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In vivo biochemistry: Applications for small molecule biosensors in plant biology

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Summary

Revolutionary new technologies, namely in the areas of DNA sequencing and molecular imaging, continue to impact new discoveries in plant science and beyond. For decades we have been able to determine properties of enzymes, receptors and transporters *in vitro* or in heterologous systems, and more recently been able to analyze their regulation at the transcriptional level, use GFP reporters to obtain insights into cellular and subcellular localization, and measure ion and metabolite levels with unprecedented precision using mass spectrometry. However, we lack key information on location and dynamics of the substrates of enzymes, receptors and transporters, and on the regulation of these proteins in their cellular environment. Such information can now be obtained by transitioning from *in vitro* to *in vivo* biochemistry using biosensors. Genetically encoded fluorescent protein-based sensors for ion and metabolite dynamics provide highly resolved spatial and temporal information, and are complemented by sensors for pH, redox, voltage, and tension. They serve as powerful tools for identifying missing processes (e.g. glucose transport across ER membranes), components (e.g. SWEET sugar transporters for cellular sugar efflux), and signaling networks (e.g. from systematic screening of mutants that affect sugar transport or cytosolic and vacuolar pH). Combined with the knowledge of properties of enzymes and transporters and their interactions with the regulatory machinery, biosensors promise to be key diagnostic tools for systems and synthetic biology.

Keywords

metabolic signaling; metabolic pathways; sugar signaling; imaging

Introduction into genetically encoded small molecule biosensors

To understand a biological process or system, we need to know the components of that process, the role of each component, the physical properties and chemical conditions, and the dynamic features such as fluxes and enzymatic activities. Biochemical and biophysical methods have brought us close to complete resolution of components and conditions, but unveiling their dynamics in living cells still poses a great challenge. To measure a dynamic process under physiological conditions we need means to visualize the actual process, e.g.

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the translocation of molecules, the conversion of one metabolite into another, or the triggering of signaling events. This visualization can be achieved by sensors that respond dynamically, e.g. to changing concentrations of a particular molecule or to modifications or structural changes of involved enzymes and complexes. As some of the first sensors in biology, pH indicators were used to visualize the activities of proton pumps [1]. Later, fluorescent sensor molecules were designed that could be injected into cells to measure levels and periodicity of calcium [2,3]. However, the way of applying such sensors by injection or other forms of membrane penetration posed a major risk of artifacts. Being minimally invasive is a critical requirement for sensors to ensure that the measured process is mostly unperturbed by the measurement itself.

The discovery, characterization and engineering of fluorescent proteins (i.e. green fluorescent protein, GFP) has played a key role in allowing many minimally invasive measurements, particularly the analysis of the localization and dynamics of proteins in live cells as well as for the development of genetically encoded small molecule biosensors [4,5]. Roger Tsien's lab developed the first prototype protein sensors, i.e. to measure caspase activity and to monitor calcium in live cells [for review see 5]. These sensors made use of Förster resonance energy transfer (FRET) between two spectral variants of GFP; shortly afterwards single fluorophore sensors based on circular permutated GFPs (cpGFPs) were developed, e.g. for calcium [6,7]. The biosensor tool set has been vastly expanded to detect sugars, amino acids, phosphate, as well as many ions and signaling intermediates (http://dpb.carnegiescience.edu/labs/frommer-lab/biosensor_database). Key features of genetically encoded biosensors are: (i) universal utility, i.e. transient or stable introduction into most organisms by transfection, transformation or protein transduction [for detailed protocols in yeast, plants and human cells see 8,9–11]; their ability to report (ii) steady state levels of ions and metabolites, (iii) dynamics with high temporal resolution, and (iv) with cellular and even subcellular information. They can be used in complex solutions and cell cultures as well as in tissues and intact organisms. Despite recent progress, many key compounds await sensors for high-resolution monitoring *in vivo*. In plant biology, the dynamics of phytohormones represent a highly attractive target for such monitoring, although some progress has been made using lower-resolution sensors, e.g. for indirect imaging of plant hormones. Reporters of either hormone responsive gene expression, such as DR5-GFP, or hormone dependent protein degradation, such as IAA28 DII-Venus have proven highly valuable tools [12,13]. Nevertheless, spatial and temporal dynamics of the full spectrum of phytohormones would ideally be monitored by FRET hormone sensors.

