BIOCHEMICAL IMAGING

Seeing the Machinery of Live Cells

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TECH VIEW

As biochemistry and genetics uncover more of the molecules responsible for life, we need to see how these molecules interact with each other in space and time as they orchestrate cellular responses. Observation of fluorescently labeled macromolecules in live cells can reveal such dynamics, both for basic studies on how cells process signals and for high-throughput screening of candidate drugs.

Live-cell imaging has received its strongest recent impetus from the development of the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* as a fluorescent label that can be incorporated into proteins by genetic fusion (*1*). The simplest way that GFP can signal physiological activation is when it is concatenated to a host protein that translocates from one cellular compartment to another upon stimulation. For example, GFP fused to glucocorticoid receptor (*2*) translocates from cytosol to nucleus upon hormone addition. GFP fusions to β-arrestin (*3*), the Cys1 domain from protein kinase C-γ (*4*), and the pleckstrin homology domain of phospholipase C-γ (*5*) move to the plasma membrane upon G-protein activation, diacylglycerol formation, and phosphatidylinositol-3,4,5-trisphosphate formation, respectively.

GFP-based indicators whose spectral properties reflect their chemical environment have also been developed. GFP mutants that reversibly lose their fluorescence upon acidification to pH 5 or 6 can be targeted to mildly acidic compartments such as Golgi or secretory vesicles, where they report local pH (*6*, *7*). A GFP spliced into a voltage-sensitive potassium channel (*8*) is a potentiometric sensor of presently modest performance but tremendous promise.

The above constructs in which a single GFP reports its conformation or environment are relatively compact and simple but have two major limitations: the readout is a simple change in intensity, which is subject to many measurement artifacts, and the sensors are hard to design rationally, because it cannot yet be predicted which stresses and strains will affect the fluorophore inside the protein shell. Another approach is to use two GFPs of different colors, chosen to permit fluorescence resonance energy transfer (FRET) from the shorter to the longer wavelength GFP. FRET is highly sensitive to the relative orientation and distance on nanometer scales between the two fluorophores and alters the ratio of their emission intensities, an ideal readout for fast imaging, flow cytometry, and confocal microscopy (*9*). GFPs linked via protease-sensitive substrate sequences exhibit FRET until the linker is proteolyzed (*10*, *11*). GFPs linked via a flexible peptide spacer from myosin light-chain kinase similarly lose FRET when Ca^{2+}-calmodulin binds to the spacer and stiffens it into an extended rod that increases the distance between the GFPs (*12*, *13*). This conformational change is an elegant way to monitor levels of endogenous activated calmodulin. Another way to concatenate the same four components is donor-calmodulin–peptide-acceptor. In this case, Ca^{2+} binding to the built-in calmodulin causes it to complex intramolecularly with the peptide, decreasing the distance between the GFPs at the amino- and carboxyl-termini and increasing FRET (*14*). Because the calmodulin and its target peptide are already fused together and preferentially interact with each other intramolecularly, the construct senses Ca^{2+} without interfering with or being perturbed by excess calmodulin or calmodulin-binding proteins (*15*). If the calmodulin-cyan GFP fusion is left unlinked to the peptide-yellow GFP fusion, the resulting model for detecting intermolecular heterodimerization (Fig. 1) gives a larger FRET change upon elevation of Ca^{2+}, but is also affected by the relative expression levels of the labeled and unlabeled partners.

