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Use of Chimeric Fluorescent Proteins and Fluorescence Resonance Energy Transfer to Monitor Cellular Responses

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Abstract—In recent years, the development of new technologies based on the green fluorescent protein and fluorescence resonance energy transfer has introduced a new perspective in the study of cell biology. Real-time imaging of fluorescent biosensors has made it possible to directly visualize individual molecular events as they happen in intact, live cells, providing important and original insights for our understanding of biologically relevant problems. This review discusses some essential methodological aspects concerning the generation and use of fluorescence resonance energy transfer-based biosensors and presents selected examples of specific applications that highlight the power of this technology. (*Circ Res.* 2004;94:866-873.)

Key Words: green fluorescent protein ■ fluorescence resonance energy transfer ■ cell signaling
■ microscopy ■ imaging

Cells are complex systems in which a multitude of biochemical reactions and molecular events take place at one time and need to be finely orchestrated to preserve the cell homeostasis and direct the cell-specific functions. In particular, the flow of information from many different cellular inputs to a diverse set of physiological functions must rely on a precise organization of the intracellular signaling networks and on their timely and coordinated activation. The study of signal transduction normally takes a reductionist approach by using model systems in which the number of variables that come into play is reduced to the minimum. This is often the only way to disentangle the complexity of the cellular environment; on the other hand, such model systems are bound to generate an oversimplified view of reality. For example, biochemical approaches provide precious information on what types of proteins are involved in a given pathway, their level of expression, and the way they interact

with each other but rarely can resolve where and when such proteins are activated within a cell. In recent years, however, thanks to the development of new technologies based on real-time imaging of fluorescent indicators, the direct visualization of individual molecular events taking place in intact cells is becoming possible. The high spatial and temporal resolution that such methodologies provide opens the possibility to accurately measure quantitative and dynamic parameters of signaling networks in their complex cellular environment.

A strong contribution to the development of bioimaging techniques has come from the molecular cloning¹ and subsequent engineering² of green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria*. GFP has several qualities that make it ideal for *in vivo* imaging. First, GFP can be expressed in a variety of cells, where it becomes spontaneously fluorescent without the need for cofactors.

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Second, because it is a protein, GFP can be tagged with an appropriate signaling peptide and expressed as such or fused to another protein in specific organelles, such as the mitochondria, the nucleus, or the endoplasmic reticulum.³ Finally, mutagenesis of GFP has generated many mutants with varying spectral properties,⁴ thus allowing imaging of several different fluorescent proteins simultaneously. Thanks to these properties, GFP has been successfully used for many years as a marker for studying gene expression as well as protein folding, trafficking, and localization. Recently, however, more sophisticated applications have been made possible by molecular engineering of GFP and the generation of active sensors capable of monitoring complex processes, such as intracellular second messenger dynamics, enzyme activation, and protein-protein interactions.^{4,5} This review will focus on GFP-based indicators that exploit fluorescence resonance energy transfer (FRET) to detect protein-protein interaction and conformational change, thus allowing direct visualization of signaling cascades. Different techniques for revealing FRET will be briefly described, and a selected number of applications will be discussed to illustrate the unique contribution that this approach can provide in the study of biologically relevant problems.

FRET-Based Indicators

FRET relies on a nonradiative, distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore. For FRET to occur, the donor-acceptor distance must be between 2 and 6 nm, the 2 fluorophores must be appropriately oriented in space, and there must be a substantial overlap (>30%) between the donor's emission spectrum and the acceptor's excitation spectrum.^{6,7} In FRET, the donor is excited by incident light, and, if an acceptor is in close proximity, the excited state energy from the donor can be transferred. This leads to a reduction in the donor's fluorescence intensity and excited lifetime and an increase in the acceptor's emission intensity. The efficiency of this process (E) depends on the inverse sixth distance between donor and acceptor, as described by the Förster's equation: $E = 1/[1 + (R/R_0)^6]$,⁸ where R_0 is the distance at which half of the energy is transferred. According to the Förster's equation, the doubling of the distance between the 2 fluorophores, for example from R_0 to $2R_0$, will decrease the efficiency of transfer from $E = 50\%$ to $E = 1.5\%$. Therefore, FRET provides a very sensitive measure of intermolecular distances and of conformational changes.