Sensor design

Small molecule fluorescent biosensors make use of conformational changes in a recognition element induced by the analytic target (analyte) as proxies for analyte concentrations. There are several different types of sensor designs and several classes of analytes with available sensors [5]. In designing a sensor for a new analyte, the selection or creation of a recognition element is the primary parameter. Recognition elements are typically chosen from proteins or protein domains that are known to directly bind or be modified by a specific analyte. The ligand recognition domains of receptor proteins are good sources for new recognition elements, with the periplasmic binding proteins of bacterial ABC transport systems proving an especially rich source for metabolite recognition elements. A variant of this approach makes use of the interaction of two domains that come together when an analyte is present. Other variants include use of a molecular spring recognition element to measure tension and co-opting intrinsic fluorophore properties to detect changes in environmental conditions such as pH or voltage.

In the simplest case, two fluorophores are fused to the recognition element at either terminus and/or internal sites to generate a FRET sensor. Because the design of new sensors is a largely empirical process, structural information for recognition elements is desirable but not an absolute requirement. These sensors are ratiometric in that they report on cognate analytes through changes in the relative intensities of the two fluorophore emissions, and thus require appropriate fluorescence detection/imaging equipment [for details see 11,14*]. The list of fluorescent proteins that form FRET pairs is ever growing, leading to a wealth of options for designing new sensors with appropriate biophysical properties (e.g. pH optimum, emission wavelength) for the intended application (http://flowcyt.salk.edu/fluo.html). Ratiometric sensors can also be generated from a single fluorophore with multiple excitation or emission maxima (e.g. the HyPer sensor has two excitation maxima, the relative strengths of which are altered by hydrogen peroxide [15]). Alternatively, single fluorophores whose fluorescence has been engineered to vary in proportion to changes in an analyte can be used to generate intensity-based sensors. Although easier to image, non-ratiometric single fluorophore intensity changes are more susceptible to artifacts resulting from variation in sensor protein level or optical parameters of the sample (background fluorescence, light scattering). Additional important parameters for any sensor include specificity, brightness, signal-to-noise ratio and a dynamic range that matches the concentrations of analyte to be examined. Typically, the dynamic range of a sensor is ~2 orders of magnitude, which means multiple sensors with different affinities are necessary if large changes in analyte concentration are to be tracked. However, the dynamic range of a recently developed lactate sensor exceeds four orders of magnitude owing to the kinetics of the recognition element [16]. Testing of such parameters is required to evaluate the effectiveness of a new sensor and to optimize existing sensors. Many of the requisite tests are best carried out on purified sensor *in vitro* (e.g. after expression in and purification from an *E. coli* host system), but *in vivo* evaluation and optimization can also be necessary to minimize interaction with off-target endogenous components and detrimental buffering of the analyte caused by the presence of the sensor, and to improve expression and/or stability of the sensor protein and signal-to-noise ratio in the cellular environment and imaging platform. For example, silencing of biosensor transgenes has been a recurring setback for *in planta* applications, but recent evidence suggests that the use of the UBQ10 promoter can greatly improve biosensor expression levels [17*].

Biosensors are complementary to both surveying ions and metabolites using mass spectrometry (ionomics and metabolomics, [18,19]) and fluxomics (an approach for estimating metabolic flux using stable isotope labeling [20]). Furthermore, biosensors can also be used in the context of other investigations, e.g. in genetic screens (see below). Yet there are still many areas in which biosensor potential has not fully been developed, and the continuing optimization of fluorophores and sensors is expected to lead to further improvements in detection range and sensitivity, as well as more options for multiplexing. Exploiting the full potential of biosensors will require accelerated methods of protein engineering (e.g. using Gateway, Gibson assembly or Golden Gate cloning; see also [21](In-Fusion® cloning, Clontech) and expression because all parts of a sensor protein (fluorophore type and site of fusion, recognition elements, linkers, etc.) are potential targets for rational or empirical modification and even small changes in any of these parts can have large impacts on sensor behavior [22,23*]. The development of plant hormone sensors is in principle feasible using recent progress in identification of hormone receptors [24]. Development of such sensors is expected to provide a significant step forward.

