Published in final edited form as:

Methods Mol Biol. 2009 ; 553: 355–372. doi:10.1007/978-1-60327-563-7_19.

Comparison of Quantitative Metabolite Imaging Tools and Carbon-13 Techniques for Fluxomics

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Abstract

The recent development of analytic technologies allows fast analysis of metabolism in real time. Fluxomics aims to define the genes involved in regulation of flux through a metabolic or signaling pathway. Flux through a metabolic or signaling pathway is determined by the activity of its individual components; regulation can occur at many levels, including transcriptional, posttranslational, and allosteric levels. Currently two technologies are used to monitor fluxes. The first is pulse labeling of the organism with a tracer such as C13, followed by mass spectrometric analysis of the partitioning of label into different compounds. The second approach is based on the use of flux sensors, proteins that respond with a conformational change to ligand binding. Fluorescence resonance energy transfer (FRET) detects the conformational change and serves as a proxy for ligand concentration. Both methods provide high time resolution. In contrast to mass spectrometry assays, FRET nanosensors monitor only a single compound, but the advantage of FRET nanosensors is that they yield data with cellular and subcellular resolution.

Keywords

Flux; FRET; nanosensor; carbon-13

1. Introduction

Metabolic fluxes underlie all biological activity, ultimately manifesting phenotype and functioning of an organism. Metabolic flux is highly dynamic and is controlled through signaling networks to acclimate appropriate cellular responses to environmental challenges. Fluxomics aims at quantifying and modeling these fluxes in the entire metabolic network of an organism, a feat that has not yet been attained, as well as the factors affecting all fluxes. Flux is measured either by determining the flow of a label (typically a radiotracer) in metabolic networks or by measuring changes in substrate and product concentrations. At present, none of the existing experimental techniques is capable of comprehensively resolving all of the metabolic fluxes of entire metabolic networks in any organism; however for apparent reasons, most progress has been made in single cell microbes. This review provides a comparison of quantitative metabolite imaging and carbon-13-based approaches for flux analysis. The particular focus is on the methods that were developed recently for metabolite imaging and how they can be used to measure the rate changes of metabolite concentrations with subcellular resolution, and how the information gained from the use of quantitative imaging can be applied to estimate net flux. For a more detailed review of FRET-based analysis of in vivo metabolite levels, cf. Okumoto et al. (1).

2. Isotope-Based Flux Analysis

Isotope tracers have proven very effective for determining pathway structure in the past (e.g., the dark reactions in photo-synthesis (2,3)) and are currently being applied to obtain comprehensive flux analysis with the aim of producing system-wide flux maps of metabolic

networks. The increased sensitivity of mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques obtained over the past years, and the development of powerful tools for data analysis, begins to make system-wide flux analysis possible in microorganisms as well as plants. ^{13}C -based flux analysis has been pioneered in microorganisms such as bacteria and yeast (4–6) but is increasingly used in plants. For recent reviews of isotope flux measurements in plants confer the special issue on fluxomics in *Phytochemistry* (7) and recent reviews in other journals (8,9).

Isotope flux measurements can be classified into two categories: steady-state analysis which measures the distribution of a label after the system has attained an isotopic and metabolic steady state, i.e., the point at which the labeling of each metabolite in a network is constant. This method is most powerful when applied in microbes, because they can easily be cultivated under such steady-state conditions. Steady-state labeling has been also been used to create flux maps of central carbon metabolism in plants (10,11) and has helped, for example, to establish a previously unknown role for Rubisco as CO_2 scavenger during oil synthesis in *Brassica napus* seeds (12). The second isotope flux measurement approach is dynamic, using time-course analysis of label distribution to calculate flux. In plants, dynamic analysis has been mainly used to characterize secondary metabolite pathways. Notable examples include the characterization of 38 fluxes involved in the production of benzenoid compounds in *Petunia* petals (13) and the regulation of phenylpropanoid biosynthesis in potato tubers (14).

