The use of FRET imaging microscopy to detect protein–protein interactions and protein conformational changes *in vivo*

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Intermolecular and intramolecular FRET between two spectrally overlapping green fluorescent protein variants fused to two different host proteins or at two different sites within the same protein offers a unique opportunity to monitor real-time protein–protein interactions or protein conformational changes. By using fluorescence digital imaging microscopy, one can visualize the location of green fluorescent proteins within a living cell and follow the time course of the changes in FRET corresponding to cellular events at a millisecond time resolution. The observation of such dynamic molecular events *in vivo* provides vital insight into the action of biological molecules.

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Abbreviations

BFP	blue fluorescent protein
BRET	bioluminescence resonance energy transfer
CaM	calmodulin
CFP	cyan fluorescent protein
FLIM	fluorescence lifetime imaging microscopy
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
RFP	red fluorescent protein
YFP	yellow fluorescent protein

Introduction

In 1948, Förster formulated the principle of FRET [1,2], a phenomenon that occurs when two different chromophores (donor and acceptor) with overlapping emission/absorption spectra are separated by a suitable orientation and a distance in the range 10–80 Å (Figure 1). In the early 1970s, after a long period of silence, groundbreaking work on FRET revealed the spatial proximity relationships of two fluorescence-labeled sites in biological macromolecules, thereby establishing the use of FRET as a spectroscopic ruler [3]. All of this early work used either fluorescent analogs of biomolecules or fluorescent reagents covalently or noncovalently attached to macromolecules as donors or acceptors of FRET [4].

Over the past decade, the use of FRET for structure elucidation became less significant, as atomic-resolution structural information on biological macromolecules was more effectively determined by X-ray crystallography or NMR spectroscopy. Recently, however, the introduction of the green fluorescent protein (GFP) to FRET-based imaging microscopy gave new life to its use as a sensitive probe of protein–protein interactions and protein conformational changes *in vivo*. This was the beginning of real-time *in vivo* imaging of dynamic molecular events, providing researchers with crucial insight into the biological mechanisms as well as the physiological functions of a cell [5–8].

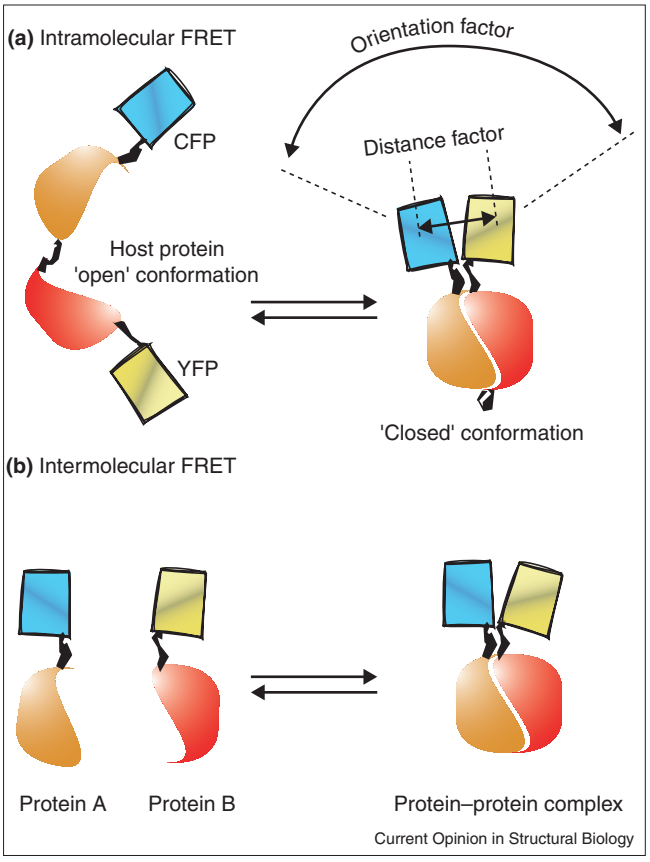
FRET meets GFP

GFP has a number of amazing properties that enable its use for *in vivo* imaging. Firstly, GFP can be expressed in a variety of cells, where it becomes spontaneously fluorescent without the aid of a cofactor [9]. Secondly, GFP can be fused to a host protein to create a fusion protein that usually retains both the fluorescence of the GFP and the biochemical function of the original host. Thirdly, fusion proteins can be targeted to specific organelles, such as the nucleus or endoplasmic reticulum, by adding an appropriate signaling peptide. Finally, and most importantly, mutagenesis of GFP has produced many mutants with varying spectral properties that can be used as donors and acceptors of FRET [9–15].

