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Fluxomics: mass spectrometry versus quantitative imaging

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Abstract

The recent development of analytic high-throughput technologies enables us to take a bird's view of how metabolism is regulated in real time. We have known for a long time that metabolism is highly regulated at all levels, including transcriptional, posttranslational and allosteric controls. Flux through a metabolic or signaling pathway is determined by the activity of its individual components. Fluxomics aims to define the genes involved in regulation by following the flux. Two technologies are used to monitor fluxes. Pulse labeling of the organism or cell with a tracer, such as ^{13}C, followed by mass spectrometric analysis of the partitioning of label into different compounds provides an efficient tool to study flux and to compare the effect of mutations on flux. 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. In contrast to the mass spectrometry assays, FRET nanosensors monitor only a single compound. Both methods provide high time resolution. The major advantages of FRET nanosensors are that they yield data with cellular and subcellular resolution and the method is minimally invasive.

Introduction

Cells and organisms dynamically acclimate their metabolism to changing conditions, such as nutrient availability, temperature or stress. Flux across the plasma membrane and through metabolic pathways is continuously optimized. Sensory systems that are coupled with complex signaling networks adjust the flux and its direction via regulatory circuits. The sensory systems measure the extracellular availability of a given metabolite and other external cues, as well as the intracellular level of the metabolite or subsequent intermediates, and send signals to regulate transporter and enzyme activities by posttranslational modification, protein turnover, or changes in the rate of their biosynthesis. At present, we know little about the receptors that detect the signals, the signaling networks that transmit the information, nor their integration. A new discipline, fluxomics (Box 1), aims to systematically analyze the fluxes occurring within a cell, and at some point even to unravel these networks in a multicellular organism. The availability of large mutant collections, RNA interference (RNAi) or overexpression line libraries, and large chemical libraries now puts us in a position to unravel these networks, provided we have the tools to measure metabolite flux, especially in high throughput. This review discusses two sets of methodologies that have been developed to measure flux: first, mass spectrometric analysis of changes in metabolite levels after pulse labeling, and second, quantitative imaging using fluorescence resonance energy transfer (FRET)-based nanosensors. These recent developments are contrasted to established isotope labeling techniques for metabolic steady-state systems.

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Box 1

Glossary

Flux	in analogy to electric current, flux is the passage of molecules (moles of a particular metabolite) through a metabolic or transport step per unit cell mass per unit time. Defined by the concentrations of compounds participating in a reaction, enzyme level and enzyme properties.
Fluxome	commonly referred to as the totality of all fluxes in a system, e.g. a cell. The term fluxome was defined by Sauer *et al.* [55] as the array of fluxes (reaction rates on a per unit cell volume or per unit cell mass basis) for all of the reactions that occur in the organism. This term is typically used in the context of pulse labeling with ^{13}C-labelled metabolites, followed by mass spectrometry to analyze multiple (typically hundreds of) metabolites in parallel. Such analyses have been expanded to the comparison of fluxes in microorganismal mutants. The term fluxome can also be used to describe the factors (gene networks) that define a specific flux rate.
Fluxomics	the discipline that analyzes the fluxome as one part of systems biology [56•]. Provides mathematically defined networks of metabolic reactions and their regulation. Fluxomics are defined here as an approach to identify the genes that affect the fluxes which control the steady state of a single metabolite (using FRET nanosensors).

The importance of fluxes

All kinds of physical systems — living cells are no exception — are governed by a dualism between potentials and flows. Potential quantities are related to energy levels whereas flow quantities are related to transport or conversion phenomena. In mechanics, potentials and flows are represented by potential energy and force (a force is a flow of momentum), in hydraulics by pressure and liquid flow, and in electrical systems by voltage and current. For example, voltage can be directly measured by using electrostatic forces, whereas current, that is the flux of electrons, can be measured using a light bulb: the number of collisions per time unit determines the intensity of light emitted. In biological networks, the dualism is given by chemical activities and reaction rates, also called metabolic fluxes. At present, the potential, for example the concentration gradient, can be determined but fluxes must be derived. The precise relation between potentials and flows is described by constitutive laws such as Hooke's law in mechanics, Ohm's law for electrical systems, Fick's law for diffusion, the Nernst equation for electrochemistry, or mass action kinetics in chemistry. However, these are just the simplest examples.

