# Genetically Encoded FRET Sensors for Visualizing Metabolites with Subcellular Resolution in Living Cells[1]

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What tools are there to determine the concentration of a particular molecular species in a physiological environment? Is it possible to visualize how its concentration varies across an organ, tissue, or cell? Is there a way to detect how metabolite levels change in response to environmental stimuli? Can these changes be monitored in real time? Can multiple analytes be measured simultaneously? Can these measurements be performed for a variety of structural and functional analyte classes? These are the central questions in the young field of metabolomics.

No currently available technology addresses these issues in a satisfactory manner. Nonaqueous fractionation is static, invasive, has no cellular resolution, and is sensitive to artifacts. Spectroscopic methods such as nuclear magnetic resonance imaging and positron emission tomography provide dynamic data, but poor spatial resolution.

The development of genetically encoded molecular sensors, which transduce an interaction of the target molecule with a recognition element into a macroscopic observable, via allosteric regulation of one or more reporter elements, may provide answers to some of the questions. The recognition element may simply bind the target, bind and enzymatically convert the target, or may serve as a substrate for the target, as in the use of a specific target sequence in the construction of a protease sensor (Nagai and Miyawaki, 2004). The most common reporter element is a sterically separated donor-acceptor fluorescence resonance energy transfer (FRET) pair of spectral variants of the green fluorescent protein (GFP; Fehr et al., 2002), although single fluorescent proteins (Doi and Yanagawa, 1999) or enzymes (Guntas and Ostermeier, 2004) are viable as well. Some molecular sensors additionally employ a conformational actuator (most commonly a peptide which binds to one conformational state of the recognition element) to magnify the allosteric effect upon and resulting output of the reporter element (Miyawaki et al., 1997; Romoser et al., 1997; Kunkel et al., 2004).

## A FAMILY OF METABOLITE SENSORS

We have recently demonstrated the applicability of the method in the absence of a conformational actuator and its generalizability to a variety of analytes. Members of the bacterial periplasmic-binding protein superfamily (PBPs) recognize hundreds of substrates with high affinity (atto- to low micromolar) and specificity (Tam and Saier, 1993). PBPs have been shown by a variety of experimental techniques to undergo a significant conformational change upon ligand binding; fusion of an individual sugar-binding PBP with a pair of GFP variants produced sensors for maltose, ribose, and glucose (Fehr et al., 2002, 2003; Lager et al., 2003). The sensors were used to measure sugar uptake and homeostasis in living animal cells, and subcellular analyte levels were determined with nuclear-targeted versions (Fehr et al., 2004). Recently, a glutamate sensor was developed using the bacterial glutamate-binding protein ybeJ as a recognition element (Okumoto et al., 2005).

## POTENTIAL OPPORTUNITIES

### Binding Specificity

One of the foremost goals is the expansion of the molecular toolbox through the conversion of additional PBP superfamily members, as well as proteins with binding specificity not seen in the PBPs. Additionally, the complementary methods of computational protein redesign (Looger et al., 2003) and directed evolution have been used to alter the binding specificity of members of these protein families, demonstrating that the repertoire may be extended beyond that found in nature.

### Detection Range

Due to the large vacuole and the largely unknown organellar compartmentalization of metabolites in plants, there is currently a great deal of uncertainty regarding cytosolic metabolite concentrations in plant cells. Thus, it will be necessary to test a family of

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sensors with dynamic ranges from 100 nM to 10 mM. Such a series of sensors has been constructed for a number of PBPs (de Lorimier et al., 2002).

### Signal-to-Noise Ratio

The sensors developed thus far provide a saturating ratio change of up to 0.3, providing a sufficient signal-to-noise ratio to facilitate in vivo measurement. Both empirical and rational techniques (Nagai and Miyawaki, 2004; L.L. Looger and W.B. Frommer, unpublished data) are capable of improving sensor signal change (mainly through fluorophore dipole reorientation), allowing the sensors to be used in novel environments.

### Sensitivity to Other Parameters

Yellow fluorescent protein is particularly sensitive to pH and halides, a property previously exploited to develop halide sensors (Kuner and Augustine, 2000), emphasizing the importance of control sensors to identify potential artifacts (Fehr et al., 2003). The use of FRET acceptor variants such as Venus or mKO, with lower pH and halide sensitivity, may improve sensor robustness (Karasawa et al., 2004). The use of thermo- and acid-stable recognition elements should further enhance sensor applicability.

### Calibration

Molecular sensors are indicators of change; because the complex cellular environment affects sensor response, the sensor has to be calibrated in situ (Fehr et al., 2003, 2004).

### Stable Plant Lines

It has thus far been difficult to generate stably transformed plant lines expressing functional sensors. A complicating factor may be the simultaneous presence of two highly homologous GFP variant sequences, which may aggravate gene silencing. Problems were observed when attempting to generate cameleon-expressing mice (Hasan et al., 2004). This issue was overcome in part by the use of sensors carrying only a single fluorophore expressed under control of a regulatable promoter.

### Effect of Nanosensors on Cellular Processes

It is conceivable that nanosensors may perturb metabolism when expressed in a living cell. This may be even more dramatic when using sensors for analytes present at low levels, e.g. signaling molecules. Programmed low-level expression may provide a remedy.

### Temporal Resolution

FRET imaging systems commonly use filter wheels and collect data at intervals of several seconds. Due to the high sensitivity of these instruments, it is possible to reduce excitation intensity to below levels that lead to strong photobleaching, permitting the use of parallel image acquisition using image splitters combined with video-rate streaming.

### Spatial Resolution

For most applications, even those requiring organellar resolution, standard epifluorescence FRET systems will suffice. For some purposes, though, it may be advantageous to follow changes in optical sections using a Nipkow spinning-disc confocal or multiphoton microscope.

### Tissue Penetration

Both epifluorescence and conventional confocal microscopy are limited with respect to sample thickness; for better penetration, multiphoton microscopy or even endoscopy may be necessary.

### Multiplexing

Several donor-acceptor protein pairs are available for use in FRET imaging. This should permit the simultaneous visualization of multiple analytes, either in the same or different cellular compartments. Alternatively, independent sensor elements may be constructed using two copies of a single fluorescent protein, using techniques such as fluorescence anisotropy decay (Squire et al., 2004).

## SUMMARY

FRET-based imaging provides an easy method to monitor analyte changes in living cells and their compartments. The generation of stable plant lines expressing optimized sensors may provide suitable tools for a variety of applications, e.g. monitoring soil contamination or the physiological status of stressed plants. It is conceivable that the technology may be applicable at the level of fields or ecosystems using LIDAR (light detection and ranging) detection.

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