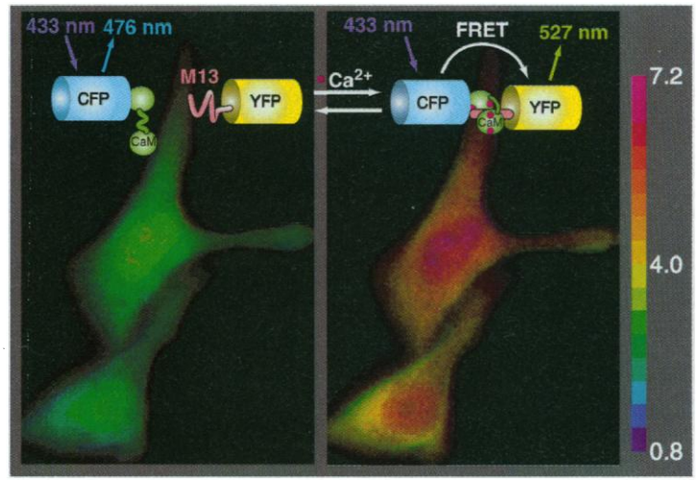

Fig. 1. Imaging by FRET of induced protein-protein interaction in individual live cells. Cyan-GFP-labeled calmodulin (CFP-CaM) and yellow-GFP-labeled calmodulin-binding peptide (M13-YFP) were coexpressed in HeLa cells (*14*). Pseudocolor hues from blue to magenta indicate increasing ratios of yellow to cyan emissions, resulting either from increased FRET (see overlaid schematic) or excess of YFP over CFP. The left panel shows two cells before stimulation, while the right panel shows the same cells after elevation of cytosolic Ca^{2+} by 0.1 mM histamine.

The very smallest protein tag would be a single unnatural amino acid, incorporated by synthesizing an aminoacylated transfer RNA that can suppress a nonsense codon engineered into the target gene (*16*, *17*). Currently, the main limitations on this elegant technique are the difficult synthesis of the aminoacylated transfer RNA, the need to microinject this molecule into single cells such as oocytes, and the imperfect suppression efficiency. However, much progress is being made toward overcoming these problems (*18*).

Much larger labels include the phytofluors, which are plant apophytochromes loaded with phycoerythrobilin instead of the normal phytochromobilin. So far, the smallest useful protein fragment has 514 amino acids. In situ reconstitution with exogenous phycoerythrobilin works well in yeast and plant cells (*19*) to yield fluorescence at 580-nm wavelength. Single-chain antibodies constitute another possible ligand-binding domain, whose size (25 kD) is comparable to GFP. Such antibodies expressed in endoplasmic reticulum or Golgi can reversibly bind fluorescein-labeled haptens added extracellularly and serve as an alternative to targeted GFPs for measurement of organellar pH (*20*).

So far, the smallest taggable domain built from natural amino acids consists of the six residues –Cys–Cys–Xaa–Xaa–Cys–Cys–, in which Xaa is any amino acid and the four cysteines must not be oxidized or metal bonded. This tiny domain can either be spliced into an existing α-helix or appended to the

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host protein as part of a short alanine-rich helix. It is recognized in situ by 4′,5′-bis(1,3,2-dithiarsolan-2-yl)fluorescein, a fluorescein carrying two arsenic substituents. This membrane-permeant label is nonfluorescent until it finds the tetracysteine domain, whereupon each arsenic grabs a pair of cysteines and the rigidly bound dye becomes highly fluorescent. The labeling works when the dye is administered extracellularly to transfected mammalian or bacterial cells, together with micromolar concentrations of 1,2-ethanedithiol to prevent arsenic toxicity and minimize background staining, and is largely reversible by millimolar concentrations of dithiol (*21*).