The dualism between substance concentrations, or more precisely chemical potentials, and metabolic fluxes is expressed by the fact that one part is causal for the other. On the one hand, chemical potentials of reactants constitute the driving forces for fluxes. On the other hand, metabolic fluxes change the potentials of metabolite pools. Usually, flows in physical systems are closely related to system functions (i.e. dynamics), whereas potentials rather describe their stock-keeping aspects (i.e. statics). In the field of biochemical networks, in particular, metabolic fluxes are the ultimate manifestation of the cell's function under certain physiological conditions.

It might appear surprising that flux, such as that across the plasma membrane, is not optimized for a wide spectrum of conditions. The plasma membrane provides a limited compartment [1], however, and a given transporter (at least if not modified), has defined kinetic properties, such as high affinity (typically coupled with low capacity) optimized for importing nutrients present in low abundance or high capacity (typically coupled with low affinity). Thus, when nutrient availability or demand change, either a different set of transporters is required or the properties of the transporter have to be adjusted to allow for optimal flux [2–4]. The same is essentially true for many isozymes. It is thus important to determine both the flux potential and the actual flow, and to compare the system's behavior under different conditions, for example, different levels of nutrient availability.

In recent years, fluxome analysis under steady-state conditions has become a widely used tool. It is applied to characterize an organism [5,6], to diagnose the effect of genetic manipulations [7], to compare the behavior of one organism under different physiological conditions [8], to detect the presence of certain metabolic pathways [9,10], to compare mutant libraries [11••], or to monitor different growth phases [12]. This tool has been applied to all classes of organisms, including bacteria, unicellular eukaryotes, animal and plant cells, and even whole organs. Some recent reviews overlook the general methodology at the state of the art [13•,14–17]. Particular problems of plant fluxome analysis have been addressed by Ratcliffe and Shachar-Hill [18] and by Schwender *et al.* [19], and its application in plants is exemplified in a number of papers [20–22].

Methods in fluxomics

Fluxes are usually determined under metabolic steady-state conditions. There is, however, no reason why fluxomics should be restricted to this case. In fact, the rapid redirection of fluxes under dynamic conditions is an important capability of living organisms. Consequently, a new aspect in fluxomics is the consideration of time-dependent fluxes. As we discover below, the experimental methods required to determine these fluxes are quite different from steady-state methods. Interestingly, some current developments are combining methods from dynamic and steady-state approaches to reach new ambitious goals.

Although, the description of living cells by fluxes seems to be more natural than a description by substance concentrations, cells have predominantly been characterized by their intracellular pool sizes. The reason is rather simple: direct measurements of flows are possible in other physical systems but there is almost no known measurement procedure that directly yields information on metabolic reaction rates (an exotic exception might be the light emission in the luciferase reaction [Figure 1a]). This makes fluxomics a rather singular discipline in the 'omics' field.

As an example that illustrates this difference, electrical currents can be measured not only directly by magnetic induction but also indirectly from the voltage change of a capacitor. In the latter case, the signal must be differentiated with respect to time to obtain the time-dependent current. For an electrical system, this is usually not crucial because the measured signal has a high precision. This situation is completely different in the biological case, where measurement noise is significant and a direct differentiation leads to error amplification. To solve this problem, only initial velocities are calculated or signal smoothing algorithms are applied (Figure 1b). However, this helps only in case of a high data density or slowly changing pool sizes. If both approaches are not applicable, a model-based data evaluation is preferred in which a constitutive law is assumed (e.g. Michaelis–Menten kinetics) and only the parameters of this law are estimated (Figure 1c). This strongly

reduces the required amount of measured data, but the interpretation of the data is 'prejudiced' by the assumed law.

