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MINI-REVIEW

# The benefits of being transient: isotope-based metabolic flux analysis at the short time scale

**Katharina Nöh · Wolfgang Wiechert**

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**Abstract** Metabolic fluxes are the manifestations of the co-operating actions in a complex network of genes, transcripts, proteins, and metabolites. As a final quantitative endpoint of all cellular interactions, the intracellular fluxes are of immense interest in fundamental as well as applied research. Unlike the quantities of interest in most *omics* levels, in vivo fluxes are, however, not directly measureable. In the last decade, $^{13}$C-based metabolic flux analysis emerged as the state-of-the-art technique to infer steady-state fluxes by data from labeling experiments and the use of mathematical models. A very promising new area in systems metabolic engineering research is non-stationary $^{13}$C-metabolic flux analysis at metabolic steady-state conditions. Several studies have demonstrated an information surplus contained in transient labeling data compared to those taken at the isotopic equilibrium, as it is classically done. Enabled by recent, fairly multi-disciplinary progress, the new method opens several attractive options to (1) generate new insights, e.g., in cellular storage metabolism or the dilution of tracer by endogenous pools and (2) shift limits, inherent in the classical approach, towards enhanced applicability with respect to cultivation conditions and biological systems. We review the new developments in metabolome-based non-stationary $^{13}$C flux analysis and outline future prospects for accurate in vivo flux measurement.

K. Nöh · W. Wiechert (✉)
Institute of Bio- and Geosciences 1: Biotechnology 2,
Forschungszentrum Jülich GmbH,
Leo-Brandt-Strasse,
52428 Jülich, Germany
e-mail: w.wiechert@fz-juelich.de

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## Introduction

The in vivo physiological state of a cell is controlled and maintained by a complex network of interacting molecules. The main players in this involved system are the metabolic fluxes—the rates of metabolic inter-conversions—reflecting the integrative quantitative endpoint of the diversity of all cellular processes, the cell's phenotype. Due to the fundamental importance of metabolic fluxes, the neologism *fluxomics* or synonymously metabolic flux analysis (MFA) has been coined. Fluxomics nowadays has gotten a fixed place in the concert of *omics* technologies forming the backbone of systems biotechnology. Briefly, MFA aims at the quantification of intracellular metabolic fluxes in the biochemical networks and has evolved into several model-based approaches to assess not directly measureable in vivo fluxes within cell populations.

MFA as outlined in this review relies on the fundamental precondition that in vivo fluxes are measured in a metabolic pseudo-steady state, i.e., all fluxes and metabolite concentrations are (at least approximately) constant over time. To assess steady-state flux distributions from measured extracellular rates, several approaches based on a description of cellular metabolism by stoichiometric models have been developed over the last decades (Stephanopoulos et al. 1998; Vallino and Stephanopoulos 1993; Varma and Palsson 1994; Niranjan and San 1989). High flux resolution of the central metabolism augmented by biosynthetic pathways,

however, requires the more sophisticated framework of isotope-labeling experiments (ILE) (Sauer 2006; Wiechert 2001). The most common technique is $^{13}$C-based and takes advantage of differences in carbon utilization by alternative metabolic paths. After feeding $^{13}$C-labeled substrate(s), the isotopic tracer is propagated through the metabolic network. Detectable labeling patterns, typically those of central metabolism intermediates, free and proteinogenic amino acids, constitute an "isotopic fingerprint" from which the real flux distribution can be reconciled. The result of a $^{13}$C-MFA is a *metabolic flux map* giving detailed insight into the relative activity of intracellular processes. Figure 1 shows flux maps for an *Escherichia coli* wild-type strain and two knockout mutants obtained from experimental data described in Ishii et al. (2007).

