Live-cell fluorescent biosensors for activated signaling proteins

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A new generation of live-cell fluorescent biosensors enables us to go beyond visualization of protein movements, to quantify the dynamics of many different protein activities. Alternate approaches can report post-translational modifications, ligand interactions and conformational changes, revealing how the location and subtle timing of protein activity controls cell behavior.

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Abbreviations

CFP	cyan fluorescent protein
FLIM	fluorescence lifetime imaging microscopy
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
TIRF	total internal reflectance fluorescence
YFP	yellow fluorescent protein

Introduction

With the discovery of GFP, fluorescent proteins became readily accessible, spurring a revolution in the development of new microscopy approaches, software and imaging equipment to derive information from live-cell images. We are now witnessing a 'second wave' of techniques that go beyond characterization of protein localization. The changing level and location of a wide range of protein activities can now be quantified *in vivo*. In this review, we will provide a brief overview of these exciting new approaches, highlighting the advantages and potential applications of each. We will focus on signaling proteins, where visualization of protein activation is especially important. New biosensors have shown how proteins can produce essentially opposite behavior through localized activation of a small proportion of total cellular protein, or through differences in rapid activation kinetics.

Protein–protein interactions via intermolecular FRET

Perhaps the greatest impact on our ability to study protein activity *in vivo* stems from the development of GFP mutants that can undergo fluorescence resonance energy transfer (FRET). The physical basis and practical side of FRET microscopy have been reviewed elsewhere [1,2], but simply put, when two fluorophores are brought close together (usually 80 Å or less), excitation of the donor fluorophore leads to emission from the other, acceptor, fluorophore. The two fluorophores in close proximity have a unique fluorescence spectrum, which can be imaged separately from either fluorophore alone. This is used to visualize and quantify the location and concentration of interacting species in living cells (Figure 1). FRET is possible only for fluorophores with specific fluorescence properties, including overlap of the donor emission and acceptor excitation spectra.

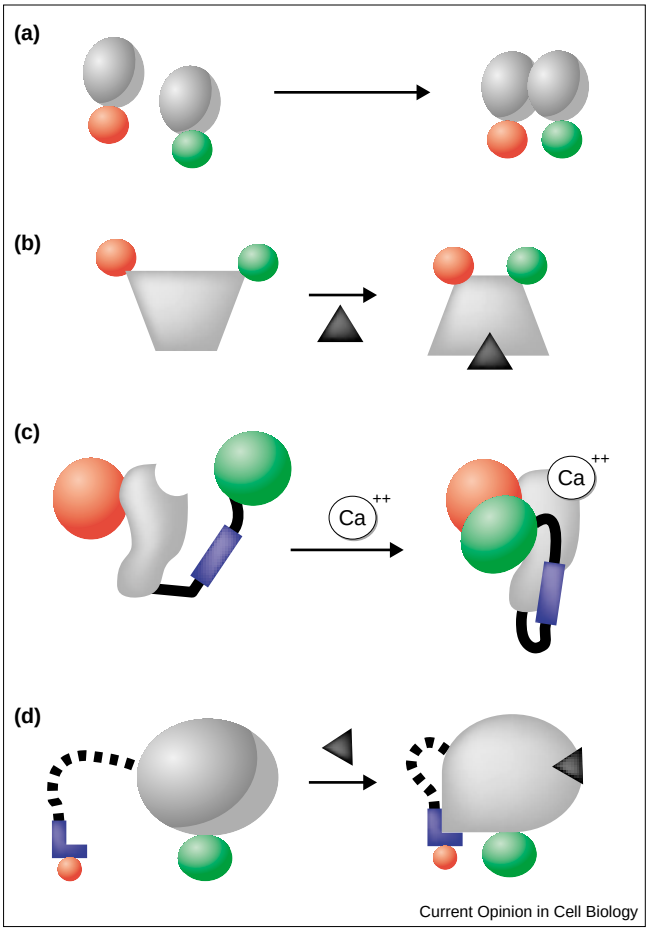
FRET has been used to study protein–protein interactions in many fields of cell biology, including signal transduction, where protein–protein interactions can serve as powerful readouts of signaling activity. For example, Devreotes and co-workers [3••] quantified interaction of heterotrimeric G-protein α and β subunits during chemotaxis, effectively using this interaction as a readout of receptor activation. Receptor oligomerization has also been studied, although with greater difficulty, as the same receptor was labeled with donor or acceptor GFP variants, and mixed in the same cell. This diminished the measurable FRET because of binding between like-labeled receptors; but FRET from donor–acceptor pairings was sufficient to reveal ligand-induced capping and oligomerization of the thyrotropin receptor [4].