One pair of fluorescent proteins originally used for FRET was a BFP (blue fluorescent protein) donor and a GFP acceptor. The BFP donor is a GFP mutant with a Tyr66His mutation that creates excitation and emission peaks around 383 and 447 nm, respectively [10,16]. The most commonly used GFP acceptor is also a GFP mutant — with a Ser65Thr mutation that eliminates an undesirable excitation peak of wild-type GFP at 395 nm and creates single excitation and emission peaks at around 488 and 509 nm, respectively [10–13]. However, BFP has the lowest quantum yield and is the most bleachable of all GFP mutants [10,15] and, as its excitation peak is in the ultraviolet (UV) range, cellular autofluorescence and scattering are a significant source of noise with the BFP–GFP pair [17,18].

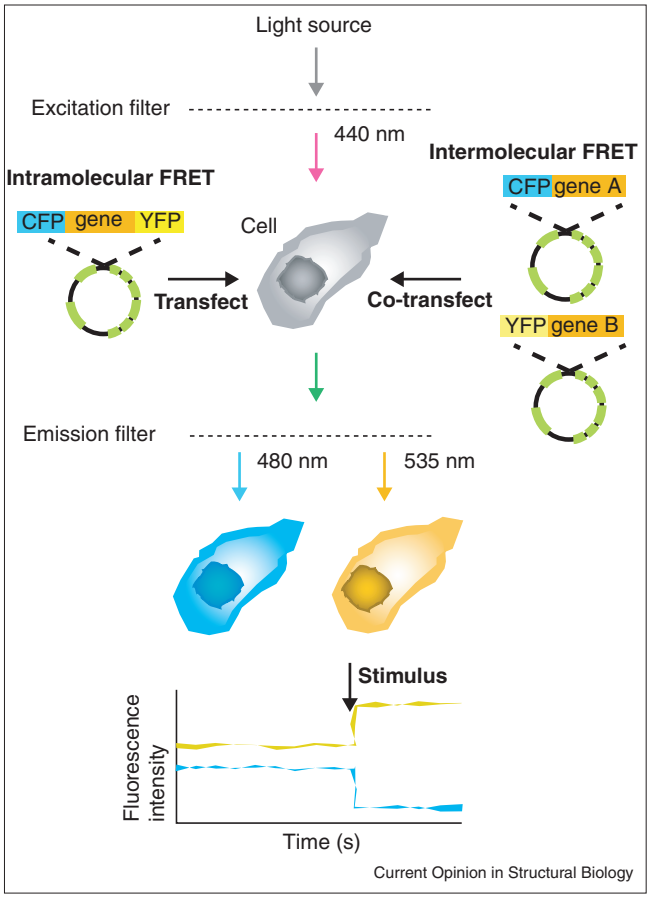
To address the above-mentioned problems, GFP mutants with longer wavelengths are now often used as FRET partners: CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein). CFP contains a Tyr66Typ mutation [10] that creates excitation and emission peaks of 436 and 476 nm, respectively. YFP was rationally designed from the GFP crystal structure [19] — it was suggested that a Thr203Tyr mutation would create a π–π stacking interaction between Tyr203 and Tyr66, thereby red-shifting the spectra and creating excitation and emission peaks of 516 and 529 nm, respectively [14]. Additionally, the CFP–YFP pair allows FRET to be detected at larger distances, as the calculated R_0 (the distance at which 50% energy transfer occurs) between randomly oriented chromophores is 5.2 nm, whereas for BFP–GFP, it is only 4 nm. [17]. More

