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Genetically encoded FRET-based biosensors for multiparameter fluorescence imaging

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The phenomenon of Förster (or fluorescence) resonance energy transfer (FRET) between two fluorescent proteins of different hues provides a robust foundation for the design and construction of biosensors for the detection of intracellular events. Accordingly, FRET-based biosensors for a variety of biologically relevant ions, molecules, and specific enzymatic activities, have now been developed and used to investigate numerous questions in cell biology. An emerging trend in the use of FRET-based biosensors is to apply them in combination with a second biosensor in order to achieve simultaneous imaging of multiple biochemical parameters in a single living cell. Here we discuss the particular technological challenges facing the use of FRET-based biosensors in multiparameter live cell fluorescence imaging and highlight recent efforts to overcome these challenges. In addition, we survey recent applications and provide an outlook on the future opportunities in this area.

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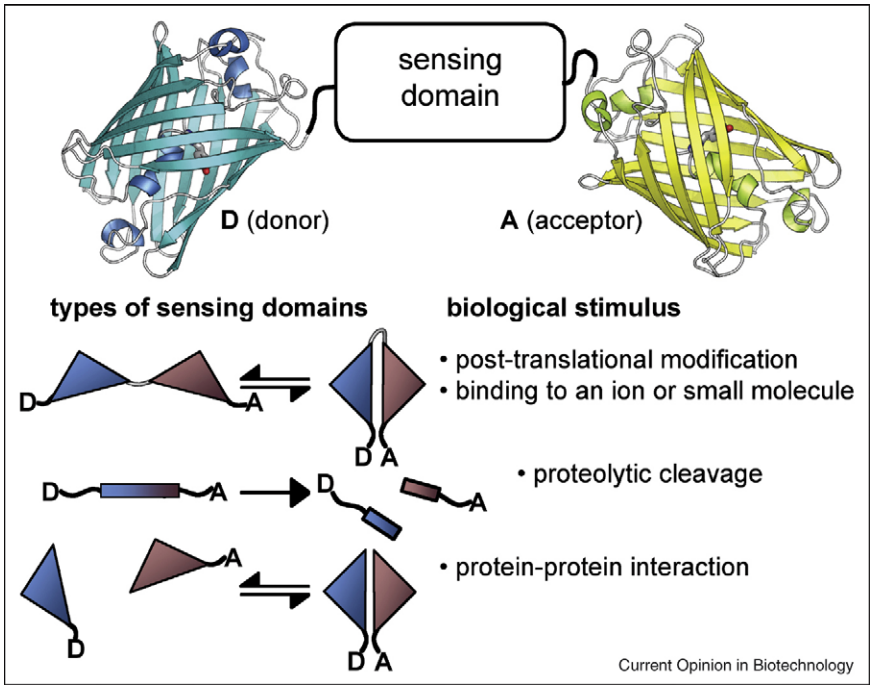
Introduction

With the first examples of recombinant functional expression of a fluorescent protein (FP) in 1994, a seed was planted in the fertile minds of the research community [1,2]. In the ensuing years, this seed has grown into a healthy tree of applications of FP-enabled technologies. One particularly vibrant branch of this tree corresponds to efforts to develop FP reporters that are able to 'sense' a specific subcellular condition and report on that condition with a corresponding change in fluorescent brightness and/or color [3]. These genetically encoded reporters, commonly (and appropriately) known as biosensors, have been applied to the investigation of a variety of subcellular conditions including second messenger dynamics, protein–protein proximity, and enzyme activation, among others [4••]. As the toolbox of FP-based biosensors has continued to fill, some researchers have begun to ask whether it might be possible to combine the use of multiple biosensors to achieve detection of multiple parameters simultaneously in a single cell [5]. The aim of this review is to provide an overview of the successes to date and the future challenges in performing multiparameter imaging with FP-based biosensors either alone or in combination with synthetic indicators and other fluorophores.

The fundamental design strategy that makes many of these FP biosensors possible is the modulation of intramolecular or inter-molecular Förster (or fluorescence) resonance energy transfer (FRET) efficiency [4••]. The basic designs of FP FRET-based biosensors are illustrated in Figure 1. FRET is the distance and orientation-dependent non-radiative transfer of energy from a higher energy (more blue shifted) donor fluorophore to a lower energy (more red shifted) acceptor chromophore [6]. Experimentally, this phenomenon manifests itself as a decrease in both the donor fluorescence intensity and donor fluorescence lifetime, from either of which the experimental FRET efficiency can be extracted [7]. If the acceptor is a fluorophore (as opposed to a non-fluorescent chromophore), it will fluoresce at its characteristic emission wavelength, thus producing a ratiometric signal change. A higher ratio of acceptor to donor intensity is correlated with a higher FRET efficiency that, in turn, is attributed to a shorter distance or more favorable relative orientation between the fluorophores. The currently preferred FRET pair of FPs (Box 1) is the cyan FP (CFP)/yellow FP (YFP) couple [8] that has been used in the vast majority of FRET-based biosensors.

