Development and use of fluorescent nanosensors for metabolite imaging in living cells


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Abstract

To understand metabolic networks, fluxes and regulation, it is crucial to be able to determine the cellular and subcellular levels of metabolites. Methods such as PET and NMR imaging have provided us with the possibility of studying metabolic processes in living organisms. However, at present these technologies do not permit measuring at the subcellular level. The cameleon, a fluorescence resonance energy transfer (FRET)-based nanosensor uses the ability of the calcium-bound form of calmodulin to interact with calmodulin binding polypeptides to turn the corresponding dramatic conformational change into a change in resonance energy transfer between two fluorescent proteins attached to the fusion protein. The cameleon and its derivatives were successfully used to follow calcium changes in real time not only in isolated cells, but also in living organisms. To provide a set of tools for real-time measurements of metabolite levels with subcellular resolution, protein-based nanosensors for various metabolites were developed. The metabolite nanosensors consist of two variants of the green fluorescent protein fused to bacterial periplasmic binding proteins. Different from the cameleon, a conformational change in the binding protein is directly detected as a change in FRET efficiency. The prototypes are able to detect various carbohydrates such as ribose, glucose and maltose as purified proteins in vitro. The nanosensors can be expressed in yeast and in mammalian cell cultures and were used to determine carbohydrate homeostasis in living cells with subcellular resolution. One future goal is to expand the set of sensors to cover a wider spectrum of metabolites by using the natural spectrum of bacterial periplasmic binding proteins and by computational design of the binding pockets of the prototype sensors.

The demand for novel approaches in metabolite imaging

Despite the increasing progress in the field of metabolite transport, we poorly understand the regulatory networks controlling metabolism. Furthermore, major components of the transport machinery (especially efflux systems for most ions and metabolites) are still unknown. Moreover, biochemical pathways are often distributed between several compartments and even neighbouring or distant cells. Thus, metabolite levels in groups of cells and different tissues are not necessarily uniform, as elegantly shown for glucose and lactate in liver or for nitrate in plant leaves [1,2]. In the past, metabolite levels were analysed as average concentrations over whole tissues or organs since measuring spatiotemporal metabolite distribution with high resolution was difficult. Many approaches only have limited resolution, often require disruption and fractionation of tissue and thus are static. A number of recent studies have addressed these limitations. Cell sap sampling from single cells was a major advance permitting measuring metabolite levels with cellular resolution and correlating it with gene expression [3,4]. Using an enzyme-based approach, sucrose and glucose concentrations in thin sections of plant tissue were determined [5–7]. To analyse metabolite levels with subcellular resolution, tissue extracts were partitioned. Using apoplasmic wash fluids average metabolite levels in the apoplasm were measured, whereas non-aqueous fractionation was used for compartmental analysis within cells [8,9]. Still, none of the above approaches permits real-time measurements in living cells. On the other hand, most techniques enabling dynamic measurements only offer limited spatial resolution. Positron emitting tracers in combination with PET (position emission tomography) imaging have a spatial resolution in the range of 1 mm [10]. Using water suppression strategies the in-plane resolution of 1H NMR micro-imaging is several hundreds of micrometres within slices of several hundred millimetres in thickness [11]. 13C-imaging with indirect detection techniques of 13C-labelled compounds achieves a similar spatial resolution [11]. Imaging at this resolution provides important new perspectives; however, it is not sufficient to detect differences in metabolite levels at the cellular or subcellular level. Substantially higher resolution was achieved by fluorescence microscopy using fluorescent probes combining subcellular resolution with high sensitivity and great versatility. Moreover, the use of chromophore pairs suitable for fluorescence resonance energy transfer (FRET) was shown to greatly enhance the ability to visualize a number of cellular processes.
FRET as a tool

FRET refers to a quantum mechanical effect between a fluorescence donor and a suitable acceptor. Basic requirements for FRET are an overlap between the donor emission and the acceptor excitation spectra, as well as the close proximity of the chromophores. Energy transfer is non-radiative and depends on the inverse sixth power of fluorophore distance. Thus, bringing the fluorophores closer together leads to decreased donor chromophore emission while the emission from the acceptor chromophore increases. The Förster distance ($R_0$) is a unique property of a FRET-pair and defines the distance where transfer is 50% efficient. $R_0$ is a function of the spectral overlap between donor emission and acceptor excitation spectra, the quantum yield of the donor in the absence of the acceptor and the relative orientation of donor and acceptor chromophore transition dipoles. Typically, the Förster distance is between 20 and 60 Å and thus in the range of protein dimensions [12]. Assuming a randomized orientation of the transition dipoles before energy transfer, FRET was used as a ‘microscopic ruler’ to determine distances between molecules [13].

