Toward metabolome-based ^{13}C flux analysis: a universal tool for measuring *in vivo* metabolic activity

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Abstract

Intracellular metabolic rates cannot be directly assessed from metabolome concentrations and vice versa. For most biological questions, stable isotope tracers must be administered and tracked to effectively determine metabolic fluxes by means of numerous computational steps. Although flux analysis targets the same analytes as metabolomics, priority is given to measuring their exact isotopic distribution rather than their concentration. In the first part of this chapter, I describe principles and issues of current ^{13}C flux analysis methods, following the entire process from experimental design, to detection of isotopic distributions, and data interpretation. Notably, current practice largely relies on the labeling patterns of protein-bound amino acids, because of their abundance and stability. In the second part, I focus on achievements, challenges, and opportunities of metabolome-based ^{13}C flux analyses, which are emerging in response to the need to tackle larger networks, higher cells, and to improve both spatial and temporal resolution.

1 Introduction

Physiological phenotypes of cells are macroscopic manifestations of their metabolic activity, that is determined by all molecular fluxes through metabolism, i.e. the fluxome (Hellerstein 2003; Sauer 2004). In nature, the fate of a cell between growth and senescence, or even life and death, is linked to its metabolic capacity to utilize heterogeneous substrates that are encountered. Whenever cellular functions have to be adjusted, for example upon shifts in external conditions, after mutations, or upon aberrant growth such as in tumors, the fluxome has to be adapted to support growth. To a large extent, adjustment of the fluxome is realized in central metabolism. These primary pathways are at the crossroad of catabolism and anabolism, and catalyze the largest metabolic fluxes in the cell. They form an intertwined reaction network capable of rearranging carbon and nitrogen from a wide range of substrates to fuel growth. Oxidation of the cofactors NADH and NADPH in respiration and biosynthesis, respectively, is flexibly balanced by modulation of fluxes through alternative routes in central metabolism.

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Table 1. Exemplary applications of ^{13}C metabolic flux analysis

Objective of study	Type of analysis	Analytes	Platform	Organisms	Representative References
Quantitation of intracellular fluxes	Isotopomer balancing	Amino acids	GC-MS	Microorganisms	(Dauner et al. 2001)
		Amino acids	NMR	Microorganisms	(Petersen et al. 2000; Dauner et al. 2002; van Winden et al. 2003)
				Plants	(Schwender et al. 2003; Sriram et al. 2004)
		Primary metabolites	LC-MS	Microorganisms	(van Winden et al. 2005)
			NMR	Human cells	(Forbes et al. 2006)
		$^{12}CO_2/^{13}CO_2$	Online MS	Microorganisms	(Hoon Yang et al. 2006)
	Flux ratio analysis	Amino acids	GC-MS	Microorganisms	(Fischer and Sauer 2005)
		Amino acids	NMR	Microorganisms	(Maaheimo et al. 2001)
	^{13}C constrained MFA	Amino acids	GC-MS	Microorganisms	(Hua et al. 2006)
Unraveling of metabolic reaction network	Flux ratios	Amino acids	GC-MS	Microorganisms	(Cannizzaro et al. 2004; Fuhrer et al. 2005)
Unraveling of enzymatic mechanism	Isotopomer balancing	Primary metabolites	LC-MS	Microorganisms	(Kleijn et al. 2005)
Investigation of redox and energy metabolism	^{13}C constrained MFA	Amino acids	GC-MS	Microorganisms	(Sauer et al. 2004)
	Isotopomer balancing	Amino acids	GC-MS	Microorganisms	(Wittmann and Heinzle 2002)
			NMR	Microorganisms	(Dauner et al. 2001)
Profile metabolic changes	SIDMAP	Amino acids and secretes	GC-MS	Human cells	(Boren et al. 2001; Boros et al. 2003)
	Fluxome profiling	Amino acids	GC-MS	Microorganisms	(Zamboni and Sauer 2004)

How are fluxes regulated? Metabolic fluxes are the integrated result of (i) all catalytic activities of enzymes as set by kinetic properties, and concentrations of educts, products, cofactors, ions, or protons; and (ii) all non-linear regulatory interactions at the transcriptional, translational, post-translational, and allosteric level, which all influence amount and state of enzyme (Hellerstein 2003; Sauer 2004). An essential consequence is that metabolic fluxes cannot be directly quantified solely from metabolites concentrations or vice versa. To realize this with good confidence, detailed enzyme modeling together with exact data on protein amounts and modifications, and metabolite concentrations would be a precondition. For this purpose, a palette of techniques is available to reveal concentrations, interactions, and kinetic parameters. (1) The concentration of cell components is determined by transcriptomics, proteomics, and metabolomics, although several specific methods are usually necessary to obtain complete information, for example on protein levels and modification state, or on chemically diverse metabolites. (2) Some approaches exist to discover the binding between proteins (Cusick et al. 2005) or of proteins to DNA (Hoglund and Kohlbacher 2004; Bulyk 2006). Unfortunately, they can hardly be used to quantify their strength, and the comprehensive identification of interactions between DNA, transcripts, proteins, and small molecules is still far out of reach. (3) Estimation of *in vivo* kinetic parameters can be done with stimulus-response experiments (Vaseghi et al. 1999). The drawback of such procedures is that these experiments are demanding, performed locally for a reduced number of parameters, and require a priori knowledge of all possible interactions: for mid-sized and large networks, the task rapidly becomes prohibitive.

The general lack of detailed regulation and kinetic information has two main consequences. First, today's omics data can, at most, provide constraints on metabolic fluxes. For example, the absence of a protein or lack of transcription can be used to exclude that it is catalytically active. Analogously, the combination of metabolome data and thermodynamics knowledge can delineate directionality of reactions in a given state, but is insufficient to precisely assess metabolic fluxes (Kümmel et al. 2006). Second, the experimental workflow is preferably reversed: metabolic fluxes are measured together with concentrations to infer changes in enzyme activity or concentration (Wu et al. 2005), or overlap with proteome or transcript data to discover regulation circuits (Krömer et al. 2004; Shimizu 2004).

Metabolic fluxes are monitored by feeding organisms with substrates enriched in stable (i.e. non-radioactive) isotopic tracers such as ^{13}C, ^{2}H, ^{18}O, ^{34}S, or ^{15}N. Physiologists extensively employed similar labeled substrates for decades to track local metabolism of nutrients or monitor polymerization and degradation of biopolymers such as lipids, DNA, or proteins in animals and cells (Hellerstein 2003; McCabe and Previs 2004; Bequette et al. 2006), and are nowadays also employed to lead drug development (Turner and Hellerstein 2005). Only in the last decade, developments independent from physiology led to ^{13}C-based metabolic flux analysis for microbes. These methods were initially developed for purposes of strain optimization in industrial biotechnology (Stephanopoulos 1999), but have found large application and consensus in systems biology (Blank et al. 2005; Fischer and Sauer 2005; Koffas and Stephanopoulos 2005) (Table 1). In contrast to the methods utilized with animals that focus on local activities, novel ^{13}C metabolic flux

analysis methods were devised to comprehensively assess carbon fluxes in large metabolic networks. Owing to the fact that microorganisms are rarely differentiated and able to grow on single carbon sources under carefully controlled conditions, an arsenal of ^{13}C flux methods was established to quantify the intracellular fluxome with different networks, substrates, and culture conditions. Modern ^{13}C flux analyses consequently enabled to investigate - from a global perspective - the link between cellular redox equilibrium, generation of energy equivalents, and metabolic phenotypes.

