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Shining light on signaling and metabolic networks by genetically encoded biosensors

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Abstract

Fluorescent labels have revolutionized cell biology. Signaling intermediates and metabolites can be measured in real time with subcellular spatial resolution. Most of these sensors are based on fluorescent proteins, and many report fluorescence resonance energy transfer. Because the biosensors are genetically encoded, a toolbox for addressing cell biological questions at the systems level is now available. Fluorescent biosensors are able to determine the localization of proteins and their dynamics, to reveal the cellular and subcellular localization of the respective interactions and activities, and to provide complementary data on the steady state levels of ions, metabolites, and signaling intermediates with high temporal and spatial resolution. They represent the basis for cell-based high-throughput assays that are necessary for a systems perspective on plant cell function.

Introduction

Primary goals of the post-genomic era are the assignment of functions to each of the genes encoded by a given genome, and their integration into metabolic and regulatory networks. While transcriptomics and proteomics are progressing rapidly, the collection of other essential information for building these network maps — the mapping of protein activities and of the small molecule intermediates and ions they act on — is being developed. Analyses that are based on mass spectrometry provide a glimpse of the total metabolite and ionic inventory, but lack the temporal and spatial resolution that are necessary to provide local concentrations and flux rates *in vivo*. Classical biochemical approaches are very useful in quantifying metabolite levels and in determining when a protein is made and where a protein might reside in the cell at any given time. However, these approaches are extremely limited in their ability to resolve where and when a protein is active, or when and where it interacts with other proteins or with their substrates. Such detailed spatial and temporal information is essential for building realistic network maps, particularly as proteins, metabolites and ions might simultaneously fulfill diverse roles in multiple pathways, requiring the tight control of the timing and location of their activity [1•]. Understanding homeostasis, metabolic regulation, and cellular signaling thus requires novel concepts and technologies.

Fluorescent ligands and proteins have revolutionized bioimaging, and comprise a new set of tools for addressing cell biological questions at the systems biology level. The ability to genetically encode fluorophores, such as the *Aequorea victoria* green fluorescent protein (GFP), has yielded significant advantages for *in vivo* studies. These include the capacity to introduce probes into a wide variety of organisms and/or cell types, to control the timing and level of probe expression, and to target probes to specific cellular compartments. Perhaps most

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importantly, the genetic encoding of optical probes enables methods for high-throughput analysis of protein and cell function [2••]. The development of a large number of novel fluorescent proteins (FPs), which might be derived from corals and anemones or generated by mutagenesis, has accelerated the development of the fluorophore tool kit by providing the researcher with a selection of spectral variants and fluorophores that have useful properties such as photoconversion [3–9].

Genetically encoded fluorophores offer several types of tools that can be used to probe molecular behavior in living cells. As simple fusion tags, FPs can help to identify protein localization and dynamics. Split FPs can be used to test for protein–protein interactions *in vivo* (Figure 1) and to create small peptide tags. Peptides that translocate in response to cellular signals and modification can be used to assay metabolites and protein activity [10••]. Finally, the (*in vivo*) combination of FPs with a second fluorophore provides novel applications that rely on fluorescence resonance energy transfer (FRET). FRET detects rearrangements of the relative orientation and distance of two fluorophores within the 1–10 nm range, thus providing extraordinary spatial resolution and vast possibilities for measuring protein–protein interactions or the creation of small molecule biosensors. Here, we review recent advances in the development and application of genetically encoded biosensors, and discuss how they might be applied to extend our understanding of metabolic and regulatory networks through novel high-throughput analyses.

High-resolution imaging of the dynamic distribution of proteins

Given the organization of protein functions into large complexes, the degree of cellular compartmentation of metabolism, and the many compartments in plant cells, we need to determine the subcellular localization and dynamics of each protein as a first step toward understanding functional networks. Such analyses are made possible in living cells, and at unprecedented spatial and temporal resolution, by combining genetically encodable fluorescent tags with advanced imaging tools such as Nipkow spinning disc or multi-photon confocal microscopy. Because these techniques are applicable in living cells, it is possible not only to probe the localization of a protein but also to assay the kinetics of protein and protein-complex formation and degradation, such as the treadmilling of cytoskeletal proteins [11] and protein secretion and turnover [12]. Refinement of protein localization is aided by FP spectral variants that permit co-localization to the subdomains of organelles [13], and by the use of FPs with variant pH sensitivity to simplify imaging of the targeting, turnover, or translocation of proteins that are found in more than one cell compartment, such as transporters and signaling proteins [14,15••]. High-throughput studies of protein expression and localization that utilize fusions of FPs to full-length proteins have been performed in yeast and animal cells and have recently been initiated in plants [16].