The generation of GFP mutants with distinct excitation and emission spectra,² as well as the molecular cloning of new fluorescent proteins from coelenterate marine organisms,^{9,10} has provided several fluorophores that can serve as FRET donor/acceptor pairs. Originally, the green and blue mutants, GFP and BFP, have been used for FRET applications.¹¹ However, BFP is the least bright and the most prone to photobleaching (the irreversible destruction of the fluorophore on illumination) of all GFP mutants. Furthermore, it requires excitation in the ultraviolet range with consequent generation of cell autofluorescence and scattering, thus significantly increasing the background noise. At present, the best pair for FRET consists of the

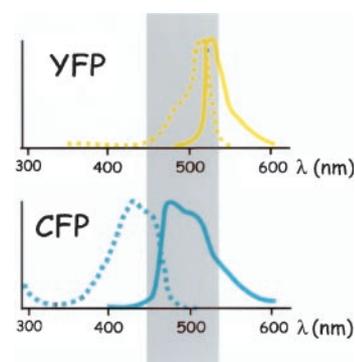


Figure 1. Excitation (dotted line) and emission (continuous line) spectra of YFP and CFP. Gray box highlights the overlap between CFP emission and YFP excitation spectra.

cyan and yellow mutants, CFP and YFP (see Figure 1). CFP is much brighter and more photostable than BFP.¹² The first YFP mutants showed a marked sensitivity to H^+ and Cl^- ions. These properties, although successfully exploited for directly measuring intracellular pH^{13,14} and Cl^- concentration,¹⁵ represent a serious source of artifacts in some FRET applications. Therefore, YFP has been additionally engineered to generate a new variant (citrine) that overcomes these problems and furthermore shows greater photostability.¹⁶ Another mutant of YFP is Venus, a very bright and fast-maturing variant.¹⁷ A lot of effort is being placed on the search for more red-shifted fluorescent proteins (RFPs) to be used as FRET acceptors in combination with a GFP donor.¹⁸ RFPs would provide greater tissue penetration and minimize tissue autofluorescence background; however, additional improvement of the existing proteins will be necessary for their useful application in FRET experiments.¹⁹ The major limitation of the original dsRed⁹ is that it forms tetramers and therefore can tetramerize any cellular protein to which it is fused.²⁰ This can lead to large aggregation of the fusions or, if the cell protein is resistant to tetramerization, to lack of fluorescence (our unpublished observation, 2004). By a combination of site-directed and random mutagenesis, a monomeric variant of this RFP has been generated (mRFP-1)¹⁸ in which most of the problems of dsRed have been overcome. However, mRFP-1 performance as a FRET acceptor remains seriously hampered by the very long tail of its excitation spectrum on the short wavelength side, leading to direct excitation of the acceptor when exciting the donor (see below).

GFP-based FRET indicators follow 2 basic designs (Figure 2): unimolecular indicators, in which 2 protein-interacting domains are sandwiched between CFP and YFP, and bimolecular indicators, in which the fluorophores are fused to 2 independent domains whose interaction depends on ligand binding or a conformational change of 1 of the domains. In general, unimolecular sensors may be preferable because a single, unimolecular probe is less likely to interact with bystander partners. Such interaction may interfere with endogenous reactions and thus affect cell physiology and reduce the probe sensitivity. Unimolecular constructs have the additional advantage of containing equimolar amounts of