In this chapter, I first present the principles of metabolic flux analysis and the corollary methods that were designed to map reaction velocities in microbes with ^{13}C-labeling patterns of protein-bound amino acids. In the second part, I focus on the extension to metabolome-based ^{13}C metabolic flux analysis, that holds promise to become a universal tool to monitor the fluxome from microorganisms to animals for purposes of systems biology, understanding metabolic control in health and disease, or drug development.

2 Fundamentals of metabolic flux analysis

Metabolic flux analysis aims at measuring *in vivo* activity of metabolic reactions. In contrast to concentrations, rates are per se not directly measurable. *In vitro*, the rate of a reaction is determined via interpretation of measured concentration profiles of the substrates and products. Similarly, one can extend this approach and quantify the reaction rates in sequential and even branching reaction networks by monitoring the concentration profiles of substrates, intermediates, and products. The rate of every single reaction is then obtained by a set of material balances, one for each compound in the reaction chain. *In vivo*, however, it is experimentally impossible to measure concentration profiles for all metabolites in a cell that encompasses thousands of compounds. This problem is obviated when metabolic fluxes are measured in a metabolic steady state, meaning that fluxes and intracellular metabolite concentrations are constant over time. When this precondition is fulfilled, all intermediates pools are by definition invariant over time and in the case of linear, non-converging, non-cyclic pathways metabolic fluxes are calculable from the time profiles of all substrates and end products, while the concentrations of all balanced intermediates are neglectable.

Stoichiometric balancing has an additional inherent flaw that normally impairs complete flux estimations and that is associated to the topology of the biochemical reaction network. In most cells, especially in central carbon metabolism, alternative biosynthetic routes and reaction cycles exist and generate redundancies. Such redundancies cannot be unequivocally resolved by stoichiometric balancing, because each one introduces an additional degree of freedom where an infinite number of flux maps lead to identical overall balances. To obtain a unique solution, one approach is to select the flux distribution that satisfies all stoichiometric constraints and also maximizes an arbitrarily chosen objective function of network operation, e.g. maximize ATP overproduction or growth yield (Varma and Palsson

1994; Kauffman et al. 2003). The outcome of optimization corresponds to the most-likely flux estimate according to the arbitrary assumptions. Systematic studies have demonstrated that the chosen paradigm of network operation can differ between organisms, mutants, and environmental conditions (Küpfer et al.2007; Segre et al. 2002). Thus, objective functions have to be carefully selected to avoid biased and erroneous results.

The inherent uncertainty of *in silico* predictions and their discordance with empirical observations evidenced the importance of experimental metabolic flux determination. This was brought about by the introduction of isotopically labeled substrates. Depending upon which pathways are active in catabolism and anabolism, atoms from the substrate are scrambled and rearranged following the schemes of enzymatic reaction mechanisms. The labeling patterns of metabolites are then detected by either mass spectrometry (MS) or nuclear magnetic resonance (NMR), and quantitatively reflect partitioning of substrate through metabolic routes. They provide information independent from stoichiometric balances, and with a properly designed tracer substrate they serve to distinguish the fluxes through alternative pathways or reaction cycles. In general, ^{13}C-tracers enable to effectively resolve the redundancies occurring in central carbon metabolism, where all catabolic and anabolic pathways diverge from. In contrast to the highly interconnected central carbon metabolism, the peripheral metabolism is composed by mostly linear biosynthetic routes (e.g. amino acids or nucleotide synthesis). Since these pathways are utilized to synthesize the building blocks for growth, their *in vivo* flux is estimated with good precision by stoichiometry with detailed models of biomass composition.

Although ^{13}C metabolic flux analysis enables monitoring of pathway activity *in vivo* in most cases, it is important to stress that (i) quantitative analysis is only possible in minimal media, (ii) technical difficulty increases exponentially when multiple carbon substrates are utilized, (iii) it is not possible to discriminate between pathways or reactions that do not differ in the scrambling of labeled atoms, i.e. between isoenzymes.

3 Principles of labeling experiments

For a labeling experiment, cells are first grown on naturally labeled substrates until metabolic steady state. Once this prerequisite is fulfilled, isotopically enriched nutrients can be administered to the cells. In batch cultures, this is done either by spiking the tracer substrate to the medium, or by diluting exponentially growing cells in fresh, labeled medium. Harvesting and resuspending is preferably avoided because handling perturbs metabolic steady state. In continuous or fed-batch cultures, the feed is switched from naturally labeled medium to an equivalently concentrated tracer-enriched solution. Ideally, these operations should provoke an immediate step change of the tracer fraction in the culture medium. Although such rapid shifts can easily be attained in well-stirred systems, enrichment of label within the cellular metabolome will take considerably longer (Fig. 1). The reason

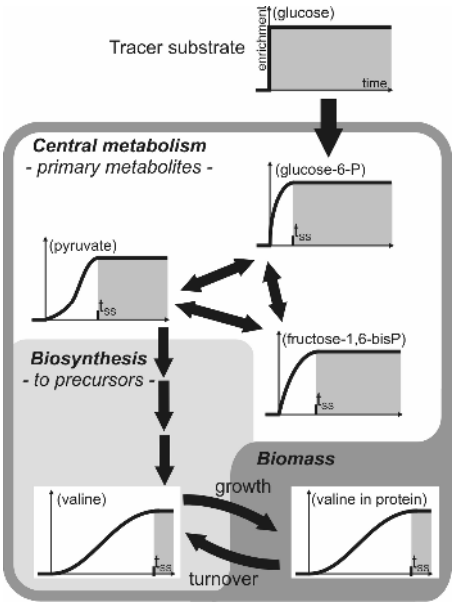

Fig. 1. Progressive propagation of labeling through intermediate pools in experiments with stable isotopic substrates. Each plot exemplarily shows time profiles of label enrichment for species of central metabolism, peripheral biosynthetic pathways leading to biomass precursors, and biomass components. Exemplary names are indicates in brackets. t_{SS} is the time necessary to attain isotopic stationarity, it is specific for each pool, and it sets the minimum labeling time that has to be respected for stationary computational methods to be applied. Delayed onset of isotopic steady state is typically observed far from tracer substrate uptake, in large pools, or when biomass turnover occur. Refer to the text for more detailed explanations.

is that starting from the entry point of the tracer, the label has to propagate through the metabolic network and progressively replace unlabeled intermediates. This is an important phenomenon, because routine application of ^{13}C flux analysis is so far solely possible from the labeling patterns of metabolites in isotopic steady state, i.e. with time-invariant labeling patterns at the time point of sampling. In theory such an isotopic steady state is never attained, but due to analytical imprecision isotopic equilibrium is experimentally observed within minutes to hours.

The time after which such an isotopic steady state is achieved depends upon the turnover rate of each pool, which is directly proportional to the flux through the pool and inversely to the concentration: larger pools slow down the process, higher fluxes accelerate it. A general and intuitive consequence is that the closer an intermediate is to the original tracer substrate, the faster it will reach isotopic steady state. Thus, for ^{13}C glucose tracers, flux analysis based on the labeling pattern of glycolytic intermediates requires shorter labeling times than with tricarboxylic acid (TCA) cycle intermediates. Biomass compartments (e.g. proteins) exhibit the longest isotopic transients, whose duration is roughly proportional to

the inverse of the growth rate. Similarly slow label uptake can also be observed for the free pool of corresponding precursors (in the same example the amino acids) when biomass turnover interferes with quick onset of isotopic steady state (Fig. 1) (Grotkjaer et al. 2004).