The present review focuses on the comparison and classification of different fluxomics methods for dynamic and static conditions and their interrelations. The flow-potential viewpoint will serve as a guideline. As the dual quantity of fluxes is given by the metabolite pool sizes, methods from metabolomics play an important role here by supplying the raw data for flux determination.

Flux analysis under highly dynamic conditions

The classical idea of measuring enzyme kinetic data from initial slopes of NAD signals immediately leads to indirect flux analysis methods that are based on time-resolved pool-size signals. A well-known class of experiments to investigate metabolic networks under highly dynamic conditions are represented by stimulus–response experiments ([23•,24; Figure 2a). Here, an external stimulus is imposed on the system by suddenly raising the extracellular concentration of a substance at time zero. The intracellular pathways immediately respond to the stimulus, usually within seconds or minutes. Under these highly dynamic conditions, the most difficult analytical problem is clearly the reliable quantitative measurement of intracellular pool sizes within short time intervals and with minimal interference with cellular functions.

Two methods have been developed to obtain dynamic flux information from measurements of pool size. In the first, a pulse stimulus in combination with rapid sampling serves to draw samples from a cell culture at high sampling frequency, up to several samples per second [23•,25]. Subsequently, each sample is rapidly inactivated, for example by cold methanol quenching. Cell disruption and separating the intercellular metabolites allows pool sizes to be determined using modern high performance liquid chromatography (LC)-MS instruments [26]. In essence, this is a technical improvement over the method originally introduced to biology by Calvin and Benson [27] to determine the carbon path in photosynthesis. The reliability of this procedure is still a controversy, however, because of the leakage of cell membranes when using methanol quenching. Moreover, it does not provide spatial information when applied to tissues or organs. Neither does it provide subcellular spatial resolution, which is of primary relevance in eukaryotic systems due to their high level of compartmentation. Finally, because of the destructive nature of this approach, the analysis of tissues is limited to parallel sampling. Thus, to date, fluxomics had been restricted to the analysis of unicellular systems in the population average.

An exciting new alternative to this method is given by the direct expression of sensors for the concentration of molecular metabolites in the cell. Certain proteins, specifically chemoreceptors, respond to ligand binding with a conformational change [28]. Protein conformation can be measured directly, using fluorescence resonance energy transfer [29]. FRET reporters in which recognition elements from diverse bacterial chemoreceptors are combined with green fluorescent protein (GFP) variants have permitted the development of genetically encoded flux-sensors for a variety of small molecules, such as calcium, phosphate, carbohydrates (ribose, glucose, maltose and sucrose) and amino acids [30,31••, 32•,33•,34,35]. These sensors can detect flux changes *in vivo* because they monitor steady-state levels of a given ion or metabolite.

A single sensor can already be used to analyze large mutant collections from any organism. The major advantage of these sensors over any other technology lies in the time resolution that they can provide (up to millisecond range as shown for calcium [36,37]). These sensors also provide high spatial resolution; for example, they have successfully been applied to

analyze metabolite levels in individual cells within intact organs [38•,39,40••]. On the one hand, a single sensor is able to monitor fewer substances than a MS approach.

Since these FRET sensors are genetically encoded, they provide subcellular resolution. This subcellular resolution is not achieved by optical methods, but genetically by targeting. The addition of targeting signals has successfully been used to monitor glucose flux across the membrane of the endoplasmic reticulum (ER) or nuclear fluxes [31••,41. Thus, local fluxes can be monitored by anchoring the sensors in specific membranes, for example at the cell surface [32••], and it is conceivable that even flux in localized domains, such as rafts, can be monitored this way. Finally, and most importantly, the sensor approach is non-destructive, making it possible to monitor an intact organ (or organism) with minimal invasion. Obviously, the expression of the sensors with a cell adds a new buffer for the analyte of interest that may affect metabolic flux.

