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Emerging In Vivo Analyses of Cell Function Using Fluorescence Imaging*

Jennifer Lippincott-Schwartz

The Eunice Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-5034; lippincj@mail.nih.gov

Abstract

Understanding how cells of all types sense external and internal signals and how these signals are processed to yield particular responses is a major goal of biology. Genetically encoded fluorescent proteins (FPs) and fluorescent sensors are playing an important role in achieving this comprehensive knowledge base of cell function. Providing high sensitivity and immense versatility while being minimally perturbing to a biological specimen, the probes can be used in different microscopy techniques to visualize cellular processes on many spatial scales. Three review articles in this volume discuss recent advances in probe design and applications. These developments help expand the range of biochemical processes in living systems suitable for study. They provide researchers with exciting new tools to explore how cellular processes are organized and their activity regulated in vivo.

Keywords

green fluorescent protein; photoactivation; signal transduction; fluorescence resonance energy transfer (FRET); zinc chemistry; nitric oxide sensing

Cells are the basic building blocks of all living systems. They rely on complex internal processes, including DNA replication and transcription, protein synthesis and transport, organelle and cytoskeleton assembly/disassembly, energy metabolism, and signal transduction pathways. These take place on the micrometer scale in membrane compartments and cytoskeletal architecture; on the tens of nanometer scale in molecular machines such as chromosomes, ribosomes, and proteosomes; and on the subnanometer scale in signal transduction and metabolic pathways. Scientists are still working on how cells of all types sense and respond to external and internal signals, and how these signals are processed to yield particular responses in a multiscale context. The challenge is immense because the details of an individual cell's small-molecule transactions can be vastly different depending on the cell's identity and physical location. And, these transactions occur in the context of complex signaling networks that underlie cell growth, death, division, and differentiation.

Fluorescence imaging, employing green fluorescent protein (GFP) from *Aequoria victoria* and its relatives, is proving to be an invaluable new tool for addressing these questions (1). GFP's paradigm-shifting advance as a molecular imaging tool draws from its fluorescence

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the capacity to produce light of a different color from the illuminating light. Occurring on the order of nanoseconds, fluorescence can monitor cellular processes over seconds, minutes, or hours. Importantly, GFP is genetically encoded by a single portable DNA sequence that can be covalently fused to a protein of interest and expressed within living cells. This makes it tailor-made for visualizing protein dynamics and biological processes by in vivo microscopy. Before GFP, researchers relied on fluorescent antibody techniques to examine the spatial distribution of proteins and nucleotide sequences, but this could only be done on dead, fixed cells or tissue sections, and so it failed to provide temporal insights into the cell's dynamic processes. With their ease of construction, precise targeting, and fluorescent read-out in living cells, GFP fusion proteins overcame these drawbacks (1).

But other advances ensured the rise of GFP from obscurity to being one of the most widely used probes for imaging the structural organization and spatiotemporal dynamics of molecules. Concerted mutagenesis diversified GFP's spectra, increasing its brightness and folding efficiencies and decreasing its oligomerization (2–4). Mutagenesis also led to the generation of forms of GFP that are photoactivable or photoconvertable (5). It was further discovered that GFP is just one member of a large family of homologous fluorescent proteins (FPs), mostly from marine corals, with different colors resulting from variations in their chromophore covalent structure and noncovalent environment (6). Directed mutagenesis of the FP from these species led to a series of red-shifted FPs, useful in deep tissue imaging owing to their long wavelengths (7). The resulting palette of FPs, covering the entire range of the visible spectrum, has allowed for simultaneous imaging of multiple sets of proteins inside cells (8).

Synergistic advances in targeting strategies, imaging techniques, instrumentation and data analysis have paralleled GFP development (9–13). Among the imaging techniques that have been particularly valuable for studying the spatial compartmentalization and temporal dynamics of FP-tagged molecules are fluorescence recovery after photobleaching, fluorescence correlation spectroscopy, FRET, fluorescence cross correlation spectroscopy, total internal reflection microscopy, fluorescence lifetime imaging, and photoactivation localization microscopy (PALM) (9, 10, 12). Varying in their axial resolution, mode of imaging, and applications, these techniques are catalyzing the study of complex molecular networks of cells in their native biological contexts.