Substantial advantages are brought about by the analysis of labeling patterns in intermediates of central metabolism. First, it decreases duration of experiments and the costs coupled to the amount of employed isotopic tracer. Second, shorter observation windows provide much more flexibility in experimental design since metabolic steady state does not have to be ensured over several hours (van Winden et al. 2005). In turn, this opens for the investigation of slow metabolic transients for which a quasi steady state can be assumed for the time span of labeling (e.g. fed batches). The analysis yields a flux map that averages pathway activities over the labeling interval. An extension is to sample the same labeling experiment at several time points during the slow flux transients to obtain time-resolved flux maps (Zamboni et al. 2005). Limitations are set by the characteristic time of monitored analytes necessary to attain isotopic steady state, which is prone to variation during metabolic transients due to changing fluxes and pool concentrations.

These underlying principles hold for every experiment involving labeling with isotopic tracers, and should carefully be considered in the design stage. In the next sections, the workflow of ^{13}C-based flux analysis from inception to evaluation and current practice is briefly reiterated.

4 Current practice of stationary ^{13}C flux analysis

4.1 Experimental design

The capability of resolving and quantifying fluxes *in vivo* is a function of (i) the tracer substrate used, (ii) the biochemical reaction network, and (iii) the analytes that are detectable. Several protocols were presented to assess a priori calculability from a dataset in the case of stoichiometric balancing (Klamt and Schuster 2002) or ^{13}C metabolic flux analysis (Möllney et al. 1999; van Winden et al. 2001; Isermann and Wiechert 2003). Notably, analytical accuracy in the detection of labeling patterns strongly influences the confidence of flux estimates. This information is frequently neglected in the aforementioned calculability tests and, thus, it is often necessary to perform more complex and detailed experiments than the simplest setup prescribed based on such tests (van Winden et al. 2001).

The selection of the tracer distribution in the substrate is paramount for effective resolution of metabolic fluxes. Basically two different strategies exist and can be combined. Positionally enriched substrates possess an uneven distribution of ^{13}C in the carbon backbone. These tracers are typically administered in the pure form, i.e. 100%, and are ideal to distinguish alternative pathways where only one branch losses or transfers the specifically labeled carbon (e.g. by decarboxylation). For example, [1-^{13}C]glucose is well suited to track fluxes in the oxidative branch of the pentose phosphate pathway (PPP) where the [1-^{13}C] atom is split to form

$^{13}CO_2$ and the resulting pentoses are label-free. In contrast, pentoses originating via the non-oxidative PPP are enriched in ^{13}C (Christensen et al. 2001). A shortcoming of positionally enriched tracers is that they are tailored for specific pathways and poorly suited for global fluxome estimates. Hence, they find wide application in networks that are highly constrained by stoichiometry and thus exhibit low degrees of freedom, or to determine the network structure in poorly characterized organisms (Cannizzaro et al. 2004; Fuhrer et al. 2005). On the other end, uniformly (fully) labeled substrates offer a larger scope in exchange for specificity. Uniformly labeled substrates are normally administered in combination with unlabeled isomers, e.g. as a 1:1 mix. When the tracer is metabolized, the carbon backbone of both labeled and unlabeled isoforms is broken and rearranged. Reactions that combine multiple carbon-containing intermediates will generate chimeric molecules with both ^{12}C and ^{13}C atoms, with characteristic labeling imprints. Examples are the transaldolase and transketolase in the non-oxidative PPP, anaplerotic reactions, or cyclic pathway such as the TCA cycle or the modular lipid biosynthesis. With uniformly labeled tracers, the essential information for pathway flux discrimination is not enclosed in the label that was lost during metabolic activity such as with positional enrichment, but in the presence of ^{13}C fine structures that reflect enzymatic scrambling specific for a pathway.

Compartments in higher cells complicate the problem in several ways: (i) additional reactions are necessary to model pathways independently for each compartment. Splitting of intermediate pools across distinct compartments considerably increases the degrees of freedom. (ii) The intracompartmental transport mechanisms are very relevant, in particular when coupled to sym- or antiport. (iii) Metabolites are measured as the sum of all compartments. When a metabolite is localized in two (or more) compartments with possibly different biosynthetic origin, the corresponding labeling patterns may differ and, thus, are typically discarded for flux calculation. Provided that the model of biochemical reactions is correct and complete, mathematical methods for the optimal selection of tracer and analytes exist (Möllney et al. 1999; Rantanen et al. 2006).

Experimental design is also influenced by the analytes that can be detected. The majority of ^{13}C-based flux studies published in the last decade was based on the labeling patterns of protein-bound amino acids because of their abundance (roughly half of total cell dry weight) that facilitates measurement of labeling pattern. High abundance is unfortunately coupled to lower turnover and, hence, short transients cannot be investigated. In the case of biomass macromolecules and the constituting monomers when turnover occurs, the inverse of the growth rate provides a rough estimate of the shortest interval that can be investigated with a stationary ^{13}C metabolic flux experiment (Wiechert and Nöh 2005).

4.2 From analytes to ^{13}C labeling patterns

Determination of carbon fluxes in isotopic steady state relies on macroscopic balances and ^{13}C labeling patterns: intermediates concentrations are superfluous unless isotopically non-stationary conditions are tackled (discussed in a later sec-

tion). During sample harvest, it is important to quench metabolism rapidly enough to avoid post-sampling artifacts. The constraints are set by the turnover of the analytes. When the protein-bound fraction of amino acids is the target, handling operations in the range of minutes are safe. In contrast, when dealing with intracellular intermediates sub-second quenching and cooling is recommended because their pools are exchanged by orders of magnitude more rapidly. In sharp contrast to metabolome experiments, quantitative and reproducible extraction of intermediates from cells is not of relevance as long as detection is not compromised by poor recoveries.

Two techniques exist to distinguish and quantify isotopic distributions, namely nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Both platforms were equally successful in providing essential information from protein-bound amino acids for the estimation of fluxes in central carbon metabolism. In NMR, 2-dimensional heteronuclear [^{13}C,^{1}H] correlation spectroscopy resolves all relevant resonances in proteinogenic amino acids without prefractionation (Szyperski 1995). Labeling patterns are inferred from characteristic spin-spin couplings that arise when neighbor ^{13}C atoms are present, and uniformly labeled tracers are therefore typically utilized. With MS, amino acids mixtures have first to be resolved by chromatographic means. Gas chromatography – mass spectrometry (GC-MS) has become the principal workhorse for mainly two reasons. First, it combines robustness, fast measurements, fully baseline-resolved amino acids, and relative low instrument and running costs. Second, it delivers extensive fragment information to unravel central carbon fluxes (Christensen and Nielsen 1999; Dauner and Sauer 2000). The latter point is crucial for ^{13}C-experiments and an important digression must be made. Each carbon atom in a molecule can be either labeled (^{13}C) or unlabeled (^{12}C). A molecule with n carbon atoms possesses 2^n possible states, called *isotopomers* (from isotope isomers). MS discriminates only the mass and is not able to distinguish between all isotopomers: those with identical weight are detected as a lumped pool. This limits calculability of fluxes when alternative pathways lead to isotopomers with equal label content. The hurdle is often overcome by inducing analyte fragmentation in the MS: from intact molecules, smaller daughter ions are generated and their isotopic distribution is measured to yield the isotopic distribution of partial carbon backbone segments or even the enrichment of single atom positions. Fragmentation in GC-MS occurs spontaneously at the interface between GC and MS when a high energy electrons beam is used to ionize the analytes. The resulting fragments enable ^{13}C flux analysis in many organisms using various tracers, such as for example [1-^{13}C], [1,2-$^{13}C_2$], and [U-^{13}C]glucose, and have contributed to the diffusion of GC-MS as preferred platform.

