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Review

Watching Proteins in the Wild: Fluorescence Methods to Study Protein Dynamics in Living Cells

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The advent of GFP imaging has led to a revolution in the study of live cell protein dynamics. Ease of access to fluorescently tagged proteins has led to their widespread application and demonstrated the power of studying protein dynamics in living cells. This has spurred development of next generation approaches enabling not only the visualization of protein movements, but correlation of a protein's dynamics with its changing structural state or ligand binding. Such methods make use of fluorescence resonance energy transfer and dyes that report changes in their environment, and take advantage of new chemistries for site-specific protein labeling.

Key words: Biosensor, dye, energy transfer, fluorescence, FRET, GFP, imaging, live cell, microscopy

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A great deal has changed since it was first shown that fluorescent proteins could be injected into living cells and studied in their native environment (1,2). The technique initially proved its great value in studies of cytoskeletal proteins, where the dynamics of large multimolecular assemblies was critical to understanding function. However, this approach was the purview of specialists who found it worthwhile to isolate, fluorescently label, and reinject proteins, then analyze their dynamics with expensive, and often self-constructed, microscope systems and software. Only some proteins could survive such treatment with biological activity intact, and the specialized cameras and computer equipment required to take full advantage of the fluorescent protein analogs were expensive and far less capable than todays tools.

A veritable revolution was ushered in by the discovery of the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (3–6). GFP not only opened the door to fluorescent fusion proteins that could be generated through relatively accessible, reproducible cloning and transfection procedures, but spurred the development of more affordable cameras, software, and complete 'turnkey' imaging systems to take advantage of the exploding interest in fluorescent analog cytochemistry. This in turn led to tremendous improvements in the capabilities of the equipment, bringing the technique very much into the mainstream, where it has become an important means to study the mechanisms of many fundamental cellular processes.

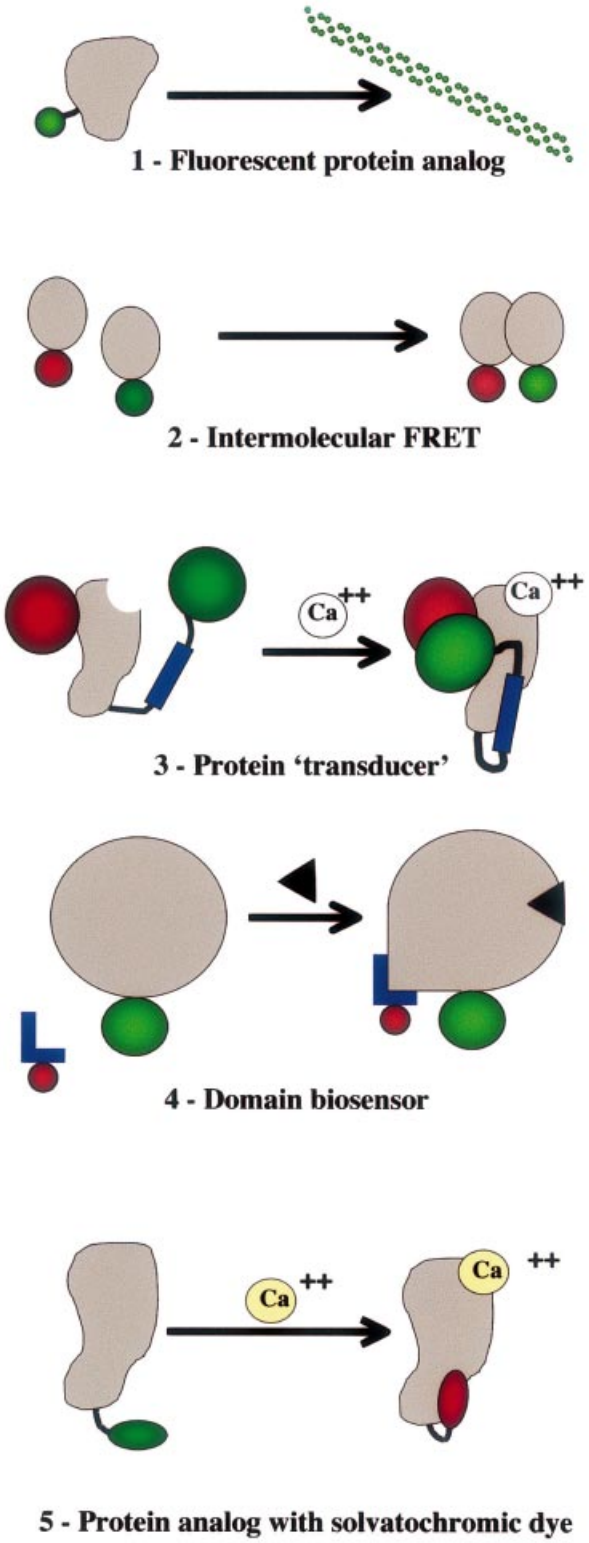
The original fluorescent analogs were purposefully designed with fluorophores that would not respond to their environment, enabling precise quantitation of subcellular concentrations. The field is now reaching beyond such tagging to analogs whose fluorescence changes to report protein activity. This was initially made possible through covalent labeling of proteins with dyes that shift their fluorescence spectra in response to changes in their protein environment (7,8), but has since been extended through the application of fluorescence resonance energy transfer (FRET) using GFP or dyes (9–11). As new approaches to fluorescent reporter proteins were developed, biophysical and optical techniques evolved in parallel to derive more information from intracellular fluorescence, even for proteins tagged with fluorophores that do not respond to environmental changes. Better ways to label proteins site-specifically and introduce dye-labeled proteins into living cells are enabling us to better use the varied capabilities of dyes as environmental reporters.