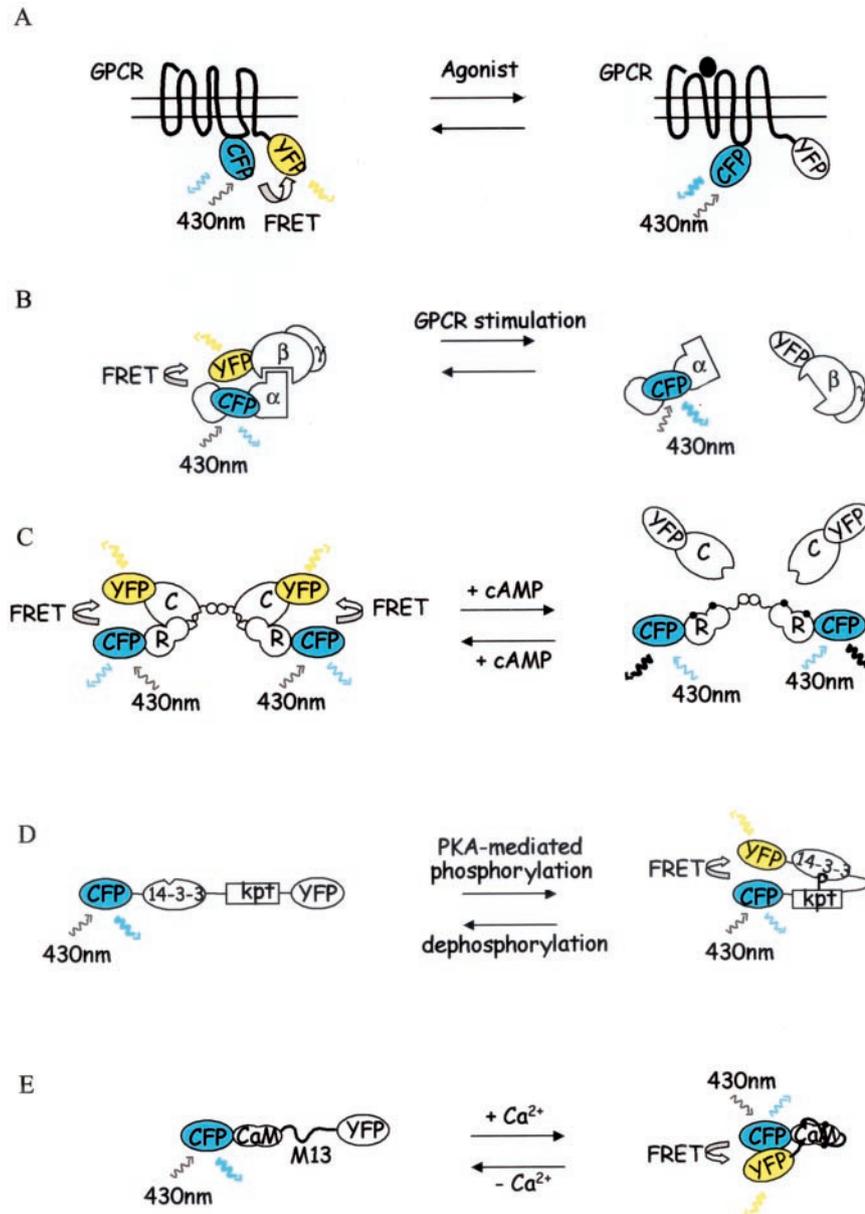


Figure 2. Schematic representation of the design of selected probes that exploit dynamic FRET and can reversibly measure signaling events in live cells. **A**, Insertion of CFP in the third intracellular loop of a G protein-coupled receptor (GPCR) and fusion of YFP to its carboxy-terminus yields a reversible sensor of GPCR activation.⁶⁷ **B**, A sensor for G protein activation was generated by fusing YFP to the amino-terminus of the G β subunit and by inserting CFP into the helical domain of G α_2 .⁶⁸ **C**, Fusion of CFP and YFP at the carboxy termini of the regulatory (R) and catalytic (C) subunits of PKA, respectively, gave a sensor for intracellular fluctuations of cAMP.⁴⁵ **D**, Sequential fusion of CFP, the phosphoserine binding protein 14-3-3, the PKA phosphorylation target sequence kemptide (kpt), and YFP provided a sensor for PKA-mediated phosphorylation.⁵³ **E**, Tandem fusion of CFP, CaM, the CaM-binding domain from smooth muscle myosin light chain kinase (M13), and YFP generated a sensor for intracellular free Ca²⁺.¹¹ Arrows indicate CFP excitation (peak at 430 nm) and emission of CFP (peak at 480 nm) and YFP (peak at 545 nm) emission.

donor and acceptor fluorophores, therefore allowing maximal exploitation of the dynamic range of the FRET changes and facilitating quantitation. However, several bimolecular FRET-based probes have been generated and used successfully.