Fluorescent dyes for imaging cellular processes

Currently, a wide variety of genetically encoded and synthetic fluorescent dyes are available. Whereas synthetic dyes need to be taken up by the cell, genetically encoded sensors can be specifically targeted to different subcellular compartments and selectively expressed in basically every kind of cells. Making use of the advantages of genetically encoded sensors Rogers Tsien’s and Atsushi Miyawaki’s groups created an efficient set of tools mostly based on fusions of suitable reporter domains to different variants of the green fluorescent protein (GFP) [14]. The earliest FRET-based sensor-protein made use of blue fluorescent protein (BFP) and GFP as reporters linked to a protease-sensitive peptide [15,16]. Proteolysis abolishes FRET by irreversibly separating the fluorophores. The first approach based on reversible conformational changes was a fusion of cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and a reporter-domain consisting of calmodulin and the Ca$^{2+}$-calmodulin binding M13-peptide, commonly known as cameleon [17]. Since then a wide variety of reporters for a number of cellular events have been developed on the basis of this principal design [18–24]. However, despite the ingenious concept of conformation-dependent FRET-based sensors, until recently, sensors for primary metabolites such as sugars were not available.

Periplasmic binding proteins (PBP)s as binding domains of FRET-based metabolite sensors

Binding domains need to meet certain criteria when considered as scaffolds for conformation-dependent FRET-based sensors. First of all, the binding domains must undergo a conformational change large enough to translate metabolite binding into a change in FRET. Moreover, the dimensions of the binding domains should provide attachment sites ideally separating the chromophores by their Förster distance, hence enabling the largest change in FRET at a given change in fluorophore distance. At best, the binding domain belongs to a large family of proteins sharing similar three-dimensional structures and covering a wide spectrum of substrates. Ultra high substrate affinity would provide an ideal basis to engineer mutant nanosensors for different detection ranges by site-directed mutagenesis.

In the search for suitable binding domains matching the criteria outlined above, we have focused our attention on PBPs, a class of diverse proteins found mainly in bacteria [25]. Although PBPs are unrelated at the primary sequence level, they consist of two similar globular domains. Typically, PBPs have a diameter of approx. 5–7 nm, and thus provide attachment sites in the range for the Förster distance of the CFP/YFP FRET-pair [26]. Specific residues in the cleft between the domains form the binding site, engulfing the substrate by undergoing a hinge-twist motion [27]. Typically, substrate affinities are in the nanomolar range. Crystal structures of more than a dozen PBPs, either with or without bound ligand, provide us with a very detailed understanding of the mechanisms of binding and the closing motion [28].

To develop a prototype nanosensor CFP and YFP were attached to the Escherichia coli periplasmic maltose binding protein (MBP). Using site-directed mutagenesis amino acids involved in maltose binding were changed producing FLIPmal-25 (fluorescent indicator protein for maltose with a $K_d$ of 25 µM) [29]. For initial analysis the prototype nanosensor was purified from an E. coli expression strain. As predicted from the positions of the termini in the crystal structures with and without bound ligand, addition of maltose led to an increase in FRET. The affinity of the purified nanosensor could be determined easily by titrating with maltose and measuring FRET using a microplate fluorimeter. Following transformation of a yeast strain that constitutively expresses a plant sucrose transporter, which also mediates active transport of maltose, the prototype nanosensor enabled real-time visualization of maltose uptake into the cytosol of individual yeast cells (Figure 1) [29].

To demonstrate the general applicability of the approach, a set of glucose and ribose nanosensors was engineered on the basis of the E. coli periplasmic glucose/galactose- (GGBPs) and ribose binding proteins (RBP)s [30,31]. Although GGBPs and RBPs are unrelated to MBPs at the primary sequence level, they have similar 3D structures. However, in the case of RBPs and GGBPs the termini are located at the proximal ends of the lobes relative to the hinge region, while in MBPs the termini are at the distal ends. Hence, upon ligand binding, in RBPs the termini move further apart, whereas in the case of MBPs they move closer together. Consistently, FRET-efficiency decreases upon binding of ribose. Similarly, glucose leads to a decrease in FRET upon binding to the glucose sensor (Figure 2). Interestingly, the maximum ratio change of all sensors is approximately 0.25 suggesting that other parameters such as chromophore dipole orientation...
Figure 1 | Uptake of maltose into the cytosol of individual yeast cells
The ratio image of an individual yeast cell is shown using pseudo-colours. Red colour is indicating high ratios, blue colour low ratios. Addition of external maltose leads to an increase in ratio as indicated by the appearance of red colour leaving out the central vacuole indicating uptake of maltose into the cytosol.