The classical scenario for $^{13}$C-based flux analysis relies on labeling enrichments observed when the isotopic labeling has become equilibrated. For this scenario, the common practice of $^{13}$C-MFA, including an experimental protocol, model-based evaluation tools, and a wide range of applications, has been reviewed quite recently (Blank and Kuepfer 2010; Dauner 2010; Niklas et al. 2010; Sauer 2006; Schwender 2008; Wiechert 2001; Wiechert and de Graaf 1996; Zamboni 2011; Zamboni et al. 2009). The present paper focuses on the youngest child in the family of $^{13}$C-MFA methods, the *isotopic non-stationary metabolic flux* analysis (INST $^{13}$C-MFA). In contrast to its conventional stationary counterpart, time profiles of metabolite labeling patterns are measured and the transient label information along with the metabolites' steady-state concentrations are used to determine the in vivo fluxes. Although the basic concepts of the method have been sketched in Wiechert and Nöh (2005), it took until Nöh et al. (2007) and Schaub et al. (2008) for the first experimental proofs. Since then, further isotopic non-stationary applications under metabolic (quasi-) stationary conditions have been conducted (Hasunuma et al. 2010; Munger et al. 2008; Zhao et al. 2008; Hofmann et al. 2008; Maier et al. 2008, 2009; Noack et al. 2010) (cf. Table 1), the bio-analytical and experimental tool box has been settled (Bennett et al. 2009; Flores et al. 2002; Iwatani et al. 2007; Luo et al. 2007; Oldiges et al. 2007; Szyperski 1995; Wittmann et al. 2002; Drysch et al. 2003; El Massaoudi et al. 2003), and the underlying theory and computational tools have been developed (Nöh et al. 2006; Young et al. 2008; Yuan et al. 2008). In this review, recent progress concerning INST $^{13}$C-MFA is reviewed. The reader is assumed to be familiar with the basic concepts of stationary $^{13}$C-MFA and its applications which can be taken from Wiechert (2002) and Zamboni et al. (2009).