FRET between molecules of receptor protein tyrosine phosphatase α tagged with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) demonstrated that this protein was regulated by dimerization [5]. FRET has also been used to trace the activity of cytoplasmic signaling proteins, including the binding of the type II regulatory subunit of protein kinase A to the A-kinase anchoring protein Ht31 [6]. Spatially localized interactions between dynamitin and MacMARCKS, the protein kinase C substrate, linked microtubule motor regulation to integrin signaling [7]. Finally, Day *et al.* [8] have used FRET in elegant studies to explore how localized transcription complex assembly regulates gene expression.

To date, the FRET pair used most frequently has been CFP and YFP, largely because of the photobleaching of blue fluorescent protein (BFP), but other mutations, together with the discovery of fluorescent proteins from other organisms, have now added to available wavelengths, improved brightness and diminished photobleaching [9•]. Importantly, it was discovered recently that intermolecular dimerization of GFPs can itself be responsible for protein–protein interaction. Mutants have been created in which this dimerization tendency can be decisively destroyed (R Tsien, personal communication) [9•].

Intermolecular FRET must be applied with great care as errors in image analysis can easily generate apparent FRET signals that are not really there. The fluorescence emission

Figure 1



Biosensor approaches. **(a)** Intermolecular FRET: FRET between a donor and acceptor fluorophore, each attached to a different protein, reports protein–protein interaction. **(b)** Intramolecular FRET: two fluorophores are attached to the same protein, where changes in distance between them reflect alterations in protein conformation, which in turn indicates ligand binding or post-translational modification. Abrogation of intramolecular FRET can be used to indicate cleavage. **(c)** Protein 'transducer'. A protein is engineered to produce a large change in the distance between an attached donor and acceptor upon ligand binding. Ideally, the transducer will have uniform distribution and a constant ligand affinity. This is sometimes ensured by perturbing the protein's normal regulation using point mutations. In this example, calcium binding generates a hydrophobic pocket to which the blue peptide binds. Peptide binding brings the two GFP mutants together, producing FRET [25]. **(d)** Domain/antibody biosensor. A protein or antibody fragment (blue) binds only to the activated state of the protein. The protein fragment bears a dye which undergoes FRET when it is brought in close proximity to the GFP on the protein. In some examples, the domain is part of the same polypeptide chain as the protein (dashed line).

and excitation of the donor and acceptor fluorophores must overlap, so it is very difficult to design microscope filters able to separate FRET from the signals resulting simply from direct excitation and emission of each fluorophore. The standardization and image-correction procedures required to overcome this have been described in valuable practical papers [10–12]. Negative controls are essential to demonstrate that FRET localizations are real. These have included bleaching the acceptor fluorophore to increase fluorescence from the donor, and/or showing that non-interacting protein mutants do not produce FRET [13,14••]. Image-correction procedures can be greatly simplified by imaging the lifetime of fluorescence emission from the donor and acceptor dyes, rather than FRET intensity, because these lifetimes are affected by FRET. Fluorescence lifetime imaging microscopy (FLIM) [1,15,16] still requires relatively specialized equipment, but commercially available systems were offered recently for the first time.