The computer evaluation of dynamic experiments to estimate the time-dependent intracellular fluxes between the metabolite pools must currently rely on the assumption of a reaction kinetic mechanism (i.e. constitutive laws) for all of the involved transport and reaction steps. This gives rise to a differential equation model that describes the dynamics of intracellular pool sizes. By fitting this model to the measured data, the *in vivo* parameters of the enzyme kinetics are estimated ([42•]; Figure 3). Finally, the time-dependent metabolic fluxes (as a function of time) can be directly computed from the fitted model [43,44•].

^{13}C metabolic flux analysis in steady state

^{13}C metabolic flux analysis (^{13}C-MFA) is currently the best-established fluxomics technology. In contrast to flux analysis under highly dynamic conditions, the ^{13}C-MFA method always requires the assumption of metabolic steady state for the entire duration of the experiment. This limits the possible applications, but on the other hand, no assumptions on reaction kinetics are needed to derive the fluxes from the available measurements. Clearly, in this situation, knowledge of the intracellular metabolite pool sizes does not help to determine the fluxes.

This major source of information for computing the fluxes is the isotope labeling of substrates that are fed into the system (usually with ^{13}C but other isotopes are discussed [45•]). After switching the feed to a labeled substrate, the isotopes are distributed over the intercellular network by metabolic activities (Figure 2b). After some time, both the metabolic fluxes and the fluxes and fractions of labeled material in the metabolite pools can be shown to have reached a steady state [44•]. This fractional information can be obtained using NMR or MS. Interestingly, once again, the intracellular fluxes have to be determined from a concentration-like quantity.

The measured fractions of isotopic labeling in intracellular pools form the basis of a rather complex mathematical model that describes the distribution of labeled material over cellular metabolism and relates the unknown fluxes with the given measurements. This is not a real biological model but rather a physical model that provides probabilistic rules describing the distribution of isotope label. Thus, the validity of this complex model is non-critical, in contrast to mechanistic reaction kinetic models for biochemical networks in a dynamic metabolic state.

By fitting the isotope distribution model to the measured data, intracellular fluxes can finally be determined [17]. Additional information, given by the knowledge of forward and backward fluxes in bidirectional reaction steps, is generated by this method. This information can also be obtained from dynamic models that are based on reaction kinetics, if all reversible reaction steps are consequently modeled with reversible reaction kinetic

formulae. This, in turn, increases the number of parameters to be estimated from the rapid-sampling data.

^{13}C metabolic flux analysis on the ultra-short time scale

The standard ^{13}C-MFA method takes rather long experimental durations, approximately 2–3 times the doubling times of the microorganism, because the label must first accumulate in the biomass before being measured. In many cases, it is not possible to keep a living system in a metabolic steady state for such a long time. Examples in which this has been achieved, however, include cells in transient growth phases, cells in industrial fermentation conditions (fed batch) or genetically unstable recombinant cells. Application to slowly growing or non-growing cells is of particular interest for plant physiology. An application that is unique to plants is given by studies on C1-metabolism in photoautotrophic plants because, in this case, the isotopic steady state contains no information on metabolic fluxes if the substrate carries only a single carbon atom [46].

Nevertheless, most physiological states of a cellular system can be considered to be in at least a *quasi* metabolic steady state, which means that, for short time durations (minutes to hours), metabolism is approximately in a steady state. In this case, a novel ^{13}C-flux analysis method can be applied that does not rely on the steady-state assumption for label enrichment in metabolite pools. By contrast, the enrichment of labeled material in the metabolite pools is now observed under isotopically transient conditions ([47]; Figure 2c).

The new isotopically non-stationary method represents an interesting fusion of the methods for MFA under highly dynamic conditions that are based on rapid sampling and ^{13}C-MFA under steady-state conditions. Metabolite pools are kept constant but must be measured because they represent the system's capacity for labeled material.