As researchers have exploited FP biosensors to investigate ever more detailed and subtle aspects of cellular physiology, the demand for methods that enable monitoring of multiple biological parameters simultaneously in a live cell has increased [5]. Multiparameter imaging experiments are uniquely well suited to addressing questions of correlation (e.g., does event A always precede B?) and kinetics (e.g., what delay separates events A and B?) in individual cells. In this review we focus on recent efforts to use FP FRET-based biosensors in multiparameter fluorescence imaging experiments, though a variety of other approaches have proven highly effective [5]. We begin by discussing the practical obstacles facing researchers interested in performing such experiments. This is followed by a series of recent examples where researchers have overcome these obstacles using a variety

Figure 1



Types of biosensors based on FP FRET-pairs. The basic requirement for a FRET-based biosensor is that the distance (or orientation) between two suitable FPs (refer to Box 1) is modified by a biological stimulus. The 'sensing domain' is designed to act as the connection between the biological stimulus and the change in distance (or orientation). Some generic examples of sensing domains are shown in the lower portion of the figure. For a more thorough discussion of the various design strategies and specific examples, see the recent review by VanEngelenburg and Palmer [4••].

of different approaches including: engineering biosensors based on new FP FRET pairs, spatial separation of spectrally identical biosensors, non-ratiometric biosensor imaging, and the combination of FP FRET pairs with small molecule indicator dyes (Table 1). We speculate that multiparameter fluorescence imaging, particularly as applied to second messenger and kinase dynamics [9] in cell signaling, will continue to grow in popularity as these enabling technologies grow ever more refined.

Practical considerations for multiparameter fluorescence imaging

The primary challenge of designing and implementing a multiparameter fluorescence imaging experiment is the judicious allocation of the limited resource of spectral wavelengths to different parameters. In the case of FPs, this resource is ultimately limited by the excitation and emission profiles of available FPs [10], which are confined to the relatively narrow band of visible wavelengths (~400–650 nm). This limitation is exasperated by the broad excitation and emission profiles of FPs and the fact that, for ratiometric imaging of FRET-based biosensors, two FPs of different hue are required for each parameter to be measured. Although ratiometric imaging intrinsically demands a greater allocation of spectral resources (i.e., it requires a greater range of wavelengths for each biosensor) it is generally preferable to intensiometric imaging since it is inherently quantitative and less prone to artifacts owing to concentration differences. Fortunately, by taking advantage of the full spectrum of FPs currently available it is (only just) possible to allocate wavelengths such that imaging of up to four dynamic processes in live cells is now an experimental reality [11••]. By contrast, imaging of three or four passive FP labels is a relatively straightforward endeavor [12].

Multiparameter ratiometric imaging with dual FRET pairs

Given the advantages of FP FRET-based biosensors and ratiometric imaging, it is apparent that the ideal approach to imaging of two parameters in a single cell would be to use two spectrally distinct FP FRET pairs. Although this conclusion has undoubtedly been apparent to many researchers in the field for some time, it has only recently been achieved. One of the key breakthroughs that made this breakthrough possible was the dramatic expansion of the FP palette with the discovery of FPs in reef Anthozoa [13] and subsequent efforts to optimize them for imaging applications [14].

In what can only be described as a multiparameter imaging tour-de-force, Piljic and Schultz recently achieved simultaneous fluorescence imaging of four parameters in a single cell [11••]. This feat was made possible, partly, by

Box 1 Considerations in designing a multiparameter imaging experiment with a FP FRET pair.

The FP FRET pair(s) should have:

- spectral overlap, donor quantum yield (Φ_D), and acceptor extinction coefficient (ε_A) sufficient to provide a Forster radius (R_o) that is ~4 nm or greater;
- minimal direct excitation of the acceptor at the wavelength used to excite donor;
- sufficient separation of emission peaks for ratiometric imaging with bandpass filters;
- sufficient acceptor quantum yield (i.e., $\Phi_A \geq 0.5$) for strong sensitized emission. For FRET-FLIM, Φ_A can be 0, but ε_A must still be large (i.e., $\varepsilon_A \geq 50\,000\ M^{-1}\ cm^{-1}$);
- closely matched values of $t_{0.5}$ for maturation (i.e., <1 h at 37 °C) for both the donor and acceptor. The FRET change will be diminished in cases where the donor matures substantially faster than the acceptor.