FRET can be especially valuable when coupled with techniques for visualization of single molecules. Total internal reflectance fluorescence (TIRF) microscopy restricts fluorescence to roughly 100 nm above the surface on which cells are attached, a 'two-dimensional' microscopy in which fluorescence of single membrane-associated molecules can be followed over time.

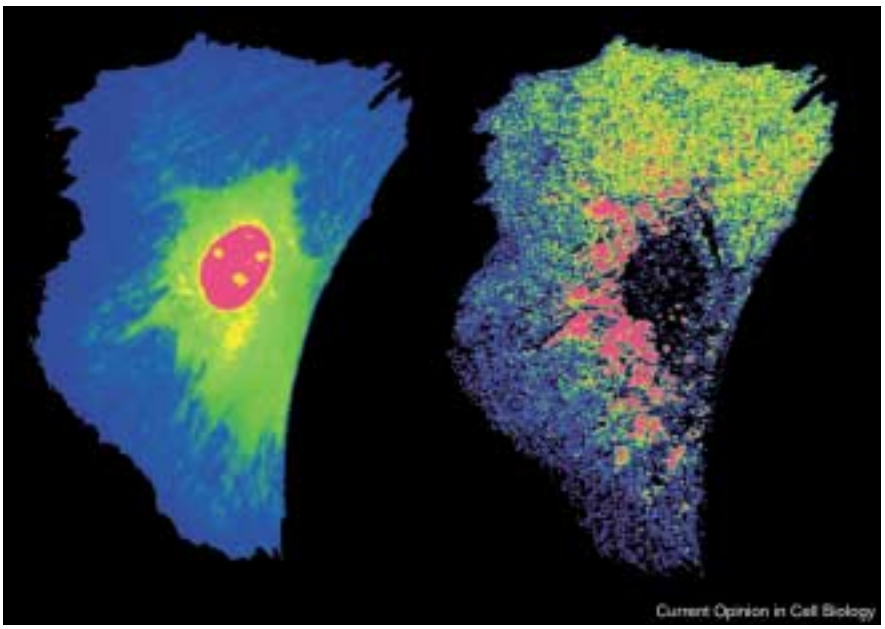
Visualizing individual molecules of Ras and downstream targets undergoing FRET has revealed that protein–protein interactions lead to restricted diffusion of membrane molecules. This is likely to be important in maintaining spatial information during localized receptor stimulation (A Kusumi and T Kobayashi, personal communication). FRET between single molecules of fluorescently tagged epithelial growth factor (EGF) and EGF receptor (EGFR) has been used to follow the dynamics of EGFR activation [17••].

Single-molecule imaging has the potential to characterize activation of the many important signaling molecules that occur in very low abundance, or are activated only in small numbers. The bright FRET signals required for TIRF are difficult to obtain using GFP mutants, and are usually obtained using synthetic dyes. The enhanced sensitivity provided by FRET between GFP and a dye can also be used where only a small quantity of a protein complex is formed [14••] or when placement of GFP at a protein's terminus perturbs biological activity. One of the proteins undergoing FRET is sometimes labeled using a fluorescent antibody [18], allowing detection of endogenous protein.

Intermolecular domain/antibody biosensors

A rapidly growing number of examples demonstrate that intermolecular FRET is a practical approach to studying interactions between two proteins. In contrast, studying changes involving single proteins (i.e. conformational change, phosphorylation, or small ligand binding) requires different approaches. Two fluorophores might be attached to the same protein to report conformational changes through *intra*molecular FRET (Figure 1b), but the conformational changes of most proteins are not sufficient to produce FRET changes that are detectable *in vivo*. Not only must two fluorophores be attached at the few positions that produce detectable FRET; they also must not perturb biological activity. This approach has been applied successfully in only a few cases, usually by using less perturbing