In some applications, either the donor or the acceptor fluorophores have been linked to lipids. In this way, FRET measurements have been used to detect either protein interactions with phospholipid bilayers²¹ or protein interactions with the plasma membrane.²²

FRET Imaging Techniques

Several FRET microscopy techniques are available, each with its own advantages and disadvantages that need to be carefully considered, although the choice of a given method often is the result of a compromise between the requirements dictated by the specific biological application and the access to the appropriate instrumentation. Wide-field microscopy is

the simplest and most widely used technique. FRET is typically measured as the ratio of acceptor emission to donor emission on excitation of the donor, giving a value that is proportional to the degree of physical association between the 2 fluorophores. One of the major drawbacks of wide-field microscopy is the generation of out-of-focus signal. This can be a serious problem, especially in cases in which relatively thick samples are inspected and when the goal of the experiment is to study molecular events that take place in restricted volumes within the cell. Laser scanning confocal microscopy^{23,24} solves this problem and, by collecting serial optical sections from thick specimens, allows resolving FRET signals in 3 dimensions. A major limitation of confocal microscopy is the availability of standard laser lines of defined wavelength that normally do not allow one to resolve FRET. A recent technological advance, however, has introduced multiphoton confocal microscopy that, by using a tunable laser in the 700- to 1000-nm range, allows the

excitation of a wide variety of fluorophores with higher axial resolution, greater sample penetration, limited photobleaching of the chromophore, and reduced damage of the sample.

The intensity-based FRET techniques described above suffer from contamination of the FRET images with unwanted bleedthrough components because of the incomplete separation of the donor and acceptor excitation and emission spectra. When using CFP/YFP, for example, excitation of CFP is associated with partial direct excitation of YFP, which therefore will emit independently of FRET. Even more important is the bleedthrough of CFP emission in the YFP channel, which can contribute to >50% of the FRET image. The degree of crosstalk between fluorophores must be assessed for each individual imaging system, and careful choice of filter sets can minimize bleedthrough. Moreover, once the degree of crosstalk has been measured, it can be accounted for in the offline image-processing phase.^{25,26} Recently, a new algorithm has been developed that removes both the donor and acceptor bleedthrough signals and corrects the variation in fluorophore expression level, generating a true FRET signal.²⁷

In a recent technical advance, by applying a spectral deconvolution approach, it has been possible to excite simultaneously several GFPs and record, pixel-by-pixel, the emission spectrum from each of them through a 32-channel spectrophotometer. By subsequent mathematical modeling, it has been possible to determine the contribution of each fluorophore to each pixel, and separation of the signal of FITC from the nearly identical signal of GFP has been reported.²⁸ Fluorophore crosstalk is a particularly serious problem when looking at steady-state, intermolecular FRET. In this situation, the intracellular molar ratio between donor and acceptor is difficult to control, and different concentrations of the 2 fluorophores may be misinterpreted as FRET. Such a problem is completely overcome if the intermolecular FRET sensor and the experimental set up allow monitoring of dynamic FRET. In this case, it is possible to establish whether a change in donor to acceptor fluorescence is a true change in FRET by monitoring donor and acceptor fluorescence intensity over time; a true FRET change corresponds to a symmetric change of donor and acceptor fluorescence intensity (Figure 3).

Another approach for imaging steady-state FRET consists in collecting the donor emission before and after photobleaching of the acceptor. If FRET is present, elimination of the acceptor by photodestruction releases the energy transferred from donor to acceptor with consequent brighter emission from the donor. This method is very simple and can be used in any laboratory equipped with a simple commercial fluorescence microscope. However, the correct interpretation of the results obtained is not always straightforward, especially if FRET efficiency is low.^{29,30} An alternative method consists of measuring FRET via donor photobleaching.³¹ This technique exploits the fact that photobleaching is proportional to the excited-state lifetime of the fluorophore. Because FRET reduces the lifetime of the donor's excited state, its photobleaching rate decreases proportionally.