Despite its many advantages, the use of GFP as a tag for protein localization also has shortcomings, the most significant being the large size of the tag, which might affect the folding, solubility, function or interactions of the protein. Smaller fluorescent dyes (e.g. Fluorescein Arsenical Helical binder [FIAsh], Resorufin Arsenical Helical binder [ReAsH], merocyanines and aminophenoxazine maleimide) can be added via chemical coupling or, *in vivo*, via small tetra-cysteine tags [1••,17,18]. However, the necessity of a second component, and their permeability and availability to the cellular compartment could limit their application. Self-associating fragments of GFP have been developed as an alternative to labeling by chemical dyes [19••]. A small peptide consisting of the carboxy-terminal amino acids of GFP serves as a protein tag that confers fluorescence when co-expressed with a modified peptide that provides the other ten β -strands of the GFP.

The cellular localization of protein–protein interactions

The above-mentioned co-localization studies hint at physical interactions but direct proof for complexes is needed. Although interactions between proteins are identified systematically using yeast two-hybrid systems [20,21], spatio-temporal information and modifications that are present in the native system are not addressed. FPs enable methods to probe stable and transient protein interactions *in situ*. For example, FPs can be split into two halves and fused to potential interaction partners. In contrast to the FP-fragment tag described above, these free FP halves do not readily associate (Figure 1). However, when brought into close proximity by interaction between their fusion partners *in planta*, a functional FP is reconstituted [22–24]. Use of FP fragments from spectral variants allows the relative affinities of multiple potential interaction partners to be measured [25•]. This method is very sensitive, but the quasi-irreversible reconstitution of GFP is unsuitable for analyses of complex disassembly [22].

Protein interactions can also be detected by FRET between two spectral FP variants [26–28] or between an FP and a chemical dye [1•,17]. FRET is defined by non-radiative transfer of energy from a donor molecule to an acceptor fluorophore [29]. Candidates are fused to fluorophores that have overlapping emission and excitation spectra. The relative emission of the acceptor is measured upon donor excitation. When the fusion partners interact, the acceptor fluorophore is brought into vicinity of the donor and the acceptor is excited by resonance energy transfer. The interaction is detected by increased emission of the acceptor, concomitant with a decrease in donor emission. Various techniques beyond the basic analysis of the relative emission ratio are available for reliable determination of FRET changes [29]. Recent improvements in FRET FP tools include the Citrine or Venus variants of yellow fluorescent protein (YFP) acceptors, which have reduced pH- and halide-sensitivity [4], and new FP pairs that have higher rates of energy transfer and dynamic range (CyPet and Ypet [30]) or larger spectral separation to reduce detector channel ‘cross talk’ (Midorishi cyan fluorescent protein [miCy] and monomeric Kusabira orange fluorescent protein [mKO] [6]). FRET signals can often be small relative to background, but unlike fluorescence complementation, FRET interactions are freely reversible and thus allow dynamic analysis of proteins interactions. Split GFP and FRET assays thus have complimentary strengths and both techniques should open the door for new high-throughput analyses of the subcellular location of protein–protein interactions.

Localizing the activities of proteins

The tools described above allow the systematic localization of tagged proteins and the identification of interactors, but protein activity might be regulated differentially within subdomains of the cell [1•,30]. Thus, we need to measure when and where proteins are active. Biosensors for protein activity fall into two major classes: FRET activity sensors and translocation sensors.

FRET activity sensors are either unimolecular, for which changes in activity lead to intramolecular rearrangements, or bimolecular, for which the recruitment of a second protein leads to FRET (Figure 2). Unimolecular FRET sensors for reversible protein phosphorylation combine a phosphorylation motif with a domain that recognizes the phosphorylated polypeptide (e.g. a 14-3-3 protein). Binding of the recognition domain to its phosphorylated target leads to a conformational rearrangement that is detectable by a change in FRET between flanking FPs. A variety of protein kinase sensors [31,32•,33,34] have been created and used successfully to visualize specific phosphorylation events. The conceptually much simpler protease sensors are composed of a specific protease recognition motif that is flanked by fluorophores; thus protease activity is detectable as loss of FRET [35–37].