Figure 2



Activation of the GTPase Rac in a living motile fibroblast. These images contrast the localization and activation of Rac in the same cell. The localization of Rac is visualized on the left, using the fluorescence of an attached GFP. The right hand image shows activated Rac (right), quantified using FRET between GFP–Rac and a domain from p21-associated kinase (PAK) that binds only to activated Rac. Warmer colors indicate higher levels of activation. A broad gradient of Rac activation is visible at the leading edge of the moving cell, together with even higher activation in juxtanuclear structures. Only a specific subset of the total Rac generates FRET. This pool of activated protein is sterically accessible to downstream targets such as PAK.

small dyes rather than GFP [19]. Success has been obtained with GFP variants attached to the termini of the mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2), however. For this protein, conformational changes induced by phosphorylation produced a relatively small FRET decrease that was detectable in living cells, revealing Crm1-dependent nuclear export of the activated protein upon induction of stress pathways [20]. This approach is now more feasible, as modern cameras and instrumentation have greatly increased the sensitivity of FRET detection. Intramolecular FRET has been used with great success in proteins modified to act as 'transducers', reporting the intracellular concentrations of their ligands. In these biosensors, regulation of localization and affinity is purposefully eliminated to determine accurately ligand concentration.

A new approach to visualizing normal protein activity has proven very versatile, having already been used to provide information about protein phosphorylation, conformational change and ligation of small molecules or ions. Fluorescently labeled antibodies or protein domains that bind only to a specific state of the protein (i.e. a phosphorylated or activated conformation) produce a FRET signal when they interact with their labeled targets in living cells (Figure 1d). This reveals the location and level of activation.

Bastiaens, Parker and co-workers [21] used antibodies to visualize activation of protein kinase Cα. More recently, Bastiaens and co-workers [22••] injected cells expressing GFP–ErbB1 with dye-labeled antibody against the phos-phorylated form of this protein. FRET was used to quantify both the overall kinetics and location of phosphorylation, revealing that localized receptor binding led to a wave of further activation originating at the site of initial stimulation.

Using a protein domain rather than an antibody, our group examined the spatio-temporal dynamics of Rac activation [14••]. When the Rho-family GTPase Rac is activated by binding to GTP, it undergoes a conformational change leading to downstream effects on multiple signaling pathways. The p21-binding domain from the downstream kinase PAK binds only to the activated conformation of Rac. This domain, labeled with dye, was injected into cells expressing Rac–GFP. FRET between the domain and Rac showed that Rac was activated in a broad gradient at the leading edge of migrating fibroblasts (Figure 2), and recent results have also shown a specific role for Rac in tail retraction (G Bokoch and K Hahn, unpublished data).

The Achilles heel of such domain/antibody biosensors is that they may be restricted from reaching their targets in some subcellular locations. The targeted protein can be hidden in multiprotein complexes or organelles, or there may be competition between the biosensor and native ligands. The locations where biosensor binding is sterically blocked can be identified using constitutively active GFP-tagged mutants. With such mutants, FRET would show the same distribution as the constitutively active protein if there were no steric blocking, but in the case of Rac there were clear locations where activated material was not generating FRET (K Hahn, unpublished data).

Such steric blocking may, in reality, be an important advantage of the new biosensors, revealing steric regulation of protein activity. In studies by Schwartz and colleagues,

the Rac activation biosensor was used to characterize where PAK was sterically restricted from access to activated Rac. This was shown to be an important form of biological regulation controlling cell polarization, mediated by integrins (MA Schwartz *et al.*, personal communication). Thus, the domain/antibody sensors can be used to study *effective* protein activation — the controlled access between activated proteins and downstream targets — rather than to map overall activation. In several studies, cells were fixed to provide access of the antibody to its target [22••,23•]; although valuable, this cannot follow the changing behavior of a protein in the same cell over time.