Apart from the intensity-based methods described above, more sophisticated technologies for measuring FRET are also available. Fluorescence lifetime imaging microscopy takes

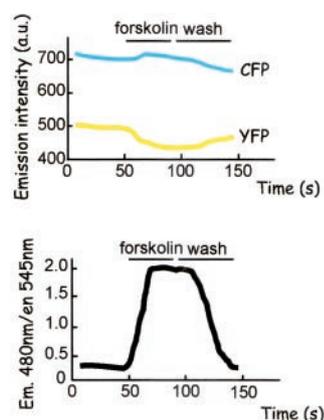


Figure 3. Time course recording of the donor (CFP) and acceptor (YFP) emission intensities and of the CFP/YFP emission ratio. In this case, values represent the average measured in a region of interest drawn in a selected cell transfected with the cAMP sensor shown in Figure 2C and challenged with a cAMP-raising stimulus. As shown, the signal is completely reversible after stimulus washout.

advantage of the fact that FRET results in a shortening of the donor's lifetime; by subtracting the fluorescence lifetime of the donor alone from the lifetime of the donor in the presence of the acceptor, the efficiency of FRET can be measured.^{32,33} Another technique is fluorescence correlation spectroscopy, in which spontaneous fluorescence intensity fluctuations are measured in a microscopic volume and energy transfer efficiency of freely diffusing single molecules can be accurately measured.³⁴

Steady-State FRET

Because FRET occurs when GFPs are at a distance of 6 nm or less of each other, FRET microscopy can resolve GFP-tagged proteins within few nanometers, well below the resolution power of optical microscopes (200 nm). Therefore, steady-state FRET imaging has been used as a powerful tool to detect protein-protein interactions. Compared with the conventional biochemical precipitation techniques, this approach is free from the limitations that derive from the use of detergents to solubilize complexes from cells and poor yield of proteins in immunoprecipitates and furthermore offers the advantage of providing information on the existence and localization of the protein complex in intact, living cells. The difficulties of this approach derive from the possibility of generating chimeric proteins in which the fused GFPs do not interfere with binding of the interacting domains while still maintaining close enough spacing and the appropriate orientation for FRET to occur. Furthermore, as mentioned above, steady-state FRET measurements are not always straightforward to obtain.²⁹ An additional possible complication derives from the spontaneous tendency of GFPs to dimerize, a feature that very much depends on where the GFPs are expressed and to what partners are fused.²⁰ This may not be a problem when measuring interactions between proteins in the cytosol, but when assessing interactions in a potentially crowded, 2D space such as a biological membrane, GFP oligomerization can substantially contribute to or even create the interactions observed, resulting in artifactual FRET.³⁵ In fact, the weak

dimerization tendency of GFPs ($K_d=0.1$ mmol/L in solution³⁵) was shown to generate a concentration-dependent FRET between plasma membrane-targeted GFPs³⁵ and to be sufficient to induce stacking of endoplasmic reticulum membranes that were otherwise incapable of doing so.³⁶ To solve this problem, monomeric GFPs have been generated by mutagenesis of selected amino acids in the hydrophobic patch at the GFP dimer interface.^{35,36}

In one application, steady-state FRET imaging was used to demonstrate a Ca^{2+} -independent preassociation of Ca^{2+} -calmodulin (CaM) to Ca^{2+} channels,³⁷ a mechanism that would ensure selective and fast responsiveness of Ca^{2+} channels to local Ca^{2+} influx. This has been a largely debated issue for its important implications on Ca^{2+} -dependent control of physiological events, such as neurotransmitter release, synaptic plasticity, and heart contraction,³⁸ but previous *in vitro* studies have yielded contradictory results, probably because of the nonphysiological conditions required to extract the channels.

