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Imaging protein molecules using FRET and FLIM microscopy

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Förster (or fluorescence) resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) have moved center stage and are increasingly forming part of multifaceted imaging approaches. They are complementary methodologies that can be applied to advanced quantitative analyses. The widening application of FRET and FLIM has been driven by the availability of suitable fluorophores, increasingly sophisticated microscopy systems, methodologies to correct spectral bleed-through, and the ease with which FRET can be combined with other techniques. FRET and FLIM have recently found use in several applications: in the analysis of protein–protein interactions with high spatial and temporal specificity (e.g. clustering), in the study of conformational changes, in the analysis of binding sequences, and in applications such as high-throughput screening.

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Abbreviations

FLIM	fluorescence lifetime imaging
FRET	Förster (fluorescence) resonance energy transfer
GFP	green fluorescent protein
SBT	spectral bleed-through
TIRFM	total internal reflection fluorescence microscopy

Introduction

In the dynamic cellular environment, proteins and other cellular components undergo many processes — all primarily designed to maintain cellular homeostasis. Biomedical methods have unlocked many cellular pathways, and continue to do so. However, the desire to capture microsecond or nanosecond cellular changes and interactions in living cells in their natural environment has led to the development of increasingly sophisticated imaging technologies [1–5]. In particular, the need for high spatial and temporal specificity has fostered the application of one-photon and two-photon/multiphoton Förster (or fluorescence) resonance energy transfer (FRET) imaging. In this approach, suitable fluorophores ('donors' and 'acceptors'; see Table 1) are associated with proteins or other components of interest. FRET occurs only when the components come into close proximity and the energy from the donor is non-radiatively transferred to the acceptor [6••,7,8••,9]. The transfer efficiency between donor and acceptor can be monitored using confocal or multiphoton microscopy, but some signal contamination has to be taken into consideration and the measurements corrected accordingly (discussed later). The necessity for these corrections can be avoided using fluorescence lifetime microscopy (FLIM), although this is a technically challenging technique. The fluorescence lifetime of a molecule is a measurement of the rate of decay of the emission, which is a property of the individual fluorophore; thus, the fluorescence lifetime is unaffected by the change in probe concentration or excitation intensity. The fluorescence lifetime is influenced by changes in the cellular environment, such as changes in pH and ion concentration, and is also effected by FRET. In a combined FRET-FLIM approach, the occurrence of FRET is measured by monitoring the change in donor lifetime in the presence and absence of acceptor. As a result, FRET-FLIM has some significant advantages over intensity-based FRET approaches (discussed below).

Measuring either sensitized acceptor emission, donor quenching or donor lifetime provides a method for estimating the energy transfer efficiency [10–15]. In turn, this value can give us information on the proximity of molecules under study and expand our knowledge of cell processes. In this review we describe the basics of FRET and FLIM and its application to studies of protein–protein interactions. We demonstrate that the rate of energy transfer from donor to acceptor is inversely proportional to the number of unquenched donor molecules and describe the importance of detector spectral sensitivity in estimating the energy transfer efficiency.

FRET methodology

FRET, at its most basic, determines the proximity between labeled components within the nanometer range [16]. Depending on the target to be imaged, a range of microscopy systems can be employed for intensity-based FRET studies. For example, changes in the cell nucleus or non-polarized cells might well be analyzed with widefield microscopy [17], whereas thicker specimens (>100 μm) may benefit from the use of two-photon/multiphoton microscopy [18•]. Polarized cells, or cells where discrete focal planes are desirable, could use confocal microscopy [8••], but investigation at the cell surface

Table 1

FRET fluorophore pairs for FRET and FLIM studies.

Donor	Acceptor	References
BFP	GFP	[65]
BFP	YFP	[17]
CFP	YFP	[6••,7,9,11,12,14,15,29,30,33,36–38,41,44,46–48,50,51,54,55,59•]
YFP	YFP	[52]
GFP	Rhod-2	[2]
FITC	Rhod-2	[2]
Cy3	Cy5, Cy5.5	[31••]
Alexa488	Alexa555	[6••,8••,18•,60]
FITC	Alexa546	
	Cy3	
Alexa350	Alexa488	[32]
	Alexa594	
Fluorescein	Cy5	[45]
Fluorescein	Rhodamine	[34]
	Texas Red	[49]
Rhodamine	NBD	[58]
DCIA	NBD	[57]
IAEDANS	DABCYL	[61]
Tryptophan	Dansyl	[64]

A general description of FRET-FLIM donor–acceptor pairs is given in [2–5,10,13,16,26••,40,42,43]. BFP, blue fluorescent protein; CFP, cyan fluorescent protein; DABCYL, 4-((-4-(dimethylamino)-phenyl)-azo)-benzoic acid; DCIA, dichloroisonicotinic acid; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; NBD, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] ethanesulfonate; YFP, yellow fluorescent protein.

might best be done using total internal reflection fluorescence microscopy (TIRFM) [19] (also see the review by Schneckenburger in this issue). FRET-FLIM requires a more specialized set-up, as described in the literature [11,12,20–22].

FRET occurs when two fluorophores (donor and acceptor) have sufficiently large spectral overlap, a favorable dipole–dipole orientation, proximity of 1–10 nm and a large enough quantum yield [9,16,23–25]. Upon energy transfer, donor fluorescence is quenched and acceptor fluorescence is increased (sensitized), resulting in a decrease in donor excitation lifetime. The spectral overlap between donor and acceptor fluorophores that allows FRET to occur is also the cause of FRET signal contamination, termed spectral bleed-through (SBT). SBT refers to that part of the donor emission spectrum that overlaps with acceptor emission (donor SBT) and the part of the acceptor absorption spectrum that is excited by the donor wavelength (acceptor SBT; Figure 1). There are several methods to avoid, minimize or correct SBT contamination in intensity-based FRET, each having specific limitations depending on the level of sensitivity desired [6••,8••,26••,27••]. In some cases the FRET signal is also contaminated by the acceptor wavelength exciting the donor; however, this contamination is usually extremely low and within the noise level [27••].