Intramolecular domain/antibody biosensors

Tsien and others [24] showed earlier that intramolecular FRET between a protein and an attached peptide ligand could be used to engineer protein 'transducers' which report the concentrations of second messengers and ions [25]. In these studies, the protein was altered to maximize FRET changes and eliminate any biological activity other than the desired response to ligand binding (Figure 1c). In a promising extension of this approach, Mochizuki *et al.* [26••] produced a domain biosensor of Ras activation using a Raf domain that binds only to activated Ras. YFP was attached to the amino terminus of Ras; the other terminus carried the Raf domain bound to CFP. This biosensor is easier to use than the intermolecular biosensors described above. Not only is it entirely genetically encoded, it also has the important advantage of greatly simplifying quantitation and image analysis. The relative concentrations of the donor and acceptor remain constant throughout the cell, so that a simple ratio of emission from the donor and acceptor can be used as a readout of activation [25]. The authors characterized the changing localization of Ras activation during growth factor stimulation of neurons, and, using a similar Rap biosensor, discovered differences in growth-factor-induced activation of Ras and Rap. This demonstrated success opens the door to similar genetically encoded indicators for structurally related small GTPase molecules. As the approach requires derivatization of both termini of the protein, caution must be used with targets that require access to the termini for biological regulation.

Biosensors using a covalently attached domain are less likely to miss activation events due to steric blocking of biosensor binding. The attached domain will compete more effectively with native ligands. For both intramolecular and intermolecular biosensors, however, experiments must be interpreted with caution, because the domains or antibodies can compete for the very interactions that produce normal subcellular localization. Ideally, dominant-negative or activating mutations of the protein should still produce the expected effects on cell behavior in the biosensor. Fixed cells can be used to show that the biosensors have normal protein localization where activation is observed, or that proteins which normally regulate localization produce the expected effects on the biosensors.

The changing concentrations of phosphinositides in different subcellular membranes has been monitored with great success simply by following the changing localization of lipid-binding pleckstrin homology (PH) domains expressed as GFP fusion molecules [27]. These studies are easier to perform than FRET experiments, but the approach has been difficult to apply to many protein activation events. In many cases, the absolute specificity of FRET is important. FRET will detect only the tagged protein, even when the domain used has multiple binding partners. Subtle changes in biosensor localization produced by protein activation can be difficult to detect because they must be quantified against a background of the same fluorescence 'color'.

Enzyme substrates

Fluorogenic enzyme substrates can be used to visualize the activity of signaling enzymes. If diffusion of the substrate can be restricted, this can show the location of the activity, or in any case whole-cell integration can be used to examine the overall kinetics of a reaction. This was used recently to follow the kinetics of protein kinase A activation *in vivo*. Phosphorylation altered FRET between GFP mutants on a specific substrate peptide derived from the cAMP-responsive element binding protein (CREB) [28•]. Peptide substrates of caspases have been used similarly [29,30]. GFP mutants were attached to either end of the substrate so that cleavage abrogated FRET and led to increased donor fluorescence.

Conclusions

The use of new biosensors has allowed us to start shedding light on the dynamics of protein activation in living cells. They exemplify versatile approaches that can be extended to many unexplored protein behaviors. In the near future, combining biosensor readouts with traditional cell manipulations and cell mutagenesis promises to reveal the function and regulation of activation dynamics. High-throughput/high-content live-cell screening will apply statistical analysis to thousands of individual cells, eeking out subtle significant biosensor behaviors despite cell-to-cell heterogeneity [31].

There remain many important molecules that are out of reach of current approaches, providing the next challenges for biosensor development. Some proteins are sterically inaccessible to domain/antibody biosensors, or are severely perturbed when such biosensors compete with normal protein-binding interactions. Other classes of molecules cannot be appropriately derivatized for any form of FRET biosensor. For such cases, new methods are on the horizon. Dyes designed for live-cell imaging can be attached directly to the protein of interest, where they will respond directly to conformational changes or post-translational modification (K Hahn, unpublished data). Such dyes can alternately be attached to domain biosensors to report the activation state of endogenous, untagged proteins. There have been important developments in site-specific protein labeling [32••,33] and in loading dye-labeled proteins into cells [34,35•,36],

which should bring such methods into the realm of practical, everyday research tools. Certainly, the future is bright for understanding the dynamic three-dimensional organization of signaling.

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