Table 2. Summary of metabolic flux analysis methods for experiments with stable isotopic tracers.

Approach	Pros	Cons
NET FLUXES		
Isotopomer balancing	- integrates all available information - calculates net and exchange fluxes - universal framework is available that do not need adaptation for new networks or tracers	- requires correct network and physiological data - cumbersome troubleshooting
^{13}C-constrained metabolic flux analysis	- simple and fast computation	- exchange fluxes not calculated
RELATIVE FLUXES		
Flux ratios analysis	- direct evidence for pathway activity - independent from measured rates - fast, unsupervised	- tedious design of new equations - implicit assumptions on reversibility that might do not hold after severe genetic perturbations.
PROFILING		
Fluxome profiling	- independent from any model - suitable for complex media - applicable with any tracer (^{13}C, ^{15}N, ^{2}H and combinations)	- qualitative - large number of replicas/samples needed
SiDMAP	- optimized for mammalian cells and glucose	- qualitative - requires multiple experiments to obtain a complete analysis

For emerging applications based on free metabolites, MS is currently superseding NMR owing to its superior sensitivity, simpler hyphenation to chromatography, and optional fragmentation capabilities. MS methods are increasingly profiting from the continuous progresses made in liquid chromatography (LC) and capillary electrophoresis (CE) that bring about baseline separations of the majority of central carbon and other polar metabolites pivotal to unravel fluxes. Flux analyses can build directly upon MS-metabolomics with minor adjustments made to prioritize precise estimation of mass distributions before concentrations (cf. 5.2 and 5.3.1).

4.3 From ^{13}C labeling patterns to fluxes

A variety of computational approaches to interpret ^{13}C labeling blueprints have bloomed driven by the need to address well-defined questions or hypotheses in

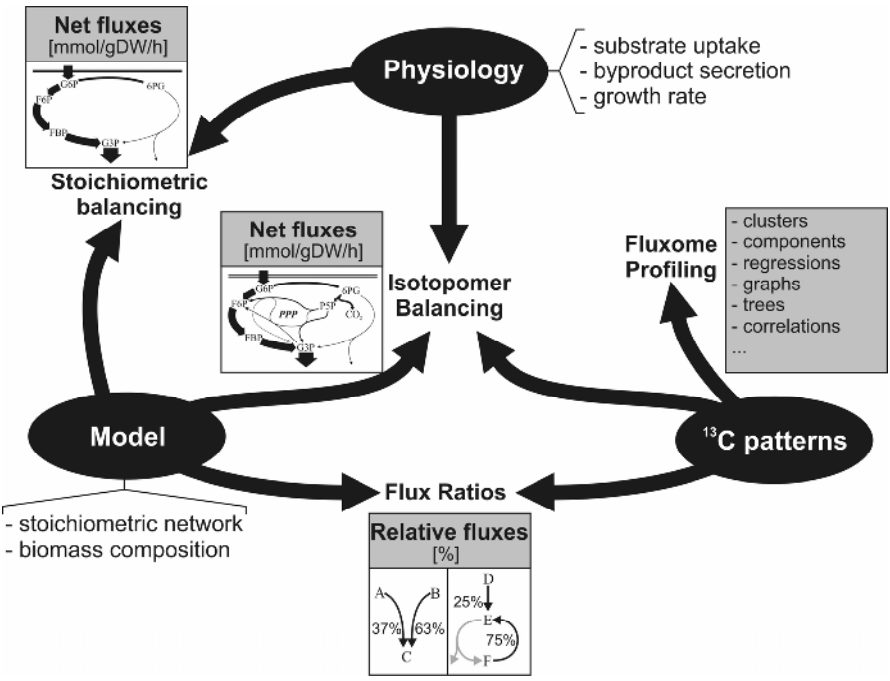

Fig. 2. Flow chart of data integration alternatives in ^{13}C metabolic flux analysis. Inputs and outputs are shown in black ellipsoids and grey boxes, respectively.

highly heterogeneous biological systems. Extensions and perhaps simplifications had to be introduced to face the sometimes scarce availability of measurements, ill-defined networks, and analytical imprecision. Three very different sets of information are utilized to estimate fluxes:

- Physiology: extracellular rates of substrate uptake and product formation, growth rate.
- Model of biochemical network: including - for each reaction in the system - stoichiometry, assumptions on the irreversibility, and the mapping of single atom positions between educts and products.
- ^{13}C labeling patterns: from NMR, MS, or both.

I define three major clusters of methods on the basis of which of the above information domains are utilized and combined to investigate metabolic fluxes (Fig. 2 and Table 2).

4.3.1 Isotopomer balancing

Isotopomer balancing is the natural extension of the stoichiometric balancing approach (cf. 2) to include ^{13}C data. It requires and concomitantly integrates extracellular fluxes, network model, and ^{13}C patterns. A network model is the basis for the balance equations. In contrast to simple stoichiometric balancing where a

single balance is constructed for each metabolite, here one equation is drawn for each isotopomer (Schmidt et al. 1997; Zupke et al. 1997; Klapa et al. 1999; Dauner et al. 2001). As the number of additional equations necessary for each metabolite increases exponentially with the number of carbon atoms, the resulting system of linear equations becomes much larger, but the same is true for the variables (from metabolites to isotopomers) and the system remains underdetermined. Fluxes are resolved iteratively: first, a semi-random flux distribution is generated, and is then used to simulate the labeling pattern in intermediates that would result from it. The simulated isotopomer fractions are in turn used to generate synthetic MS or NMR signals, which are compared to the experimental findings. Until a satisfactory match is attained, the cycle is repeated with a new flux distribution that is derived from the previous ones with some rational plan to accelerate convergence and increase the probability of reaching the global optimum. The finally obtained solution constitutes the flux map that best explains the labeling patterns within the constraints set by the network topology and the measured rates.

Isotopomer balancing is the most comprehensive strategy for data interpretation as it simultaneously integrates all available data. This kind of global analysis has the merit that it exploits the maximum possible information from the dataset. The drawback is that the flux estimate is severely biased by incomplete or erroneous network models and physiological data. In case of bad fits, the whole flux solution has to be rejected. Expertise and time are needed to pinpoint the inconsistencies in model or measurements. Calculation is complex and computationally expensive, and special derivatives of isotopomer fractions such as cumomers (Wiechert et al. 1999) or bondomers (van Winden et al. 2002), were demonstrated to effectively improve the process. Antoniewicz et al. recently introduced a novel approach to reduce the number of systems variables by at least one order-of-magnitude while preserving a full description of the isotopomers. This decomposition in so-called *elementary metabolite units* dramatically simplifies the equation system and thus accelerates solving, and will most likely constitute a cornerstone for the rapid analysis of non-stationary experiments or of concomitant ^{2}H, ^{13}C, ^{18}O, and ^{15}N labeling in large networks (Antoniewicz et al. 2006). Notably, a detailed statistical analysis is crucial to correctly weight the outcomes (Antoniewicz et al. 2006).

