Recent advances in technology for measuring and manipulating cell signals

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Signal transduction research has made some glowing progress in the past 12 months. Recent advances in fluorescent proteins, small molecule fluorophores and imaging technology are generating new ways to investigate signal transduction.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
BFP	blue fluorescent protein (a mutant of GFP)
BRET	bioluminescence resonance energy transfer
CaM	calcium/calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CFP	cyan fluorescent protein (a mutant of GFP)
cp	circular permutation
FP	fluorescent protein
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
IP_3	inositol-1,4,5-trisphosphate
LRET	luminescence resonance energy transfer
NMDA	*N*-methyl-D-aspartate
PHD	pleckstrin-homology domain
PK	protein kinase
YFP	yellow fluorescent protein (a mutant of GFP)

Introduction

Progress in understanding signal transduction, especially in complex tissues such as those found in the central nervous system (CNS), depends increasingly on decoding the spatial localization and temporal dynamics of intra- and extracellular signals. We need both to image endogenous fluctuations in messenger concentrations and to mimic or suppress such signals in order to determine whether they are sufficient and/or necessary for a biological response. The most generally applicable and popular techniques with a high spatiotemporal resolution are optical in nature. Fluorescent indicators typically report messenger concentrations by alterations in the amplitude or wavelength distribution of their excitation or emission spectra — in this way, biochemistry affects the light output of the fluorescent indicator. Conversely, caged compounds photochemically release messengers or chelators upon light absorption — therefore, light input can control biochemistry. In this review, we will focus mainly on recent advances in the fluorescence and uncaging techniques that have been used to image and manipulate signal transduction at the level of single cells; such techniques may also have the flexibility to work inside thick tissues or eventually whole organisms.

Fluorescent proteins

In the last few years, perhaps the biggest advance in technology for the optical imaging of signal transduction pathways has been the introduction of the green fluorescent protein (GFP) from the jellyfish *Aequorea*, which enables genetic encoding of strong visible fluorescence. Over the last several years, both random and semirational mutagenesis have produced GFP variants with new colors, improved folding, extinction coefficient (the strength of light absorption per molecule), quantum yield (the proportion of absorbed light that ends up as fluorescence), and/or altered pH sensitivity [1]. Through genetic manipulations, hundreds of proteins have been fused to GFPs to allow monitoring of their expression and trafficking. Currently, there are three general approaches by which GFP fusions can report rapid intracellular dynamics: translocation, biochemical modulation of GFP spectra, and fluorescence resonance energy transfer (FRET).

Translocation

Many signaling proteins or motifs translocate from one compartment of the cell to another as part of their activation mechanism. The most common and rapid transitions are between the plasma membrane and the adjacent cytosol. Four recent examples of this type of translocation will be discussed. First, the activation and deactivation of protein kinase C βII, G-protein-coupled receptor kinase, and β-arrestin can be monitored by the translocation of GFP fusions to and from the plasma membrane in response to substance P [2]. Second, a GFP-tagged pleckstrin-homology domain (PHD) from phospholipase C-δ1 adheres to phosphatidylinositol-4,5-bisphosphate (PIP_2) on the plasma membrane but detaches when that lipid is metabolized to inositol-1,4,5-trisphosphate (IP_3). Because detachment can be evoked by microinjection of IP_3 and prevented by overexpression of IP_3-phosphatase, translocation seems to report IP_3 levels rather than PIP_2 breakdown. If so, this GFP–PHD is the first fluorescent indicator for intracellular IP_3 dynamics [3••]. Oscillations and spatial waves in GFP–PHD translocation indicate that IP_3 levels oscillate and propagate in synchrony with the cytosolic Ca^{2+} increases, contravening the widespread dogma that receptor-stimulated Ca^{2+} oscillations and waves are mediated by monotonic IP_3 elevations. Third, in cultured neurons, cytosolic Ca^{2+} transients cause reversible translocation of GFP-tagged calcium/calmodulin-dependent protein kinase II (CaMKII) to postsynaptic densities. Such translocation is controlled by many factors including autophosphorylation, calcium/calmodulin (CaM) binding, and F-actin affinity [4]. Fourth, GFP tagging has been used to monitor the translocation of AMPA receptors from intracellular membranes to the surface of dendritic spines and into clusters in dendrites in organotypic slice cultures.