In another application, FRET imaging has been used to study the association in a macromolecular complex of the multiscaffolding A-kinase anchoring protein AKAP-79, protein kinase A (PKA), and the protein phosphatase calcineurin (CaN).³⁹ Such a multiprotein signaling complex is localized to excitatory neuronal synapses, where it is recruited to glutamate receptors by interaction with membrane-associated guanylate kinase scaffold proteins. This mechanism is thought to play an important role in the modulation of synaptic plasticity. The assembly of multiprotein signaling complexes containing receptors, protein kinases, protein phosphatases, and their substrates would provide compartmentalized signal transduction that would ensure optimal control of the specificity, duration, and amplitude of the signal.⁴⁰ From their studies, the authors concluded not only that AKAP79, PKA, CaN, and membrane-associated guanylate kinase are assembled in a macromolecular complex in intact cells but also that, by FRET imaging, CaN and PKA bind to AKAP 79 within 5 nm of each other, thus extending our understanding of the level of molecular compartmentation of signaling proteins that is made possible by scaffolding proteins.³⁹

Dynamic FRET

Local detection of protein-protein interactions with nanometer resolution is only one of the amazing applications of FRET-based biosensors. Indeed, GFP-tagged proteins have been developed that can monitor activation of signaling components or generation of second messengers as the process happens within a living cell, allowing the dynamics of such events to be recorded in real time and space.

The first sensors based on dynamic FRET to be developed were probes for measuring Ca^{2+} -CaM⁴¹ or free Ca^{2+} fluctuations.¹¹ In the latter case, the general design of the sensor consists in the tandem fusion of CFP, CaM, the CaM-binding domain from smooth muscle myosin light chain kinase (M13), and YFP (Figure 2E). After an increase of Ca^{2+} concentration, the CaM component binds Ca^{2+} and preferentially wraps around the fused M13 peptide. This conformational change results in a decrease of the distance between the

2 GFP mutants and, therefore, an increase in FRET. These Ca^{2+} sensors, named cameleons, have been subsequently modified and targeted to specific subcellular compartments and have been used to monitor, for example, Ca^{2+} dynamics that occur locally at the secretory vesicle surface,⁴² in caveolae,⁴³ or in the nucleus,⁴⁴ a result difficult to obtain with conventional Ca^{2+} indicators such as Fura-2.

Probes based on dynamic FRET have been developed also for other soluble intracellular second messengers, such as cAMP⁴⁵ and cyclic GMP (cGMP).⁴⁶ In the case of cyclic nucleotides, the conventional tool available for studying their intracellular fluctuations has been radioimmunoassay on cell lysates with which concentrations can be measured accurately but that provide rather poor time resolution and no information on how the dynamics of these messengers may differ in different compartments of the cell. A sensor for cAMP has been generated by genetically fusing the catalytic (C) subunit of PKA to YFP and the regulatory (R) subunit of PKA to CFP.⁴⁵ When cAMP is low, the GFP-tagged PKA forms a heterotetramer in which CFP and YFP are close enough for FRET to occur. When cAMP levels rise, YFP-C dissociates from CFP-R and FRET disappears (Figure 2C). By using such a sensor, it is possible to demonstrate that cAMP generated via β -adrenergic receptor stimulation does not behave as a freely diffusible second messenger but is compartmentalized (Figure 4).⁴⁷ This hypothesis was formulated more than 20 years ago⁴⁸ and has profound implications for heart physiology and pathophysiology.^{49,50}

Another interesting set of fluorescent indicators have been developed for visualizing protein kinase activity. These reporters include an appropriate phosphorylation substrate peptide or domain linked to a phospho-amino acid-binding domain flanked by CFP and YFP (Figure 2D). Following this general design, indicators for different tyrosine kinases, such as Src, the epidermal growth factor receptor, and the insulin receptor,^{51,52} as well as for serine/threonine kinases such as PKA⁵³ and protein kinase C,⁵⁴ have been developed. In particular, by using this approach, it was possible to reveal that oscillatory protein kinase C-mediated phosphorylation occurs at the plasma membrane that is strictly controlled by Ca^{2+} oscillations and dependent on a tight spatial coupling of kinase and phosphatase activities.⁵⁴ The cycles of phosphorylation/dephosphorylation of the sensor recorded in these experiments show a frequency as high as 1.5 oscillations per minute, a time resolution very difficult to obtain with conventional biochemical assays based on radioactive phosphorus or phosphor-specific antibodies. Furthermore, this approach allows detection of phosphorylation/dephosphorylation events with a very high spatial resolution and can potentially reveal differences in enzyme kinetics at sites that are indistinguishable with the light microscope.