The scope of biological phenomena now being monitored using fluorescent imaging is understandably enormous and continues to expand. In this volume, three articles highlight where this field is moving (14–16). In the review article by Mehta & Zhang (14), the authors discuss how FP technology can be used to understand the complex networks of biochemical processes that shape large biological phenomena. They begin by describing strategies for designing FP reporters that can "speak" the language of the cell. These include those fused directly to a protein to report on its location and turnover, those designed to undergo changes in their fluorescent signal to report on conformational changes or intermolecular interactions within a protein of interest, and those containing a sensory element to detect accumulation or degradation of a small molecule. Mehta & Zhang then discuss how these types of FP reporters can be used to decipher the behavior of tagged signaling molecules and their spatiotemporal organization.

In a typical signaling pathway, receptors on the plasma membrane detect extracellular cues and mediate the production of intracellular second messengers, which regulate the activity of signaling enzymes and downstream transcription factors. FP reporters, in combination with different imaging approaches, enable the detection of specific pools of each of the components in such a system. For example, reporters in which a protein of interest is tagged with a genetically encoded FP can be used to address the kinetic properties of a protein

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within a signaling pathway, such as whether the protein is free to diffuse within a membrane bilayer or is attached to a matrix, and whether it is undergoing exchange between compartments or on/off a substrate (9). This can be achieved using photobleaching techniques, whereby an area of the cell is photobleached with a high-intensity laser pulse and the movement of unbleached molecules from neighboring areas into the bleached area is recorded by time-lapse microscopy (10). Alternatively, a protein of interest can be tagged with GFP variants whose spectral properties change with time or are photoactivatable. Examples of these variants are the fluorescent timer protein and the photoactivatable proteins, including photoactivatable GFP (PA-GFP), Kaede, and KFP1 (5). In the case of PA-GFP, it displays little initial fluorescence under excitation at the imaging wavelength but increases its fluorescence up to 100-fold after activation by irradiation at a different wavelength (17). This allows direct highlighting of distinct pools of molecules within cells. Because only photoactivated molecules are fluorescent, the temporal expression pattern and turnover of a protein can be readily studied independently of newly synthesized proteins. In both photobleaching and photoactivation, therefore, the overall dynamics of an FP fusion protein can be determined without perturbing other pathways or cell function.

Use of FPs in fluorescent imaging methods such as FRET has enabled protein-protein interactions to be spatially and temporally resolved (11). FRET measurements can be made in real time in living cells, enabling transient protein-protein interactions over short periods of time to be assessed. These types of interactions are often missed in classical biochemical approaches that are dependent on populations of cells exhibiting sufficient, isolatable protein-protein interactions to be measurable. In FRET, one reporter is a donor fluorophore, the other reporter is a longer wavelength acceptor fluorophore, and the readout is energy transfer from the donor to the acceptor. The working scale of FRET is less than or equal to 100 Å . Because GFP variants are available that undergo FRET, and these can be attached to different proteins to test for their interaction, they have permitted the inter- and intramolecular distances in proteins to be probed (11). This, in turn, has allowed the mapping of protein-protein interactions within cells. Miyawaki (15), a pioneer in FRET studies using FPs, discusses the latest developments in probe design and analysis for FRET in his review article. Focusing on hetero-FRET, the transfer of energy between two different fluorophores, Miyawaki presents theoretical and practical comparisons of bimolecular and unimolecular FRET constructs. He then describes technical aspects of FRET, including quantitative aspects of intensity- and lifetime-based FRET using either FPs or chemical fluorophores, and FRET imaging in live versus fixed cell samples.

Signal transduction networks operate like complex computation systems, with regulatory interactions between signaling molecules forming interlinked circuits that control the dynamics of signal flow. FP reporters can be used to detect and visualize these interactions and mechanisms (18, 19). This is achieved by monitoring the production of intracellular second messengers, which regulate the activity of signaling enzymes and downstream transcription factors. A common strategy is to place a conformationally sensitive protein, such as a genetically encoded calcium or PKC activity reporter, between an FP FRET pair (14). Results from such experiments have been combined with mathematical modeling to map out signal flow through signaling pathways, revealing whether a pathway exhibits negative feedback, bistability, or oscillatory signaling dynamics (13, 18–20). Furthermore, new developments in spectral separation of FP biosensors have enabled coimaging of molecules in different cross regulatory signaling pathways (i.e., PKA and cAMP) (13, 19–22).