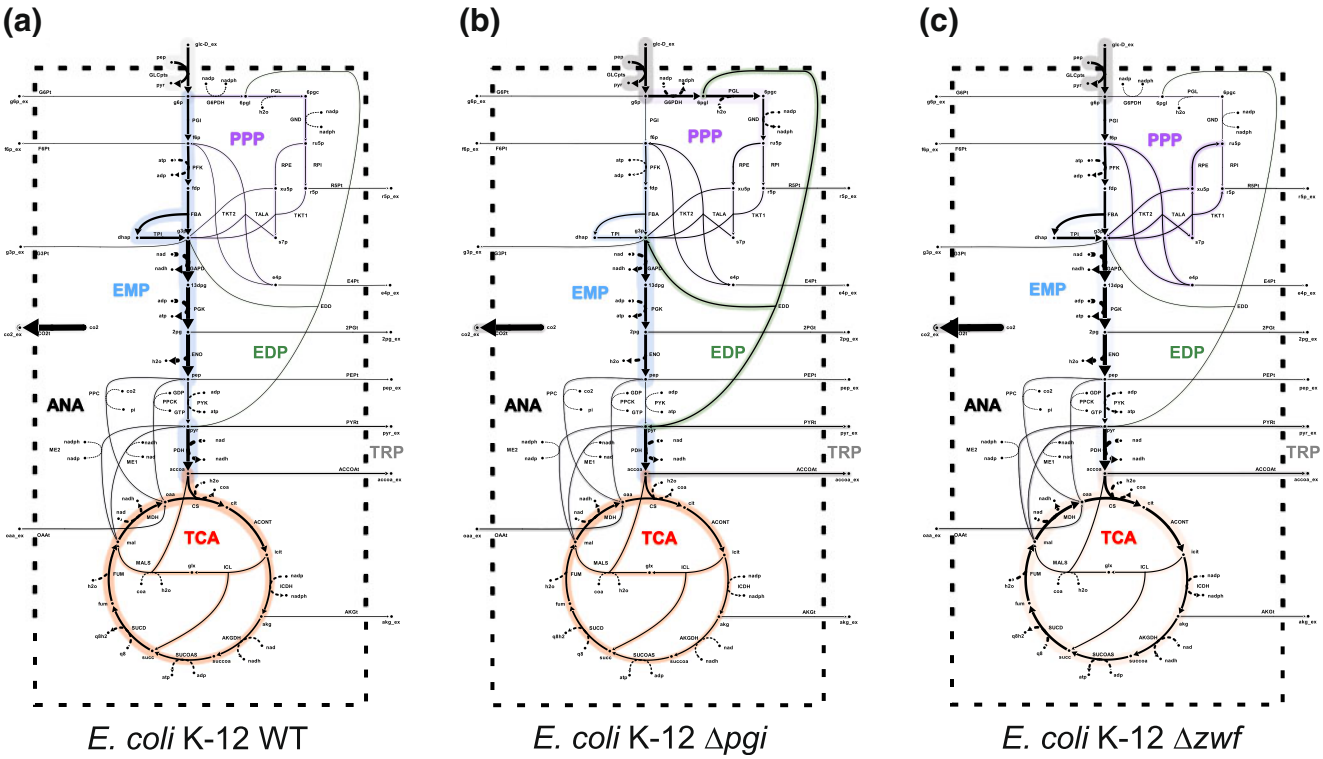


**Fig. 1** Flux maps unravel re-routing fluxes in the metabolic central carbon pathways of *E. coli* K-12 as result of genetic modification: wild-type (**a**), *pgi* (knock-out of first glycolytic reaction, **b**), and *zwf* (knock-out of first pentose phosphate pathway reaction, **c**) mutant strains (from *left* to *right*, data taken from Ishii et al. (2007) and visualized with Omix, [www.13cflux.net/omix])

**Table 1** Summary of recent applications of non-stationary $^{13}C$ labeling experiments under metabolic steady-state conditions

| Reference | Organism | Objectives | Experiment | Analytics | Model |
|---|---|---|---|---|---|
| Hasunuma et al. (2010) | *Nicotiana tabacum* (leaves) | Determination of in vivo turnover rates in primary metabolism of higher plants under different $CO_2$ concentrations to reveal the influence of the photosynthesis rate on the turnover rate of photosynthetic pathway intermediates | Gas exchange photosynthesis chamber freeze-clamp<br>99% $^{13}CO_2$<br>Expose leaf with $O_2$ containing 200 or 1,000 ppm $CO_2$<br>Stabilization time of photosynthesis 30 min<br>Duration: >36 h | CE-MS/MS<br>Quenching: liquid nitrogen<br>14 pool sizes<br>77 mass isotopomers<br>Isotopic steady state:<br>Glycolysis and PPP: O(10 min)<br>Amino acids: >36 h | Metabolic turnover is calculated from the initial slope of the $^{13}C$ fraction in the time frame of 0.5–2 min |
| Hofmann et al. (2008)/ Maier et al. (2008) | Hepatic cells (HepG2 HB-8065™) | Identification of metabolic fluxes in hepatic central metabolism by INST $^{13}C$-MFA | 6-well dishes with $10^6$ cells/well<br>Medium: William's E ++<br>40% naturally, 20% 1-$^{13}C$, 40% U-$^{13}C$ glucose<br>Samples: 7 (in triplicates)<br>Duration: 3 h | GC–MS (TCA intermediates)<br>LC–MS (sugar phosphates)<br>Quenching: hot air followed by boiling water<br>14 pool sizes<br>343 (47–51 per sample time)<br>Isotopic steady state: glycolysis and PPP: 30 min, TCA: >3 h | 40 fluxes<br>25 balanced metabolites<br>860 isotopomers |
| Maier et al. (2009) | Primary rat hepatocytes (male Wistar rat) | Quantification of statin effects on hepatic cholesterol synthesis by INST $^{13}C$-MFA | 6-well dishes with $10^5$ cells/cm$^3$<br>Medium: William's E ++<br>Steady state after 4 h 100% U-$^{13}C$ glucose<br>Samples: 3 (in triplicates)<br>Duration: 12 h | GC–MS (cf. Hofmann et al. 2008)<br>11 pool sizes<br>Mass isotopomers of TCA intermediates, phosphoenolpyruvate, 3-phosphoglycerate, cholesterol, lathosterol<br>Isotopic steady state:<br>Central metabolic intermediates: < 4 h (before first sample time)<br>Cholesterol pathway intermediates: >12 h | 73 reactions<br>47 balanced metabolites<br>950+253 balance equations |
| Munger et al. (2008) | Confluent human MRC-5 fibroblasts (ATCC) | Quantify changes in metabolic activity induced by human cytomegalovirus (HCMV) | Medium: Dulbecco modified Eagle medium (DMEM)<br>100% U-$^{13}C$-glucose, 100% U-$^{13}C$-glutamine, [1,2-$^{13}C$] glucose [3-$^{13}C$] glucose<br>Samples: 8<br>Duration: 120 min | Quenching: cold methanol<br>LC–MS/MS (ESI): 43 mass isotopomers 17 pool sizes<br>Isotopic steady state: glycolysis: <5 min;<br>PPP: <10 min;<br>TCA, ATP, UTP: >120 min | 69 differential equations<br>Model parameters: 12 fluxes, 4 uptake and excretion rates, 4 pool sizes |