To our knowledge, 13C-FLUX is currently the most complete and freely available software tool that offers rigorous ^{13}C-based balancing for generalized networks from both NMR or MS experiments (Wiechert et al. 2001). Alternatively, NMR2Flux computes fluxes in plants from 2D-NMR spectra of protein-bound amino acids (Sriram et al. 2004). Isotopomer balancing has been used to quantify fluxes for example from amino acids in microorganisms with NMR (Marx et al. 1996; Petersen et al. 2000; Emmerling et al. 2002; van Winden et al. 2003) and MS data (Fischer and Sauer 2003; Klapa et al. 2003), from free metabolites with MS (van Winden et al. 2005; Kleijn et al. 2006), or in plants with NMR of amino acids (Sriram et al. 2004).

4.3.2 Flux ratios

The isotopomer balancing approach outlined in the previous section sets strict requirements in terms of input data (Fig. 2). Initially driven by the need to analyze fluxes also in absence of physiological data, *metabolic flux ratio analysis* was developed to directly decipher ^{13}C labeling patterns (Szyperski 1995). Briefly, metabolic flux ratios quantify the relative fluxes of alternative pathways at the node (metabolite) of convergence. For this purpose, analytical equations are developed first for each branch point of interest. Each analytical equation is designed to take advantage of the labeling features that best discriminates between the theoretical ^{13}C blueprints of converging pathways. In central metabolism, about 10 independent flux ratios can be determined from amino acids for ^{13}C-glucose experiments with bacteria or yeast using either NMR (Szyperski 1995; Maaheimo et al. 2001) or MS data (Christensen et al. 2001; Fischer and Sauer 2003; Blank and Sauer 2004). For the broadly used flux ratios from ^{13}C experiments and GC-MS data, a detailed protocol is given in (Nanchen et al. 2006). Single flux ratios are calculated from the mass distributions of typically only 1-3 intermediates (or inferred from amino acids) and absolutely no kind of measured rate is required. The power of ratios lies in their local nature that renders them less susceptible to possibly erroneous models or measurements, and in the fact that they provide direct evidence for the operation of a particular pathway *in vivo*. In addition, the rapid and almost completely unsupervised computation of flux ratios enables high-throughput - and yet quantitative - flux studies. The major drawback is the initial time invested for development or adaptation of the analytical equations for new tracers or modified metabolic networks. Flux ratios were, for example, used to identify new pathways or unexpected cross-activity (Fischer and Sauer 2003; Zamboni et al. 2004), characterize unknown networks (Fuhrer et al. 2005), demonstrate metabolic robustness and suboptimal operation of *Bacillus* (Fischer and Sauer 2005), and to investigate adaptive evolution of metabolism (Hua et al. 2006).

In the so-called *^{13}C-constrained metabolic flux analysis*, flux ratios can be used to solve the problem of undetermined stoichiometric balances, because they provide additional, independent constraints to reduce the solution space (Fischer et al. 2004). If at least one flux ratio is available to fix each degree of freedom in the metabolic network, a unique flux map can be calculated by means of a linear system or least-square fit for fully and overdetermined systems, respectively. Results from ^{13}C-constrained metabolic flux analysis and isotopomer balancing are consistent (Fischer et al. 2004). Yet, the latter provides more detailed information with respect to the exchange fluxes in bidirectional reactions. These are neglected or implicitly assigned when developing the analytical equations to calculate flux ratios. In knockout mutants with severe growth defects, these tacit assumptions may not hold and lead to wrong ratio estimates and, in turn, erroneous net fluxes from ^{13}C-constrained metabolic flux analysis. Nevertheless, ratios-constrained net flux analyses are a robust tool for both large-scale (Blank et al. 2005) and detailed studies of cellular carbon, redox, and energy metabolism (Zamboni et al. 2003; Blank et al. 2005; Hua et al. 2006). For experiments on glucose minimal medium,

software packages for metabolic flux ratio and ^{13}C-constrained metabolic balance analysis are freely available (Zamboni et al. 2005).

A related approach is the so-called *stable isotope based dynamic metabolic profiling* (SIDMAP), that - akin to metabolic flux ratios analysis - interprets ^{13}C-patterns according to a metabolic model without measured extracellular rates. It features a collection of analytical equations that were tailored to monitor specific changes in carbon metabolism of mammalian cells grown on [1,2-$^{13}C_2$]glucose and analyzed by GC-MS of biomass or secreted products. The complex composition of culture medium impairs large-scope fluxome quantitation. Nevertheless, this approach affords a specialized profiling tool to, for example, capture metabolic responses in tumoral cells or to lead targeted drug design (Boren et al. 2001; Boros et al. 2003; Marin et al. 2004).

4.3.3 Fluxome profiling

In analogy to data mining methods applied to other omics data, multivariate analysis can be used to explore large datasets of ^{13}C labeling patterns (Zamboni and Sauer 2005). This approach of fluxome profiling features the unique chance to infer structural and quantitative information from raw labeling data without any a priori knowledge of the biochemical reaction network.

What can be discovered in ^{13}C labeling patterns? A first proof-of-concept study with bacterial cultures and a variegated set of tracers and conditions was presented by our lab (Zamboni and Sauer 2004). The working hypothesis was that the absence or presence of pathway activity is reflected in the label fingerprints of metabolites. By purely unsupervised statistical techniques, this work (i) demonstrated that it is indeed possible to separate the overlapping signatures of independent pathways, (ii) proved that signatures are consistent with biosynthetic routes, (iii) showed that structural knowledge on biosynthesis of metabolites can be deduced from covariating patterns, (iv) showed that mutants can be clustered according to metabolic changes, and (v) mapped the effect of transcriptional regulators on metabolic activity. Current efforts aim at developing robust tools of machine learning and expertise to systematically scavenge all relevant features in large datasets. Albeit in progress, first results reveal that for each dataset the number of stable (not sensitive to algorithm parameters or to *in silico* superimposed noise) pathway signatures is well defined and sometimes exceeds the number of those calculable with the established metabolic flux ratio equations. This suggests that novel, still latent blueprints of metabolic activity are contained in the data in addition to those disclosed by today's metabolic flux ratio analysis.

Beyond the qualifiers obtained, for example, from hierarchical clustering or classification trees, it is obviously desirable to obtain quantitative insights on metabolic fluxes. In fact, quantitative estimators for flux partitioning ratios were successfully derived from unsupervised methods such as independent component analysis (Zamboni and Sauer 2004), but for some flux ratio no matching estimator could be identified. Supervised methods such as regressions or adaptive neural

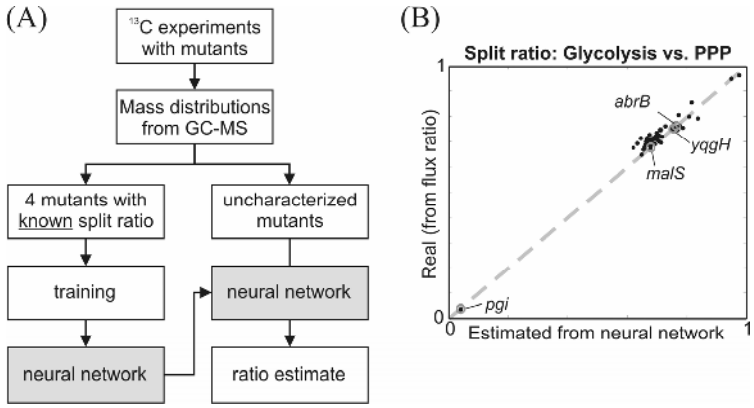

Fig. 3. Quantitative determination of glycolysis-to-PPP split ratio in *Bacillus subtilis* knockout using supervised machine learning and no a priori knowledge of metabolism. (A) Schematic representation of approach. Mutants were grown individually on a mix of 50% [U-^{13}C] and natural glucose. The mass distributions of 4 mutants were used to train an adaptive neural network to estimate the flux ratio. (B) The graph shows the validation of the trained neural network: for each mutant the estimated flux split estimated by the neural network is compared to the real value calculated from a model-based analytical equation. Circles and dots indicate the mutants used for training and validation, respectively. The dashed diagonal indicates perfect predictions.

networks can possibly fill this gap as shown exemplarily in Figure 3, but the general applicability and utility of supervised machine learning with ^{13}C labeling patterns is still questionable and has to be assessed in systematic studies.