Figure 1



Intramolecular and intermolecular FRET. **(a)** Intramolecular FRET can occur when both the donor and acceptor chromophores are on the same host molecule, which undergoes a transition, for example, between 'open' and 'closed' conformations. In each square box corresponding to CFP or YFP (shown in cyan or yellow, respectively), a diagonal line represents the chromophore. The amount of FRET transferred strongly depends on the relative orientation and distance between the donor and acceptor chromophores: the parallel orientation and the shorter distance (<100 Å) generally yield larger FRET. **(b)** Intermolecular FRET can occur between one molecule (protein A) fused to the donor (CFP) and another molecule (protein B) fused to the acceptor (YFP). When the two proteins bind to each other, FRET occurs. When they dissociate, FRET diminishes.

Figure 2



FRET imaging microscopy experiment. In FRET experiments, a single transfection (intramolecular FRET) or co-transfection (intermolecular FRET) of the constructs must first be performed. The occurrence of FRET can be observed by exciting the sample at the donor excitation wavelengths while measuring the fluorescence intensities emitted at wavelengths corresponding to the emission peaks of the donor versus those of the acceptor. If the acceptor and donor are at a favorable distance and orientation, donor emission intensity decreases (CFP, cyan) while the acceptor emission (YFP, yellow) intensity increases.

recently, novel YFPs and red fluorescent proteins (RFPs) have been cloned from corals [20], producing yet another FRET pair, GFP–RFP [21].

Intramolecular FRET to monitor protease cleavage, calcium signaling and phosphorylation

Intramolecular FRET can be measured when both the GFP donor and the acceptor are fused to the same host molecule (Figures 1a and 2). One of the first demonstrations of this technique was performed by Mitra *et al.* [22], who fused a BFP and GFP in the same molecule, separated by a flexible polypeptide linker containing a Factor X_a protease cleavage site. When incubated with Factor X_a, cleavage of the linker was followed by a decrease in FRET. Subsequently, Heim and Tsien [16] demonstrated a similar phenomenon with a 25-residue linker containing a trypsin cleavage site. The first biologically significant example occurred when a linker containing the caspase-3 cleavage site (DEVD) was fused between a BFP–GFP or a CFP–YFP pair [23,24]. When the HeLa cells were irradiated with UV light to induce apoptosis, a fivefold change in the fluorescence emission ratio was observed. This construct was then used to screen novel apoptosis-inducing agents [25,26].

Romoser *et al.* demonstrated the use of intramolecular FRET to measure Ca^{2+}-calmodulin (CaM) signaling by fusing the CaM-binding domain [27,28] from smooth muscle myosin light chain kinase (M13) [29] between BFP and GFP [30,31]. In the Ca^{2+}-saturated medium, CaM binds Ca^{2+} and wraps around the M13 peptide, causing the GFP mutants to move further apart and resulting in a decrease in FRET. This hybrid protein returns to its resting state when the Ca^{2+} level drops, thereby illustrating

that changes in FRET can report the dependency of Ca^{2+}-CaM activity on intracellular Ca^{2+} concentration.

In the same year, Miyawaki *et al.* [14] independently created novel Ca^{2+} indicators named cameleons, which consist of a tandem fusion of CFP, CaM, M13 peptide and YFP. Upon an increase in Ca^{2+} concentration, the CaM component of the hybrid protein binds Ca^{2+} and preferentially wraps around the fused M13 peptide. This conformational change results in a decrease in the distance between the two GFP mutants and, therefore, an increase in FRET [14,21,32,33•]. As cameleons include CaM within the engineered molecule, they are much less inert to endogenous CaM and therefore monitor Ca^{2+} changes more specifically. More recently, we have rationally designed a new class of cameleons that offer a larger dynamic range in physiologically significant levels of Ca^{2+} concentrations in the cytosol [34•]. Other GFP-based Ca^{2+} indicators have used a single circularly permuted GFP [35] fused with CaM and an M13 peptide [36,37].