Methods that avoid the need for SBT correction are the widely used acceptor photobleaching technique and the less frequently used donor photobleaching [28]; however, photobleaching is unsuitable for use in live cells or time series. A recent paper comparing different FRET methodologies draws several conclusions in relation to the problem of SBT: that acceptor photobleaching is in good accord with other methods, if bleaching is complete (a problem in live-cell imaging); that post-image acquisition SBT correction methods vary in establishing energy transfer efficiency; and that for best results donor-to-acceptor ratios should be within the range 0.1–10 [26••]. Another method for handling SBT is spectral unmixing [29]. This is an image analysis technique in which the spectral imaging software compares the experimentally derived emission data with a previously recorded reference spectrum for that fluorophore. The use of quantum dots as donors might also reduce SBT. It is beyond the scope of this review to discuss SBT correction further, and instead readers are referred to the available literature [6••,8••,26••,27••]. Suffice it to say that SBT is an important concern and, depending on the sensitivity required, the level of the FRET signal, and the requirement for distance estimates, employing one of the available methods is likely to be effective. By contrast, the use of FRET-FLIM avoids the problem of SBT altogether [30].

Energy transfer efficiency

In one recent method to remove SBT and to establish energy transfer efficiency [6••,27••], seven images were required to remove the donor SBT (DSBT) and the acceptor SBT (ASBT) from the contaminated or uncorrected FRET (uFRET) and to obtain the processed FRET (PFRET) image (Figure 2), as shown in Equation (1).

$$\mathrm{PFRET} = \mathrm{uFRET} - \mathrm{DSBT} - \mathrm{ASBT} \quad (1)$$

The non-radiative rate of energy transfer efficiency from donor to acceptor molecule (E) can be estimated [6••]

$$E = 1 - I_{DA}/I_D = 1 - I_{DA}/[I_{DA} + \mathrm{PFRET}] \quad (2)$$

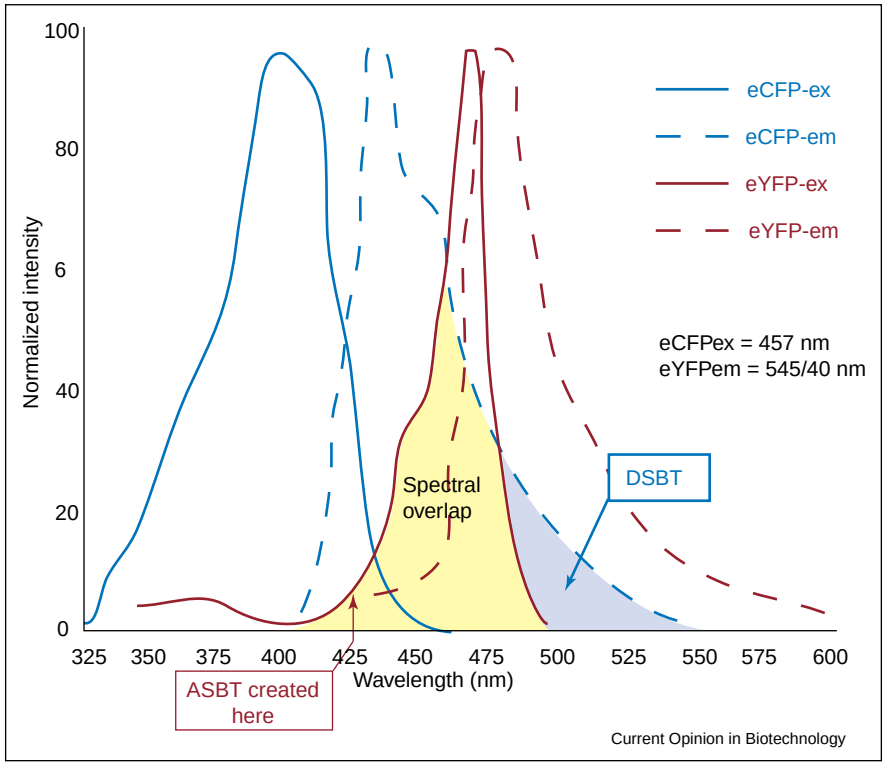
$$I_{DA} + \mathrm{PFRET} = I_D = \mathrm{uD} \quad (3)$$

where uD is the unquenched donor and I_{DA} and I_D are the intensity of the donor in the presence and absence of acceptor, respectively. Using Equations (2) and (3), the following can then be derived:

$$E = \mathrm{PFRET}/\mathrm{uD} \quad (4)$$

As shown in Equation (4), the energy transfer from the donor to the acceptor can be expressed as a percentage of the total unquenched donor fluorescence and is called 'energy transfer efficiency' (E%). E% should correctly be called 'apparent' E%, as the computation is based on total

Figure 1



A FRET pair (enhanced cyan [eCFP] and yellow [eYFP] fluorescent protein) with sufficient spectral overlap. Excitation (solid line; ex) and emission (dashed line; em) spectra of donor (eCFP) and acceptor (eYFP). The requirement of FRET for sufficient spectral overlap (yellow) occurs at the expense of SBT, as indicated in the figure. ASBT, acceptor spectral bleed-through; DSBT, donor spectral bleed-through.

donor fluorescence, including non-FRET donors (i.e. donor molecules that did not participate in the energy transfer process). As a 'spectroscopic ruler', E% measures the average distance between fluorophores using R_o (the distance at which E% is 50%, termed the Förster distance) [25]. E% decreases rapidly with increasing distance between fluorophores. Where there is a consistent spatial arrangement between fluorophores (e.g. in fusion proteins or stable macromolecular assemblies), the distance measurement is likely to be more accurate than in co-localizations of components where distances vary (e.g. clusters).