Here we will give a brief overview of these developments. Rather than focus on the rapidly expanding list of applications where tagged proteins have been used to follow protein localization, we will describe new fluorescent protein approaches that reveal changing protein structure or ligand binding. The exciting new biophysical techniques that are partner to these new fluorescent protein analogs must also be set aside in this review. We hope this overview will provide a useful starting point for those wishing to harness these newly accessible techniques, or develop approaches of their own.

Fluorescent Analog Cytochemistry-Tagging Proteins to Quantify Changing Localization *In Vivo*

The great majority of recent fluorescent protein analogs have been made using GFP rather than covalent labeling with dye (Figure 1, Scheme 1). The ability to clone and transfect fluorescent proteins is not only more convenient, but provides access to proteins that cannot be isolated and labeled or reintroduced into their native environment, including membrane-spanning proteins, or many targeted to cellular compartments. The original wild-type GFP protein was greatly improved through alteration for mammalian codon usage and

introduction of point mutations to enhance brightness and photostability (12–15). The tremendous range of GFP applications, described in other reviews (16–20), attests to the value of the approach.



Covalent labeling with dyes also remains important and valuable, even for simply tagging proteins, either because attachment of a GFP mutant to either the N or C terminus perturbs function, or because fusion with GFP blocks expression (21). There are now numerous commercially available dyes that have overcome problems associated with fluorophores such as fluorescein or rhodamine. The new dyes have improved brightness and, most importantly, greatly improved water solubility (22–24). They address fluorescein's poor photostability and rhodamine's tendency to generate labeled proteins of varying brightness and fluorescence wavelengths.

Dyes must be attached where they least perturb biological activity, often a matter of empirical variation of dye position. Even when an appropriate position for dye attachment can be predicted, site-specific labeling can be difficult, depending greatly on the structure of the protein. As reviewed elsewhere, selection of reactive groups on the dye and control of the reaction pH permit selective labeling on cysteines, on lysine amines, or on the N terminus (25–27). Proteins have been modified through mutagenesis to generate only a single exposed cysteine, which can be selectively labeled using iodoacetamide or maleimide reactive groups near neutral pH. Selective labeling has been accomplished by reacting the dye with a protein–ligand complex to mask reactive sites that are important to ligand interactions (28). In some cases, a specific lysine can show an unusually low pKa due to its hydrophobic environment or proximity to other positively charged residues. Therefore, using lysine reactive groups at low pH has sometimes resulted in selective labeling (29). Dyes have