Among the promising new directions discussed by Mehta & Zhang (14) are probes for monitoring GTP hydrolysis (23) and cell cycle events (24, 25). Also of value are biosensors in the range of 650–900 nm for deep tissue imaging and bright FPs for imaging at small

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spatial scales as in the superresolution techniques of stimulated-emission-depletion microscopy and PALM (4–6, 8). Probes that can perturb discrete biochemical activities provide additional options. One strategy is to use small molecules that can be induced to form dimers (23). By changing a protein's distribution or interactions, these probes can be used to drive specific biological activities at selected times and places in cells. Another exciting strategy involves optically inducible switches (26, 27). These employ light to discretely activate signaling molecules. Unprecedented new FP tools thus are continually becoming available for probing and uncovering the spatial and temporal details of biological processes on a variety of scales.

Inorganic species, such as zinc and nitric oxide, help drive physiological processes or when uncontrolled trigger pathology. Zinc-binding proteins, for example, are encoded by approximately 10% of the human genome, with the origins and consequences of zinc signals central to human physiology and pathology (28, 29). Likewise, the biosynthesis and potentiation of nitric oxide is important to physiological homeostasis, playing an important vasodilatory role in the cardiovascular system (30). How zinc and nitric oxide participate in biological signaling networks, however, has not been fully elucidated. A valuable means to study the generation, accumulation, and translocation of these metal ions and other inorganic agents is using small-molecule fluorescent probes (31, 32). These probes can respond in a direct and selective manner to specific inputs with both spatial and temporal resolution. Lippard and colleagues (16) address this topic in their review article on fluorescence imaging of mobile forms of the divalent zinc cation and the gaseous nitric oxide radical.

Available turn-on sensors for biological mobile zinc are numerous, offering excellent methods for visualizing zinc ions inside cells with high spatial and temporal resolution (16).Most zinc indicators employed in fluorescence microscopy experiments are intensity-based sensors, usually associated with fluorescein, that respond to zinc coordination with an increase in fluorescence emission intensity. Ratiometric indicators are also available. These can be used to determine zinc concentrations in dual excitation and dual emission experiments, supplying valuable information on transient zinc levels in live cells or tissues. Other promising ratiometric systems are based on zinc-induced modulation of intramolecular FRET.

Probes to monitor nitric oxide, in contrast to those for zinc, have been especially challenging to generate because of nitric oxide's gaseous nature, complex chemistry, limited water solubility, and diverse biological concentrations (33). Promising fluorescent probes, as discussed by Lippard and colleagues (16), include organic probes in which the oxidation product of nitric oxide reacts with a functional group to modulate its fluorescence. It is also possible to use transition-metal-mediated nitric oxide reactivity for nitric oxide sensing. In addition, numerous constructs have been devised that use genetic encoding of nitric oxide–reactive proteins. These often contain transition-metal nitric oxide–reactive sites. An example is a fusion of two mutant GFPs joined by a sequence that functions as a nitric oxide indicator. The FRET response between the mutated GFPs in response to the conformational change in the nitric oxide indicator upon reaction with nitric oxide provides a visible readout.

The fluorescent probes for biological zinc and nitric oxide are helping to clarify the multiple pathways in which these inorganic species are interconnected in biology. Because of zinc/nitric oxide synergism, the neuronal, cardiovascular, and immune systems have all benefited from such probes. Zinc accumulates in axons, perikara, and interneurons under various stimuli, and treatment of neurons with high concentrations of exogenous nitric oxide leads to Zn(II) release from intracellular stores (34, 35). To clarify how this cross talk occurs, Lippard and colleagues (16) recommend more sensitive and selective probes for zinc and

nitric oxide in a palette of colors. Ratiometric probes that span a wide range of zinc affinities with large dynamic ranges are also needed, as are probes that detect nitric oxide reversibly.

The reviews in this volume from the labs of Lippard, Miyawaki, and Zhang clearly illustrate the value of FP and sensors in providing a comprehensive understanding of biological phenomena. Describing emerging reporter technologies for characterization of native biochemistry, the reviews are tantalizing reminders that real-time visualization of a wide range of biochemical processes in living systems is now a reality and offers a means to obtain unique insights into the spatial organization and dynamic regulation of intracellular signaling networks underlying biological processes.

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