Quantifying and visualizing molecules over time at the cellular and subcellular level

Advancing technologies have long driven major progress in determining – at cellular and even subcellular resolution - gene expression levels [25], ion and metal distributions [26,27], membrane composition [e.g. using nanoSIMS, 28], or metabolites [29]. High spatial resolution measurements can reveal patterns that lead to the discovery of novel physiological processes [30]. Yet subcellular compartments are often not accessible to current methods. For example, organelles such as the endoplasmic reticulum can be biochemically enriched using differential centrifugation gradients, but the isolated organelle is then out of its native cellular context. Thus, we require tools for measuring with high resolution in native environments under physiological conditions.

Genetically encoded fluorescent protein based sensors can be targeted to subcellular compartments to reveal subcellular patterns (Figure 1). For example, glucose produced from G6P in the ER lumen was measured by targeting glucose sensors to the ER [22]. The data from these sensors indicate that the residence time of GLUT glucose uniporters during their transit via the ER to the plasma membrane is sufficient to provide high capacity ER import and export of glucose [31]. Alternatively, biosensors can be targeted to the cell surface, e.g. to monitor glutamate release triggered by depolarization of hippocampal neurons [32]. Subcellular analysis of calcium dynamics in plant cells has been facilitated by Cameleon biosensors [33*], and an array of Cameleons targeted to different subcellular compartments is now available [17*]. In addition to subcellular spatial resolution, biosensors combined with appropriate imaging platforms permit time-course analysis with near real-time temporal resolution. Rapid dynamics of analytes are only revealed at such higher resolutions. Plant growth techniques that allow for continuous imaging under controllable environmental conditions also facilitate advanced study of analyte dynamics over time (Figure 2).

In vivo application of biosensors

High resolution measurements can open up previously undetectable patterns to primary observation, which can lead to new understanding of plant physiological processes. For example, the early discovery of differential calcium accumulation in different leaf cell types [26] led to functional models of calcium flux and regulation through the leaf [30]. The spatial (sub-cellular) and temporal (real-time) resolution afforded by biosensors has similarly led to deeper understanding of processes like the calcium oscillations required for symbiosis signaling in root hair cells. Initial observations that calcium oscillations in root hair cells were concentrated in the perinuclear region [34] were reproduced using Cameleon calcium sensors [33*]. However, the downstream signal transducer calmodulin-dependent protein kinase (CCaMK) is nuclear-localized [35,36], suggesting that nucleo-cytoplasmic calcium oscillations, rather than perinuclear calcium oscillations, are involved in the symbiotic signaling pathway of legumes. The presence on the inner nuclear membrane of two proteins required for symbiotic calcium oscillations - the cation channel DMI1 and the sarco/endoplasmic reticulum calcium ATPase SERCA – provides further evidence that nucleo-cytoplasmic calcium oscillations are important in legume symbiosis signaling [37**]. Observation of calcium oscillations in the nucleus was made possible through the use of nuclear localized Cameleon [38], and biosensor imaging combined with flux modeling indicates that calcium is released from the inner nuclear membrane [37**]. Thus, sub-cellular monitoring of a regulator (calcium oscillations) provided key support for a more detailed model of how that regulator controls a process (symbiosis signaling). High temporal resolution imaging of calcium oscillations using Cameleon sensors also led to important insights. For example, the finding that number of calcium spikes is more important than duration of spiking for symbiosis signaling was arrived at using time-course analysis of

plants expressing Cameleon [33*]. Cameleon biosensors have been widely adopted for *in vivo* analysis and have led to insights into the role of calcium in pollen tube growth [39], root mechano-sensing [40], guard cell regulation by ABA [41,42] and JA [43], and root response to treatment with aluminum [44]. Furthermore, metabolite biosensors have revealed *in vivo* metabolite dynamics and transport in intact roots [45,46**]. Additional sensors have been applied to measuring redox status, reactive oxygen species and pH *in planta* [for review, see 47**]. However, the majority of available biosensors (http://dpb.carnegiescience.edu/labs/frommer-lab/biosensor_database) have yet to be applied to *in planta* studies.