Enzyme activity and metabolite levels can be measured with the help of protein domains whose location changes in response to a signal, such as movement from cytosol to nucleus or plasma membrane. Simple fusion of a translocating polypeptide to a fluorophore generates a translocation sensor (Table 1). For example, the translocation of a pleckstrin domain of mammalian phospholipase C δ serves as a probe for phosphatidylinositol-3,4,5-trisphosphate (PIP₃) and phospholipase activity [38]. Similarly, a tandem repeat of the C1 domain of mammalian protein kinase C γ translocates to the plasma membrane in response to 1,2-diacylglycerol [15•]. This strategy also yielded a plethora of activity sensors for Rho small GTPases and G-protein activation [1•,39,40]. Such sensors can be used to dissect the dynamics of complex signaling pathways. For example, translocation sensors have been used to demonstrate that insulin activates phosphatidylinositol 3 (PI3)-kinase, and the resulting increase in PIP₃ activates the plasma membrane translocation of the glucose transporter GLUT4 [41].

A major advantage of these sensors is their suitability for ‘omics’ approaches: systematic fusion of putative translocation domains to GFP and analysis of conditional translocation provides multiparallel analysis tools for a wide range of cellular processes [10•], whereas systematic RNA interference (RNAi) combined with robotic imaging can be used to unravel the underlying signaling network [2•].

FRET biosensors for the detection of steady-state ion and metabolite levels

Biosensors for small molecules, such as signaling intermediates, ions and metabolites, exploit allosteric linkage between ligand binding and the conformation of a ‘recognition element’. Binding-coupled structural changes are transduced into altered properties of a ‘reporter element’ (Figure 3).

The prototype sensor for calcium makes use of conformational changes in calmodulin that are induced by calcium binding [42]. Subsequent versions employed a conformational actuator to magnify the allosteric effects [32•,43]. Today, more than a dozen calcium-sensor variants are available [44], some carrying a single fluorophore and many utilizing two [45]. Systematic comparison of the dynamic properties of these sensors suggests that they differ *in vivo* [46–48]. Using similar strategies, sensors have been engineered for ligands of the G-protein-coupled receptors cAMP [40], cGMP [49] and PIP [50].

FRET metabolite sensors use the conformational change of periplasmic binding proteins (PBPs) upon binding to their substrates [51]. The best-studied family member, maltose binding protein, was fused at its termini to engineered forms of cyan fluorescent protein (ECFP) and yellow fluorescent protein (EYFP) to generate a maltose sensor. The initial version did not respond but engineering provided a functional maltose sensor that was successfully deployed to image maltose homeostasis in yeast [52]. Out of a large number of PBPs that have been tested as recognition elements, several are non-functional. However, functional glucose, ribose and glutamate sensors have permitted the analysis of sugar homeostasis in the cytosol and nucleus of mammalian cells and the analysis of glutamate release from neurons [51,53•].

The maximal ratio change between YFP and CFP emission (the difference in ratio between apo and saturated form) observed for all of the initial FRET-based PBP sensors was comparatively small, about 0.25. A combination of rational and empirical engineering approaches led to enhanced allosteric linkage between ligand binding and fluorophore rearrangement and thus improved sensitivity [54•]. The results also suggest that the FRET change depends both on fluorophore distance and the relative orientation and rotational freedom of the reporters: rotational freedom decreases if linkers are eliminated or if the FP is fixed in the backbone of the PBP. These improved sensors have now been introduced into

plants and are being used to develop high-throughput systems for the analysis of the metabolome [51].

Biosensors for pH, halides, redox state, and membrane potential

Fluorescent proteins are sensitive to pH, halides, and redox state. Sensitivity to pH has been used to develop pH sensors, such as the pHluorins [55]. EYFP is particularly sensitive to pH and halides, and simple CFP–YFP or CFP–citrine tandem fusions can serve as a FRET sensors for H⁺ and halide ions [56]. Variants of GFP that have enhanced redox sensitivity (roGFP) visualize the oxidation state of the indicator and thus the redox potential [57]. This group of sensors could be used as controls to exclude artifacts that result from the effects of ions and redox on biosensor output.

Finally, the sensitivity of the FP chromophore to the conformation of the β -barrel cage that surrounds it was fortuitously exploited to create a membrane voltage sensor. The FlaSh sensor (not to be confused with the biarsenical dye FlAsH), which was created by fusion of a truncated GFP ‘reporter domain’ to a voltage-gated K⁺ channel ‘detector domain’, is fast enough to allow imaging of action potentials [58]. It will exciting to see if the conformation sensitivity of FP chromophores can be used to produce other unimolecular reporters for protein activity and cell physiology.