**Table 1** (continued)

| Reference | Organism | Objectives | Experiment | Analytics | Model |
|---|---|---|---|---|---|
| Noack et al. (2010) | *Corynebacterium glutamicum* (DM 1730) | Comparison of stationary and non-stationary 13C-MFA for central metabolic pathways | Fed-batch<br>$\mu_{max}$=0.2 $h^{-1}$<br>Rapid sampling rotary plane<br>Medium: CGXII<br>1 L working volume<br>12% $^{12}$C-, 9% 1-$^{13}$C-, 79% U-$^{13}$C glucose<br>Samples: 18 samples in 40 s for non-stationary analysis; 4–5 samples between 2,000 and 4,000 s for stationary analysis | Quenching: cold methanol<br>LC–MS/MS<br>standard addition method<br>5 technical replicates<br>Stationary analysis: 65 mass isotopomers<br>Non-stationary analysis:<br>18 pool sizes<br>1,332 mass isotopomers<br>Isotopic steady state:<br>Glycolysis and PPP: O(min)<br>TCA: <30 min<br>Amino acids: <50 min | 22 balanced metabolites<br>43 reactions<br>Model parameters: stationary: 24 fluxes, non-stationary: 46 fluxes and pool sizes |
| Nöh et al. (2007) | *Escherichia coli* K-12 | INST $^{13}$C-MFA: proof-of-principle of ultra-short experiments | Chemostat D=0.58 $h^{-1}$<br>Rapid sampling rotary plane<br>2 L working volume<br>Medium: Luria–Bertani (LB)<br>20 samples in 16 s<br>Stationary state after 8.9 h<br>18% $^{12}$C-, 39% 1-$^{13}$C-, 43% U-$^{13}$C glucose | Quenching: cold methanol<br>LC–MS/MS<br>Standard addition method<br>17 pool sizes<br>660 mass isotopomers (60 per sampling time point)<br>Isotopic steady state:<br>upper glycolysis: O(10 s)<br>Lower glycolysis and PPP: O(100 s), TCA: O(10 min) | 86 fluxes<br>23 metabolites<br>Model parameters: 43<br>Remark: 11 of 20 time points are evaluated<br>Sampling time points and substrate mixture are determined by a priori experimental design |
| Schaub et al. (2008) | *Escherichia coli* K-12 W3110 | Integrative analysis of stationary and transient labeling data to determine intracellular central metabolic fluxes | Chemostat, D=0.10 $h^{-1}$<br>1.5 L working volume<br>Medium: M9<br>Steady state after 6.5 residence times<br>40% naturally, 40 % 1-$^{13}$C, 20 % U-$^{13}$C glucose | Quenching: heat exchanger<br>LC–MS: central metabolic intermediates<br>1 mL sample volume triplicates<br>5 samples in 589 s<br>1 sample at 65 min<br>9 partly lumped pool sizes<br>90 mass isotopomers (transient and stationary) | 41 fluxes<br>16 metabolites<br>550 isotopomers<br>Model parameters: 12 fluxes and reversibility factors<br>Remark: pool sizes were not fitted |