The major advantage of ^{13}C flux measurements is that it allows the determination of net fluxes in a network and, in some cases, provides the individual forward and backward fluxes of bidirectional steps using the information embedded in the isotopomer distribution (15). For this purpose, isotope-based flux analysis requires mathematical models that represent the possible isotopic states of the metabolic network. The distribution of fluxes is then estimated as a best fit of intracellular fluxes to the actually measured isotope distributions and physiological fluxes in and out of the cell. The main challenge in flux analysis of plants (and other eukaryotes) using isotopes comes from the complexity of the metabolic networks arising from different cell types and the subcellular compartmentalization of metabolism.

Another challenge is that analysis of isotope experiments relies on the current structural understanding of the networks involved: in plants these are only known accurately for a few pathways in primary metabolism. Even for primary metabolism the subcellular compartmentalization of the pathways is not always clear and is still being revised as apparent from, for example, the recent discovery of a plastidic maltose transporter and maltose metabolizing cytosolic glucosyltransferase, both of which are essential for starch degradation in leaves (16–18). Another example of the limited understanding of metabolic compartmentalization in plants is the debate on sucrose transport in and out of vacuoles, which contributes to carbon storage in leaves, in stems of sugarcane, and in roots of sugar beet (19,20). Only recently one of the sucrose transporters SUT4 was localized to the tonoplast membrane (21), although it remains unclear how exactly SUT4 contributes to vacuolar sucrose accumulation.

Our current understanding of the compartmental distribution of metabolites relies mostly on the destructive analysis of whole organs. Compartmentalization of metabolic reactions and metabolite flux within and between cells can only be understood if the cellular and subcellular flux of the metabolites can be established by non-destructive dynamic monitoring techniques. Therefore the application of methods for the non-destructive determination of metabolite fluxes in subcellular compartments and different cell types is of major importance. A comparison of extractable in vitro enzyme activities and steady-state in vivo fluxes in *B. napus* embryos showed no clear correlation between the two (22),

emphasizing the necessity for developing non-invasive in vivo analysis techniques with cellular and subcellular resolution.

3. Imaging-Based Flux Analysis

As an alternative to the isotope-based flux methods, metabolite imaging-based flux analysis, which measures dynamic changes in metabolite concentration, provides both cellular and subcellular resolution. The development of Förster resonance energy transfer (FRET)-based nanosensors was the first step toward in vivo flux measurements (23). Genetically encoded FRET sensors enable both the analysis of steady-state concentration of metabolites and dynamic changes in response to perturbations in living tissue with high temporal and, most importantly, subcellular resolution. FRET sensors report conformational changes of proteins (recognition elements) as a change in the rate of energy transfer between two coupled fluorophores (reporter elements) (24). Thus when the recognition element changes conformation in response to analyte binding, a change in the FRET efficiency reports a change in analyte levels. Importantly, such FRET sensors report changes in steady-state levels over time, e.g., glucose nanosensors, after addition of glucose to a cell or an intact organ, provide information on the sum of the rate of influx and the rate of metabolism. The principle of inferring flux information from these metabolite nanosensors is thus based on the analysis of dynamic responses of metabolite concentrations when the composition of the external medium is manipulated. Apparently the sensor reports only a single metabolite or the change in any of the flux components that affect the steady state.

The concept for genetically encoded FRET sensors was originally developed 10 years ago for measuring calcium by Persechini's and Tsien's groups (25,26). In short, a calmodulin was fused between two fluorescent proteins (e.g., cyan and yellow variants of the green fluorescent protein, GFP). When the cyan FP in the fusion protein is excited with 435 nm light, a fraction of the energy will be transferred to the yellow FP provided the yellow FP is in Förster distance (50% energy transfer at ~5 nm distance). When Ca^{2+} binds to calmodulin, the domain undergoes a conformational change which results in a change in FRET and thus into a change in the ratio of emission of the two fluorescent proteins. Miyawaki et al. (25) used an additional actuator, a calmodulin-binding domain to increase the conformational rearrangement of the binding moiety. FRET nanosensors are essentially ratiometric dyes that provide for quantitative measurements and, since they are DNA encoded, analyses can be performed in any type of transiently or stably transformed cells. Moreover, the addition of targeting sequences allows targeting of the fusion proteins to specific cellular compartments. Subsequent imaging of compartment-specific fluxes then does not require high-resolution microscopy due to the specific localization of the sensors. Based on this concept, a variety of nanosensors have been developed for small molecules (phosphate, carbohydrates, and amino acids) using bacterial periplasmic binding proteins or transcriptional regulators as the backbone (27–35) (Table 19.1). All published FRET sensors developed by the Frommer lab can be obtained from Addgene (<http://carnegiedpb.stanford.edu/research/frommer/nanosensors/index.html>) for a nominal fee.