For multiparameter imaging applications you must be able to:

- excite each FRET pair (or additional fluorophore) at a wavelength where the other FRET pair (or fluorophore) is not efficiently excited;
- or, collect emission from each FRET pair (or additional fluorophore) at wavelengths where the other FRET pair (or fluorophore) does not fluoresce;
- or, use subcellular targeting to physically confine each FRET pair (or additional genetically encoded fluorophore) to distinct and non-overlapping regions of the cell.

combining a CFP/YFP-based biosensor with a orange FP (mOrange)/red FP (mCherry)-based [14] biosensor. These two FRET pairs are spectrally distinct and can be uniquely excited in each other's presence: the CFP/YFP pair with a 405 nm laser line and the mOrange/mCherry pair with a 561 nm laser line (Figure 2a). The authors converted a previously reported FRET biosensor for assembly of annexin (CYNEX4) [15] to one based on mOrange and mCherry (ORNEX4). The ORNEX4 biosensor was imaged with two spatially resolved CFP/YFP-based biosensors (see below). The major limitation of this particular set of dual FRET pairs for multiparameter imaging is that the spectral properties of the mOrange/mCherry pair are less than ideal (Box 1). For example, a number of researchers have reported that mCherry gives very poor sensitized emission owing to its relatively low quantum yield (Figure 2a) [16•,17••]. Also, the excitation profiles of mOrange and mCherry are not very well separated, and the 561 nm excitation line would excite both donor and acceptor with similar efficacy. A 532 nm laser line that would preferentially excite mOrange over mCherry resulted in unacceptable levels of YFP bleed-through into the mOrange emission channel. Despite these issues, the authors were able to deftly combine these spectrally distinct FRET pairs with spatial separation and a spectrally distinct Ca^{2+} indicator to achieve simultaneous imaging of four parameters in single cells.

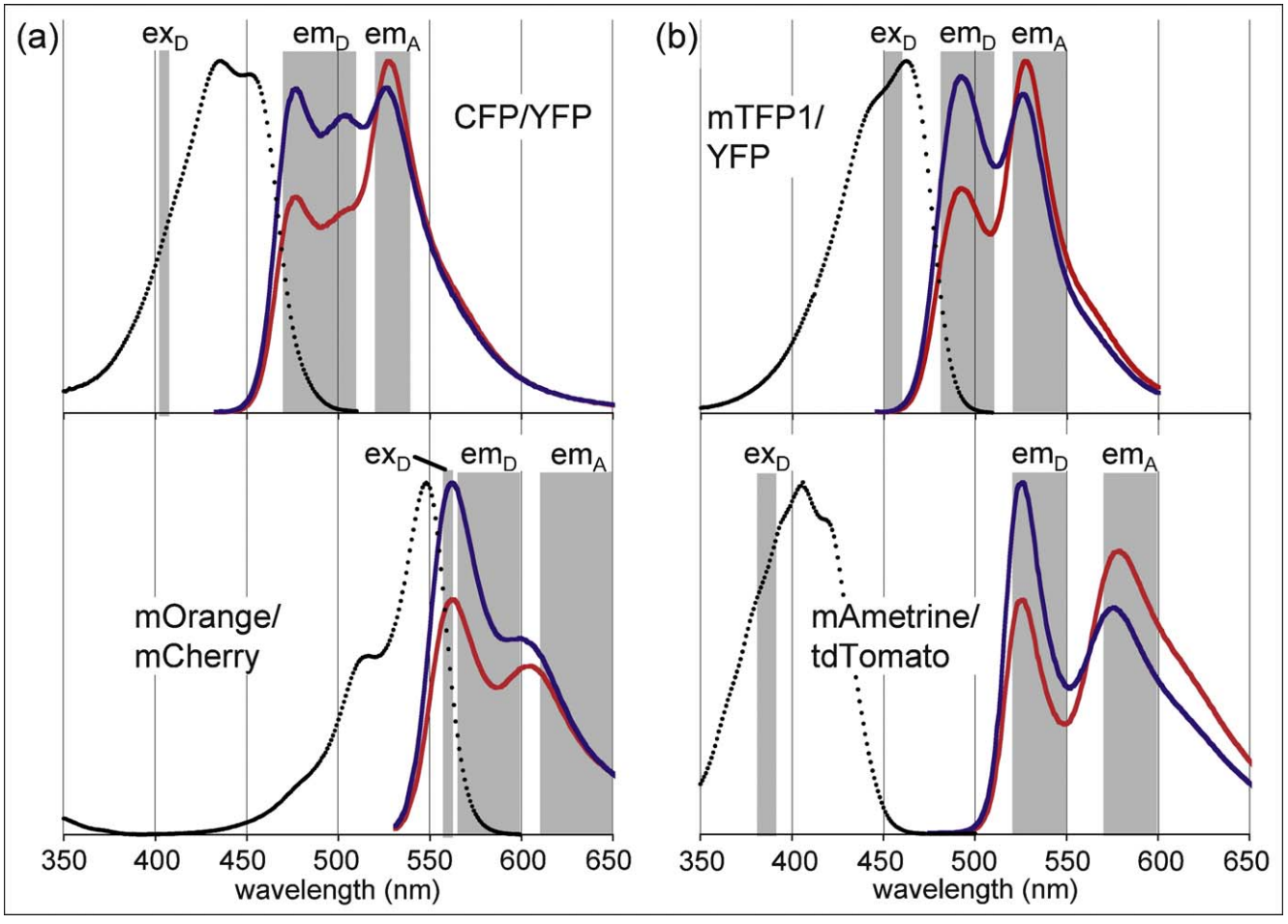
Faced with the poor performance of the currently available monomeric red and far red FPs as FRET acceptors, Ai *et al.* tackled the challenge of developing spectrally distinct FRET pairs from a somewhat different angle [17••]. Their approach took advantage of the distinct excitation profiles of two FRET donors: the cyan-fluorescing mTFP1 (which was paired with a YFP), and the newly developed yellow-fluorescing mAmetrine (which was paired with the very bright orangish-red-fluorescing tdTomato as the acceptor [14]) (Figure 2b). mTFP1 is preferentially excited with a 450–460 nm bandpass filter while mAmetrine is preferentially excited with a 381–392 nm filter. The acceptors of both FRET pairs have quantum yields that are among the highest for all monomeric (keeping in mind that tdTomato is composed of a