Figure 1: Approaches to study protein behavior in living cells. (1) Proteins tagged with GFP or dyes incorporate into cell structures, mimicking the behavior of native protein and revealing changing protein localizations in real time. Fluorophores used in such studies are ideally not sensitive to their environment, so that localized intracellular concentrations can be quantified. (2) Using dyes or GFP mutants with different fluorescence wavelengths, protein–protein interactions can be monitored using FRET. When the two fluorophores are brought in close proximity, a unique fluorescence spectrum is generated. (3) Proteins can be used as transducers, to report the binding and concentration of their ligands. Here, two GFP mutants are brought together when calcium binding generates a hydrophobic pocket. The blue peptide, fused to the calmodulin, binds to the hydrophobic pocket, bringing the two GFP fluorophores close enough to generate FRET. (4) A small protein domain or antibody can be used to 'sense' a specific conformation or post-transtlational modification of a protein. The domain binds only to a specific conformation of the targeted protein. The localization of the biosensor can sometimes be used to follow the position of the targeted protein state. For more quantitative studies and enhanced specificity and selectivity, techniques such as FRET between biosensor and target (shown here) can be used. (5) Solvatochromic dyes can be attached to proteins where they respond to conformational changes with a change in their fluorescence spectrum. In this example, the dye was modified to bind to a hydrophobic pocket produced when calmodulin was activated by calcium binding.

even been attached via cleavable linkers to protein ligands which directed labeling to a particular site, or modified with side chains to produce affinity for specific protein regions (7,25,93).

Several dyes can be attached to the same protein for enhanced brightness, and new dyes have been modified with charged sulfonate groups and other side chains to prevent dimerization and quenching, but attachment of multiple dyes is not often used when it is important to keep protein activity intact. In a novel alternate approach, multiple dyes were attached to a rigid carbon framework bearing a single protein-reactive group, in effect generating a single multidye cluster, which is very bright but nonetheless significantly smaller than GFP (30).

There are now multiple approaches to incorporate labeled proteins into living cells, including injection, fusion with peptides leading to cell uptake (31,32), and transient rupture of the cell membrane for uptake of molecules dissolved in the extracellular fluid (i.e. bombardment with beads (33), electroporation (34), passage through a syringe (35), osmotic shock (36)). Nonetheless, many proteins are very difficult to tag or to successfully reincorporate into cells, and further developments to extend the capability of GFP are still most likely to find widespread application.

Multiple Colors: Synchrony of Protein Dynamics, and Studying Protein–protein Interactions with FRET

Imaging more than one protein in the same cell has been used for years to examine protein interactions, or the coordination of proteins working together to generate a cellular behavior. There are numerous examples of simultaneous imaging using proteins labeled with dyes. Dyes are commercially available in a broad range of wavelengths, allowing ready separation of fluorescence from at least five analogs in the same cell (37). Point mutations in GFP have been used to shift its wavelengths, making it possible to image two separate proteins expressed as GFP fusions (6,38–42), but such mutations have not been able to generate the broad variety of wavelengths seen in synthetic molecules.

Using two different GFP mutants in the same cell, quantitative studies of the relative dynamics of two proteins have been performed, but they require careful image analysis to correct for 'bleedthrough' of the fluorescence from one GFP mutant into the image of the other. Microscope filters designed for specific GFP mutants and correction algorithms to compensate for bleedthrough are commercially available (38). The wavelengths of GFP continue to be extended through mutagenesis studies, and different genetically encoded fluorophores from other organisms are now being discovered and enhanced (94). The CFP and YFP mutants appear to have found the most favor to date, although researchers have also reported success with combinations of BFP/GFP (43,44), and other mutants appearing in only single reports and requiring use of more complex image correction (38). BFP/GFP was largely supplanted by CFP/YFP due to the relatively poor photostability of BFP (45).