Therefore the combination of in vivo metabolite imaging techniques and mass spectrometry-based fluxomics is likely to be required to understand the dynamics of metabolic systems. For a comparison of the two approaches, cf. Table 19.2.

4. Comparison of FRET Sensor and Carbon-13-Based Fluxomics

When integrating ^{13}C -data, extracellular fluxes, and biosynthetic requirements with computer models, ^{13}C -based fluxomics can reach high pathway coverage for those parts of the metabolism where a particular tracer molecule is converted and suitable analytes are

available to track the resulting isotope patterns of these conversions. The flux distribution is typically identified by iterative fitting of fluxes to the measured data, whereby the difference between observed and simulated isotope spectra is minimized (36). Essentially, this is a parameter fitting procedure where the relation between unknown fluxes and measured data is described by mathematical models of varying complexity. Most published data sets were obtained from (quasi) steady-state growth in glucose media, while other substrates remained largely unexplored although they are principally amenable to the current methods. Its strength, in particular with respect to FRET sensors, is the ability to resolve fluxes through several competing or diverging pathways such as in the ubiquitous central metabolism. If one accepts the limitation that cells are cultured in a stable steady state, appropriate isotope experiments typically resolve the distribution of flux between competing pathways with an accuracy of about 5% (5,36). While standard ^{13}C -flux methods are based on relatively tedious experiments and data analysis, a simplified method based on a direct and local interpretation of selected labeling patterns – so-called flux ratio analysis – enables high-throughput monitoring of intracellular flux distributions (37,38).

A major limitation of most current methods is that they rely on the detection of isotope patterns in amino acids bound in cell proteins, which requires that these amino acids be actually synthesized from a labeled source molecule, thus precluding the analysis of non-growing cells or cells cultivated in complex media. A second major disadvantage that also relates to pattern detection in proteinogenic amino acids, is the restriction of current ^{13}C -methods to steady-state conditions. Analyzing metabolism under biologically relevant dynamic conditions requires different methods. One of these is the detection of isotope patterns in free intracellular intermediates, where an isotopic steady state can be attained within minutes to a few hours, enabling dynamic analyses at this time scale. To achieve higher dynamic time resolution, alternative methods that measure during the period of isotopic instationarity are currently under development (39,40). The down side is that these methods will be even more tedious than the above ^{13}C -flux methods. With the exception of certain in vivo NMR experiments with a relatively low resolution and sensitivity, essentially all ^{13}C -methods are destructive.

FRET sensors typically analyze a single metabolite at a time and they cannot detect flux changes unless there is a change in the concentration of the metabolite. Multiplexing is possible by either targeting sensors to different compartments and analyzing the cellular regions separately or using sensors with separated spectral properties. Even when there is an observable rate of concentration change of a metabolic intermediate the way in which the change relates to flux has to be studied on a case-by-case basis. The FRET change reflects the sum of total flux change, which consists of all possible components affecting the influx and efflux of the metabolite. In vivo glucose measurements in *Arabidopsis* roots using glucose FRET sensors illustrate the point (Fig. 19.1) and (41). The rate of glucose concentration change in the cytosol can be calculated from the slope of the FRET change. This rate reflects the influx (import/uptake and synthesis) and efflux (export, subcellular transport, and metabolism) of glucose in the cytosol of *Arabidopsis* root cells, provided the perfusion system is not limiting. Additional experiments are required to establish how much each of these components contributes to the measured rate. This typically involves manipulation of the system with genetic or chemical (specific inhibitors) tools and/or the use of isotopes.