Table 1

Spectrally distinct FP FRET-pairs and fluorophores used for multiparameter fluorescence imaging.

FRET pair (imaging method)	2nd FRET pair or additional fluorophores (imaging method)	References
CFP and YFP (emission ratiometric)	mOrange and mCherry (emission ratiometric)	[11••]
mTFP1 and YFP (emission ratiometric)	mAmetrine and tdTomato (emission ratiometric)	[17••]
CFP and YFP (emission ratiometric)	mTagRFP and mPlum (FLIM)	[21•]
YFP and tHcRed (FLIM)	CFP and tHcRed (FLIM)	[20]
YFP and DsRed (donor intensiometric)	CFP and DsRed (donor intensiometric)	[23]
CFP and YFP (emission ratiometric)	Single red fluorescent fluorophore such as Cy3, TMR, or mRFP1 (intensiometric)	[30,32–34]
YFP and DsRed (emission ratiometric)	CFP (intensiometric)	[31]
CFP and YFP (emission ratiometric)	Fura-2 (excitation ratiometric)	[29,37–39,40••,42]
CFP and YFP (emission ratiometric)	Indo-1 (emission ratiometric)	[43•]
CFP and YFP (emission ratiometric)	Fura red (intensiometric)	[29]
CFP and YFP (emission ratiometric)	mOrange (intensiometric) and Fura red (intensiometric)	[11••]

Figure 2



Spectrally distinct FP FRET pairs that have been used for dual ratiometric imaging. On each chart is represented the excitation spectrum of the donor (black dotted line) and simulated emission spectra (accounting for the quantum yield of the acceptor) for the FRET pair at FRET efficiencies of 40% (blue line) and 60% (red line). Excitation bandpass filters or laser lines (ex_D), donor emission filters (em_D), and acceptor emission filters (em_A), are represented by gray lines or boxes. **(a)** The CFP/YFP plus mOrange/mCherry FRET pairs used by Piljic and Schultz [11••]. **(b)** The mTFP1/YFP plus mAmetrine/tdTomato FRET pairs used by Ai *et al*. [17••].

tandem fusion of a normally dimeric FP) FPs [12]. The combination of the mTFP1/YFP and mAmetrine/tdTomato FRET pairs for multiparameter imaging was validated by construction of two caspase-3 biosensors that were used to accurately determine the delay between the onset of caspase-3 activity in the cytoplasm and the nucleus during apoptosis. Control experiments with non-proteolyzable analogs of the FRET constructs demonstrated that changes in the emission ratio of one pair did not influence the emission ratio of the second. One consideration with respect to application of these FRET pairs is that the spectral separation of the excitation profiles is not perfect and thus there is some inter-FRET pair crosstalk. Specifically, ~3% of the mAmetrine signal contributes to mTFP1/YFP FRET channel and ~14% of mTFP1/YFP FRET signal contributes to mAmetrine channel. However, the authors demonstrate that the intensity correction for this crosstalk is relatively trivial and may not even be necessary in cases where the concentrations of the biosensors are closely matched and the FRET changes are large. It is likely that most of the existing CFP/YFP-based biosensors could be converted to these new FRET pairs and mixed and matched to enable a wide variety of two parameter imaging applications.