This migration is evoked by tetanic stimulation and requires synaptic activation of NMDA receptors. Modulation of AMPA receptor content at synapses in this manner may account for why some synapses are 'silent' and how they become potentiated [5•].

For proteins that translocate as part of their activation mechanism, imaging of GFP fusions is a simple and apparently rather reliable assay, which often reveals a much faster and more complete migration than was previously suspected from cell fractionations and immunocytochemistry. However, some cautions and limitations are worth noting. High spatial resolution is required, so nearly all studies to date have been performed in thin, cultured cells examined in small numbers. The quantitative extent of translocation may vary with levels of fusion protein expression and imaging resolution. Response kinetics may be limited in some cases by the time required for the engineered protein to diffuse to the membrane, rather than the intrinsic biochemistry. Potential perturbations resulting from the GFP tag itself are difficult to assess because there are so few other methods for comparison. In one relatively carefully conducted comparison, live-cell imaging of GFP–CaM not only confirmed the results of previous immunolocalization studies of endogenous calmodulin, but also revealed additional localization of calmodulin to the plasma membrane or cortical regions adjacent to the cleavage furrow during mitosis [6].

Biochemical modulation of GFP spectra

Even though the fluorophore of GFP is deeply buried within an apparently rigid cylindrical shell, a surprising number of biochemical parameters can modulate the fluorescence of GFP mutants [1]. (We restrict our discussion to spectroscopic effects on mature GFPs, rather than alterations of the amount of GFP through modifications of transcription, translation, or degradation.) The fluorescence of many yellow mutants of GFP (YFPs) is quenched by acidic pH, with apparent pK_as (the pH at which 50% of the fluorophores in a sample of GFP are protonated) ranging from 5 to 8. The pK_as of some YFPs are further modulated by concentrations of certain anions, of which Cl^- is the most physiologically relevant [7]. These proteins have now been used in live cells to assay Cl^- ion transporters such as the cystic fibrosis transmembrane conductance regulator (CFTR), defects in which result in cystic fibrosis [8•].

A more flexible and general strategy for making GFP sensitive to other influences is either to insert GFP inside other proteins, or to insert other proteins into GFP. Insertion of wild-type GFP into a particular location within the *Shaker* K^+ channel resulted in a chimera whose fluorescence decreased by a few percent upon depolarization with a slow time course comparable to that of C-type inactivation of K^+ channels [9]. Conversely, insertion of β-lactamase into position 172 of GFP produced a fusion whose fluorescence increased roughly 1.6-fold on binding β-lactamase inhibitory peptide (BLIP) [10]. In two other examples, either the calcium-binding protein calmodulin or a zinc-finger motif from zif268 was inserted in place of Tyr145 of YFP [11••]. These chimeric proteins increased in fluorescence nearly 8-fold or 1.7-fold, upon binding either Ca^{2+} or Zn^{2+}, respectively. The mechanism for the fluorescence enhancement was that the metal ion decreased the pK_a of the YFP, so that at appropriate intermediate pH values, metal binding mimicked alkalinification in enhancing chromophore deprotonation and fluorescence. The YFP with CaM insertion ("Camgaroo") was functional as an intracellular Ca^{2+} indicator that gave desirably large fluorescence changes inside HeLa cells; however, it required relatively high intracellular Ca^{2+}, was vulnerable to pH interference, and lacked a second wavelength for ratiometric readout [11••]. Other insertions of peptides into GFP have been shown to be fluorescent [12], but dynamic modulation of GFP properties by the inserts was not reported.

Another way to demonstrate and exploit the surprising tolerance of GFPs to modular rearrangements is circular permutation — in other words, the use of molecular biological techniques to encode and express proteins whose natural amino- and carboxyl-termini are linked via a spacer while new amino- and carboxyl-termini are created elsewhere in their primary sequence. Circularly permuted GFPs (cpGFPs) were made by screening libraries of random permutants [11••] and by design [13], which together have found 17 locations at which new termini could be created while retaining fluorescence. In almost all cases, cpGFPs are less stable and more pH sensitive than standard GFP, so cpGFPs will not replace normal GFPs in standard applications. However, there are three niches in which cpGFPs may be advantageous [11••]. First, since cpGFPs have different amino- and carboxyl-termini from standard GFPs, they offer a new way to fuse fluorescent proteins to other proteins. cpGFPs might, therefore, allow one to make a functional fluorescent fusion protein if a standard GFP fusion protein is not functional. Second, circular permutation changes the orientation of the GFP chromophore relative to the fusion partner. Therefore, if a particular GFP-fusion protein is lacking in fluorescence resonance energy transfer (FRET; see next section) to or from another fluorophore, a cpGFP might be worth trying in place of the normal GFP. Third, inserting cpGFPs into a conformationally flexible protein might confer the same type of fluorescence sensitivity observed when proteins are inserted inside GFP. The topologies of these two fusion strategies are similar, as the GFP is linked through its former midsection to the foreign protein. This might allow fluorescence-sensing of structural changes in proteins such as transmembrane proteins whose amino- and carboxyl-termini are too far apart from each other to be inserted inside a GFP.