Figure 2 | Emission spectra of a purified glucose sensor
Emission spectra of FLIPglu-170n in the absence and presence of glucose show that FRET decreases upon binding of ligand. w/o glucose, without glucose.

Figure 3 | Ribose dynamics in mammalian cells
Upon addition of external ribose (grey bar) the ratio decreases inside the cytosol indicating uptake of ribose. Following withdrawal of the external supply the ratio slowly increases until it reaches its initial value.

also contribute to the observed ratio changes. Taken together, it appears feasible to design a wide spectrum of nanosensors exploiting the natural variety of PBPs and related proteins.

Measuring sugar levels in mammalian cells already gave us new insights into sugar homeostasis. Using FLIPglu-600µ, a glucose nanosensor with a $K_d$ of 600 µM, free glucose was detected in the cytosol of African green monkey derived COS-7 cells [30]. Metabolism of glucose occurred rapidly keeping cytosolic levels at approximately 50% of external supply under physiological conditions. Measuring glucose levels in the presence of cytochalasin B, an inhibitor of mammalian glucose transporters, suggests that the decrease in cytosolic glucose upon removing the external supply is caused by metabolism rather than export. Similar results were obtained with ribose: upon addition of external supply ribose accumulates in the cytosol, where it is slowly metabolized rather than exported from the cell (Figure 3) [31]. A major advantage of genetically encoded nanosensors is their suitability for subcellular analyses. Using appropriate targeting signals, the nanosensors can be easily directed to different subcellular compartments. Using a nuclear glucose sensor, it was shown that nuclear and cytosolic glucose levels in COS-7 cells are tightly coupled and very similar [32]. Currently, glucose levels in the endoplasmic reticulum (ER) are analysed to characterize the mechanisms controlling glucose homeostasis.

Future applications
As outlined above, the nanosensors enable compartmental analyses of metabolite levels, metabolic activity and intracellular transport in cell cultures transiently or stably expressing the nanosensors. To better understand how neighbouring cells within a certain tissue, and how different tissues forming an organ, functionally interact, imaging will be performed on tissue slices derived from transgenic organisms expressing the sensors. However, measuring metabolite levels in cells several layers deep in tissue puts new technical challenges on the application of the nanosensors. Fluorescence from cells outside the focal plane, shading effects by overlying cells as well as absorption of donor fluorophore emission by acceptor fluorophores in overlying tissue will affect the signal. Especially in plants, potential problems can arise from background fluorescence and the possible effect of factors on the FRET signal other than ligand binding, i.e. alteration of fluorophore properties or conformational changes caused by ionic conditions or pH. To ameliorate these difficulties, the expression of the nanosensors can be restricted to a fraction of the cell, i.e. by directing the sensors to nuclei as shown for COS-7 cells [32]. Alternatively, the nanosensors can be selectively expressed under the control of tissue-specific promoters. Despite the nanosensor’s suitability for

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in vivo imaging applications, the comparatively small maximum ratio change limits the sensitivity and dynamic range of the current versions. Although a set of mutant nanosensors may be generated to cover the full physiological range of concentrations, the nanosensors will be further improved by increasing the maximum ratio change as has been done over time for the cameleon [33]. Furthermore, more sophisticated imaging technologies than simple ratiometric measurements, such as fluorescence lifetime imaging microscopy (FLIM), Nipkow spinning disk confocal microscopy, multiphoton imaging technologies than simple ratiometric measurements, deconvolution-based approaches can be used to obtain images with higher spatial resolution [34,35].

With the development of genetically encoded nanosensors, significant progress has been made towards real-time, high-resolution imaging of metabolites. Thus, further exploitation of the natural spectrum of PBPs in combination with re-modelling the binding sites of existing sensors by computational design together with new developments in imaging technology will contribute to a better understanding of transport and compartmentalization in multi-cellular organisms [36–38].

References

Received 17 September 2004