FRET-FLIM for multiparameter imaging

Like ratiometric imaging, fluorescence lifetime imaging (FLIM) records an inherently quantitative and concentration independent signal, but it does so with substantially less use of the valuable resource of spectral wavelengths. For this reason, FLIM is particularly useful when applied to the imaging of multiple FRET-based biosensors [18]. Indeed, since FRET-FLIM imaging

generally only interrogates the lifetime of the FRET donor, there are less constraints on the choice of acceptor (Box 1). Specifically, the acceptor chromophore need not provide any sensitized emission and, in the case of multiparameter imaging of FRET pairs, the acceptor does not need to be spectrally distinct [19]. The primary drawbacks of the FRET-FLIM strategy are that the equipment is not as widely available as standard widefield and confocal microscopes, and (depending on the method used and the application) the rate of image acquisition could be a limiting factor.

In an example that nicely illustrates some of the advantages listed above, Peyker *et al.* used FRET-FLIM for multiparameter imaging of activation (i.e., binding to a Ras-binding domain (RBD)) of two Ras GTPase isoforms [20]. H-Ras and K-Ras were labelled with YFP and CFP, respectively, and the RBD was fused with the far-red (and relatively dim) FP tHcRed. By imaging of the lifetime of both CFP and YFP the authors were able to visualize the activation of both isoforms simultaneously and show that subcellular localization was the dominant factor in determining activation kinetics.

Another twist in the use of FRET-FLIM is to combine it with ratiometric imaging of a second FRET pair. Grant *et al.* combined ratiometric imaging of a CFP/YFP-based Ca^{2+} biosensor (yellow cameleon 3.6) with FRET-FLIM imaging of an intermolecular H-Ras biosensor analogous to those described above but based on a red FP (mTagRFP)/far red FP (mPlum) FRET pair [21•]. The authors noted that the poor photostability of the mTagRFP variant limited the number of images that could be acquired, though this problem could potentially be ameliorated by switching to the 10-fold more photostable TagRFP-T variant [22•].

Two FRET pairs with the same acceptor

If one is willing to sacrifice the benefits provided by ratiometric imaging or FLIM, simultaneous imaging of two FRET-based biosensors can be achieved by measuring only the donor fluorescence intensity for two FRET pairs that incorporate distinct donors but the same acceptors. For example, Kawai *et al.* used two FRET probes, one based on YFP paired with the *Discosoma* red FP (DsRed) and one based on CFP/DsRed, to simultaneously track caspase-3 activation in TNF-α induced cell death [23]. Efforts to ratiometrically image both FRET pairs in a single cell were thwarted by the fact that the 488 nm laser line used for YFP excitation also directly excited DsRed (present in both pairs) and thereby abolished the ratiometric response. The authors successfully circumvented this limitation by performing intensiometric imaging of the increase in donor fluorescence upon proteolysis of the FRET construct. This work illustrates a drawback of using non-ratiometric biosensors for multiparameter sensing. As the cells entered apoptosis, an increase in the intensity of the CFP and YFP channels was observed, consistent with the loss of FRET due to proteolytic release of the DsRed acceptor. However, the intensity in the DsRed channel also increased, indicating that the increasing concentration of the fluorophores within the shrinking volume of the cell probably accounted for some of the increase in the CFP and YFP signals. This complication would have been avoided with a ratiometric (and therefore concentration-independent) biosensor signal.