**Table 1** (continued)

| Reference | Organism | Objectives | Experiment | Analytics | Model |
|---|---|---|---|---|---|
| | | | Duration: 4.86 h | GC–MS: proteinogenic amino acids<br>1 sample at 4.86 h<br>24 mass isotopomers (stationary)<br>Isotopic steady state: glycolysis and PPP: O(100 s), TCA: O(5 min) | |
| van Winden et al. (2005) | *Saccharomyces cerevisiae* CEN.PK113-7D | Serial stationary $^{13}$C-MFA based on mass isotopomer measurements of primary metabolites | Chemostat D=0.10 $h^{-1}$<br>4 L working volume<br>1 mL sample volume | By-products: HPLC | 21 fluxes<br>8 balanced metabolites |
| | | | Medium: defined<br>Steady state after 7 residence times | LC–MS<br>5 replicates | Model parameters: 9 fluxes |
| | | | 100% 1-$^{13}$C glucose and 100 % U-$^{13}$C-ethanol | 40 mass isotopomers of 10 metabolic intermediates | |
| | | | Samples: 2<br>Duration: 60 min | Isotopic steady state: glycolysis and PPP: >60 min due to turnover with large unlabeled glycogen storage pool | |
| Zhao et al. (2008) | *Penicillium chrysogenum* (DS17690) | Determination of the split-ratio between the oxidative branch of pentose phosphate pathway and glycolysis | Chemostat D=0.06 $h^{-1}$<br>1 L working volume | Quenching: cold methanol | 8 fluxes<br>6 metabolites |
| | | | 2 mL sample volume<br>Medium: chemically defined | By-products: HPLC<br>LC–MS<br>triplicates<br>IDMS correction 2 pool sizes, 21 mass isotopomers | Model parameters: 5 fluxes, 1 pool size |
| | | | Steady state after 5 residence times 100 % U-$^{13}$C-gluconate<br>Samples: 14=28×1 mL, pooled | Isotopic steady state: PPP: 1 h | |
| | | | Duration: 5 h | | |

### Prospects and challenges of INST $^{13}$C-MFA

In three points, INST $^{13}$C-MFA is superior to the established isotopic stationary flux analysis method which makes the new approach very promising for future applications:

1. The time needed for the non-stationary ILE is significantly shortened: in case of *E. coli* and *Corynebacterium glutamicum*, for instance, from the order of tens of hours to sub-minute scale (Noack et al. 2010; Nöh et al. 2007; Zamboni 2011) (cf. Table 1). Progress in (mainly) MS-based analytics enabled the quantification of pool sizes of central metabolic intermediates along with their labeling patterns instead of analyzing the cell protein. This enables transient metabolic views (for example in (fed-) batch cultures) to be observed.
2. For organisms that metabolize monocarbonic compounds, for example $CO_2$ in plants or $CH_4O$ in yeasts, stationary $^{13}$C-MFA will give no information about metabolic fluxes at all. This changes when the isotopic non-stationary viewpoint is taken (cf. Fig. 2) (Shastri and Morgan 2007).
3. Last but not least, the non-stationary method has the potential to verify (or disprove) assumptions that are usually made in stationary $^{13}$C-MFA (Noack et al. 2010; Nöh et al. 2007; van Winden et al. 2005).

Summarizing, INST $^{13}$C-MFA will enable a more direct insight into metabolic processes, significantly broadens the