In addition to their involvement in investigating protease cleavage and Ca^{2+} signaling, fluorescent indicators were designed using intramolecular FRET to examine the phosphorylation of the transcription factor cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) [38]. This construct consisted of the kinase-inducible domain (KID) of CREB flanked by GFP and BFP. The phosphorylation of a specific serine residue in the KID domain by cAMP-dependent protein kinase A (PKA) [39,40] induced a FRET-detectable conformational change, which can be used as an *in vivo* screening system for kinases. Another fluorescent indicator was created to study the phosphorylation of the adaptor protein CrkII, which was sandwiched between CFP and YFP. CrkII is involved in signal transduction of the tyrosine kinase receptors when phosphorylated on its Tyr221 residue [41]. This phosphorylation induces an intramolecular interaction between the SH2 domain and Tyr221 within CrkII, which brings CFP and YFP closer and, therefore, allows FRET to occur [42]. Most recently, using a similar fusion construct, Mochizuki *et al.* [43•] were able to monitor the cellular signaling of G proteins of the Ras family, which cycle between GDP-bound (inactive) and GTP-bound (active) forms.

Intermolecular FRET to visualize protein–protein interactions

Intermolecular FRET can occur when the GFP donor and the acceptor are on different macromolecules (Figures 1b and 2); however, this form of FRET is more difficult to observe because the stoichiometry of acceptors to donors can vary with transfection efficiencies, and also the donor and acceptor host proteins may not be constitutively bound *in vivo*. Optimal conditions occur when all the donors are paired with an acceptor, as any unpaired protein adds noise to the signal. Additionally, if the distance or orientation between the pairs are unfavorable, FRET may not occur, even if the two proteins form a complex [33•]. A successful FRET system yields information about the location and exact timing of the interaction that is not available from conventional detection systems such as co-immunoprecitation, cross-linking, yeast two-hybrid, phage display or unlinked noncomplementing mutant detection [44].

Despite the aforementioned experimental requirements, a number of excellent applications have been reported. An early example of GFP-based intermolecular FRET occurred with Bcl-2 and Bax, which play a crucial role in apoptosis, as well as in the initiation and progression of human cancer [45–47]. A direct interaction in the mitochondria between Bcl-2 and Bax was shown when a GFP fused to the N terminus of Bcl-2 and a BFP fused to the N terminus of Bax were co-expressed in the same cell [48]. Similarly, biochemical studies were done to reveal the formation of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes that are involved in synaptic vesicle formation [49,50]. When the N termini of the two core proteins in the SNARE complex, SNAP-25 and synaptobrevin, were fused to CFP and YFP, respectively, FRET was observed [51]. Both these examples used a mathematical correction method [52] to deduce the stoichiometry of acceptors to donors for quantifying FRET efficiency.

Llopis *et al.* [53] demonstrated ligand-dependent FRET in HeLa cells between CFP tagged to the retinoic acid receptor (RAR) and YFP tagged to the nuclear receptor interaction domains of the coactivators steroid receptor coactivator-1 (SRC-1) and peroxisome proliferator-activated receptor binding protein (PBP). The RAR nuclear receptor activates transcription by binding to a coactivator (SRC-1 or PBP), which can be induced by hormone stimulation [54–56]. FRET was proved convincingly by following a time course of CFP and YFP emission intensities, during which CFP emission was significantly quenched by YFP after stimulation. Subsequently, acceptor bleaching experiments further demonstrated FRET, whereby the donor emission (CFP) increased upon acceptor photobleaching (YFP) [33•]. In a similar way, van der Wal *et al.* [57] showed phosphatidylinositol bisphosphate metabolism on the plasma membrane in response to an external stimulus by tagging CFP and YFP to pleckstrin homology domains of phospholipase C (PLC). Most FRET fusion reported in the literature occurs at either the N or C terminus; however, Janetopoulos *et al.* [58•] showed that it is possible to fuse CFP and YFP mutants to a loop region of the α subunit and β subunit of G-protein heterotrimers, respectively. This construction allowed the observation of G-protein-mediated cell signaling, during which the G-protein heterotrimer dissociates and associates upon the addition and removal of stimulants such as chemoattractants, hormones and neurotransmitters [59].