Biosensors for gene discovery

Biosensors can be used to identify missing components related to the metabolism, regulation, or transport of the analyte, either in a heterologous system or in the native environment. For example, a FRET sensor for sucrose led to the identification of proteins that carry out a previously uncharacterized transport step in phloem loading – sucrose efflux from the mesophyll. Sucrose is made in mesophyll cytosol, but imported into the cytosol of phloem companion cells by a proton sucrose cotransporter of the SUT family. Thus, efflux from the mesophyll is required. A human cell line that expresses a sucrose sensor but does not import sucrose was used to screen heterologously expressed Arabidopsis proteins for sucrose transport activity. An analogous approach identified AtSWEET1, a glucose uniporter and the first member of the SWEET family of transporters [48*]. The screen for sucrose transporters led to the characterization of AtSWEET11 and AtSWEET12 as sucrose uniporters involved in net sucrose efflux from the mesophyll [49*]. When expressed in a native cell environment, a biosensor can be used to systematically identify components and processes that affect the analyte. Fluorimeter-based assays with FRET sugar sensors were successfully used to identify sugar transporters that can function immediately after exposure of starved yeast cells to glucose [50,51*]. Similar assays were successfully deployed to identify genes that affect cytosolic or vacuolar pH in yeast [52,53]. These results prove that biosensors can be widely applied in genetic screens provided imaging technologies of suitable throughput are available.

Future applications of biosensors

Today it is possible to track gene expression at the cellular level using cell-type specific sorting of protoplasts [54] or from RNA in affinity purified nuclei or ribosomes [25,55], protein abundance and localization with subcellular resolution using GFP fusions, and small molecule/metabolite dynamics with subcellular resolution using biosensors. Some biosensors can also track other protein dynamics such as phosphorylation events or receptor activation [for review see 5]. In the future, we anticipate biosensors to track these dynamics with even greater spatial and temporal resolution, and even to report directly on critical biophysical mechanisms such as enzyme or transporter activities. A biosensor approaching such direct reporting of transport activity tracks pH changes at the site of Cl^-/HCO_3^- exchangers [56], but an ideal tool would detect conformational changes during the transport cycle. In addition to reporting on fine scale activities, current and future biosensors will be able to readily report with high resolution on dynamic molecules and processes that regulate fundamental check points in cell control. Biosensor-facilitated measurement of such dynamics (e.g. calcium oscillations) will permit the dissection of their regulation and co-regulation by multiple signals and inputs. The development of integrated imaging platforms like the RootChip presents a first step towards enabling technologies for integrated and high-resolution analysis of various inputs on Arabidopsis lines that express biosensors [46**], possibly even in medium to high throughput. Future directions include new applications of biosensors already expressed in plants (e.g. metabolite tracking at sites of nutrient

exchange), new applications of biosensors that have not yet been expressed in plants (e.g. cell-cycle control measurement with FUCCI [57], and new applications of sensors yet to be developed (e.g. mapping phytohormone dynamics with biosensors). We would like to note that in addition to protein-based sensors described here, there are many other approaches, e.g. RNA-based sensors like Spinach and aptamer sensors as well as a suite of non-genetically encoded chemical dyes that can be used as complementary tools for monitoring ions and metabolites *in vivo* [58**].

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Abbrev

FLIP	fluorescent indicator protein
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
eCFP	enhanced cyan fluorescent protein
eYFP	enhanced yellow fluorescent protein
LWL	long wavelength fluorophore
SWL	short wavelength fluorophore
cpGFP	circular permutated GFP

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Highlights

- Biosensors provide information on location and dynamics of substrates of enzymes and transporters
- Biosensors provide information on regulation of enzymes/transporters in the native environment
- Biosensors provide highly resolved spatial and temporal information on ions and metabolites
- Biosensors help identifying unknown processes and molecular components, and signaling networks