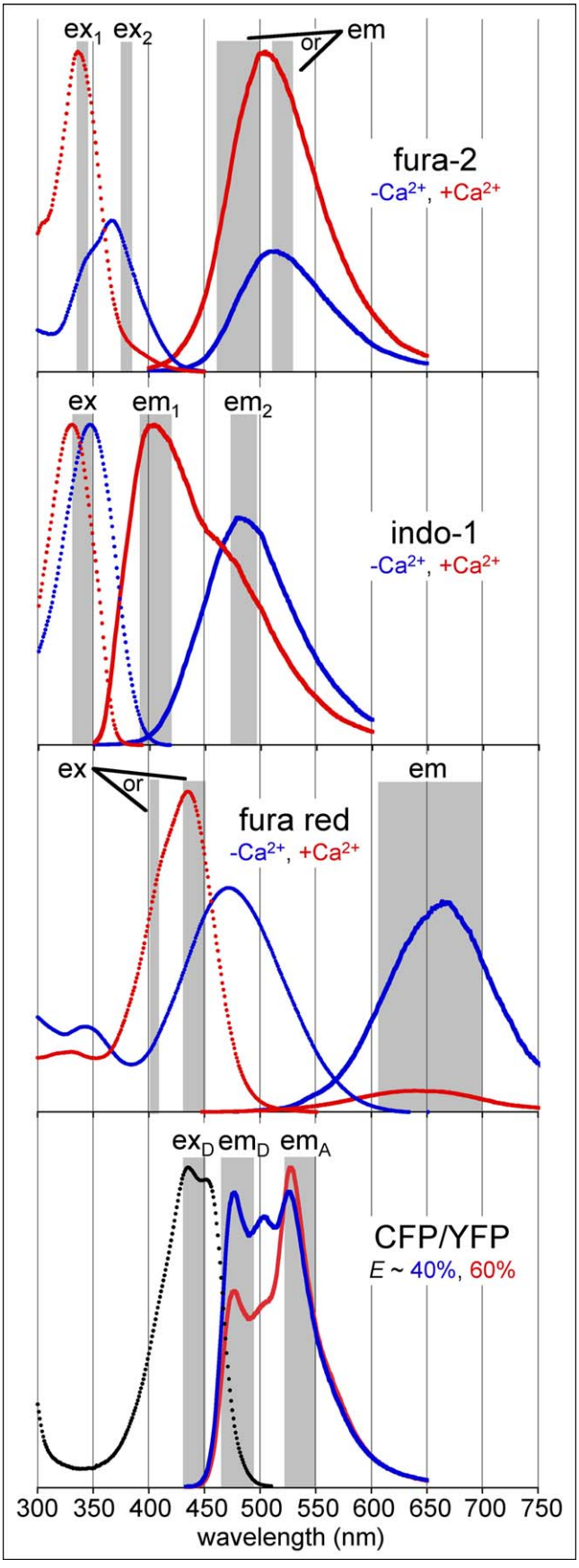
Dual parameter FRET probes

In certain cases it has proven possible to perform multiparameter imaging with a single FRET pair construct that exhibits distinct ratiometric changes in response to two different stimuli. Brumbaugh *et al.* introduced this concept with their report of a single CFP/YFP FRET probe that is sensitive to both PKC and protein kinase A (PKA), responding with either a FRET increase or decrease, respectively [24]. The obvious advantage of this design is that it is possible to image two parameters using only a relatively small window of the spectral range. However, it is unclear how generally applicable this strategy could be and deconvolving the contributions made by each kinase to the overall FRET change may pose a substantial challenge. This problem is somewhat less of a concern with the dual parameter FRET biosensor for caspase-3 and caspase-6 activities created by Wu *et al.* [25•]. This biosensor consists of a fusion of three different hues of FP (CFP, YFP, and the red FP mRFP1) with the caspase-3 substrate between CFP and YFP, and the caspase-6 sequence between YFP and mRFP1. Activation of these effector caspases in apoptotic cells was monitored by flow cytometry using both a 406 nm and a 488 nm laser line to excite CFP (for CFP/YFP FRET) and YFP (for YFP/mRFP1 FRET), respectively. FRET between three different hues of FP have also been used to detect formation of ternary protein complexes using intermolecular FRET from CFP to YFP to mRFP1 in living cells [26,27].

Spatial separation in lieu of spectral separation

Yet another solution for multiparameter imaging with genetically encoded biosensors is to use spatial separation of two spectrally identical FRET-based biosensors in lieu of spectral separation. For example, DiPilato *et al.* simultaneously imaged the response of two different CFP/YFP-based biosensors in a single cell: a plasma membrane localized Epac1-based cAMP reporter (pmICUE1) and a nuclear localized PKA activity reporter (NLS-AKAR) [28]. Piljic and Schultz also successfully imaged two spatially resolved CFP/YFP-based biosensors: the protein kinase C (PKC) biosensor CKAR [29] targeted to the plasma membrane, and the cytosolic calcium/calmodulin-dependent protein kinase IIα biosensor (CYCaMKIIα) [11••]. The advantage of the spatial separation approach is

Figure 3



that the imaging of both biosensors can be truly simultaneous as one pair of images (as opposed to four sequential images) can capture the donor and acceptor intensities for both FRET pairs. A limitation of this approach is that it can only be used for investigation of spatially resolved compartments.