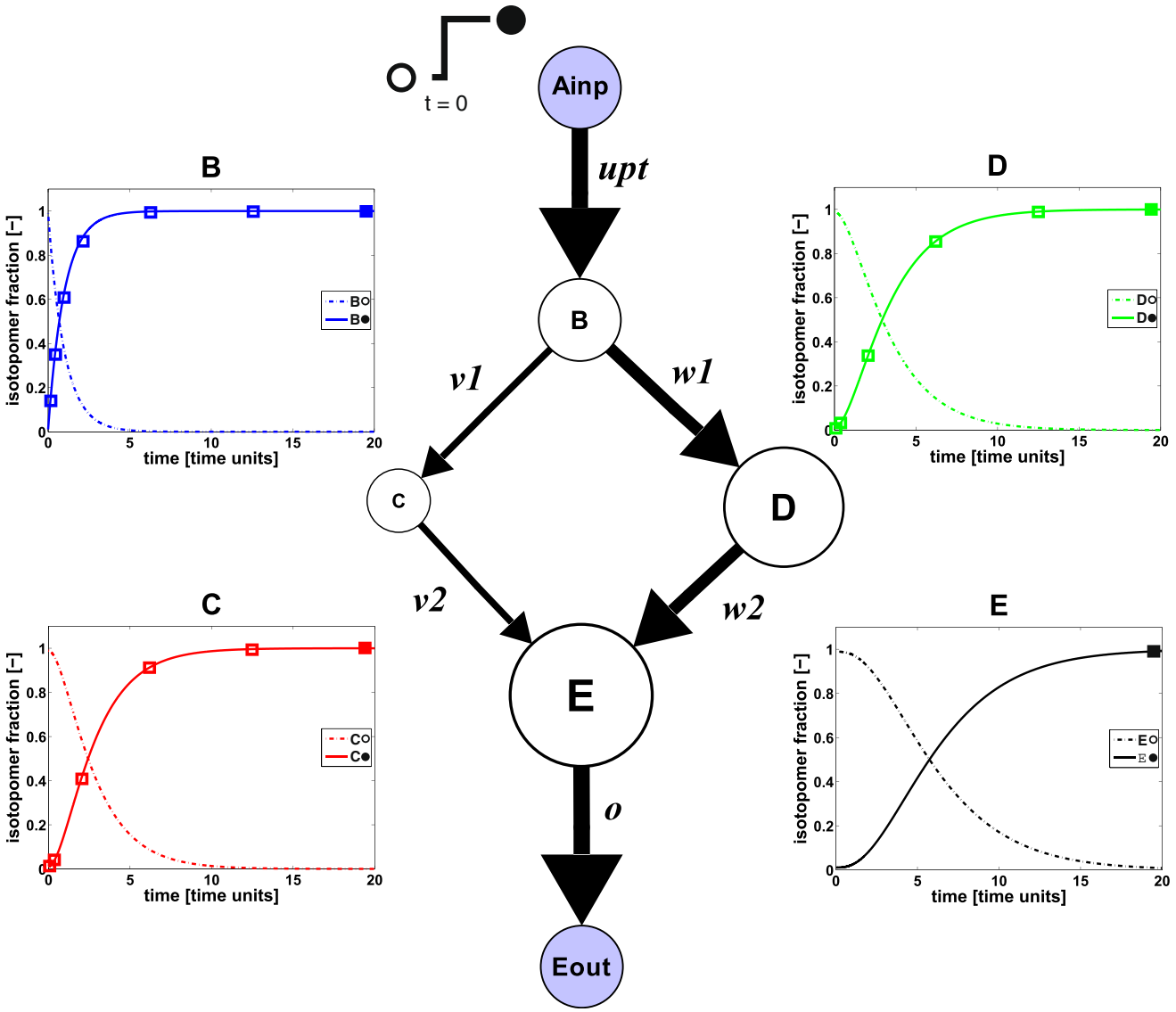


**Fig. 2** A simplistic example of alternative pathways. Labeling enrichment over time for parameters $upt=o=1.0$; $v_1=v_2=0.3$; $w_1=w_2=0.7$; $B=1.0$; $C=0.6$; $D=2.1$; $E=3.0$ is shown. Label pattern measurements are indicated by *boxes* (*unfilled*: isotopic transient, *filled*: isotopic stationary state). The sizes of the *circles* and *arrows* scale with the pool sizes and flux values, respectively

range of application areas, and allows strengthening the theoretical fundament of MFA.

In contrast to most other *omics* technologies, $^{13}$C-MFA cannot directly measure the wanted quantities, i.e., the in vivo metabolic fluxes (Wiechert 2007). Instead, these quantities have to be inferred from the available data, i.e., isotope-labeling patterns as well as uptake and secretion rates, relying on a mathematical model. This model is based on the knowledge of the biochemical network, the transitions of carbon atoms for all reactions in the network, a closed carbon balance (by balancing all uptake and production rates), and some mostly physical assumptions concerning net flux directions (Vallino and Stephanopoulos 1993). Notably, no fixed assumptions on in vivo enzyme kinetics, enzyme expression levels, cofactors and energetic requirements, or strain-specific biomass constitutions are necessary, although these quantities may be readily integrated as bounds constraining a physiologically sensible flux space.

An environment for INST $^{13}$C-MFA requires several ingredients:

1. An experimental platform for well-controlled cell cultivation equipped with a rapid sampling and fast metabolic quenching device;
2. A bio-analytical platform for quantitative metabolomics and the measurement of isotopic label enrichment in low-concentrated intermediates of cellular metabolism;
3. Computational tools for modeling isotope-labeling networks, simulation of ILEs, and their experimental design as well as data pre- and post-processing.