Moreover, it is important that PFRET should be corrected for the spectral sensitivity of the detector (g^{-1}) for both donor and acceptor channel images [27••]. The new E_n and r_n are then given by:

$$E_n = 1 - I_{DA}/[I_{DA} + g^{-1}(\mathrm{PFRET})] \quad (5)$$

$$E_n = 1 - I_{DA}/[I_{DA} + \mathrm{PFRET} * ((\psi_{dd}/\psi_{aa}) * (Q_d/Q_a))] \quad (6)$$

where Q_d and Q_a are the quantum yield of the donor and acceptor, respectively, and ψ_{dd} and ψ_{aa} are the collection efficiency in the donor and acceptor channel

$$(\psi_{dd}/\psi_{aa}) = \{(\text{photonmultiplier tube gain of donor channel}/\text{photonmultiplier tube gain of acceptor channel}) \times (\text{spectral sensitivity of donor channel}/\text{spectral sensitivity of acceptor channel})\} \quad (7)$$

Equation (6) is the final energy transfer efficiency equation. The distance between donor and acceptor molecule (r) is estimated using Equation (8):

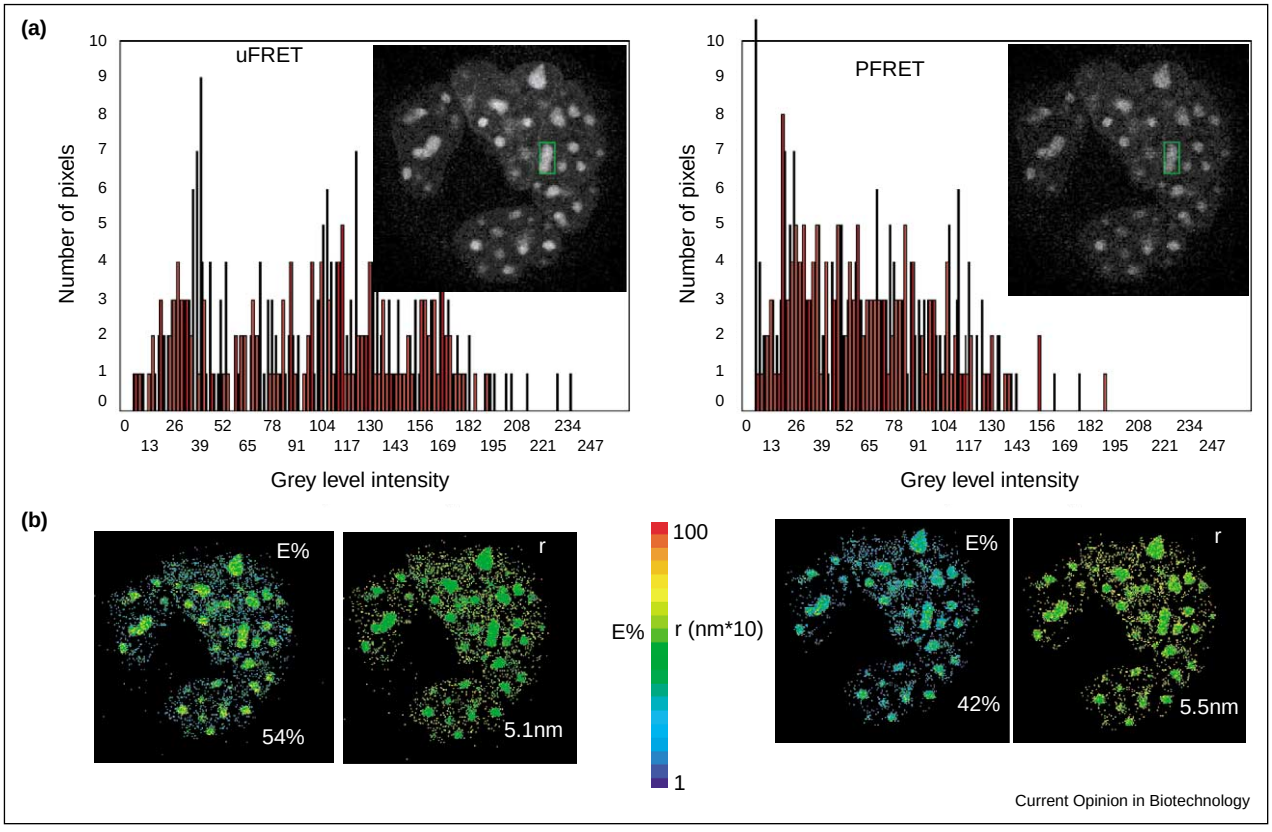
$$r_n = R_0\{(1/E_n) - 1\}^{1/6} \quad (8)$$

Applications of FRET

The burgeoning number of publications on the use of FRET is a testament to the utility of this microscopy technique, which is increasingly used as a starting point to investigate cellular structure and function in four dimensions. More quantitative analyses can then follow FRET analysis.

If the aim of a study is proof of proximity between components, which conventional fluorescence microscopy cannot unequivocally provide, the occurrence or

Figure 2



Intensity-based FRET data analysis. The data analysis for the CCAAT/enhancer-binding protein α expressed in GHFT1-5 cells using Biorad Radiance confocal/multiphoton microscopy. The donor–acceptor pair comprised cyan and yellow fluorescent protein. Seven images were required for PFRET data analysis [6,27]. The histograms clearly demonstrate the implementation of acceptor and donor SBT correction. This correction also accounts for variation in fluorophore expression levels within the cellular images. **(a)** The uncorrected FRET (uFRET) and processed FRET (PFRET) image and their respective histograms. **(b)** The two-dimensional distribution of efficiency (E) and distance (r) images. Before correction E = 54% and r = 5.1 nm; after SBT correction E = 42% and r = 5.5 nm.

lack of FRET is a sufficient indicator (after appropriate correction for SBT) without the need for additional computations. Qualitative FRET has enjoyed widespread use in establishing co-localization, cellular organization, conformational changes and the like. Box 1 provides some examples.

Box 1 Qualitative FRET analysis: examples of recent applications.

The distribution of potassium channels [45]
Co-localization of vacuolar H^+-ATPase subunits in living plant cells [46]
The discovery of binding partners linking gap junctions to the submembrane cytoskeleton [47]
Providing direct evidence for the formation of a ternary kinase–scaffold–phosphatase complex [48]
The use of three-fluorophore FRET to show RNA conformational changes and binding to ribosomal S15 in one assay [49]
Investigating conformational changes of a transcription factor [50]
Showing in intact cells that mammalian G-protein subunits undergo molecular rearrangement rather than dissociation [51]
The characterization of lipid rafts – addressing a controversial scientific question [52]
High-throughput screening assays [53–56]

Increasingly, quantitative analyses are being carried out using FRET based on the calculation of E% and distance estimates. Quantitative FRET studies can provide information on clustered versus random distribution and can be used to investigate spatial arrangements. Specialized approaches, such as single-molecule and three-color FRET (or a combination of the two), are also being explored [31••,32]. Box 2 provides some examples.