Simultaneous imaging can correlate protein dynamics, but cannot demonstrate a direct interaction between two proteins. At best, one can say that different proteins are closer than the resolution limit of the light microscope, which is roughly 0.5 microns. To demonstrate close protein association, FRET has proven to be a very powerful tool. In FRET, when a donor fluorophore is brought in close proximity to an acceptor fluorophore (generally less than 100 angstroms for fluorophores suitable for *in vivo* applications), the donor does not emit at its characteristic wavelengths, but transfers the energy to the acceptor, which then emits as it would had it been directly excited (46–48). Thus, two dyes in close proximity generate a unique fluorescence spectrum that can be imaged separately from that of either dye alone. FRET is very sensitive to the distance between the two fluorophores, falling off with the sixth power of the separation between them (49,50)

Among FRET applications used to study the activity of biologically active protein analogs, use of intermolecular FRET between fluorophores on different proteins (i.e. to see protein–protein interactions) will likely find the most widespread application (Figure 1, scheme 2). In contrast, intramolecular FRET, in which two fluorophores are attached to the same protein to monitor conformational changes, is much more difficult. Few naturally occurring conformational changes alter the separation between attached dyes sufficiently for detection *in vivo*. Furthermore, only in limited cases will attachment of two fluorophores at the appropriate positions result in an analog with fully intact biological activity. In the single example where intramolecular FRET was used to monitor the conformational changes of a biologically intact protein within living cells, fluorescein and rhodamine were attached to myosin II at positions where phosphorylation of the protein led to a 26% change in the ratio of two emission wavelengths (51,52). Despite this relatively small change, careful image analysis enabled visualization of localized myosin II phosphorylation in specific subcellular regions during cell motility.

In contrast, for detecting protein–protein interactions using intermolecular FRET, each protein need only be labeled with a single fluorophore, making it much easier to generate biologically active analogs. Furthermore, sufficient FRET changes can be generated over a broader range of attachment points (53–58). New GFP mutants with spectral overlap appropriate for FRET have made it possible to readily generate analogs for this valuable approach (6,59–66). Dyes currently provide substantially better FRET spectral properties than the GFP mutants, but the majority of new *in vivo* FRET applications involves GFP, presumably because of increased accessibility of labeled proteins. There are still relatively few examples of FRET used *in vivo* to examine the activity of proteins with purportedly intact biological activity.

The BFP-GFP pair has been used to examine transcription factor interactions (67), and CFP-YFP to examine cAMP-dependent protein kinase binding to A-Kinase anchoring proteins (61). The technique has also found application in high throughput drug screening, where protein interactions can be examined in cell lines stably transfected with two GFP fusion proteins.

Even the best GFP mutants have strongly overlapping spectra that cause the FRET signal to be contaminated by light from the donor fluorophore emission. FRET images from current mutants require very careful correction for this 'bleedthrough' from direct excitation. The intensity of the real FRET signal and the bleedthrough can be similar. Furthermore, artefactual fluorescence signals resulting from bleedthrough occur in the same place as at least one of the interacting proteins, so such signals often resemble the desired results! Real FRET can be verified using controls that include bleaching the acceptor fluorophore to generate an increase in donor fluorescence, or showing that biologically inert mutants of the interacting proteins no longer undergo FRET.

While it remains daunting to generate a biologically active protein analog that reflects its activity through intramolecular FRET, a different application of intramolecular FRET holds great promise. Here, labeled proteins are used not to examine protein function, but as components in biosensors reporting the concentrations of protein ligands (68,69). New calcium concentration indicators, generated using GFP mutants to produce FRET, first exemplified this application (11,66). The C-terminus of calmodulin was modified with a short peptide that bound to the protein only when the protein had bound calcium (Figure 1, scheme 3). CFP or BFP was fused to this short peptide, and GFP or YFP was attached to the other terminus of the protein. When the protein bound calcium, the peptide moved to its binding site, bringing the two GFP mutants together and increasing FRET. Here the protein was severely modified to generate the large conformational changes required for sufficient FRET changes. Use of proteins as building blocks in FRET-based sensors such as this is more practical than generating biologically active protein analogs using intramolecular FRET. In the transducer there was no need to maintain any biological activity other than calcium binding, so two bulky GFP mutants could be used. Intermolecular FRET has also been used to build transducers of small ligand concentration. The two subunits of cAMP-dependent protein kinase separate upon binding to cAMP. In a FRET-based cAMP concentration indicator, which has been built using either dyes (10) or GFP mutants (70), separation of fluorophores on the two subunits reports cAMP binding. A caveat with this approach is the possibility that the dissociated subunits will bind to native, unlabelled subunits when ligand levels drop, thus altering the indicator response upon repeated changes in ligand concentration. Intramolecular FRET has also been used to report phosphorylation of a kinase A substrate donor, and revealed its localized kinase activity (71).