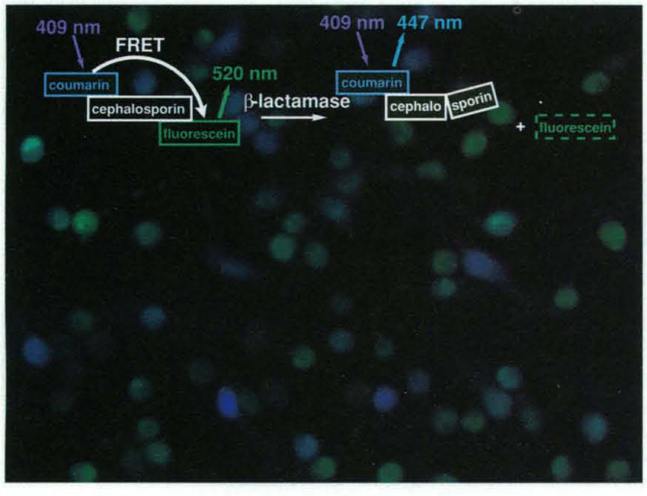

Fig. 2. Imaging of reporter gene expression in individual live cells. Rat basophilic leukemia cells were transfected with a β-lactamase reporter gene construct and subcloned by fluorescence-activated cell sorting for maximal responsiveness to cytosolic Ca^{2+}. Artificial Ca^{2+} oscillations were then produced by repetitive uncaging of an inositol-1,4,5-trisphosphate analog (*23*). After waiting 3.5 hours to allow β-lactamase expression, the cells were loaded with a membrane-permeant fluorogenic substrate (*22*). Cells not expressing β-lactamase fluoresce green due to FRET from the coumarin to the fluorescein, whereas cells containing β-lactamase fluoresce blue because the enzyme cleaves the cephalosporin linker holding the two fluorophores in proximity (see overlaid schematic).

Protein labeling is not the only area where molecular biology and small fluorophores can usefully synergize. GFP is a relatively slow and insensitive reporter of gene expression, because it lacks enzymatic amplification. A promising alternative is β-lactamase, the bacterial enzyme that cleaves penicillins and cephalosporins. New membrane-permeant fluorogenic substrates are cleaved by β-lactamase and change their emission color from green to blue because of disruption of intramolecular FRET (*22*). Gene expression can now be sensitively imaged at the single-cell level in large populations of nonpermeabilized mammalian cells (Fig. 2) at expression levels several orders of magnitude lower than those required for GFP. Fluorescence-activated cell sorting allows rapid isolation and subcloning of cells with optimal or unusual expression patterns (*22*, *23*).

Spectroscopic monitoring of live-cell biochemistry with designed molecules should continue to expand in at least four directions:

1) *More target analytes and macromolecules*. Small molecules can be made detectable by tagging their native receptors to monitor the trafficking, contacts, and conformational changes in the receptors. Can FRET be used to fish out and image novel protein interactions, analogous to coimmunoprecipation or the two-hybrid screen? The current problem with FRET for this challenging task is that spectral cross-talk between the donor and acceptor, together with uncertainties in relative expression level, produce a high and variable background even in the absence of true protein interaction (*14*).

2) *More readout modes and chemical versatility*. Site-specific attachment of small molecules to proteins should permit assessment of local changes in conformation, polarity, and rotational mobility of the host protein domain. Incorporation of chromophores that photochemically generate singlet oxygen permits local photoconversion of diaminobenzidine into an osmophilic polymer for electron microscopic visualization of the chromophores (*24*). Likewise, photochemical generation of hydroxyl radical should enable rapid ablation of the labeled protein, a knockout with high temporal and spatial precision (*25*). The same attachment technologies should aid protein purification, immobilization, and crosslinking.

3) *Subcellular and submicroscopic localization*. The ability to target indicators by molecular biology means that the effective spatial resolution for biochemical imaging is no longer limited by the resolution of the microscope. For example, if Ca^{2+} indicators can be accurately targeted to the mouth of Ca^{2+} channels or to synaptic active zones by appropriate fusions, one should be able to monitor the local Ca^{2+} within nanometers of those privileged locations, even if the resolution of the optical microscope is hundreds of times worse.

4) *Transgenic organisms*. Transfectable indicators should permit in vivo imaging of molecular processes even in organisms previously unsuited to physiological recording. But how will we see inside tissues or organisms too thick or opaque for conventional fluorescence imaging? Multiphoton excitation may help in some cases, because the excited state is produced by infrared light, which penetrates much further than ultraviolet or visible photons, and it tolerates scattering of emitted photons. Fortunately, GFPs are good fluorophores for multiphoton excitation (*26*). Chemiluminescence can also give images from live animals, albeit with lower spatial and temporal resolution (*27*, *28*). Detailed views deeper than a few millimeters into tissue may require techniques such as magnetic resonance imaging together with magnetically detectable synthetic molecules that mate with specific protein domains in vivo.

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