Conclusions and outlook

Fluorescence-based technologies have empowered us with a rapidly growing tool kit with which to image essential properties of living cells: protein localization and activity, ion and metabolite levels, redox state, and membrane potential. Modern imaging technology allows the output of these sensors, and thus the molecules and properties that they measure, to be detected with extraordinary spatial and temporal resolution. Many of these technologies have been developed in animal systems but are increasingly adopted for plants; for example, calcium imaging with cameleons is well established in plants [59].

We know that steady-state metabolite levels or signaling intermediates are affected by uptake rates, synthesis and conversion, compartmentation and export. Each step might be allosterically regulated and rates can be regulated by events that affect enzyme or transporter amounts in response to external and internal stimuli. To determine the underlying metabolic and signaling networks, topology maps need to be established that encompass the subcellular localization of metabolites, ions and enzyme or transporter activities, as well as the flux rates. When applied in high-throughput systems, the tools described in this review will provide a ‘cellomics’ approach to plant function for the first time.

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Figure 1. Fluorescent biosensors for protein–protein interactions. Models for protein–protein interaction biosensors using (a) full-length fluorescent proteins or (b) the split-GFP to reconstitute fluorescent proteins with different spectral properties. Color code: fusion protein (red), ECFP (cyan), EYFP/citrine/Venus (yellow).

Figure 2. Fluorescent biosensors for detecting and localizing protein activities. **(a)** Sensors for detecting kinase activity, **(b)** protease activity, and **(c)** GTPase activation. Color code: ECFP (cyan), EYFP/citrine/Venus (yellow), recognition element (red), MiCy (turquoise) and mKO (orange).

Figure 3.

Fluorescent biosensors for metabolites. **(a)** The calcium sensor cameleon, which consists of the calcium-binding domain of calmodulin fused to myosin light chain. **(b)** The sensor for cAMP, which uses a guanine nucleotide exchange factor (GEF) as a reporter element. **(c)** Sensor for the catalytic subunit and regulatory domain of a protein kinase A. **(d)** The sensor for inositol phosphate/phosphatidylinositol bisphosphate uses the phosphatidylinositol phosphate binding domain of ActA from *Listeria*. **(e)** FLIP nanosensors for maltose, glucose, ribose and glutamate that use PBPs. **(f)** FLIIP nanosensors (for glucose and glutamate) in which CFP was inserted in a permissive site of the periplasmic binding protein. **(g)** Sensors for pH (CFP–YFP tandem) or for pH and halide (clomeleon) that use a fusion between CFP and YFP. **(h)** The voltage sensor FIAH. Color code: ECFP (cyan), EYFP/citrine/Venus (yellow), recognition element (red), MiCy (turquoise) and mKO (orange).

Table 1
Unimolecular and bimolecular sensors

Sensor name	Substrate	Reference(s)
Single fluorophore sensors		
eGFP-PH _{PLC} ^δ	Phosphatidylinositol bisphosphate	Reviewed in [60]
CFP-AktPH	Phosphatidylinositol trisphosphate	[41]
eGFP-C1 ₂ -PKC _γ	1,2-diacylglycerol	[60]
LIBRA	Inositol trisphosphate	[61]
SH2-GFP	Tyrosine phosphorylation	
pHluorin	pH	[55]
Camgaroo	Calcium	Reviewed in [62]
Pericam	Calcium	Reviewed in [62]
FlaSh	Voltage	[58,63]
FRET sensors		
CIM-FP	Calcium	[42]
Cameleon	Calcium	[44]
Cameleon	Calcium	[5]
Clomeleon	pH and halides	[64]
Troponeon	Calcium	[45]
CFP-PKA-YFP	cAMP	[40]
CFP-Epac-YFP	cAMP	[40]
Cygnets 2.1	cGMP	[49]
AKAR, BKAR, CKAR	Protein kinases A, B, and C	[31,32••]
Phocus	Kinase signaling	[33]
CAY	Phosphorylated phosphoinositides	[50]
LF ₂ C/YR	GTPase	[39]
FLIPmal	Maltose	[52]
FLIPglu	Glucose	[51]
FLIPrib	Ribose	[51]
FLIPgluΔ13	Glucose	[54••]
FLIIPglu	Glucose	[54••]
FLIPE	Glutamate	[53••]
FLIPE	Glutamate	[54••]
SCAT3/SCAT9	Caspase	[65]
MiCy-DEVK-mKO	Caspase	[6]
CFP-YFP tandem	pH	[56]
roGFP	Redox potential	[57]
Cdc42 signaling	Cdc42 signaling	[66]