Fluorescent Probes

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Über-Responsive Peptide-Based Sensors of Signaling Proteins**

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An intricate intracellular web of biochemical pathways is responsible for the remarkable adaptability of life. Signaling pathways enable cells to preserve the intracellular conditions required for life by recognizing and responding to environmental change. However, these pathways can also act in a confrontational capacity, literally turning on the host organism. Cancer cells divide at inappropriate times, in inappropriate places, and, like their bacterial counterparts, develop ingenious biochemical mechanisms to defeat therapeutic agents. Biochemistry in the 20th century was primarily devoted to the isolation of proteins and their subsequent characterization in vitro. However, a deeper understanding of the relationship between protein action and cellular behavior is best obtained by studying the protein of interest in its natural environment. The biochemistry of the cell, as opposed to the biochemistry of the test tube, drives adaptability in the face of environmental challenges, be they natural or artificial. Our understanding of cellular biochemistry has been greatly assisted by extraordinary advances in fluorescence, particularly in the areas of technology and molecular biology. However, recent progress from a third discipline, namely synthesis, offers a glimpse of the shape of things to come.

Green fluorescent protein (GFP) and its many genetically altered constructs have revolutionized cell biology.[1] Appending GFPs to the N or C terminus of any given protein is straightforward, as is the expression of the newly constructed species in living cells. Does a protein change its location as a function of the cell division cycle? How rapidly is it degraded or what is its intracellular diffusion rate? GFPs have been used to detect dynamic chemical changes to the appended protein as well. For example, constructs containing FRET-paired GFP analogues (typically cyan fluorescent protein and yellow fluorescent protein; FRET = fluorescence resonance energy transfer) have been designed that serve as sensors for a variety of bioactive agents, such as cAMP,[2] Ca^{2+},[3] and protein kinases.[4] However, although the impact of fluorescent proteins cannot be overstated, GFPs are not without limitations. GFPs are large and thus can alter the biological behavior of the protein to which they are appended (Figure 1). Their large size also precludes localization of fluorescence to a specific subdomain (e.g. adjacent to the active site) on the protein under study. Furthermore, with the exception of proteolysis or some other form of degradation, GFPs are generally unresponsive to changes in their local environment. Indeed, even FRET changes in doubly labeled constructs are typically less than 50%. By contrast, small fluorophores are less likely to perturb biological activity, they provide a greater degree of spatial precision with respect to placement and orientation, and they can be remarkably environmentally responsive.

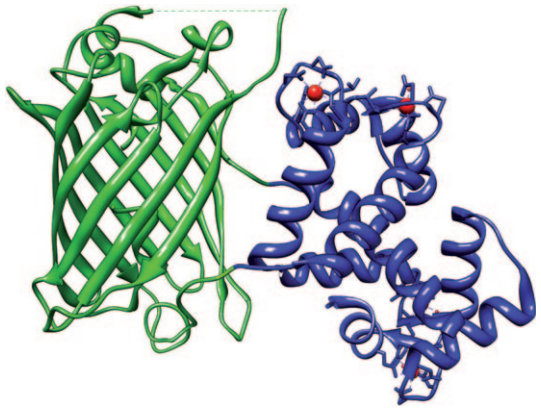

Figure 1. Crystal structure of the genetically encoded GFP-based Ca^{2+} sensor GCaMP2.[5] GFP is highlighted in green, the Ca^{2+}-binding protein component calmodulin is shown is blue, and Ca^{2+} is red.

A number of methods have been developed for attaching fluorophores directly to proteins in living cells.[6] The general strategy typically involves genetically appending a short amino acid sequence to the protein of interest, which serves as the attachment site for a synthetic, but cell-permeable, fluorophore. Schultz's nonsense-mediated suppression tech-

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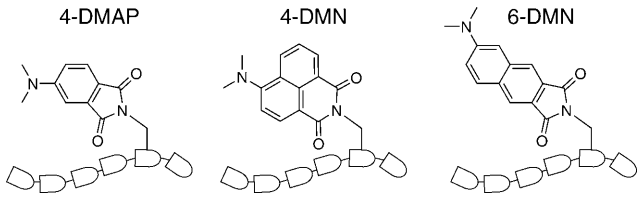
nology offers an even greater degree of spatial resolution, incorporating a fluorophore at specific amino acid positions.[7] However, relatively few fluorophores have been introduced into proteins by these methods. In short, genetic and biochemical approaches are simply unable to compete with the versatility and scope of organic synthesis. For example, the range of useful GFP variants pales in comparison with the large number of commercially available synthetic fluorophores. Furthermore, cell-based synthesis of artificial protein constructs raises the specter of perturbing the endogenous biochemistry of the cell.