The advantage of FRET sensors is their applicability for in vivo determination of cellular, tissue-specific, and subcellular metabolite concentration changes (29,30,41), measurement of steady-state concentration of metabolites (35,41), and screening of signaling networks affecting metabolite concentrations in vivo (Haerizadeh and Frommer, unpublished). Apparently, even imaging at low magnification can provide cellular resolution (41). Since

the sensors are genetically encoded, they can be targeted to subcellular compartments as demonstrated for the glucose sensor, which by fusion to a nuclear targeting sequence was successfully employed to measure nuclear glucose flux (29), or by fusion to an ER targeting and retention sequence could be used to monitor glucose flux across the ER membrane (31). Exocytosis of glutamate was monitored by targeting and anchoring the glutamate sensor to the cell surface (35). Apparently, extracellular analysis can simply be performed by adding purified sensor to the cells or tissues of interest (42).

These attributes qualify FRET sensors uniquely for studies of how and when the concentration or net flux of a metabolite varies across an organ, tissue, or cell. Another great advantage of FRET sensor technology is their applicability to large-scale screens of chemicals or mutant collections. In the case of single cells, fluorescence microplate readers may be used instead of imaging to analyze FRET responses of a large number of samples in a short time (43).

The use of FRET sensors already provided new insights into metabolic processes. FRET sensors with different affinities for glucose were used to show that the cytosolic glucose concentration in soil-grown roots can drop to <100 nM in the absence of external glucose supply in *Arabidopsis* roots (41). This estimate is much lower than the previous estimation of cytosolic glucose concentrations in heterotrophic tissues (potato tuber) measured using nonaqueous fractionation (NAF) to provide subcellular resolution (44). Concentration estimations using disruptive extraction and analysis methods rely on estimations of cellular compartment volumes. Farré et al. estimated the volumes from electron microscopy pictures of cellular cross sections (44). The sensors were also used to carefully characterize the protonophore-insensitive accumulation of glucose and sucrose in root tips of *Arabidopsis* (45). FRET sensors measure steady-state levels and detect concentration change directly in vivo and are therefore superior tools for the analysis of factors affecting metabolite concentrations in the cell of interest. They may even allow more accurate estimation of subcellular compartment volumes when combined with NAF analysis of total metabolite amounts in subcellular compartments. FRET sensors also provide a tool to test for the potential metabolite oscillations, as used to analyze cytosolic calcium waves (46).

A detailed comparison of the specific advantages and drawbacks of FRET sensor and ^{13}C -flux technologies is presented in Table 19.2.

5. In Vivo FRET Imaging in Arabidopsis – A How to Guide

FRET can be measured either in a fluorimeter or by imaging. Many excellent overviews over the use of FRET in biology have been published (47–50). Quantitative analysis of FRET data derived from imaging approaches has been used most extensively to determine changes in calcium in neurobiology. Several excellent how-to-guides have been published (51–53). While written for applications in the animal field, the technical approach is highly similar for plants as are the challenges, e.g., how to carry out analyses in live organs. The reader is thus referred to these reviews for details in the methodology. FRET sensors for calcium and fluorescent indicators for pH have also been used by a small number of plant labs (46,54–57). Thus here, mainly aspects relating to metabolite imaging will be covered.

5.1. Expression of FRET Sensor Constructs in Plants

Glucose FRET sensors have successfully been used to monitor glucose levels in intact roots and in leaf slices of *Arabidopsis* (41). Stable transgenic *Arabidopsis* lines for the FRET sensors of interest are created using standard transformation protocols. Most calcium imaging studies have been carried out in guard cells (46). Apparently, FRET sensors are subject to gene silencing in *Arabidopsis* (41). This has not precluded the analysis in guard

cells since these cells, at least when mature, are protected from gene silencing (58). Thus to be able to monitor FRET sensors in other tissues, gene silencing has to be overcome. This can be achieved either by the use of gene silencing mutants (41) or by analyzing young seedlings at stages before silencing has reduced fluorescence below levels necessary for obtaining high-quality FRET images. Alternatively, it may be possible to use cell-specific or regulated promoters to circumvent gene silencing.