Live-cell FRET microscopy can usefully be combined with a range of techniques including fluorescence correlation spectroscopy (to investigate diffusion [33]), anisotropy measurements (to investigate structural relationships of molecules [34,35]), TIRFM (for analysis of specimen surfaces [19,36]), and FLIM (see below). Potentially, other microscopy approaches are feasible using FRET as a platform for establishing proximity and for linking quantitative parameters with whatever

Box 2 Quantitative FRET analysis: examples of recent applications.

Application of FRET to measure energy transfer efficiency (E%)

Quantifying protein–lipid binding selectivity [57]

Monomer–dimer equilibrium suggests that transmembrane peptide helices are parallel [58]

E% was used to measure cardiomyocyte contraction in response to titrated Ca^{2+} concentrations [59•]

Determining the clustered distribution of membrane receptors by correlation of E% to donor/acceptor levels [8••,60]

Measuring the binding stoichiometry of two membrane components [61]

Using FRET–PCR for genotyping [62]

Application of FRET to measure distance (r)

Measuring the spatial separation between nucleotide binding and phosphorylation domains of Ca-ATPase [63]

Combining FRET with molecular dynamics simulations defines the otherwise heterogeneous donor–acceptor distances [64]

Spatial analysis: comparison of E% between receptors at the cell surface and the interior, as well as the fraction of either [29]

Comparing the formation of multimeric complexes in the cytoplasm versus the nucleolus [65]

Distance distribution studies [11,25]

system is most suitable to analyze cellular structure and function. For example, spectral sensitivity correction for donor and acceptor channels was implemented for estimating E% and distance measurements for C/EBPα (CCAAT/enhancer binding protein α) dimerization. The study employed a cyan fluorescent protein and yellow fluorescent protein FRET pair and was carried out in live GHFT1-cell nuclei [6••,27••] (Figure 2).

FRET-FLIM

A fluorophore is not only characterized by its emission spectrum, but also by its unique lifetime. The fluorescence lifetime (τ) is defined as the average time that a molecule remains in an excited state before returning to the ground state. In practice, the fluorescence lifetime is defined as the time in which the fluorescence intensity decays to 1/e of the initial intensity (I_0) immediately following excitation (i.e. 37% of I_0). FLIM is independent of changes in probe concentration, excitation intensity and other factors that limit intensity-based steady-state measurements. Instrumental methods for measuring fluorescence lifetimes can be divided into two major categories: frequency domain [22] and time domain [11,14,21,37,38]. Either method can be used in one-photon or two-photon FRET-FLIM microscopy. With the time-domain method (or pulse method), the specimen is excited with a short pulse and the emitted fluorescence is integrated in two or more time windows [11]. The relative intensity capture in the time windows is used to calculate the lifetime decay characteristics. As an alternative to the time-domain method, the frequency-domain method uses a homodyne detection scheme and requires a modulated light source and a modulated detector. The excitation light is modulated in a sinusoidal fashion. The fluorescence intensity shows a delay or phase-shift with respect to the excitation and a smaller modulation depth [25].

The fluorescence lifetime is influenced by changes in the cellular environment including the occurrence of FRET, which shortens or changes the excited state lifetimes. FLIM allows the measurement of dynamic events at very high temporal resolution (ns). FRET-FLIM provides direct evidence of interactions between proteins. Moreover, as only the donor is monitored, no SBT correction is needed. By measuring the donor lifetime in the presence and the absence of acceptor one can accurately calculate the distance between the donor- and acceptor-labeled proteins.

Whereas one-photon or two-photon FRET produces an 'apparent' E% (i.e. the energy transfer efficiency calculated on the basis of all donors, both FRET and non-FRET), the double-label lifetime data in one-photon or two-photon FRET-FLIM usually exhibits two donor lifetimes: quenched and unquenched (FRET and non-FRET), allowing a more precise estimate of distance based on FRET donors only [7,12]. The unquenched lifetime might be sufficiently accurate for many situations, but the quenched lifetime might be vital for establishing comparative distances between biomolecules. Moreover, the intensity-based FRET methods cannot distinguish between an increase in FRET E% (i.e. coupling efficiency) and an increase in FRET population (concentration of FRET species); FRET-FLIM methodology can resolve this issue using multicomponent analysis [7].

Applications of FRET-FLIM

FRET-FLIM microscopy has been used to characterize intranuclear dimer formation for the transcription factor C/EBPα in living pituitary GHFT1-5 cells [7,11]. Shown in Figure 3, dimerization reduces the donor lifetime as a consequence of FRET, resulting in different lifetime distributions (τ_{DA} = 1.87 ns). On bleaching the acceptor molecule (using illumination of acceptor wavelength 514 nm), the donor molecule returns to its natural lifetime (τ_D = 2.52 ns). This clearly demonstrates the occurrence of FRET. FRET-FLIM can separate FRET from non-FRET donors on the basis of lifetime distributions, resulting in more realistic measurements. E and r can be calculated using Equations (9) and (10)