A sophisticated method of quantifying FRET is fluorescence lifetime imaging microscopy (FLIM), in which the lifetimes

of the donor alone (τ_D) and also in the presence of the acceptor (τ_{DA}) are measured (as opposed to measuring emission intensity) [8,60]. If FRET occurs, τ_{DA} will be different from τ_D and this difference can be used to calculate FRET efficiency. The major advantage of FLIM is that it permits an internally calibrated measurement of FRET. Also, as only donor emission is monitored, factors that affect the quantum yield of the acceptor can be disregarded. Verveer *et al.* [61••] used the FRET between a GFP fused to the C terminus of ErbB1 (ErbB1–GFP) and a Cy3-labeled antibody to the phosphotyrosine (pTyr72) of ErbB1 to determine the characteristic lifetime of the phosphorylated and unphosphorylated forms of ErbB1–GFP. ErbB1 is a tyrosine kinase receptor that is thought to be activated by a ligand-induced dimerization on the plasma membrane [62]. However, these studies revealed a ligand-independent mechanism for the activation of ErbB1. Additionally, they demonstrated the flexibility in designing such experiments, as FRET can occur between combinations of GFPs and synthetic dyes such as Cy3 and Cy5.

Finally, another technology for monitoring protein–protein interaction is bioluminescence resonance energy transfer (BRET), which relies on the same principles as FRET, except that the donor is a bioluminescent macromolecule (*Renilla* luciferase) that acquires luminescence when activated by a cofactor [63]. Xu *et al.* successfully applied the BRET method to assay the interaction between proteins encoded by the circadian clock genes kaiA and kaiB from a strain of cyanobacterium *Synechococcus* sp. [64,65]. Subsequently, Angers *et al.* [66] demonstrated that BRET could be used as a tool to study constitutive protein–protein interactions *in vivo* between a β_2 adrenergic receptor (β_2AR) fused to *Renilla* luciferase and another fused to YFP. β_2AR is a G-protein-coupled receptor that forms a constitutive dimer; however, it displays an increase in BRET upon receptor stimulation by isoproterenol, a hydrophilic agonist. One disadvantage of BRET is that *Renilla* luciferase generates a broad emission peak that substantially overlaps with the YFP emission and, therefore, contributes to a low signal to noise ratio for the system [63].

Conclusions and future directions

Fluorescent imaging technology now offers numerous benefits to the expanding field of structural biology. High-resolution techniques such as X-ray crystallography and NMR spectroscopy determine three-dimensional structures of biological molecules, whereas fluorescence imaging technology, along with other imaging methods, unveils both temporal and spatial information on molecular structures in living cells. A combination of new and existing techniques provides a more comprehensive picture of studied biological molecules from the cellular level (approximately micrometers) to the atomic level (approximately angstroms). High-resolution structural information can also help molecular cell biologists in the successful design of fluorescently active constructs used in *in vivo* imaging, as exemplified in CaM/GFP-based Ca^{2+} indicator studies [14,34•]. The knowledge of several high-resolution structures of CaM in complex with target peptides has provided vital information about the approximate distance between fused FRET partners.

FRET imaging microscopy has been proven to be an extremely useful tool in the detection of protein–protein interactions and protein conformational changes in a single cell. Future directions of FRET imaging include biologically interesting applications involving cellular events coupled to specific molecular signaling processes; imaging in thick tissues or organisms using multiphoton excitation microscopy [67,68]; and detection of single-molecule FRET using evanescent wave microscopy [69].

More specifically, future biological applications of FRET imaging microscopy may include the simultaneous observation of a series of reversible molecular processes in living cells. We may, for example, soon find it possible to monitor a signaling cascade using different FRET markers: a tyrosine receptor–ligand interaction; conformation-dependent activation and autophosphorylation of the receptor in the cytoplasmic domain; docking of a signaling protein to the phosphorylated receptor; assembly of a cytosolic protein signaling module and activation of intracellular effectors; and activation of the transcription of certain gene(s). In addition, two-photon excitation microscopy has been successfully used to detect the three-dimensional localization of fluorescent proteins exhibiting FRET in different tissues of various organisms [70]. Also, the total reflection method has been employed to detect EGFR signaling on the surface of living cells [71•]. The future of FRET imaging microscopy is bright, as microscope technology advances and possible applications of GFP-based FRET become infinite.

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