For all of the metabolite sensors developed so far a series of affinity mutants are available (e.g., for FLIPglu (41), FLIPE (35), and FLIPPi (32)). It is recommended to use several affinity mutants to exclude artifacts due to changes in other parameters that may either affect the fluorophores or the recognition element. If the FRET change is due to a change in analyte levels, the response curves should shift according to the affinity of the sensors used (cf. (41)). If affinity mutants of the sensor, which typically differ only in a single amino acid, show identical responses, additional controls such as analysis for pH shifts may be necessary. pH shifts can be monitored using fluorescent indicator proteins expressed in control plants (56).

FRET is analyzed by determining the relative fluorescence intensity of the two fluorophores, typically YFP and CFP. The fluorescence intensity is measured with either a fluorimeter or a fluorescence microscope.

5.2. Instrumentation for Imaging-Based FRET Metabolite Analysis in Plants

The FRET sensors for metabolites described here contain a recognition element fused to two spectral variants of GFP. FRET between CFP and YFP can be measured using a variety of methods such as fluorescence lifetime imaging (FLIM), fluorescence recovery after photobleaching (FRAP), anisotropy decay or simply by ratiometric analysis of the relative fluorescence intensities of FRET donor (CFP) and FRET acceptor (YFP). Due to the fixed molar ratio of the two fluorophores, the simplest method, i.e., ratiometric analysis of emission intensities, is sufficient for most applications. The signal-to-noise ratio of the described metabolite FRET sensors is sufficient to use “poor human’s FRET”, i.e., simple recording of the emission intensities at two wavelengths. More sophisticated approaches may be recommended that correct for bleed-through (direct excitation of the acceptor at excitation wavelength) or for changes in sensor levels or proteolysis of the sensor (by normalization to acceptor amount obtained by recording acceptor emission at the acceptor’s excitation wavelength) (59). It is important to note that a ratio change cannot necessarily be attributed to a change of FRET, e.g., during photobleaching or due to interference of other parameters; the two GFP variants may differ in their sensitivity to photobleaching or other parameter changes or changes in the focal plane may mimic a FRET change. Inspection of the raw data (individual fluorescence emission intensities and correction as described above) will help identify potential artifacts.

Due to the low intrinsic noise of metabolic signals such as glucose (Fig. 19.1 and (60)), the relatively slow rate changes compared to calcium spikes together with the ability to express the sensors to high levels in stably transformed plants allow the use of simpler acquisition systems. Since the sensors can be targeted genetically to subcellular compartments, epifluorescence imaging is sufficient for most cases. Since spatial resolution is not relevant, essentially a single or few pixels per cell are sufficient, thus allowing pixel binning to enhance the signal-to-noise ratio. It is also possible to record FRET using a confocal microscope, e.g., to observe spatial differences inside a cell.

For ratiometric FRET analysis the following instruments are required: a microscope stand with fluorescence optics, a fluorescence excitation light source, appropriate filters, a filter switching device or image splitter and a digital camera for acquisition of emission, a

perfusion system to be able to change the analyte levels in the perfusion medium, and software for driving the instruments. A complete and workable epifluorescence FRET imaging system suitable for metabolite imaging can be assembled for below \$50,000. Apparently, if a suitable microscope and camera are available, a FRET imaging system can be assembled at minimal cost. Factors that determine the cost include quality of the stand, number of objectives, sensitivity of the camera, the use of free or commercial software, and the versatility of the devices such as fast multi-wavelength acquisition and computer-controlled perfusion. Notwithstanding, systems are available that enable spectral imaging to obtain full spectra of donor and acceptor as well as background fluorescence. Such data can then be used for spectral unmixing to obtain reliable FRET data even in cells with significant fluorescence background (61).