Fluxome profiling, based on either supervised or unsupervised procedures, is still in its infancy and hence, in contrast to the well-established approaches of isotopomer balancing and flux ratio analysis, it is only possible to speculate on its practical applications. With this in mind, principally two advantages unique to fluxome profiling call for further development. First, fluxome profiling can handle labeling data from experiments with higher cells because it is compatible with virtually any network (unicellular – multicellular), isotopic tracer (^{13}C, ^{2}H, ^{18}O, ^{15}N, and combinations), and medium composition (Zamboni and Sauer 2004). Second, multivariate statistics afford a very simple basis for comparing different omics data. For example, if it is true that metabolic fluxes reflect the integration of all interactions between and within metabolites, proteins, RNA, etc., it can be expected that statistical correlations and anticorrelations between metabolic fluxes and concentration of species in the different layers will contribute to identify the loci were control is exerted and the mechanism how regulation occurs (Weckwerth et al. 2004; Morgenthal et al. 2006).

5 Toward metabolome-based ^{13}C flux analysis

Flux measurements published in the last decade were originated almost exclusively from ^{13}C data of protein-bound amino acids or secreted metabolites, because of their large abundance that facilitates both sampling due to the low turnover and ease of detection. As witnessed by the considerably number of studies, this approach has undoubtedly maturated to a robust tool suited for addressing various questions. Nevertheless, there are several reasons that call for true metabolome-based ^{13}C flux analyses:

- Cells without *de novo* amino acid (or protein) biosynthesis may be analyzed, e.g. higher cells, microbes grown in rich media or resting.
- Identifiability of fluxes is increased by monitoring of ^{13}C patterns in metabolites that are not precursors of proteinogenic amino acids. In addition, the risk of erroneous or ambiguous mapping of atoms between precursors and metabolic end products is circumvented.
- Labeling experiments are shortened because isotopic steady state is attained earlier. This leads to lower costs and enables the analysis of systems that cannot be kept long in metabolic steady state.
- Slow metabolic shifts (in the range of minutes to hours) become observable, as long as a metabolic steady state can be approximated throughout onset of the isotopic steady state in intracellular metabolites.

The full potential of metabolome-based ^{13}C flux analysis to tackle such conditions and questions can be unleashed only with direct measurements of intermediates in proximity of the pathway of interest.

5.1 Experimental proof-of-concept

Two landmark studies of cellular fluxes based on ^{13}C-patterns of primary metabolites have been published so far, both by van Winden and coworkers (van Winden et al. 2005; Kleijn et al. 2006). In the first one, baker's yeast was grown in glucose-limited continuous cultures, and at metabolic steady state the culture was fed with 100% [1-^{13}C]glucose. After 40 and 60 min of labeling, two cell aliquots were harvested rapidly, quenched, and central carbon metabolites were extracted and measured by liquid chromatography (LC)-MS. Isotopomer balancing (cf. 4.3.2) was successfully used to fit fluxes in glycolysis and PPP to the ^{13}C labeling pattern of ten intermediates. This study demonstrates the feasibility of metabolome-based flux analyses, and contributes further relevant observations. First, comparison of labeling pattern at the two time points of sampling confirm that already after 40 min the majority of metabolites is in isotopic steady state. Exceptions are discussed below. Second, the direct comparison of labeling patterns in reactants at both sides of every bidirectional reactions indicates which metabolite pools are equilibrated, and thus, which reversible enzymes operate in forward and backward direction at rates that are much higher than the apparent net metabolic flux, that is the difference of the two. Third, turnover of the storage carbohydrate glycogen

was found to interfere with rapid onset of isotopic steady state in glucose-1-phosphate and glucose-6-phosphate, so that after 60 min isotopic steady state is not yet achieved. When a turnover reaction between glycogen and glucose-1-phosphate is introduced into the model, the result is a worse confidence interval for the flux split between glycolysis and the PPP. This is caused by the fact that both [1-^{13}C] label loss in the oxidative PPP and variable inflow of unlabeled hexose-phosphates from glycogen produce hardly distinguishable increases in unlabeled fractions of intermediate. Two solutions can obviate to the problem of large pools disturb onset of isotopic steady state. As anticipated by the authors in the above study, one option is to label for a longer period of time. The drawback is that extensive time is probably necessary to obtain isotopic equilibration of the large glycogen pool. Alternatively, differently labeled substrates can be adopted to experimentally assess the exchange of large reservoirs. For the aforementioned example, [1,2-$^{13}C_2$] or [U-^{13}C]glucose would have served to estimate more precisely the glycolysis-to-PPP split in the same span of time, because they enable concomitant quantitation of the collateral turnover of glycogen.

Indeed, the second and more recent metabolome-based ^{13}C flux study by the same lab affords determination of the flux split between oxidative PPP and glycolysis in filamentous fungi by an analytical equation that calculates the flux ratio from the isotopic mass distribution of tree intermediates close to the node (Kleijn et al. 2006). This study shows that the results obtained analytically are consistent with isotopomer balancing but more accurate, and demonstrates for the first time the potential of metabolome-based ^{13}C flux ratio analysis (cf. 4.3.2).

5.2 Analytics: lessons from metabolomics

The trivial analogy between metabolomics and metabolome-based ^{13}C flux analysis in terms of analytes is reflected by the similar experimental workflow in the steps from cells harvest to analysis. Hence, current best practices for accurate flux studies include the use of rapid sampling devices, immediate quenching of metabolism, tailored chromatographic separation to possibly reduce matrix effects, and highly-sensitive detection. MS is actually preferred to NMR in the detection of ^{13}C labeling in free metabolites due to the higher sensitivity. In addition, chromatographic separation becomes compulsory to capture the ^{13}C distributions of structurally similar metabolites as it often occur in the same pathway, for which MS is prioritized because on-line interfacing to GC, LC, or capillary electrophoresis (CE) is well established.

The topic of analytical separation introduces a relevant question: which of the MS-compatible platforms frequently used in metabolomics (i.e. GC, LC, and CE) is the most suited for metabolome-based ^{13}C metabolic flux analysis? For the specifics of the intermediates of interest, i.e. phosphorylated sugars and carboxylic acids in glycolysis, PPP, and TCA cycle, all three modes can be used for separation and subsequent MS detection. Here I survey these separation techniques, while the specifics of MS detection are addressed in the following sections.

For GC-MS acquisition, volatile derivatives of polar compounds are obtained after methoxymation and silylation and separated with simple protocols amenable to high-throughputs (Strelkov et al. 2004; Koek et al. 2006). The strength of this method is that it is generally suited to detect other classes of compounds such as alcohols, amines, amino acids, or purines. Although it suffers from derivatization efficiencies varying for the different classes (Koek et al. 2006), this does not affect the measurement of isotopic distributions because they do not depend on absolute concentrations. To increase the amount of sample introduced onto the column, temperature programmable injectors can be used to inject up to 1000x larger volumes. Notably, the benefits are marginal when low and highly concentrated analytes elute closely or overlapping, because overloading of the more abundant compound causes peak broadening and often detector saturation. Due to the extensive fragmentation that is normally caused by electron impact ionization, GC-MS spectra are very complex and identification of analytes relies on spectral databases of compound libraries (Schauer et al. 2005).