Fluorescence resonance energy transfer (FRET)

FRET is a quantum-mechanical phenomenon of radiationless energy transfer between two fluorophores. It is

dependent on the proper spectral overlap of the donor and acceptor (i.e. the emission spectrum of the donor should overlap sufficiently with the excitation spectrum of the acceptor, but the excitation spectra of the donor and acceptor should not overlap excessively, and the emission spectra of the donor and acceptor should also not overlap excessively), their distance from each other, and the relative orientation of the chromophores' transition dipoles [14]. The efficiency of FRET falls off as the inverse sixth power of distance beyond a few nanometers, so measurements of FRET monitor proximity over typical macromolecular dimensions about two orders of magnitude closer than colocalization via standard optical imaging resolution. FRET is detectable by quenching of the donor emission, sensitized emission from the acceptor, decreased excited-state lifetime, or increased resistance to bleaching of the donor. Within the known GFP mutants, the best pair for FRET comprises the cyan and yellow mutants CFP and YFP [1], because the initial pair, blue mutant fluorescent protein (BFP) and GFP, suffers from the relatively poor brightness and photostability of the BFP. FRET has become a very useful tool enabling one to measure intermolecular interactions between pairs of host proteins fused to two spectral mutants of GFP, or intramolecular conformational changes in single proteins bracketed by the two GFPs.

Tandem fusions of CFP, CaM, a CaM-binding peptide, and YFP constitute genetically encoded Ca^{2+} indicators in which Ca^{2+}-dependent binding of CaM to the target peptide increases FRET from CFP to YFP. Such molecules ("cameleons") were recently improved by reducing the pH-sensitivity of the YFP [15]. The molecules were verified to be rather indifferent to competing CaM-sensitive target enzymes, presumably because the $(Ca^{2+})_4$–CaM prefers to interact with the peptide already fused to it. Cameleons were shown [16] to be quite suitable for two-photon excitation, an elegant form of laser illumination in which pairs of infrared photons pool their energy to excite fluorophores that would normally require ultraviolet or blue photons for their excitation. Because two-photon excitation is a nonlinear optical effect that requires extremely high photon fluxes, it occurs preferentially at the focus of a laser beam rather than where the beam is out-of-focus. Two-photon excitation inherently confers optical sectioning properties without the use of an observation pinhole as in confocal microscopy, and it is particularly advantageous in thick scattering tissues such as the CNS. Therefore, genetically targetable indicators are complementary to multiphoton excitation because the genetic encoding allows the indicator to be expressed at virtually any location or depth within intact organs or animals, while the optical technique greatly increases the depth from which useful signals can be recovered. Cameleons were targeted to the cytosolic surface of secretory granules in PC12 cells and MIN6 pancreatic beta cells by fusion to phogrin, a vesicle protein [17]. Granules next to the plasma membrane showed somewhat larger Ca^{2+} responses than those by deeper granules or untargeted cameleons, though all the optical responses were significantly smaller than expected from the previously reported properties of cameleons *in vitro* or in HeLa cells.

If the GFP mutants are linked by just the CaM-binding peptide, then binding of unlabeled $(Ca^{2+})_4$–CaM straightens the peptide and decreases FRET. When the previously used BFP and GFP are replaced by the superior CFP–YFP pair, the improved chimeras give adequate signals in stably transfected cells and permit measurements of endogenous levels of free $(Ca^{2+})_4$–CaM as a function of free Ca^{2+} [18•].

A first-generation transfectable indicator has just been reported [19•] for cAMP, the most ubiquitous second messenger other than Ca^{2+}. The type IIβ isoform of the regulatory subunit of cAMP-dependent protein kinase (PKA) was fused to BFP while the catalytic subunit of PKA was fused to GFP. When these constructs are cotransfected into cells, significant FRET is observed, which is reversibly disrupted by elevation of cAMP as expected. In this study, however, rapid bleaching of the BFP was a major problem, so analogous constructs with CFP and YFP are being developed.