Multiparameter imaging with a FRET pair and a passive fluorophore

In cases where researchers are interested in imaging a single FRET-based biosensor together with a second passive (as opposed to dynamic) fluorescent label, the challenge of spectral separation is substantially decreased. Generally speaking, a red FP is the preferred color for combining with a CFP/YFP FRET pair. For example, Kitano *et al.* developed a new CFP/YFP biosensor (Raichu-Rab5) to detect the activity of Rab5, a small GTPase that is a key regulator in the endocytotic pathway [30]. The authors performed multiparameter imaging of Raichu-Rab5 together with an mRFP1-actin fusion in order to correlate the activation of Rab5 with the process of actin disassembly. In another notable example, Kawai *et al.* simultaneously imaged a YFP/DsRed biosensor for caspase activity and a cytochrome C-CFP fusion to correlate the release of cytochrome C from mitochondria with the onset of caspase-3 activation [31].

In certain cases it is sufficient (or necessary) to use a synthetic fluorophore rather than a genetically encoded FP in combination with a CFP/YFP FRET pair. As with FPs, the synthetic fluorophore must be spectrally distinct from the CFP/YFP FRET pair and red fluorescent labels are therefore preferred. Some key examples of red fluorescent labels imaged in the presence of CFP/YFP include: tetramethylrhodamine methyl ester as a probe of mitochondrial membrane potential [32]; Bodipy FL C5-ceramide (presumably the red fluorescent excimer) as a marker for golgi localization [33]; and Cy3 labeled oligonucleotide as an siRNA cotransfection marker [34].

Multiparameter imaging with a FRET-pair and a synthetic Ca^{2+} indicator

Perhaps the most successful and highly exploited strategy to date in ratiometric multiparameter fluorescence imaging

Spectra of the fura-2, indo-1, and fura red Ca^{2+} indicators that have been used in combination with the CFP/YFP FRET pair for multiparameter imaging. For each of the Ca^{2+} indicators the absorbance (dotted lines) and emission spectra (solid lines) for both the Ca^{2+}-free (blue) and Ca^{2+}-saturated (red) species are represented. The CFP/YFP spectra and bandpass filters and laser lines for excitation are represented as in Figure 2. For fura-2, the two excitation bandpass filters used for ratiometric imaging are designated as ex_1 and ex_2, respectively. For indo-1, the emission bandpass filters for ratiometric imaging are designated em_1 and em_2, respectively. Filters and laser lines for fura-2 [40••,42], indo-1 [43•], fura red [11••,29], and CFP/YFP represent those combinations that have actually been used for multiparameter imaging applications with FP FRET pairs. Original spectra data points were downloaded from www.invitrogen.com.

has been to combine the use of a CFP/YFP FRET biosensor with a synthetic Ca^{2+} indicator. Fortunately, several popular Ca^{2+} indicators, including fura-2 and indo-1 [35], either absorb or fluoresce at wavelengths that are distinct from that of the CFP/YFP pair (Figure 3). A number of researchers have exploited this fact and designed multiparameter fluorescence imaging experiments in which the spectral window of ~400–600 nm has been allocated to the CFP/YFP FRET pair while spectral windows in either the near-UV (for excitation) or the far red (for emission) have been allocated to Ca^{2+} indicators. Some of the specific concerns in combining the use of FPs with calcium indicators have previously been discussed [36].

The single most popular Ca^{2+} indicator to date for combining with a CFP/YFP FRET pair (bottom panel of Figure 3) is fura-2 (top panel of Figure 3). Owing to the large change in absorbance maximum upon binding to Ca^{2+}, fura-2 is amenable to excitation ratiometric imaging by alternating between excitation wavelengths of ~340 and ~380 nm. It has been used for multiparameter excitation ratiometric imaging of Ca^{2+} together with CFP/YFP biosensors that report the activity (or concentration) of: PKC (CKAR), phospholipase C (CYPHR), and diacylglcyerol (DAGR) [29]; endoplasmic reticulum Ca^{2+} [37]; cAMP (Epac1-camps) [38]; the serine/threonine phosphatase calcineurin (CaNAR1) [39]; and myosin light chain kinase [40••]. To validate a new Ca^{2+} indicator (F2C) based on CFP/YFP, Takatsuka *et al.* used simultaneous ratiometric imaging of fura-2 and intensiometric imaging (sensitized emission only) of the FRET pair [41].