Epifluorescence microscopes are well suited for whole tissue analysis as well as single cell analysis. Apparently, fluorescence intensity drops when tissues deeper inside an organ are analyzed. However, when analyses are performed in roots expressing the sensor in all cell types as described by Deuschle et al. (41), it is not possible to determine cellular responses reliably. The use of specific promoters active only in certain cell layers provides an alternative to the use of confocal microscopes in order to obtain tissue layer or subcellular resolution. Confocal microscopes have to be used with caution as changes in focal plane due to swelling or shrinking of the cells as a consequence of changes in the composition of the perfusion medium may lead to artifacts. Tracking of the z-stacks may be required to verify that the same z-section is analyzed. The apparent advantage of confocal microscopes is that they reduce the background fluorescence originating from tissues outside the region of interest.

Several parameters affect the signal-to-noise ratio and thus the quality of the data as well as the detection range, e.g., fluorescence intensity over background and signal change of the sensor. Therefore a lot of effort has been invested in improvements in sensor responses (27,60,62). On the other hand, typically, the higher the emission intensity, the higher the signal to noise. Due to the occurrence of photobleaching, apparently too high excitation light may be damaging. At low magnification, the amount of excitation light from a normal fluorescence light source is limiting, thus lower angle/high-magnification lenses, high-intensity light sources (Hg band at 435 nm of mercury lamps, high-power Xenon lamps, high-power LED lights or lasers), high-transmission filters (such as high-throughput modified magnetron sputter-coated filter sets), and high-sensitivity cameras with on-chip multiplication gain have proven advantageous. If high excitation leads to photobleaching, one may reduce excitation intensity by neutral density filters, reducing the frequency of acquisition while increasing integration times for acquisition or camera gain.

To monitor responses of FRET glucose sensors (containing eCFP as donor and Venus or eYFP as acceptor) we mount roots of intact seedlings in a perfusion chamber (e.g., P1 Warner Instruments, USA) and on a stage adapter (41,60). A wide spectrum of open and closed perfusion chambers suitable for different applications is available from different companies. Ratio imaging is performed on an inverted fluorescence microscope (DM IRE2, Leica) with a QuantEM digital camera (Roper) and a 20× oil objective (HC PL APO 20×/0.7IMM CORR, Leica, Germany). Essentially, any high-quality inverted microscope can be used for this purpose. Dual emission intensities are simultaneously recorded using a DualView with a dual CFP/YFP-ET filter set (high-transmission modified magnetron sputter-coated filter sets ET470/24m (470 indicates emission wavelength, /24 indicates bandwidth); ET535/3, Chroma, USA) and Slidebook software (Intelligent Imaging Innovations, Inc., USA). The Dualview (or similar image splitter from other companies) enables simultaneous recording of both emission wavelength without mechanical filter switching. For most metabolic imaging studies, filter wheels that automatically switch

between the two emission wavelengths are equally suitable. Software for FRET image acquisition is available from a variety of commercial vendors, as scripts from individual labs, or can be implemented using the free software package ImageJ (rsb.info.nih.gov/ij/). The use of EM-gain cameras may be advantageous when analyzing low-fluorescence samples or when using low magnification, but in general is not crucial. Excitation (filter ET430/24x, Chroma) is provided by a Lambda DG4 light source (Sutter Instruments; <http://www.sutter.com>), which enables rapid switching between several excitation wavelengths, a feature used when normalization to YFP emission is intended. Simpler light sources are available from a variety of vendors. Images are acquired within the linear detection range of the camera and depend on the expression level. Exposure times used for measuring glucose flux vary typically between 300 and 600 ms, with software binning 2 and at an EM gain of 300. Typical values for acquisition with an non-EM gain camera such as the Coolsnap HQ (Roper) have been described (41). Fluorescence intensities for eCFP and eYFP are typically in the range of 2000–8000 and 6000–14,000, respectively. YFP, CFP, and ratio images of an *Arabidopsis* root tip are shown in Fig. 19.1. The software allows to select regions for analysis that can be freely chosen, e.g., to determine the YFP/CFP ratio of individual cells or of groups of cells (grey and black squares). Regions outside the tissue are analyzed for background subtraction (large white square). Image stacks derived from time laps analysis are used to obtain traces of the ratio over time (lower panel Fig. 19.1.) Typically the software provides an option for real-time monitoring of images for the individual channels (Fig. 19.1) and traces of the intensities for each fluorophore as well as traces of the ratio for the individual regions that were selected. Acquired image stacks can be analyzed by selecting different regions and the quantitative data can be transferred to data analysis programs for more detailed analysis (e.g., ASCII or spreadsheet export function). Some software also provide options to implement corrections, e.g., bleed-through correction obtained from cells expressing CFP and YFP alone as well as automatic background subtraction or normalization to YFP excitation/emission.