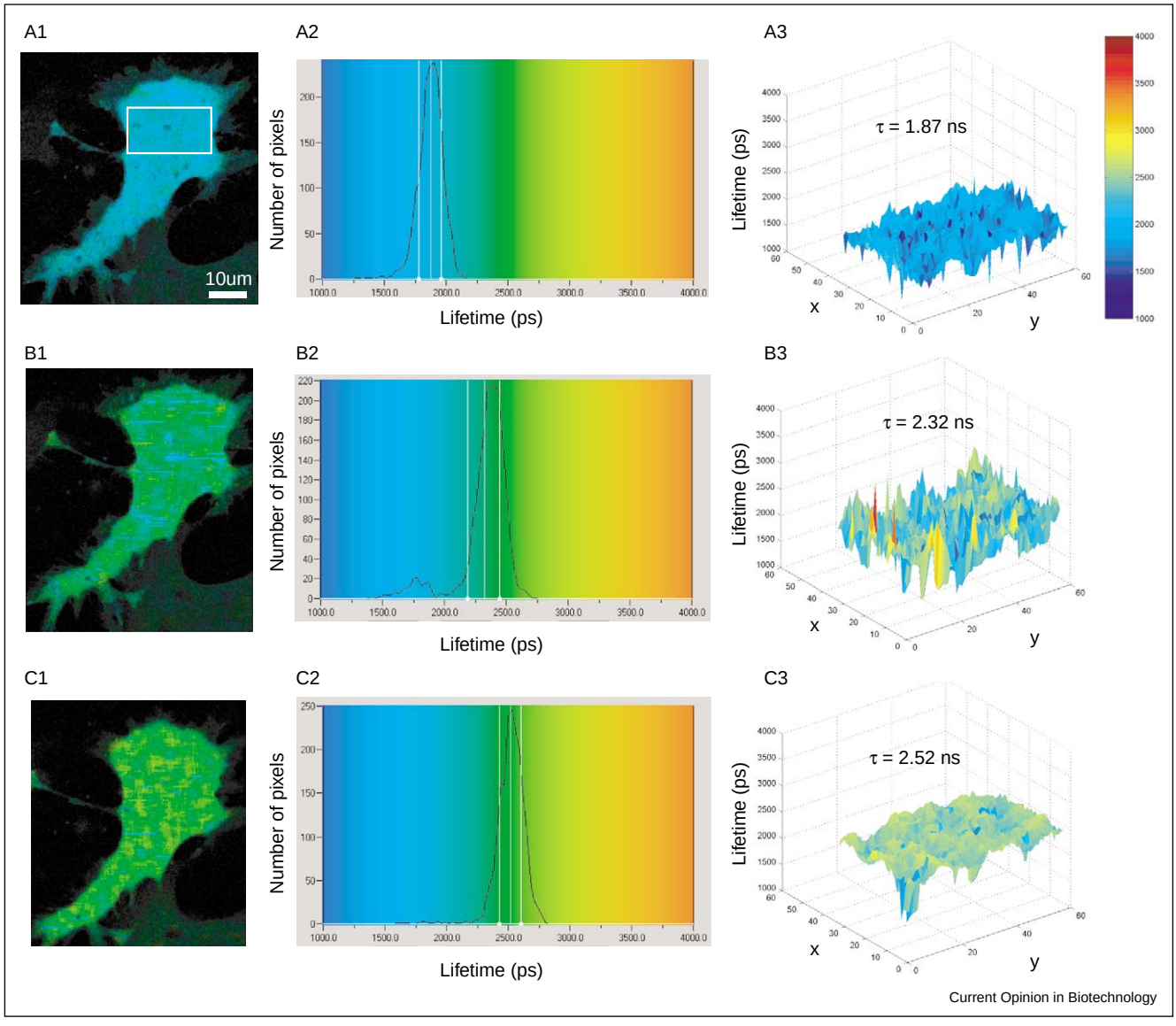
$$E = 1 - (\tau_{DA}/\tau_D) \quad (9)$$

$$r = R_0\{(1/E) - 1\}^{1/6} \quad (10)$$

where τ_D and τ_{DA} are the donor excited state lifetimes in the absence and presence of the acceptor.

FRET-FLIM technology has been used in several other recent studies to investigate cellular functions. For

Figure 3

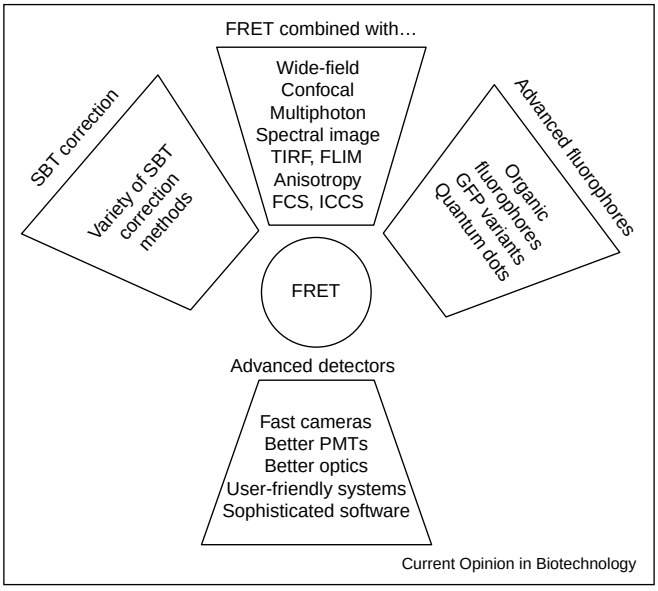


Protein localization using acceptor photobleaching FRET-FLIM microscopy. Images of cells expressing enhanced cyan fluorescent protein coupled directly to enhanced yellow fluorescent protein through a 15 amino acid linker were acquired and analyzed using FRET-FLIM microscopy [7,30]. The mean lifetime of the selected region of interest was 1.87 ns in the presence of acceptor (A1 and A2). Photobleaching of the acceptor molecule demonstrates the occurrence of FRET and the lifetime returns to its natural lifetime 2.52 ns (C1 and C2). The lifetime of the donor molecule increases by increasing the photobleaching of the acceptor molecule step-by-step, as shown in the cellular images A1, B1 and C1 and in the three-dimensional plots A3, B3 and C3. The two-dimensional lifetime distribution shift towards increase in lifetime A2, B2 and C2 clearly demonstrates the return of the quenched molecule to its natural lifetime 2.52 ns.

example, FRET-FLIM was used in a study of MCF-7 breast carcinoma cells, and revealed appreciable FRET at the plasma cell membrane and cell cortex beneath the membrane [13]. In another study, Bacskai *et al.* [14] characterized the macromolecular structures in amyloid-β plaques in tissue sections of a transgenic Alzheimer mouse model. In a further example, FRET-FLIM techniques were used to study caspase activity in baby hamster kidney cells. In this case, as a result of apoptosis, a wider distribution of altered donor lifetimes was observed for tert-butyl hydroperoxide (tBOOH)-treated cells [15,39].