Another example of the power of *in situ* imaging comes from experiments in which the activation of the Rho-family small GTPase Ras, on epidermal growth factor receptor activation, was monitored in COS-1 cells transfected with a sensor made of H-Ras and the Ras binding domain of Raf sandwiched between CFP and YFP.⁵⁵ The imaging data showed that Ras was strongly activated locally and, in particular, at the free edges of the cells and was inhibited in

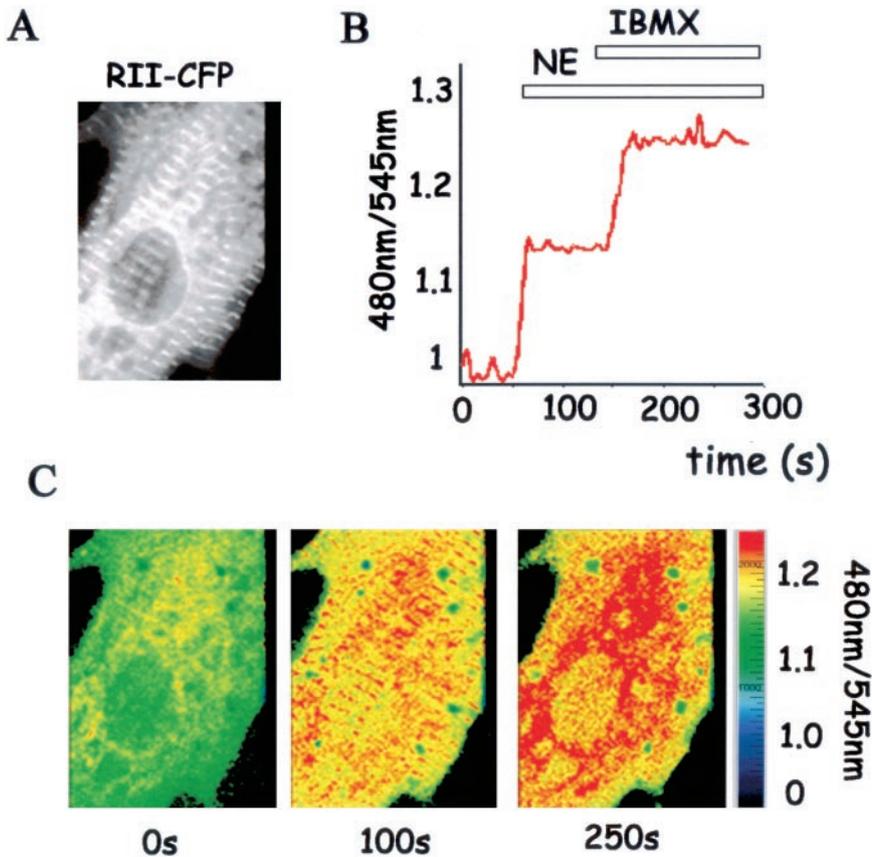


Figure 4. cAMP spatiotemporal dynamics in neonatal cardiac myocytes monitored by FRET video imaging of cells transfected with a GFP-tagged PKA. A, Localization of the RII-CFP component of the FRET-based biosensor for cAMP (see Figure 2C). The striated pattern results from anchoring of the RII subunits of PKA to AKAPs.⁴⁷ B, Kinetics of FRET changes calculated as 480 nm/545 nm emission intensities over time. Values are the average calculated over the entire cell shown in A. C, Pseudocolor ratio images of the same cell at time 0, 100, and 250 seconds showing microcompartments of cAMP generated on β -AR stimulation with norepinephrine (NE). Interestingly, cAMP levels equilibrate over the entire cell on phosphodiesterase inhibition with isobutyl-methyl-xanthine (IBMX).

the regions of cell contact, where membrane ruffling is suppressed. The same authors also showed that activation of Rap1, another small GTPase monitored with a similar probe in which Ras was substituted with Rap1, displayed a surprising activation pattern, initiated at the perinuclear region and spreading outward. From these studies, Ras and Rap1 seem to be activated in mutually exclusive compartments within the same cell, in agreement with the antagonizing effect of Rap1 on some Ras-mediated effects.