5.3. Perfusion Chamber

One of the difficulties of observing live organisms under the microscope, especially in the context of quantitative imaging, is the necessity to exclude movement under perfusion and during time lapse, while ensuring free exchange of the perfusion medium. To prevent movement, roots were mounted on coverslips using medical adhesive (stock no. 7730, Hollister). Alternatively, tracking software may be used to register images in stacks (e.g., stackreg in ImageJ).

Control of perfusion buffer composition, temperature, flow rate, and chamber volume is of paramount importance to ensure reproducible experiments. If rates will be recorded rather than just steady state, it is also important to be able to change the perfusion media surrounding the specimen at velocities that are not limiting. Minimizing the chamber volume and efficient peristaltic pump or pressurized gravity-operated systems ensures that FRET response is not limited by substrate supply. Precise event marking and knowledge of the dead volume of the perfusion system (time until new buffer reaches cells) are important for correlating the response to the change in perfusion. Computer control over the perfusion system and TTL-linked acquisition to the valves of the perfusion system increase the data quality. Root perfusions as shown in Fig. 19.1 are performed with full nutrient medium containing typical macro- and micronutrients buffered with 20 mM MES to pH 5.8 at 3 ml/min containing the molecule of interest. Apparently accessibility of the perfusion medium to the tissue is essential. Therefore roots are apparently ideal objects. For analysis of other organs such as hypocotyls, leaf, or stem, access to the perfusion medium needs to be ensured, e.g., by removing the cuticle, by using cuticle mutants, or by using organ slices (41).

5.4. Analysis of FRET Data

Baseline shifts of the FRET response can be corrected using second- or third-order polynomial fits of the ratio measured in the absence of treatment. The obtained function describes the baseline aberration (photobleaching) as a function of time during perfusion. To correct for this effect, the difference between the ratio at the beginning of the experiment $r(0)$ and the baseline aberration $f(t)$ is calculated at each time point of the measurement and added to the value of the measured ratio at the respective time point $r(t)$: $r_{\text{corr}}(t)$ or $r(t) + [r(0) - f(t)]$ (63).

Example of flux calculation from FRET slope data: the cytosolic glucose concentration can be calculated using the equation: $[\text{gluc}]_{\text{cytosol}} = K_d \times (r - 1)/(R_{\text{max}} - r)$. R_{max} is the maximum Δ ratio, which can be determined by measurement of the ratio at 95% saturation, K_d is the in vitro glucose binding affinity of the sensor, and r is the Δ ratio at each glucose concentration. The in vivo apparent $K_{0.5}$ of each nanosensor can be determined by fitting to the Michaelis–Menten equation; $r = [\text{gluc}] \times R_{\text{max}}/(K_{0.5} + [\text{gluc}])$; $[\text{gluc}]$ is extracellular glucose concentration; and r is the initial ratio change rate after glucose loading (Δ ratio/s). This calculation relies on the assumption that the sensor has the same K_d in vivo as in vitro. To determine the initial flux rate in vivo, the initial accumulation rate is calculated by using time-ratio plot 2–20 s after glucose loading (provided sensor dynamics and perfusion are not limiting).