The distance between donor and acceptor molecules is not linear and there is a possibility that more than one pair of donor and acceptor molecules can come into proximity. FRET-FLIM helps to identify the distance distribution in the dimerization of the protein molecules [11]. More-

Figure 4



Drivers of the increased use of FRET. The rapidly increasing application of FRET microscopy is driven by better technology (microscopy systems and software), by the availability of a wide range of fluorophores, by various methodologies to overcome SBT signal contamination and, most importantly, by the combination of FRET with other microscopy techniques and/or analysis tools to capture additional temporal and spatial information, such as kinetics, cluster densities and the ability to carry out high-throughput screening in drug development. FCS, fluorescence correlation spectroscopy; ICCS, image cross-correlation spectroscopy; PMT, photomultiplier tube.

over, as this methodology follows the excited state lifetime of the molecular interactions, it is possible to follow one or more interacting protein molecules in a single living cell. Using fluorescence lifetime measurements, complete quantitative characterization of the molecular interactions can be made.

Suitable fluorophores have been developed for FRET and FLIM [40–43] (Table 1). However, it has been reported that some variants of green fluorescent protein have two-component lifetimes when fused to proteins, and this may be an issue in FRET-FLIM imaging [30,44]. As the donor molecule lifetime is followed for the energy transfer process, it is important to verify whether the donor molecule has single exponential decay in the absence of acceptor.

Conclusions

There is no question that the application of FRET microscopy will continue to increase, driven by the advances outlined in Figure 4 and by the growing number of researchers that routinely use this technology. In the future, SBT and other challenges in intensity-based FRET will most likely be handled in real-time with suitable software — some commercial developments are already taking place. The use of FRET-FLIM provides many advantages and is also likely to expand; however, not every center will have a FLIM facility and one advantage of 'conventional' FRET is the opportunity to use a wide range of standard microscopy systems. Three-color fluorophore FRET systems are beginning to emerge, particularly combined with spectral imaging and spectral unmixing. Further advances will arise from the combination of the basic FRET phenomenon with other technologies such as dual-color fluorescence correlation spectroscopy, image cross-correlation spectroscopy and others. New fluorophores and, in particular, quantum dots will also expand the usefulness of FRET, both qualitatively and quantitatively. Opportunities for further mathematical modeling using E% and distance information, donor-to-acceptor ratios, knowledge of protein structure and so on, together with data gleaned from other experimental methods, will lead to detailed insights into cellular dynamics.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Stephens DJ, Allan VJ: **Light microscopy techniques for live cell imaging**. *Science* 2003, **300**:82-86.

2. Periasamy A: *Methods in Cellular Imaging*. New York: Oxford University Press; 2001.

3. Marriott G, Parker I: *Biophotonics, Part A and B. Methods in Enzymology Vol 360*. San Diego, CA: Academic Press; 2003.

4. Tsien RY: **Imagining imaging's future**. *Nat Rev Mol Cell Biol* 2003, **Suppl**:SS16-SS21.

5. Miyawaki A: **Visualization of the spatial and temporal dynamics of intracellular signaling**. *Dev Cell* 2003, **4**:295-305.

6. •• Elangovan M, Wallrabe H, Chen Y, Day RN, Barroso M, Periasamy A: **Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy**. *Methods* 2003, **29**:58-73.
Microscopy-based FRET imaging requires data analysis software to remove contamination from the FRET image. This paper describes a step-by-step algorithm to remove the SBT contamination in the FRET image collected using wide-field, confocal, and two-photon FRET microscopy systems.

7. Chen Y, Mills JD, Periasamy A: **Protein localization in living cells and tissues using FRET and FLIM**. *Differentiation* 2003, **71**:528-541.

8. •• Wallrabe H, Elangovan M, Burchard A, Periasamy A, Barroso M: **Confocal FRET microscopy to measure clustering of ligand-receptor complexes in endocytic membranes**. *Biophys J* 2003, **85**:559-571.
This paper describes the usage of confocal FRET microscopy for quantitative analysis of receptor clustering in the apical endocytic compartments in MDCK cells. Mathematical modelling was used to differentiate between clustered and random distributions of fluorophore-bound molecules on the basis of the dependence of FRET intensity on donor and acceptor concentrations.

9. Sekar RB, Periasamy A: **Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations**. *J Cell Biol* 2003, **160**:629-633.

10. Lidke DS, Nagy P, Barisas BG, Heintzmann R, Post JN, Lidke KA, Clayton AH, Arndt-Jovin DJ, Jovin TM: **Imaging molecular interactions in cells by dynamic and static fluorescence anisotropy (rFLIM and emFRET)**. *Biochem Soc Trans* 2003, **31**:1020-1027.

11. Elangovan M, Day RN, Periasamy A: **Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell**. *J Microsc* 2002, **205**:3-14.

12. Chen Y, Periasamy A: **Characterization of two-photon excitation fluorescence lifetime imaging microscopy for protein localization**. *Microsc Res Tech* 2004, **63**:72-80.

13. Peter M, Ameer-Beg SM: **Imaging molecular interactions by multiphoton FLIM**. *Biol Cell* 2004, **96**:231-236.

14. Bacskai BJ, Skoch J, Hickey GA, Allen R, Hyman BT: **Fluorescence resonance energy transfer determinations using multiphoton fluorescence lifetime imaging microscopy to characterize amyloid-beta plaques**. *J Biomed Opt* 2003, **8**:368-375.

15. Krishnan RV, Masuda A, Centonze VE, Herman B: **Quantitative imaging of protein-protein interactions by multiphoton fluorescence lifetime imaging microscopy using a streak camera**. *J Biomed Opt* 2003, **8**:362-367.

16. Jares-Erijman EA, Jovin TM: **FRET imaging**. *Nat Biotechnol* 2003, **21**:1387-1395.

17. Day RN, Voss TC, Enwright JF III, Booker CF, Periasamy A, Schaufele F: **Imaging the localized protein interactions between Pit-1 and the CCAAT/enhancer binding protein α in the living pituitary cell nucleus**. *Mol Endocrinol* 2003, **17**:333-345.