Another interesting application is the generation of biosensors that can measure intracellular fluctuations of metabolites. An example is the visualization of the dynamics of glucose uptake in COS-1 cells obtained with a probe in which CFP and YFP flank a glucose/galactose binding protein from *Escherichia coli*.⁵⁶

Applications to Basic Cardiovascular Science

A key feature of the FRET-based methodologies described above is the possibility to perform single-cell analysis and accurately resolve, both in time and in space, individual signaling events. For this reason, these methodologies are ideally suited for the study of compartmentalized signaling. Cardiac myocytes are characterized by a highly ordered architecture, and their function seems to intimately rely on the spatial organization of signaling cascades. For example, cAMP/PKA signaling seems to depend in heart cells on a strict spatial geometry of the molecular components of this pathway, including receptors, G proteins, adenylyl cyclases, cAMP, PKA, and phosphodiesterases.⁵⁷ cAMP/PKA signaling is regulated at different levels by Ca^{2+} , a key second

messenger in heart physiology, which also displays complex patterns of compartmentalized signaling.⁵⁸ The diverse and sometime opposing effects of the free radical gas NO on cardiac function have been ascribed to spatial confinement of different NO synthase isoforms, leading to differential local activation of downstream signaling pathways.⁵⁹ The NO synthase activity is primarily controlled by intracellular Ca^{2+} levels, and NO activates guanylyl cyclase to synthesize cGMP. NO can thus inhibit L-type Ca^{2+} channel activity via cGMP-dependent protein kinase phosphorylation of the channel⁶⁰ as well as via cGMP-mediated activation of PDE2 and consequent decrease of cAMP levels.⁶¹ By contrast, NO stimulates release of Ca^{2+} from internal stores via direct S-nitrosation of the ryanodine receptor.⁶² How are all such events coordinated? Although over the past decade our knowledge of the structural basis of compartmentation has advanced considerably, most of the molecular details remain to be elucidated. Real-time imaging of signaling cascades in heart cells should provide detailed quantitative and dynamic information that may help the understanding of how compartmentalized signaling allows for a specific and robust response, generates crosstalk between pathways, and integrates signals from distinct receptors.

Conclusions and Future Directions

FRET imaging microscopy has been proven to be a very useful tool in detecting protein-protein interactions and protein conformational changes in single live cells and to offer many possibilities for tracking the flow of information in the complex network of the intracellular signaling pathways.

Therefore, the combination of real-time imaging and approaches that can selectively interfere with the signaling network (ie, expression of dominant-positive and dominant-negative signaling elements or RNA interference) should provide, in the near future, important clues for the identification of relevant signaling modules.

In the future, we might expect to simultaneously monitor a series of reversible molecular processes so that entire signaling cascades or crosstalk between different pathways can be imaged at one time. In this respect, the generation of new fluorophores with distinct spectral properties will be very important, as will the refinement of acquisition techniques and offline treatment of the data. However, this prediction seems realistic if one considers the enormous progress that has been made in the last few years in this field, as shown by the small selection of probes illustrated in Figure 2. Most of the steps of the key cAMP/PKA signaling pathways have already been monitored using available technology.

Being genetically encoded, the FRET-based protein sensors can potentially be expressed in transgenic animals, offering the possibility to perform imaging of signal transduction in live, whole organisms. Multiphoton excitation microscopy allows imaging in thick tissues, and a 2-photon, miniaturized microscope for imaging the brain of freely moving rats has been reported.⁶³ Ca²⁺ transients have been successfully observed in neurons and pharyngeal muscles of intact *Caenorhabditis elegans*⁶⁴ and in the brain and larval neuromuscular junctions^{65,66} of *Drosophila melanogaster*, opening an entire new realm for the research in the field of signal transduction.

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