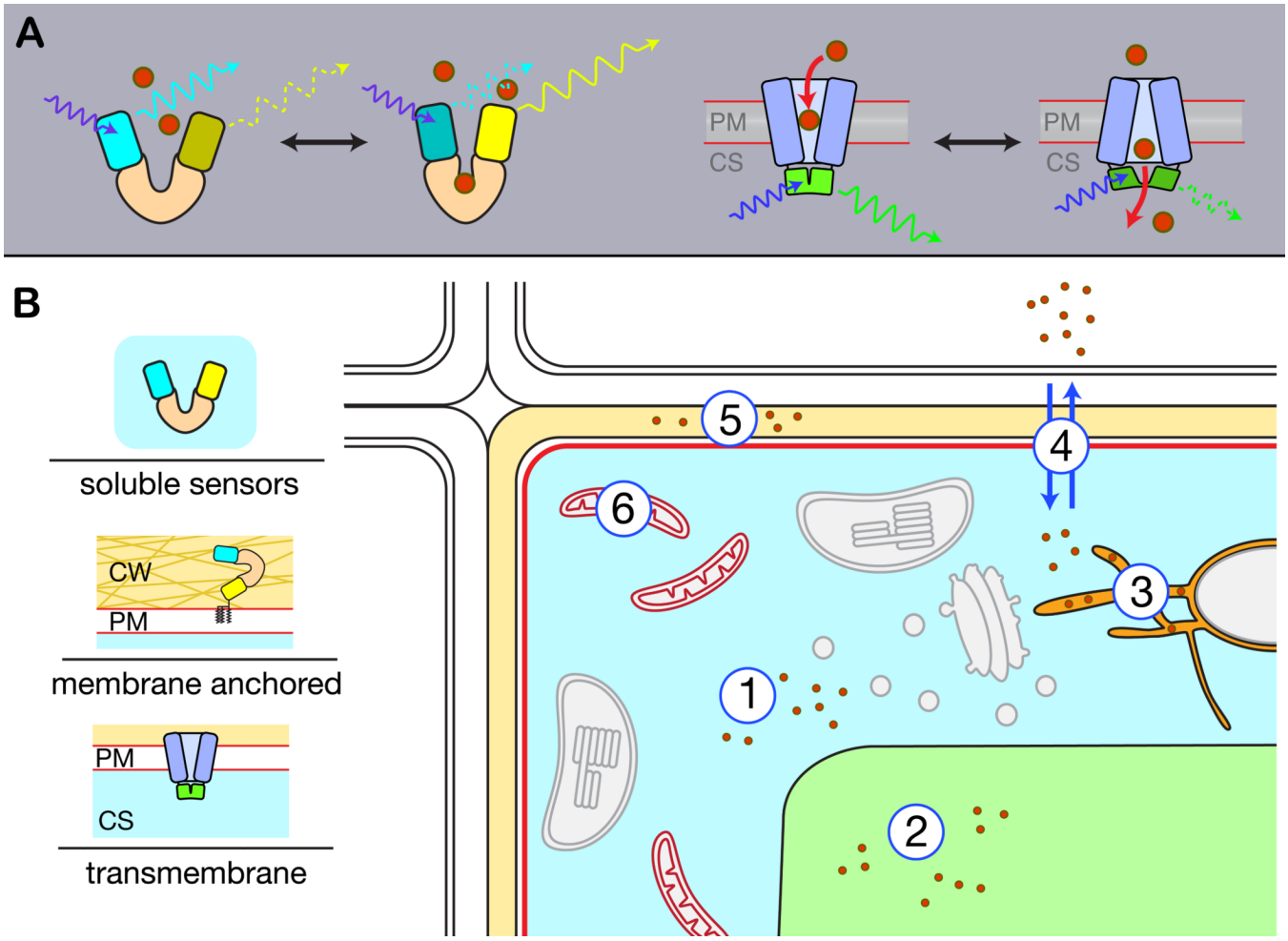

Figure 1. Biosensor design principles and their targeting to subcellular compartments

A. Left: The recognition element (orange) is fused to two spectrally overlapping fluorophores (SWL short wavelength fluorophore, e.g. cyan version of GFP (cyan barrel); LWL long wavelength fluorophore, yellow version of GFP (yellow barrel). Binding of ligand (red) results in a conformational change of the recognition element and a change in orientation and/or distance of the two fluorophores, altering FRET efficiency. Right: cpGFP is inserted into the recognition element (here: transmembrane protein, blue). Conformational changes due to transmembrane protein activity are translated into signal intensity changes of cpGFP. B. Left: Example diagrams of soluble, membrane anchored and transmembrane sensors. Right: Genetically encoded fluorescent protein based sensors can be targeted to subcellular compartments to reveal local substrate level changes or enzyme activity. Cytosol (1): untargeted, nucleoplasm with nuclear localization signal (NLS), or extranuclear cytoplasm with nuclear export signal (NES). ER (2): ER targeting sequence, retain with ER retention signal (KDEL). Vacuole (3): lumen with vacuolar signal sequence, vacuole with targeting peptide. Apoplasm (4): secrete with export sequence. PM (5): display on surface using membrane anchor, e.g. platelet-derived growth factor (PDGF) receptor transmembrane (TM) domain; membrane integral with sensing domain cytosolic. Mitochondria (6): targeting with mitochondrial signal sequence.