18. • Mills JD, Stone JR, Rubin DG, Melon DE, Okonkwo DO, Periasamy A, Helm GA: **Illuminating protein interactions in tissue using confocal and two-photon excitation fluorescent resonance energy transfer microscopy**. *J Biomed Opt* 2003, **8**:347-356.
This paper describes the usage of multiphoton microscopy for tissue FRET imaging. The authors have demonstrated the interaction between the proapoptotic protein BAD and the prosurvival protein Bcl-x_L within traumatic axonal injury following traumatic brain injury deep inside the tissue.

19. Schneckenburger H, Wagner M, Kretzschmar M, Strauss WS, Sailer R: **Laser-assisted fluorescence microscopy for measuring cell membrane dynamics**. *Photochem Photobiol Sci* 2004, **3**:817-822.

20. Clegg RM, Holub O, Gohlke C: **Fluorescence lifetime-resolved imaging: measuring lifetimes in an image**. *Methods Enzymol* 2003, **360**:509-542.

21. Gerritsen HC, Asselbergs MA, Agronskaia AV, Van Sark WG: **Fluorescence lifetime imaging in scanning microscopes: acquisition speed, photon economy and lifetime resolution**. *J Microsc* 2002, **206**:218-224.

22. Gratton E, Breusegem S, Sutin J, Ruan Q, Barry N: **Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods**. *J Biomed Opt* 2003, **8**:381-390.

23. Förster T: **Delocalized excitation and excitation transfer**. In *Modern Quantum Chemistry Part III: Action of Light and Organic Crystals.* Edited by Sinanoglu O. New York: Academic Press; 1965:93-137.

24. Stryer L: **Fluorescence energy transfer as a spectroscopic ruler**. *Annu Rev Biochem* 1978, **47**:819-846.

25. Lakowicz JR: *Principles of Fluorescence Spectroscopy*. 2nd ed. New York: Plenum Press; 1999.

26. •• Berney C, Danuser G: **FRET or no FRET: a quantitative comparison**. *Biophys J* 2003, **84**:3992-4010.
This paper compares different FRET data analysis algorithms from the literature to remove the contamination in the FRET image.

27. •• Chen Y, Elangovan M, Periasamy A: **FRET data analysis: the algorithm**. In *Molecular Imaging: FRET Microscopy and Spectroscopy*. Edited by A Periasamy and RN Day. New York: Oxford University Press. Chapter 7: in press.
This book chapter describes an advanced level step-by-step algorithm for FRET data analysis including SBT and the back bleedthrough contamination correction in the FRET image. The detector spectral sensitivity correction for the donor and acceptor channels and their quantum yields are also included to estimate the efficiency and the distance. Moreover, this book has 16 chapters in total covering various FRET microscopy and spectroscopy basics and methodology of preparation of FRET assays for intensity-based FRET and lifetime FRET imaging techniques with interesting biological applications.

28. Kenworthy AK: **Photobleaching FRET microscopy**. *In Molecular Imaging: FRET Microscopy and Spectroscopy*. Edited by A Periasamy and R Day. New York: Oxford University Press. Chapter 8: in press.

29. Nashmi R, Dickinson ME, McKinney S, Jareb M, Labarca C, Fraser SE, Lester HA: **Assembly of α4β2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons**. *J Neurosci* 2003, **23**:11554-11567.

30. Chen Y, Periasamy A: **Time-correlated single photon counting (TCSPC) FLIM-FRET microscopy for protein localization**. In *Molecular Imaging: FRET Microscopy and Spectroscopy*. Edited by A Periasamy and RN Day. New York: Oxford University Press. Chapter 13: in press.

31. •• Hohng S, Joo C, Ha T: **Single-molecule three-color FRET**. *Biophys J* 2004, **87**:1328-1337.
FRET has been demonstrated for two fluorophore models and this paper describes an estimation of more than one distance between donor and acceptor molecule using three spectrally distinct fluorophores for FRET.

32. Watrob HM, Pan CP, Barkley MD: **Two-step FRET as a structural tool**. *J Am Chem Soc* 2003, **125**:7336-7343.

33. Larson DR, Ma YM, Vogt VM, Webb WW: **Direct measurement of Gag-Gag interaction during retrovirus assembly with FRET and fluorescence correlation spectroscopy**. *J Cell Biol* 2003, **162**:1233-1244.

34. Hiller DA, Fogg JM, Martin AM, Beechem JM, Reich NO, Perona JJ: **Simultaneous DNA binding and bending by EcoRV endonuclease observed by real-time fluorescence**. *Biochemistry* 2003, **42**:14375-14385.

35. Shchyolkina AK, Kaluzhny DN, Borisova OF, Hawkins ME, Jernigan RL, Jovin TM, Arndt-Jovin DJ, Zhurkin VB: **Formation of an intramolecular triple-stranded DNA structure monitored by fluorescence of 2-aminopurine or 6-methylisoxanthopterin**. *Nucleic Acids Res* 2004, **32**:432-440.

36. Riven I, Kalmanzon E, Segev L, Reuveny E: **Conformational rearrangements associated with the gating of the G protein-coupled potassium channel revealed by FRET microscopy**. *Neuron* 2003, **38**:225-235.

37. Biskup C, Kelbauskas L, Zimmer T, Benndorf K, Bergmann A, Becker W, Ruppersberg JP, Stockklausner C, Klocker N: **Interaction of PSD-95 with potassium channels visualized by fluorescence lifetime-based resonance energy transfer imaging**. *J Biomed Opt* 2004, **9**:753-759.

38. Becker W, Bergmann A, Hink MA, Konig K, Benndorf K, Biskup C: **Fluorescence lifetime imaging by time-correlated single-photon counting**. *Microsc Res Tech* 2004, **63**:58-66.

39. Herman B, Krishnan RV, Centonze VE: **Microscopic analysis of fluorescence resonance energy transfer (FRET)**. *Methods Mol Biol* 2004, **261**:351-370.

40. Zhang J, Campbell RE, Ting AY, Tsien RY: **Creating new fluorescent probes for cell biology**. *Nat Rev Mol Cell Biol* 2002, **3**:906-918.