Analysis by LC-MS is slightly complicated by the ionic and polar character of central carbon metabolites because of the poor compatibility between MS ionization and the LC buffers commonly used for separating such anionic and hydrophilic compounds. Electrospray ionization is enhanced by solvents with high organic phase and low salt content, whereas chromatographic elution is controlled by concentrated sodium hydroxide gradients in water (van Dam et al. 2002). Interfacing to MS is then only possible with electrochemical exchangers of sodium cations-protons that are inserted in the liquid path between column and sprayer but comes at the cost of sensitivity and chromatographic resolution. Retention of ionic analytes in reverse phase LC can be mediated by hydrophobic ion pairing reagents (Huck et al. 2003). Although volatile counter-ions that are compatible with electrospray process can be used, particular care must be dedicated in instrument maintenance to loss of sensitivity and signal deterioration. A even more MS-friendly alternative is hydrophilic liquid interaction chromatography (HILIC), which exhibits improved separations of ions in high organic phases and is available in nanoscale systems, where maximum sensitivity is attained (Alpert et al. 1994; Tolstikov and Fiehn 2002; Bajad et al. 2006). In general, sensitivity in nano-LC can be further increased with preconcentration by loading large sample volumes to a short enrichment column that fully retains the analytes in a thin section. When the solvent gradient is started, a focused and highly concentrated analyte plug elutes from the enrichment column, and is separated on the analytical column. Unfortunately, the injection volumes of central carbon metabolites is still limited when their retention on commercially available phases is not sufficient to load large sample volumes without having a fraction already eluting from the enrichment column, e.g. with most HILIC material. In comparison to GC, the longer equilibration time and chromatographic separations of organic gradients reduce sample throughputs. In contrast, the milder ionization in LC-MS enables the detection of intact molecules, which produce less populated spectra and facilitates identification.

Among the three platforms, CE-MS features unsurpassed peak capacity, concomitant separation of anions and cations, and resolution of most isomers present

in central metabolism within short runs (Soga et al. 2003; Harada et al. 2006). CE as well offers the possibility to focus the analytes in large volumes by sandwiched injection techniques (Britz-McKibbin and Terabe 2003). The drawback of CE-MS measurement lies in the expertise and time necessary to obtain reproducible measurements at high-throughputs. In addition, the narrow eluting peaks limit the number of different fragmentation cycles that can be performed over a peak.

Overall, all three systems provide access to key intermediates in central metabolism and can cope with large injection volumes which are used to enhance sensitivity. To date, GC-MS and LC-MS are the preferred platform to detect labeling patterns in amino acids and central carbon metabolism, respectively. CE-MS is superior in sensitivity and enables detection of both compound classes. Nevertheless, these advantages are apparently not yet sufficient to replace GC and LC.

5.3 Current developments

To fulfill the goals of metabolome-based ^{13}C flux analysis (cf. 5), further improvements are necessary. In the following sections I address three topics that are targets of current research. The first two are of experimental nature and aim at obtaining possibly detailed and accurate labeling information from free metabolites. Both aspects are pivotal in the quest of comprehensive flux analysis for cells grown in complex media. The third topic is the extension of metabolic flux analysis to cope with the frequently occurring isotopically non-stationary systems, which will promote metabolome-based flux analyses to a universally applicable tool.

5.3.1 How to measure precise isotopic mass distributions?

The analogies between fluxome and metabolome measurements stop upon subjecting metabolites to mass spectrometry, because measuring precise mass distributions differs from measuring concentrations, and MS instruments have to be set up accordingly. In quantitative concentrations measurements, MS/MS acquisitions are the mode of choice for best signal-to-noise and high scanning rate are employed to obtain more data points on a peak and reduce interpolation errors. In contrast, detection of isotopic mass distributions such as needed for ^{13}C flux analysis is generally done with full range MS acquisitions, because for each metabolite/fragment a range of 10-15 m/z has to be scanned (or fragmented) due to the overlapping presence of naturally occurring isotopes. In complex samples, where chromatographic coelution is frequent, or with in-source fragmentation (e.g. electron impact ionization in GC-MS), selected ion monitoring looses attractiveness because at least 50-100 m/z bins have to be scanned simultaneously and complicate acquisition programs must be prepared to ensure that the correct mass range is monitored at the elution time of each analyte.

As a rule of thumb, isotopic fractions of 1 mol% (better if lower) compared to the monoisotopic mass should be precisely quantifiable to obtain fluxes with good confidence. Hence, the limits of quantitation (LOQ) for mass distributions are at

least 2 orders-of-magnitude higher than the LOQ for metabolite concentrations. Because of poor ion statistics, low abundant fractions are more prone to inaccuracy. Another consequence is that MS detectors must exhibit a wide linear dynamic range of >4 decades to effectively measure distributions in real samples where analytes are heterogeneously concentrated. If that is not the case (e.g. as in most ion traps) multiple injections of different amounts are necessary to characterize low and highly abundant species.

Low mass resolution is also detrimental for exact isotopic distributions, in particular when quadrupoles or ion traps are used for detection. High-resolving time-of-flight or Fourier Transform instruments are not affected. Resolution has to be increased to ensure that no overlap or crosstalk between neighbor m/z bins occur, also after slight calibration drifts. Unfortunately resolution comes always at the cost of sensitivity, but this drawback can be partly alleviated with slower scan speeds. As mentioned above, this is in conflict with the ideal settings for quantitative concentration measurements because less data points open for peak interpolation errors. In synthesis, detection of exact mass distributions depends on possibly high ion counts in full-range MS mode, good mass resolution, and an outstanding linear dynamic range. Due to the interdependency of these properties and the generally low abundance of free metabolites, sensitivity rapidly emerges as the major bottleneck in fluxome measurements.

5.3.2 Fragmentation: the key to obtain the labeling of single atoms

In metabolomics, fragmentation is extensively utilized for identification and selective detection. In fluxomics, fragmentation provides labeling imprints at submolecular level and eventually positional enrichment, i.e. the abundance of label at single atom positions (Fig. 4). Flux identifiability is subordinated to the ^{13}C patterns that are measurable and hence, in turn, to the fragments that can be generated. Novel fragments can enable more detailed analyses and more flexibility in the choice of the tracer. Also when equivalent fragments of the same metabolite are measured (e.g. those with the same carbon backbone), they lead to higher confidence in the flux estimation. As a general rule, it is thus desirable to obtain and detect the largest number of fragments possible.

Routine utilization of fragment data is, however, hindered by the overlap of three technical issues: (1) Fragmentation is inducible and happens when molecules collide at high energy with gas molecules or electrons, or when they are subject to strong electric fields. However, for each ion the break points can only be minimally controlled by the instrument settings. Increasing or decreasing of (collision) energy favor formation of low and high molecular weight daughter ions, respectively. It is, however, not possible to break every C-C bond at will, and some atoms are virtually not distinguishable (e.g. C_1 and C_2, or C_5 and C_6 in Fig. 4).