The construction of fluorescent sensors by nongenetic strategies enjoys the advantage of leaving the natural biology of the cell unaltered until the time of the experiment. Moreover, organic synthesis offers nearly unlimited structural versatility, thereby allowing the sensor to be fine-tuned with a level of precision that is simply not possible by genetic means. A case in point is the recent study by Imperiali and her colleagues, who described the construction of peptide-based fluorescent sensors for PDZ domains.[8] The Imperiali group posed the following question: is it possible to construct highly responsive peptide-based sensors that discriminate between closely related protein binding sites? A family of solvatochromatic dimethylaminophthalimide-based fluorophores, (4-DMAP, 4-DMN, and 6-DMN; Scheme 1) has been extensively employed by the Imperiali group to study SH2[9] and 14-3-3 domains,[10] as well as calmodulin[11] and class II MHC proteins.[12] Structural and consensus-sequence information has been used by these investigators to identify key sites of interaction between a peptide and its targeted protein domain. Specifically, a hydrophobic aromatic residue on the peptide, which becomes embedded within a lipophilic pocket upon interaction with its protein-binding partner, is an ideal candidate for replacement with a solvatochromic fluorophore. This strategy affords large enhancements in fluorescence that range from 10 to 2000 fold.



Scheme 1. The solvatochromic fluorophores 4-DMAP, 4-DMN, and 6-DMN appended to a peptide backbone.

Unfortunately, unlike SH2 or 14-3-3 domains, the binding properties of PDZ domains are not well defined, and therefore the optimal position for a solvatochromic fluorophore on a PDZ-directed peptide was, at the time the study was initiated, unknown. The initial step involved the synthesis of a peptide library based on the sequence of C-terminus residues found in Stargazin, a protein known to interact with three different PDZ domains. Residues at eight different positions were replaced by three different handles (diaminopropionic acid, diaminobutyric acid, and ornithine) and coupled to 4-DMAP. Each peptide was screened with three different PDZ domains, and an evaluation of fluorescence change allowed the ideal position and nature of the handle for 4-DMAP to be ascertained. In a subsequent optimization step, the fluorophore and handle were incorporated into sequences known to selectively bind to specific PDZ domains. This approach furnished the desired peptide-based sensors that not only distinguish between different PDZ domains but also do so with fluorescence enhancements of up to more than 200-fold.

In the interest of full disclosure, others, including our own group,[13] have described related strategies to acquire peptide-based sensors for an array of signaling molecules as well. In short, organic and solid-phase peptide synthesis, in combination with screening, has furnished an assortment of impressive sensors and probes that can be used for in vitro purposes. However, what of intracellular applications? In marked contrast to GFPs, comparatively few of these reagents have found their way into cell-based experiments. There are a number of reasons for the apparent limitation of peptide-based probes. First, the structural options inherent within systems such as those described by Imperiali are immense, which is both a blessing and a curse. Easy-to-prepare fluorophores are required for the initial library-based phase of these studies, yet these fluorophores do not necessarily possess the ideal photophysical properties for intracellular work. Second, unlike GFP-modified proteins, which are synthesized inside the cell after transfection with the corresponding gene, fluorophore-appended peptides must be introduced into the cell after their laboratory synthesis. Generally speaking, peptides are not cell-permeable, and methods for intracellular incorporation are either nontrivial (e.g. microinjection) or of questionable utility. In the latter instance, "cell-penetrating peptides" are especially controversial, as these reagents may or may not introduce their cargo through an endosomal-mediated mechanism.[14] This issue is potentially significant, as peptides that gain cellular entry through endosomes are entrapped within these small vesicles and thus not exposed to the cytoplasm. Furthermore, endosomal-embedded peptides and proteins commonly suffer proteolysis. Third, it is unclear how stable any given peptide will be in the intracellular milieu. Consequently, general methods for establishing the structural integrity of peptide-based probes, including measurements of intracellular lifetime, will be necessary. We note that biologists will ultimately decide whether a particular reagent, probe, or sensor is useful. Currently, GFPs are simple to use, as much of the chemistry (site-directed mutagenesis to prepare an array of fluorescent variants) has already been accomplished. By contrast, the rules for the design of nongenetically encoded probes are still being developed.

Finally, whether a sensor or probe is synthesized by ribosomes in a cell or by scientists in the laboratory, all potentially suffer from the cellular version of the observer effect. This principle has found application in fields as diverse as physics and psychology, and simply notes that the mere act of observation can influence the phenomenon under study. Given the extraordinary adaptability of cellular biochemistry to environmental change, it should be expected that a probe, be it large (a GFP-fusion protein, Figure 1) or small (a

fluorophore-appended peptide, Scheme 1), could elicit unintended consequences. Probe a water-filed balloon with a finger, and the physical ramifications will manifested at both the site of the probe (locally) as well as throughout the balloon (globally). In an analogous vein, the act of detecting the availability of a PDZ domain in a cell may likewise engender local and global effects since, if the probe is bound to the PDZ domain, then the endogenous protein that should be bound has been displaced. The cell may very well respond both locally and globally to this disruption in biochemical homeostasis. Fortunately, this need not be an all-or-none scenario. If the sensor is "über-responsive", then only a small fraction of the PDZ domain molecules under study need be probed to obtain a measurable response. Consequently, the cell-wide influence of the sensor, and thus the observer effect, can be kept to a bare minimum. This is the ultimate promise that nongenetically encoded agents offer. However, many challenges remain before the universal application of these reagents can be fully realized.

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