41. Chan FK, Siegel RM, Zacharias D, Swofford R, Holmes KL, Tsien RY, Lenardo MJ: **Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein**. *Cytometry* 2001, **44**:361-368.

42. Lippincott-Schwartz J, Patterson GH: **Development and use of fluorescent protein markers in living cells**. *Science* 2003, **300**:87-91.

43. Miyawaki A, Sawano A, Kogure T: **Lighting up cells: labelling proteins with fluorophores**. *Nat Cell Biol* 2003, **Suppl**:S1-S7.

44. Rizzo MA, Springer GH, Granada B, Piston DW: **An improved cyan fluorescent protein variant useful for FRET**. *Nat Biotechnol* 2004, **22**:445-449.

45. Panyi G, Bagdany M, Bodnar A, Vamosi G, Szentesi G, Jenei A, Matyus L, Varga S, Waldmann TA, Gaspar R, Damjanovich S: **Colocalization and nonrandom distribution of Kv1.3 potassium channels and CD3 molecules in the plasma membrane of human T lymphocytes**. *Proc Natl Acad Sci USA* 2003, **100**:2592-2597.

46. Seidel T, Kluge C, Hanitzsch M, Ross J, Sauer M, Dietz KJ, Golldack D: **Colocalization and FRET-analysis of subunits c and a of the vacuolar H^+-ATPase in living plant cells**. *J Biotechnol* 2004, **112**:165-175.

47. Butkevich E, Hulsmann S, Wenzel D, Shirao T, Duden R, Majoul I: **Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton**. *Curr Biol* 2004, **14**:650-658.

48. Oliveria SF, Gomez LL, Dell'Acqua ML: **Imaging kinase–AKAP79–phosphatase scaffold complexes at the plasma membrane in living cells using FRET microscopy**. *J Cell Biol* 2003, **160**:101-112.

49. Klostermeier D, Sears P, Wong CH, Millar DP, Williamson JR: **A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly**. *Nucleic Acids Res* 2004, **32**:2707-2715.

50. Zheng L, Hoeflich KP, Elsby LM, Ghosh M, Roberts SG, Ikura M: **FRET evidence for a conformational change in TFIIB upon TBP-DNA binding**. *Eur J Biochem* 2004, **271**:792-800.

51. Bunemann M, Frank M, Lohse MJ: **Gi protein activation in intact cells involves subunit rearrangement rather than dissociation**. *Proc Natl Acad Sci USA* 2003, **100**:16077-16082.

52. Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, Rao M, Mayor S: **Nanoscale organization of multiple GPI-anchored proteins in living cell membranes**. *Cell* 2004, **116**:577-589.

53. Pollitt SK, Pallos J, Shao J, Desai UA, Ma AA, Thompson LM, Marsh JL, Diamond MI: **A rapid cellular FRET assay of polyglutamine aggregation identifies a novel inhibitor**. *Neuron* 2003, **40**:685-694.

54. Chin J, Adams AD, Bouffard A, Green A, Lacson RG, Smith T, Fischer PA, Menke JG, Sparrow CP, Mitnaul LJ: **Miniaturization of cell-based β-lactamase-dependent FRET assays to ultra-high throughput formats to identify agonists of human liver X receptors**. *Assay Drug Dev Technol* 2003, **1**:777-787.

55. Nagai T, Miyawaki A: **A high-throughput method for development of FRET-based indicators for proteolysis**. *Biochem Biophys Res Commun* 2004, **319**:72-77.

56. Willard FS, Kimple RJ, Kimple AJ, Johnston CA, Siderovski DP: **Fluorescence-based assays for RGS box function**. *Methods Enzymol* 2004, **389**:56-71.

57. Fernandes F, Loura LM, Koehorst R, Spruijt RB, Hemminga MA, Fedorov A, Prieto M: **Quantification of protein-lipid selectivity using FRET: application to the M13 major coat protein**. *Biophys J* 2004, **87**:344-352.

58. Lear JD, Stouffer A, Gratkowski H, Nanda V, DeGrado WF: **Association of a model transmembrane peptide containing Gly in a heptad sequence motif**. *Biophys J* 2004, **87**:3421-3429.

59. • Ottolia M, Philipson KD, John S: **Conformational changes of the Ca^{2+} regulatory site of the Na^+-Ca^{2+} exchanger detected by FRET**. *Biophys J* 2004, **87**:899-906.

The authors use FRET as a tool to demonstrate that during excitation-contraction coupling the binding of regulatory Ca^{2+} in neonatal cardiac myocytes could activate the Na^+-Ca^{2+} exchanger.

60. Wallrabe H, Stanley M, Periasamy A, Barroso M: **One- and two-photon fluorescence resonance energy transfer microscopy to establish a clustered distribution of receptor-ligand complexes in endocytic membranes**. *J Biomed Opt* 2003, **8**:339-346.

61. Mueller B, Karim CB, Negrashov IV, Kutchai H, Thomas DD: **Direct detection of phospholamban and sarcoplasmic reticulum Ca-ATPase interaction in membranes using fluorescence resonance energy transfer**. *Biochemistry* 2004, **43**:8754-8765.

62. Suzuki G, Izumi S, Hakoda M, Takahashi N: **LTA 252G allele containing haplotype block is associated with high serum C-reactive protein levels**. *Atherosclerosis* 2004, **176**:91-94.

63. Chen B, Squier TC, Bigelow DJ: **Calcium activation of the Ca-ATPase enhances conformational heterogeneity between nucleotide binding and phosphorylation domains**. *Biochemistry* 2004, **43**:4366-4374.

64. Gustiananda M, Liggins JR, Cummins PL, Gready JE: **Conformation of prion protein repeat peptides probed by FRET measurements and molecular dynamics simulations**. *Biophys J* 2004, **86**:2467-2483.

65. Daelemans D, Costes SV, Cho EH, Erwin-Cohen RA, Lockett S, Pavlakis GN: ***In vivo* HIV-1 Rev multimerization in the nucleolus and cytoplasm by fluorescence resonance energy transfer**. *J Biol Chem* 2004, **279**:50167-50175.