Wier *et al.* have closely examined the degree of crosstalk between excitation of the CFP/YFP FRET pair and fura-2 [40••]. The longer of the two excitation filters used for fura-2 (375–385 nm) partially overlaps with the CFP excitation spectrum. Likewise, the 435–445 nm filter used for excitation of CFP partially overlaps with the absorbance of Ca^{2+}-free fura-2. However, the authors found that the crosstalk between fura-2 and CFP/YFP in their shared emission channel (460–500 nm) was no more than 5%, and this could be accounted for using a simple correction factor. Harbeck *et al.* also examined the level of crosstalk between fura-2 and a CFP/YFP FRET pair and found the crosstalk to be practically negligible [42]. It is reasonable to conclude that, while there is certainly the possibility of crosstalk between a CFP/YFP FRET pair and fura-2, whether this is significant or not depends on the specific conditions of the experiment including: filter sets; loading concentration of the acetoxymethyl (AM) ester of fura-2; expression level of the FRET construct; and the magnitude of the FRET construct ratio change. For FRET constructs with small ratio changes (i.e., only a few percent) even a small degree of crosstalk from fura-2 could possibly obfuscate the true ratio changes.

Indo-1 (second from top in Figure 3) is similar to fura-2 in that both dyes absorb in the near UV and have fluorescence emission peaks in the range of 400–500 nm. However, a key difference is that indo-1 is dual emission ratiometric (while fura-2 is dual excitation ratiometric) and thus requires only a single excitation filter at ~340 nm. Fortunately, this wavelength (like the shorter excitation wavelength for fura-2) does not substantially excite the CFP/YFP FRET pair and thus indo-1 meets the criterion of being spectrally distinct. Tay *et al.* used multiparameter imaging of the CFP/YFP troponin C-based Ca^{2+} biosensor TN-L15 and indo-1 in order to validate and calibrate the response of TN-L15 in myocytes [43•]. The authors specifically noted that fura-2 exhibited a small amount of crosstalk with CFP/YFP and thus indo-1 was preferable.

The far-red fluorescing Ca^{2+} indicator fura red (second from bottom in Figure 3) has also been used in combination with a CFP/YFP FRET pair. Owing to its dramatic change in absorbance profile upon binding to Ca^{2+}, fura red can be used as a ratiometric dual excitation indicator with alternating excitation between ~440 nm and ~490 nm. Despite the fact that both of these wavelengths overlap with the excitation profile of the CFP/YFP FRET pair, the far red emission of this dye is sufficiently separated from that of the FRET pair to enable multiparameter imaging. Accordingly, Violin *et al.* performed multiparameter imaging of Ca^{2+} and PKC-mediated phosphorylation using fura red (intensiometric) and the CFP/YFP biosensor CKAR (ratiometric) [29]. The authors used the same 430–450 nm filter for excitation of both the FRET pair and fura red, but did not incorporate a second excitation filter at ~490 nm that would have enabled ratiometric fura red imaging. It is possible that 490 nm excitation would have resulted in an unacceptable level of YFP emission in the fura red emission channel. To minimize the opposite bleedthrough problem (fura red emission in the yellow emission channel) the authors limited the amount of fura red loaded into the cells and only imaged cells with a relatively high expression level of the CKAR biosensor. In their recent multiparameter imaging tour de force, Piljic and Schultz performed intensiometric imaging of fura red, ratiometric imaging of two spatially separated CFP/YFP-based biosensors for PKC and CaMKIIα, and intensiometric imaging of an mOrange-based translocation probe, all in a single cell [11••].

Conclusions and outlook

Multiparameter imaging with FP FRET pairs, either alone or in combination with synthetic indicator dyes, is poised to emerge as a standard research practice. Researchers now have at their disposal a selection of validated tools, including various biosensor designs, spectrally distinct FRET pairs, and synthetic indicator dyes, which enables them to develop and/or employ a multiparameter imaging

strategy customized to their research needs. However, one desirable tool that is missing from the current toolbox is a robust orange/red (or far red) FP FRET pair with strong sensitized emission. This much-needed development is currently being delayed by the surprisingly difficult hurdle of engineering monomeric orange and red FPs with fast maturation, high quantum yields, and high extinction coefficients. Yet another valuable set of tools that could be added to the toolbox is synthetic Ca^{2+} indicators specifically designed to be spectrally distinct from CFP/YFP. Perhaps the synthetic chemists would have an easier time of changing the spectral properties of the indicators than the protein engineers have changing the properties of the proteins! Given the potential research rewards, it is certain that multiparameter fluorescence imaging will continue to grow in popularity and sophistication as researchers apply these methods to an ever expanding range of important biological questions.

Conflict of interest statement

REC is an author on a US patent application describing mTFP1.

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- • of special interest
- •• of outstanding interest

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