6. ^{13}C -fluxomics – How-to-Guides

Recent how-to-guides to ^{13}C -fluxomics can be found for local flux ratio analysis by Nanchen et al. (4) and for ^{13}C -flux balancing by Ratcliffe and Shachar-Hill (8).

Acknowledgments

This work was supported by grants to WBF from NIH (NIDDK, 1R01DK079109-01) and DOE (DE-FG02-04ER15542). TN was supported in part by an HFSP fellowship.

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Fig. 19.1.

Glucose-induced FRET changes in the cytosol of intact *Arabidopsis* roots. The FRET sensor FLIPglu-600 $\mu\Delta$ 13 with an affinity for glucose of 600 μ M in stably transformed *rdr6-11* *Arabidopsis* plants (41) responds to perfusion with 20 mM glucose. *Top panel:* Images of the root tip for the YFP and the CFP channels as well as the ratiometric image with pseudocolor converted to grayscale are shown (at time 0). The regions from which the quantitative data are calculated are shown on the ratiometric image as black (1) and gray (2) boxes. Data from the white box (B) was used for the background correction. Quantitative data were derived by pixel-by-pixel integration of the regions in the ratiometric image. *Bottom panel:* The graph shows the ratio of eYFP intensity divided by eCFP intensity (normalized to the starting ratio) for the two regions (gray trace corresponds to gray box (2) after background subtraction and normalization, black trace (1) corresponds to black box) at different time points (here 10 s intervals) measured over 8 min. The bars on top of the trace give the concentration and the duration of the glucose perfusion. The response is fully reversible. Note that accumulation and elimination phases show different rate constants. Also note the low noise as apparent from the smooth trace.

Table 19.1

FRET sensors for ion and metabolite analysis

Analyte	Recognition element	Sensor construct	Reference
Glucose/ galactose	Bacterial periplasmic binding protein	FLIPglu	(27,41,60)
Maltose	Bacterial periplasmic binding protein		(28,43)
Sucrose	Bacterial periplasmic binding protein		(64)
Ribose	Bacterial periplasmic binding protein		(34)
Arabinose	Bacterial periplasmic binding protein		(43)
Glutamate	Bacterial periplasmic binding protein		(27,35)
Tryptophan	Bacterial repressor protein		(33)
Arginine	Bacterial periplasmic binding protein		(65)
Calcium	Calmodulin, troponin C		(52,62)
Phosphate	Bacterial periplasmic binding protein		(32)
Other FRET sensors	Various		For review, cf., e.g.,(47,49)

Table 19.2Side-by-side comparison of ^{13}C - and nanosensor-based fluxome analyses

Application	^{13}C -fluxomics	Nanosensor-based fluxomics
Pathway coverage	High	Limited to single node
Sensitivity	> 0.1 mmol analyte per hour and g of cells (dry weight)	Depends on the K_d and dynamic range of the nanosensor: e.g., ultrahigh affinity sensors such as FLIPglu170n Δ 13 can provide nM analyte per second per cell
Information content (cells, tissues, or cell populations)	Population average	Population average, single cells, or subpopulations of cells
Compartment specific	Currently not available	Targeting allows to specifically analyze cytosolic and organellar levels and flux across intracellular membranes
Temporal resolution	0.8 s intervals (39)	Full frame acquisition is possible in 200–1000 ms intervals (cf. (51,60); small regions can be imaged at 10–30 Hz (53))
Suitability for dynamic analysis	Good	Excellent
Sensitivity to changes in other parameters	N/A	Sensor conformation may be affected by pH, ionic strength, posttranslational modification, binding to other proteins, or proteolysis (can be controlled for by using set of affinity mutants; effect of pH can be determined in vitro and corrections can be applied if in vivo pH is monitored)
Invasiveness	High	Relatively low; however the additional ligand buffer may affect the cell's physiology (can be evaluated by comparing wt and transformant using ^{13}C -fluxomics)
Suitability for high throughput	Hundreds of mutants (19)	Thousands of mutants, complete genomes possible