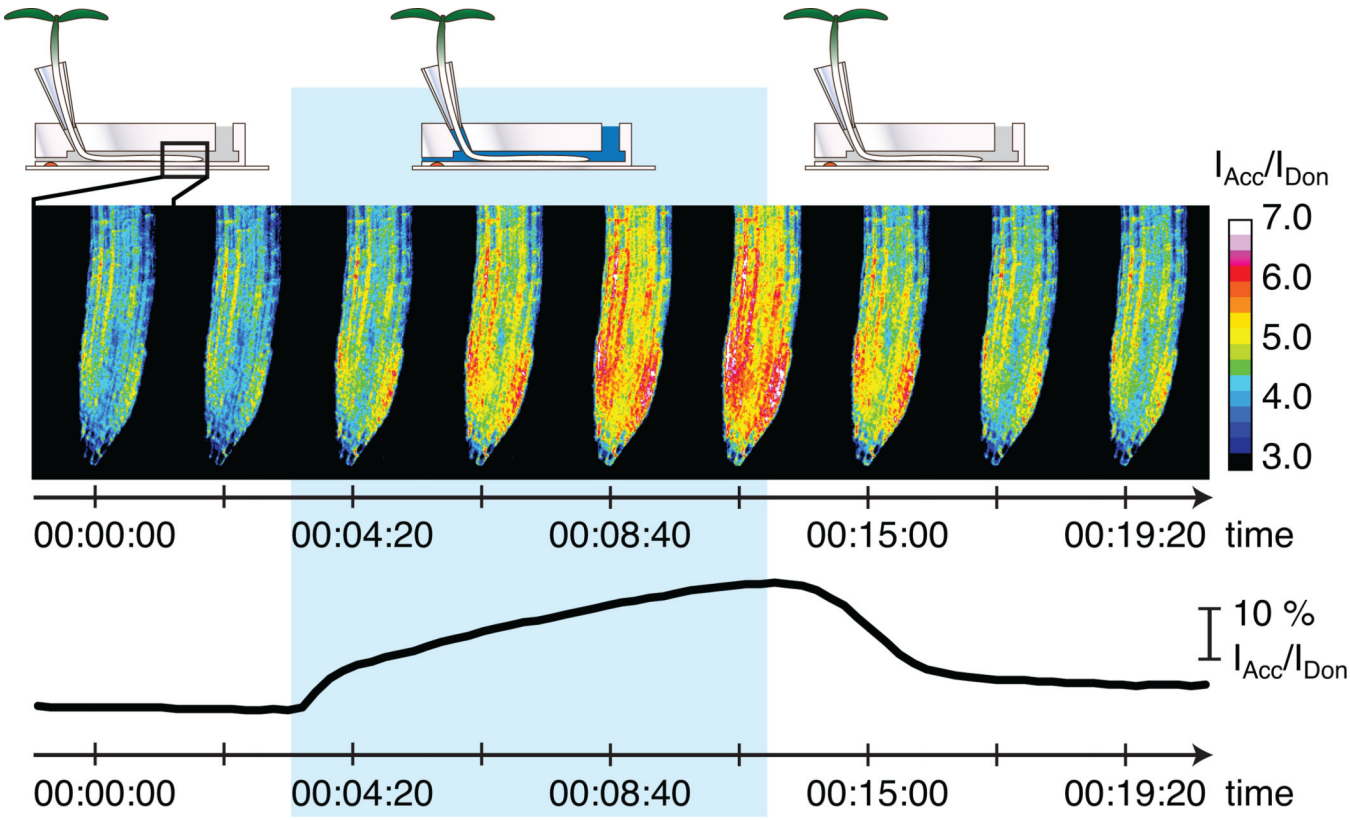

Figure 2. Time course analysis of the response of a fluorescent biosensor for glucose (FLII^{12}P) in a RootChip

Upon perfusion of a root with ligand solution (blue background) the cytosolic sensor reports intracellular increase in ligand concentration by a changed ratio of donor and acceptor intensities (here: increase in ratio I(Acc)/I(Don); for details see [10, 46**].