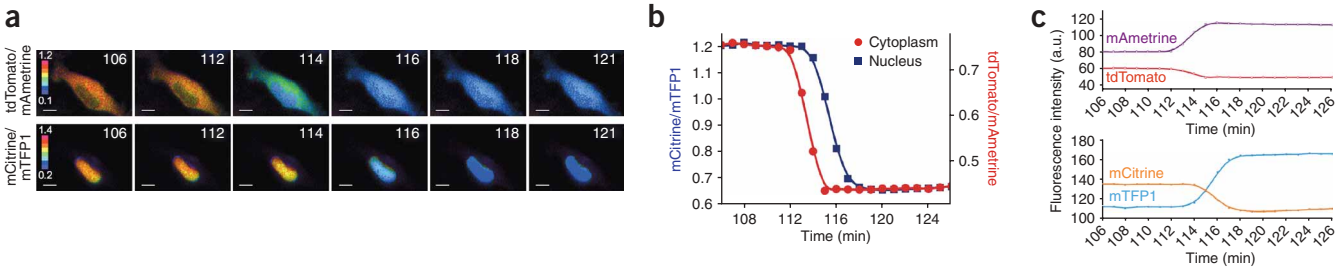

Figure 2 | Live-cell imaging with dual FRET pairs. (**a**) Pseudocolored ratio images of a representative staurosporine-treated HeLa cell expressing dual caspase biosensors at the indicated times (in minutes). mCitrine-DEVD-mTFP1 is localized to the nucleus and mAmetrine-DEVD-tdTomato is nucleus-excluded (from **Supplementary Movie 1**). Scale bar, 10 μm. Color bar represents fluorescence ratio. (**b**) Ratios plotted versus time for the cell shown in **a**. (**c**) Intensities in the 4 emission channels for the cell shown in **a**.

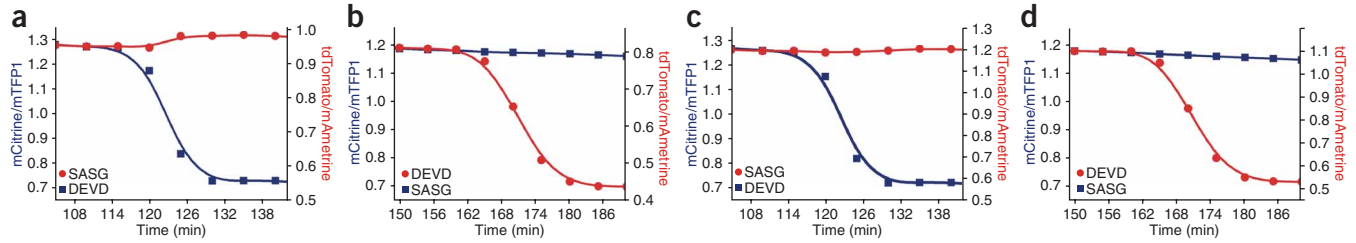

Figure 3 | Control experiments with noncleavable FRET constructs. (**a**,**b**) Ratios plotted versus time for single staurosporine-treated HeLa cells co-expressing a cytoplasmic caspase-3 FRET biosensor (DEVD linker) and a cytoplasmic noncleavable control construct (SASG linker). The cell is expressing mCitrine-DEVD-mTFP1 and mAmetrine-SASG-tdTomato (**a**) or mCitrine-SASG-mTFP1 and mAmetrine-DEVD-tdTomato (**b**). (**c**,**d**) The data presented in **a** and **b**, respectively, corrected for inter-FRET pair bleed-through using the equations described in **Supplementary Note 2**.

unchanged (time difference of 2.2 ± 1.1 min for 11 individual cells; **Supplementary Table 2** and **Supplementary Fig. 3** online). When both biosensors were nucleus-excluded, apoptosis-associated ratio changes occurred simultaneously, demonstrating that they are equivalent caspase-3 substrates (**Supplementary Fig. 4** online).

An important caveat to this dual FRET approach is that the intensities in all four emission channels should be similar (that is, within a factor of 2) to minimize spectral bleed-through. The most likely source of potentially confounding bleed-through is sensitized mCitrine emission contributing to the mAmetrine fluorescence channel in the case where mTFP1-mCitrine is expressed at a substantially higher concentration than mAmetrine-tdTomato. As mAmetrine and mCitrine are imaged using the same emission filter, the extent of the bleed-through depends only on the relative intensities of the two emission signals and the amount of cross-excitation between the donors. By convolving and integrating the excitation spectra of mTFP1 and mAmetrine with each excitation filter, we determined the amount of cross-excitation and derived a simple equation that corrects for inter-FRET pair bleed-through (**Supplementary Note 2** online). Application of this correction factor had no notable impact on the quantitative analysis of images acquired in this work (**Fig. 3c**,**d**), a result that we attribute to matched expression levels and large FRET ratio changes for both FRET constructs. However, in cases where expression cannot be matched by adjustment of transfection conditions, or ratio changes are small in magnitude, application of this correction factor may be required. We also note that control experiments using individual biosensors are essential for establishing the expected magnitude of ratio changes in the dual FRET scenario.

We note that mT-Sapphire could, in principle, be used in place of mAmetrine for this dual FRET application. However, its use would be encumbered by both decreased sensitized emission of tdTomato owing to decreased spectral overlap (**Supplementary Fig. 1**) and increased emission bleed-through with the mTFP1 channel. The latter issue could be addressed with appropriate intensity corrections, though the correction factors would be larger in magnitude and the corrections more complex if an optimal emission filter (that is, 500–530 nm) was used for mT-Sapphire. Both the mTFP1 and mCitrine intensities would appear as terms in the mT-Sapphire correction factor and vice versa. Advantages of using mT-Sapphire include greater photostability and less pH sensitivity than mAmetrine (**Table 1**). Efforts to evolve mAmetrine for improved photostability are in progress.

We described the application of dual FRET pairs for accurate measurement of the delay between the onset of caspase-3 activity in the cytoplasm and in the nucleus during apoptosis[11]. This technology should enable a broad community of researchers to interrogate the interplay and relative kinetics of multiple biological processes with a spatiotemporal precision that was previously inaccessible.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

H.A. contributed to the conceptual development, performed experiments, analyzed data and assisted in manuscript preparation; K.L.H. performed photobleaching experiments and imaging of fusion proteins; M.W.D. contributed to experimental design, analyzed photobleaching data and assisted with manuscript preparation; and R.E.C. contributed to conceptual development, data analysis and writing of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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