This isotopically non-stationary method (INST-MFA) became possible only recently because mass spectrometers are now able to determine reliable fractional label information from low-concentration pools of intermediate metabolites [48]. The processes for the computational evaluation of such transient data are substantially more difficult than those for the evaluation of data describing the isotopic steady state because the accumulation of labeled materials in the intracellular pools is now described by a dynamic model. Thus, the system of equations that describes the distribution of label over the network has to be replaced by differential equations [49•].

A first application of INST-MFA to *Escherichia coli* proved that information on all intracellular fluxes could be obtained in just 15 s, during which about 20 samples were drawn from the culture [50••]. This makes isotopically non-stationary metabolic flux analysis a promising candidate for the analysis of slowly growing plant or animal cells. Several teams are currently working to establish the new method from experimental and theoretical view-points [45•,49•,51,52].

Conclusions and new horizons

A still-speculative extension of the INST-MFA method may be the use of experiments under both metabolic and isotopic steady states (Figure 2d). An exploratory simulation study [24] has already shown that INST-MFA would significantly improve the available information on reaction kinetic parameters *in vivo* and, ultimately, on the dynamic fluxes.

Another challenging development is the model-free determination of flux information from pool-size data by smoothing and differentiation of high-density data (Figure 1b). It should

be noticed that this is already possible in other chemical disciplines, as has been demonstrated with the determination of diffusion flows [53].

Altogether, a detailed theoretical basis has been developed for fluxome analysis using ^{13}C-labeling. These mathematical tools are currently adapted for fluxome studies using the FRET flux sensors. FRET sensors can provide direct information on steady-state levels and fluxes in a specific subcellular compartment. Moreover, these sensors provide information on flux in individual cells and thus significantly expand the potential of metabolomics. The combination of FRET sensors with microfluidics and modeling promises new insights into the regulation of metabolism in response to a changing environment, and into the underlying signaling networks not only in multicellular, eukaryotic systems but also in microorganisms from *E. coli* to yeast. Both ^{13}C and nano-sensor MFA technologies will rapidly be applied to fluxomics, as exemplified in the case of yeast mutants that are affected in sugar signaling [54].

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• of special interest

•• of outstanding interest

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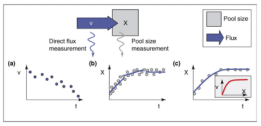

Figure 1.

Time-dependent flux determination from direct and indirect measurement data. **(a)** Direct flux measurement, the ideal case. **(b)** Computation of time derivatives from high-density pool size data. **(c)** Fitting of a kinetic model to time resolved pool size data.

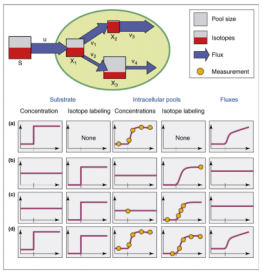

Figure 2.

Conceptionally different approaches to obtain flux information from measured pool size and/or isotope labeling data. **(a)** Rapid sampling of intracellular pools under highly dynamic conditions of a stimulus response. **(b)** Standard ^{13}C-labeling experiment under metabolic and isotopic steady-state conditions. **(c)** Isotopically non-stationary ^{13}C-labeling experiment under metabolic steady-state conditions. **(d)** Fictive combined experiment under metabolically and isotopically non-stationary conditions.

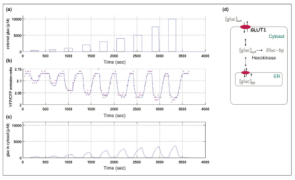

Figure 3.

Changes in glucose concentration in HepG2 cells stably expressing FLIPglu600 μ. **(a)** External perfused glucose concentration. **(b)** Measured yellow fluorescent protein (YFP)/ cyan fluorescent protein (CFP) emission ratios and simulation curve based on a kinetic model. **(c)** Cytosolic glucose concentration from simulation. **(d)** Underlying compartment model for analysis of glucose homeostasis in HepG2 cells (modified from Fehr *et al*. [31••]). Glucose is transported reversibly across the plasma and ER membranes and is phosphorylated irreversibly in the cytosol.