Labeling Proteins in Living Cells – Combining the Best of Dyes and GFP

As described above, the overriding advantages of GFP are the convenience of expressing genetically tagged proteins, and the ability to generate functioning analogs of many proteins inaccessible through extracellular labeling and reintroduction into cells. Dyes, on the other hand, have great advantages over GFP as fluorophores, including their tremendous selection of fluorescence wavelengths and ability to respond to a wide range of different environmental effects. Ideally, one could combine these by dye-labeling expressed proteins within living cells. This may in fact soon be possible.

Proteins can be fused to peptide tags which bind to a fluorescent reagent. Thus, for example, membrane-bound proteins could be expressed in the cell, and the dye introduced separately for binding to the tag. In one proven system, a tag of only six amino acids binds specifically to a cell permeable Arsenic compound bearing a fluorescent dye. The fluorescence of the compound is quenched until it binds to the peptide tag (72), greatly improving sensitivity over other approaches where labeled material must be imaged over a background of unattached dye. Proteins have also been expressed fused to an antibody fragment that binds to a dye–hapten conjugate (73). Dyes have been shown to bind directly to small peptides (95), suggesting that tags could be found that bind to membrane permeable dyes. Two tightly interacting leucine zipper peptides have been used to fluorescently label a protein *in vivo* (74). The two peptides were expressed in the same cell, one fused to GFP and the other to vinculin, but the approach could conceivably be extended to dye-labeled peptides introduced from outside the cell.

Artificial amino acid mutagenesis is a promising technique that enables incorporation of artificial amino acids into proteins during *in vitro* transcription/translation reactions. Many different synthetic, unnatural amino acid side chains, including dyes, have been incorporated into precisely selected positions in proteins using this technique (75–82). Briefly, a tRNA is charged with an artificial amino acid bearing an unnatural side chain. This tRNA is designed to read through a specific stop codon, which has been incorporated in the coding regions at the point where the unnatural amino acid is to be placed. The unnatural amino acid is incorporated in the protein during read-through of the stop codon. After extensive applications *in vitro,* this has now been accomplished within living cells, incorporating biophysical reporters into oocyte ion channel proteins. Unlike the N or C terminal tags, this approach has the potential to incorporate synthetic dyes anywhere in the protein sequence, where the various specific reporter functions of dyes can be put to good use.

Reaching Further

Extremely important protein functions that remain largely invisible to the approaches described above include protein phosphorylation and other postranslational modifications, conformational changes, and binding to small ligands that cannot be tagged with fluorophores (i.e. second messengers or ions). There are at least two new approaches which show the promise of providing general solutions to imaging a very broad range of activities, including these.

Two recent studies have demonstrated the feasibility of generating live cell biosensors from protein fragments that bind only to one structural state of a targeted protein (Figure 1, scheme 4). In one case, a biosensor that reports the activation state of the small GTPase Rac was produced using intermolecular FRET (83,96). The Rac binding domain from p21activated kinase, which binds only to activated GTP-bound Rac, was derivatized with a dye and injected into cells expressing Rac-GFP. Wherever the Rac was activated, the labeled domain bound to the Rac and generated a FRET signal. In a different application, a fluorescently labeled antibody against the phosphorylated form of protein kinase C was used (84). Rather than monitor FRET between the fluorophores on the antibody and target protein, here the effects of energy transfer on the fluorescence lifetimes of the dyes was imaged (85). Use of such small biosensors provides a very versatile way to target many different protein activities, especially using antibodies as sensors. In some cases it is possible to simply use the changing localization of the protein biosensor to examine the dynamics of the targeted protein state. However, unlike the above examples, studies relying on localization alone cannot readily quantify the changing levels of the activation state being measured, and may be much less specific if the protein sensor binds to multiple targets, including unknown proteins within living cells. For all these methods, steric restrictions or competition from binding of other molecules may prevent the sensor from finding its target in some locations.