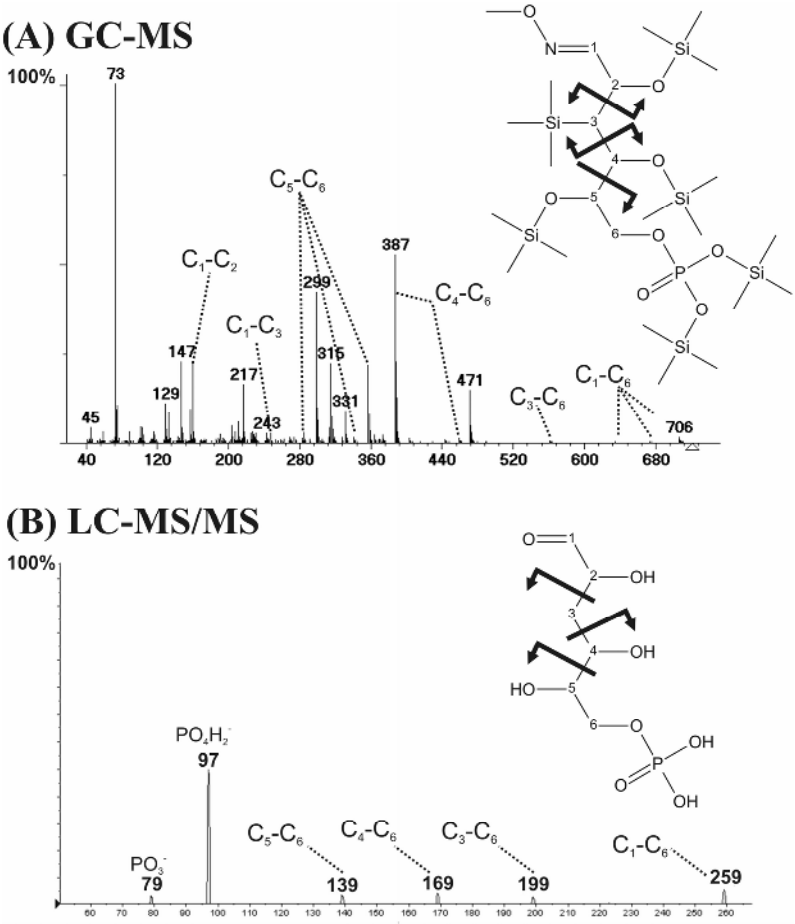

Fig. 4. MS spectra of fragmented glucose-6-phosphate. On each pane, the intact parent molecule is drawn with numbered carbon atoms. Directly measurable fragments are indicated by thick arrows. (A) Spectrum resulting by in-source fragmentation in GC-MS. The analyte was first methoximated and sylilated to obtain a volatile derivative. Many fragments are observable, but their intensity is too low to quantify isotopic distributions (e.g. C_3-C_6). (B) Spectrum provoked by collisional fragmentation in a LC-MS/MS experiment. 100% intensity corresponds to that of the parent ion (m/z -259) in absence of collisions. Sugar-phosphates are prone to break at the phosphoester bond, so that the carbon-containing fragments are underrepresented versus the non-informative phosphate ions (m/z -97 and -79). Since the charge is located on the phosphate group, only one daughter ion is observed when the carbon backbone is broken. Nevertheless, the mass distribution of the neutral complement can be calculated from that of the intact molecule. Hence, GC-MS and LC-MS/MS provide qualitatively equivalent information, and the true limitation is set by ion counts. Considering that for each fragment several m/z have to be measured, LC-MS/MS might be preferred here because no overlaps between fragments or unknown peaks occur. A decision must account for the expected mass shifts caused by ^{13}C enrichment.

(2) MS is only able to detected charged species. Hence, when a singly charged species is fragmented, two daughter fragments are formed: one is charged and one is neutral. The ionic moiety can be detected, while the neutral part is lost and invisible in the spectrum (Fig. 4). The isotopic mass distribution of the latter cannot be directly measured, but can be inferred with worse precision from those of the parent ion and the complementary ionic fragment. (3) The intensities of the fragment peaks are typically 1-2 orders of magnitude smaller than those of the parent ion because of ion loss during collisional fragmentation and redistribution of daughter ions among different masses (Fig. 4B). Hence, sensitivity becomes once more the limiting factor in the determination of accurate mass distributions.

To summarize, fragmentation is without doubts beneficial to obtain either independent information or improved confidence. Accordingly, theories were developed to deconvolute overlapped fragment spectra (Jeffrey et al. 2002; Rantanen et al. 2002). In practice, however, fragment data tends to be qualitative because of low ion counts. Since overloading of MS negatively influences resolution and accuracy, the only plausible alternative to obtain sufficient ion counts is seemingly to decouple separation and MS detection, i.e. to collect eluate fractions from chromatography and then infuse single fractions at very low rates and long times to the MS for acquisition. In addition, ad-hoc derivatization protocol can be used to provoke breakdown at different sites or increase the abundance (Price 2004).

5.3.3 Faster, cheaper, and better: non-stationary flux analysis

Another area of development is isotopically instationary ^{13}C flux analysis (Wiechert and Nöh 2005), which undertakes to perform fully-descriptive flux experiments within minutes after introduction of the labeled substrate as isotopic steady state is no longer a precondition, also when macromolecules turnover occur or large intermediate pools exist (Grotkjaer et al. 2004; van Winden et al. 2005). The so far unique strategy outlined to integrate isotopically instationary ^{13}C data is the extension of isotopomer balances to the dynamic case by replacement with ordinary differential equations. For this purpose, metabolite pool sizes are also newly introduced in the equations and fitted in an iterative procedure.

Time profiles of ^{13}C-patterns must be measured upon start of labeling to monitor the label propagation through the network. Conjoint measurement of metabolite concentrations is not strictly required. Omission, however, causes an increase in degrees of freedom, complicates the fitting procedure, and results in worse confidence intervals. Ideally, as many pool sizes as possible should be measured, and missing data can only be compensated by multiple labeling experiments (Nöh and Wiechert 2006). Notably, due to the metabolic steady state of the culture, the pool sizes are constant while the labeling pattern is still instationary. Thus, a single measurement fully describes concentrations throughout labeling. Solving the resulting highly non-linear system with thousands of ordinary differential equations is the most challenging and time-consuming step, although it can be speculated that implementation of elementary metabolite units decomposition would boost the calculation by a few orders of magnitude (Antoniewicz et al. 2006). Simulations done by Wiechert and coworkers demonstrate that the flux calculability is

tightly connected to sampling time points, total labeling duration, and tracer choice. Optimal and detailed a priori design of experiments is therefore mandatory (Nöh and Wiechert 2006).

6 Conclusions

Metabolome-based ^{13}C metabolic flux analysis is on the track to become a universal tool to quantify metabolic activity in large networks, higher cells, and complex environments. Measuring metabolic fluxes under such conditions is a challenging task that demands conjoint experimental, analytical, and mathematical skills. Know-how on aspects such as experimental design, execution, and data integration can be transferred from existing ^{13}C metabolic flux methods developed for microbes, where expertise and computation tools were established over the last decade. Nevertheless, further technical improvements are still necessary in the domains of (i) analytics to increase sensitivity of MS detection, and (ii) mathematical algorithms to efficiently cope with isotopically non-stationary ^{13}C flux experiments.

These accomplishments will eventually enable to comprehensively estimate fluxes, help unravel the underlying control mechanisms that govern metabolic fluxes, discriminate genetic mutations, assess the effect of drugs and diet on metabolism, or monitor the metabolic response in health and disease in virtually any biochemical reaction network where intermediates are accessible.

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List of abbreviations

CE: capillary electrophoresis
GC: gas chromatograph(y)
LC: liquid chromatograph(y)
MS: mass spectrometry
NMR: nuclear magnetic resonance
PPP: pentose phosphate pathways
TCA: tricarboxylic acid (cycle)