Other the past year, several reports exploiting FRET from CFP to YFP have been published; these include the following four. First, a demonstration has been made in live CHO cells of static binding of the type IIα isoform of the regulatory subunit of PKA to a peptide from an A-kinase anchoring protein [20]. Second, detection of caspase-1 and caspase-3 activation has been performed in living COS-7 cells by cleavage of tetrapeptide linkers YVAD and DEVD (single-letter amino acid codes) linking CFP and YFP [21]. Third, ATP- and microtubule-dependent oligomerization of katanin, a microtubule-severing ATPase, has been studied *in vitro* [22]. Fourth, Zn^{2+}- and nitric oxide-dependent conformational changes in the metal-binding protein metallothionein have been demonstrated in pulmonary artery endothelial cells [23].

Resonance energy transfer other than FRET between GFP mutants

PKC autophosphorylation monitored by FRET to Cy5

Resonance energy transfer is by no means limited to two GFPs as donor and acceptor. For example, activation of protein kinase Cα eventually results in autophosphorylation of Thr250, for which a phosphospecific antibody has been generated. Such autophosphorylation could be detected in live COS-7 cells by FRET from a GFP that has been fused to the PKC, to a Cy3.5 label on the microinjected antibody. Autophosphorylation of endogenous PKCα has also been detected in fixed tissue sections (including clinical pathology specimens) by FRET from a Cy3-labeled antibody bound elsewhere on PKCα to a Cy5 on the phosphospecific antibody. In this case, FRET had to reach across three intervening proteins. Because the phosphospecific antibody had to be used in large and variable excess, FRET was best detected not by the ratio of donor and acceptor emissions but by the decreased excited-state lifetime of the donor [24••].

Bioluminescence resonance energy transfer

The natural function of GFP in the jellyfish is generally believed to be as an acceptor of bioluminescence resonance energy transfer (BRET) from the Ca^{2+}-triggered photoprotein aequorin. BRET has been used to determine the kinetics of homodimerization of the circadian clock protein KaiB in cyanobacteria that are naturally responsive to light and thereby unsuited to FRET, which requires strong visible excitation. The fused donor was *Renilla* luciferase rather than aequorin, to avoid any intrinsic affinity for an *Aequorea*-derived GFP mutant; the acceptor was YFP, to increase the spectral distinction between the two emissions [25•]. Apart from the greatly reduced light levels, which are helpful during the study of photosensitive systems, the main advantage of BRET over FRET is the lack of emission arising from direct excitation of the acceptor, or autofluorescence. This reduction in background should permit detection of interacting proteins at much lower concentrations than are required for FRET. However, BRET requires the addition of a cofactor, in this case coelenterazine. Also, the low number of photons available from each bioluminescent molecule means that a greater total number of molecules, or a greater integration time, will be required for BRET than for FRET for equal numbers of photons to be collected. Thus, BRET is most advantageous for detecting very slow changes in proteins at low concentrations in large homogenous populations of light-sensitive cells, while FRET is favored for faster observations of proteins at higher concentrations in light-tolerant single cells or in high-resolution imaging. It is also not yet clear how easy it will be to extend the BRET system to mammalian cells.

Voltage-dependent FRET

Membrane potentials can be monitored by staining the plasma membrane with two fluorophores, one of which binds to one face of the membrane and cannot cross to the other side, the other of which is charged yet hydrophobic enough to translocate rapidly across the membrane in response to membrane potential. FRET from one dye to the other is stronger when both dyes are on the same rather than on opposite sides of the bilayer. Dye loading conditions require more optimization than for conventional voltage-sensitive dyes because two separate hydrophobic substances have to be delivered to the same membrane at an approximately optimal stoichiometry; however, the benefit is a ratiometric output that can be considerably more sensitive than previous sensors. This assay has been very valuable in the pharmaceutical industry for high-throughput screening for modulators of ion channels and transporters [26]. The first application to neurons in an intact behaving circuit is the detection of the phase-locking of known, and possibly novel, neuronal participants in fictive swimming in leech ganglia [27].