Another approach, more technically challenging but having the potential to overcome the difficulties associated with use of separate protein-based sensors, is derivatization of protein with dyes that change fluorescence depending on the activity of the protein: so-called 'solvatochromic dyes'. This has been used many times to examine protein behaviors *in vitro*, but has been reported only infrequently in living cells. This is most likely because dyes that have been shown to respond to protein conformational changes *in vitro* are not well suited to *in vivo* imaging. Such dyes must not only show strong fluorescence changes depending on their environment, but must be brightly fluorescent at long wavelengths that do not overlap cellular autofluorescence or kill cells. A novel dye was developed for this purpose and applied to generate a calmodulin analog, which changed fluorescence upon calcium-induced activation (7,8) (Figure 1, scheme 5). The analog was successfully used to map localized calmodulin activation in motile cells, and to trace the kinetics of overall calmodulin activation in individual cells. Here the challenge was to place the dye at a position where the changing protein conformation was likely to affect its fluorescence. This required modification of the dye with side chains producing affinity for a protein region generated only when the protein was activated. This approach suffers from the fact that extensive work must be carried out to generate a reporter analog for any given protein, including dye modifications and/or experimentation with labeling sites. A family of new dyes designed specifically for this purpose will be helpful in the future (Toutchkine and Hahn, unpublished data).

Those wishing to construct these new types of biosensors must determine the dye position where protein activity will be sensed but not perturbed, and must attach the dye site-specifically to any desired position. It is relevant to mention here important advances in protein chemistry that promise to have a great impact on our ability to overcome these obstacles. Unnatural amino acid mutagenesis, described above, has been used to label proteins with fluorescein and other dyes with absolute specificity (86). Proteins of more than 20000 molecular weight have now been made by linking synthetic peptides end to end (98). This also allows complete control of dye position and makes it possible to readily generate and screen multiple analogs of a protein with dyes in different positions. Some useful dyes cannot withstand the chemistries required for peptide and protein synthesis, or interfere with the enzyme activities required for artificial amino acid mutagenesis. In such cases it is possible to incorporate uniquely reactive amino acid side chains that can be used as handles for dye attachment when protein synthesis is complete (86,88). These groups react selectively with commercially available dyes even in the presence of cysteine, lysine or other reactive residues. Finally, it is now possible to splice synthetic peptides into large expressed proteins, not only on the termini but also in the middle of the protein sequence (87,89,90). Briefly, proteins are expressed containing an 'intein' sequence, which splices itself out of the center of the protein while joining the two termini together. By mutating the intein, the reaction can be halted at an intermediate stage, allowing incorporation of synthetic peptide, rather than one of the terminal protein portions.

Although this review has focused on methods based on modifying proteins, it should be mentioned that fluorogenic substrates have also been used in living cells to monitor enzyme activity (24,91). Small molecules designed to follow the activity of specific enzymes have been adapted for use in living cells, and proteolytic reactions have been monitored using peptides bearing donor and acceptor fluorophores (GFP mutants or dyes). In the latter case, FRET ceased upon peptide cleavage between the points of dye attachment. This was used to monitor cleavage of peptides during antigen processing (92) and activation of caspase proteases during apoptosis (60). As it has become evident that cleavage is an important mechanism for signal transduction and regulation of cell function, such analogs are likely to prove illuminating in the future.

Great optimism can be derived from the combination of new labeling chemistries and first examples of versatile biosensor methods capable of detecting almost any protein activity. When these approaches become accessible, it will be possible to quantify the changing level, location, and duration of protein activity, and to correlate these aspects for multiple proteins in the same cell. The value of such measurements for both biological research and drug screening will continue to drive development of the approach (97), bringing such protein tools to a level where individual laboratories can embark on biosensor development projects with a high certainty of success.

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