Channel conformations seen by LRET

Conformational reorganization of the voltage-sensing region of the *Shaker* K^+ channel in response to depolarization has been elegantly quantified by analysis of luminescence resonance energy transfer (LRET) from terbium chelates to fluorescein on different subunits of the K^+ channel homotetramer [28••]. By analysis of the terbium excited-state lifetimes, distances both between adjacent subunits and diagonally across the central pore could be resolved as a function of labeling site. Depolarization caused relatively small shifts consistent with rotation of the voltage-sensing helices about an axis roughly perpendicular to the membrane, rather than major translations along such an axis.

Red fluorescent protein

A genetically encodable red fluorescent protein would be extremely useful to minimize interference from autofluorescence and scattering, to provide a new color maximally contrasting with the native hue of GFP, and to serve as a FRET, LRET, or BRET acceptor from GFP, terbium chelates, or firefly luciferase respectively. The recent cloning of a family of new fluorescent proteins from non-bioluminescent anthozoan corals, especially a red variant from the coral *Discosoma*, was a spectacular breakthrough [29••]. The excitation and emission spectra of the red FP (called dsRed commercially, see http://www.clontech.com/archive/OCT99UPD/RFP.html) peak at 558 and 583 nm respectively, giving it an appearance very similar to rhodamine dyes. The primary sequence has only <30% identity to *Aequorea* GFP, but enough residues were conserved to permit its discovery by PCR and to suggest very strongly that the overall protein fold is preserved. The physical basis for the red shift awaits crystallographic structure determination. Although the published extinction coefficient and quantum yield are rather low, the more significant problem for most applications will be the lengthy time-constant for development of the mature red color, which can be of the order of days (GS Baird, unpublished observations). One hopes that mutagenesis will solve this problem in the same way that *Aequorea* GFP has been so drastically altered and improved.

Caged compounds

Beyond the mere monitoring of signal transduction pathways lies the need to control those intermediate signals to determine if they are necessary or sufficient for the biological response. Caged compounds, in which an active molecule is rendered biologically inert by a photolabile group until a flash of light is delivered, offer the highest temporal, spatial, and amplitude control of the desired perturbation. Even a molecule as complex as plasmid DNA encoding GFP or luciferase can be caged with DMNPE (1-[4,5-dimethoxy-2-nitrophenyl]ethyl) groups, which block transcription of the gene until photolyzed. The level of reporter-gene expression can be regulated by the amount of 355 nm irradiation [30]. Precise temporal and spatial control of gene expression will be a very powerful tool in many areas of biology, especially in neurobiology.

Until recently, almost all caged compounds were sensitive only to ultraviolet photons. As in multiphoton excitation

of fluorophores, uncaging using pairs of infrared photons would confer much greater three-dimensional spatial localization, and would reduce photodamage to neighboring tissue because photolysis would be confined to the focus rather than the entire double cone of the pulsed laser beam. The use of infra-red instead of ultraviolet photons would also permit much deeper penetration into scattering tissue. However, except for azid-1, a chelator that cages Ca^{2+} [31], all the common caging groups such as DMNPE are very insensitive to two-photon infra-red photolysis. Recently a new caging group, brominated 7-hydroxycoumarin-4-ylmethanol (Bhc), was shown to cage carboxylates, phosphates, and amines with high sensitivity to both one- and two-photon photolysis. Two-photon uncaging of Bhc-caged glutamate was used to make the first three-dimensionally resolved maps of the glutamate sensitivity of neurons in intact brain slices [32•]. This same synthetic strategy has been extended to cage other neurotransmitters such as GABA and messengers such as cAMP and cGMP (T Furuta, TM Dore, RY Tsien, unpublished data).

Conclusions

The ability to target genetically-encoded biosensors based on GFP to virtually any site within a cell has proven to be an extremely powerful tool, and has led to many interesting discoveries. The recent discovery of new fluorescent proteins including red emitters may expand these current applications. Likewise, small molecule fluorophores, sensors, and cages continue to evolve and fill new and interesting niches, particularly for the many roles that fluorescent proteins still cannot fill. Thus, optical technologies continue to have a bright and expanding future in studies of signal transduction.

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Bhc is the first caging group with the following properties: a high sensitivity to both one- and two-photon photolysis; solubility in aqueous solutions; and the ability to cage several classes of biologically important chemical including neurotransmitters and many second messengers. Two-photon photolysis has enabled mapping of glutamate sensitivity with optical sectioning resolution over the surface of neurons in brain slices.