This explains why INST $^{13}$C-MFA is inherently more complex than classical $^{13}$C-MFA. Particularly, a broad spectrum of experimental, analytical, and computational expertise has to be brought together. Although $^{13}$C-MFA has recently undergone several substantial developments, the number of applications as well as contributing groups is relatively low compared to those of other *omics* technologies.

## A simple example

Some basic principles of the isotopic non-stationary method and the major differences to its conventional stationary counterpart are now discussed by using a simple toy example. The corresponding network configuration is shown in Fig. 2. Here a substrate is taken up, processed via two alternative pathways, and finally combined further downstream. To keep this illustrative example as simple as possible, it is assumed that the substrate molecule (and consequently all subsequent ones) has only one single carbon atom. Under the premise that all fluxes in the metabolic network are constant, a theoretical ILE is performed by switching from unlabeled to fully labeled substrate at time zero. The objective is to unravel the unobservable ratio of fluxes between the alternative pathways from measured labeling and external flux data to produce the wanted flux map.

Usually the extracellular fluxes (in this case, the substrate uptake "*upt*") are directly measured from classical bioreactor balances and, thus, define the absolute scale for the intracellular fluxes. Nevertheless, the percentage of flux going via $v_1$ and $w_1$ cannot be determined from these data. Consequently, if no further information is available, only an ILE can yield this information. At time $t$=0 of this ILE, the percentage of labeled isotopes in all intracellular pools is 0 and the so far unlabeled substrate is exchanged by a 100% $^{13}$C-labeled one. The labeled material is taken up by the cell and distributed among the intracellular pools. As can be seen in Fig. 2, labeled material will first become observable in the metabolite pool B, subsequently in pool D, and shortly after that in the metabolite E. After 20 time units the unlabeled material is completely washed-out of the system and all intracellular pools have become fully $^{13}$C-labeled.

Operating a rapid sampling device with immediate inactivation of cellular metabolism, it is possible to measure at least a part of the transient labeling enrichment at discrete time points. Thus, direct insight into the enrichment processes is granted. The sampling time points, shown by squared markers in Fig. 2, cover the whole time frame from labeling switch until labeling enrichment becomes (quasi-) stationary. If the flux analysis approach relies on a single measurement (with or without replicates) taken under isotopic equilibrium conditions (i.e., $t \geq 20$ time units in the example), it is called *stationary* $^{13}$C-MFA. Otherwise, if MFA relies on measurements taken within the transient labeling regime, the approach is termed *isotopic non-stationary* (INST) $^{13}$C-MFA.

This already indicates that the non-stationary method is more elaborated but also yields a maximum of information due to the incorporation of several measurements instead of relying on a single snapshot in the equilibrated labeling state. However, this may still not be enough information to quantify the fluxes. The reason is that not only the absolute flux through a given metabolite pool but also its total capacity determines the time needed to observe some significant increase of labeled material. For example, Fig. 2 shows that the labeling equilibrium in pool C is reached faster than that in pool D. The reason is that the capacity of D (indicated by the sizes of the pool symbols) for labeled material is 3.5-fold higher than that of C, although the flux $w_1$ used in the simulation is more than 2-fold higher than flux $v_1$.

To get the complete picture of labeling dynamics, labeling balance equations have to be formulated. To describe the percentage of labeled material in each of the metabolite pools B, C, D, and E, the time-depending

variables $b(t)$, $c(t)$, $d(t)$, and $e(t)$ are used. Furthermore, for the (constant) metabolite pool sizes (or synonymously, total amounts) the quantities $B$, $C$, $D$, and $E$ are introduced. The system is then described by a set of stoichiometric equations

$$\begin{aligned} &\text{B}: && upt = v_1 + w_1 \\ &\text{C}: && v_1 = v_2 \\ &\text{D}: && w_1 = w_2 \\ &\text{E}: && v_2 + w_2 = o \end{aligned} \tag{1}$$

and dynamic labeling balances

$$\begin{aligned} &\text{B}: && B \cdot \frac{d\,b(t)}{dt} = upt \cdot a - (v_1 + w_1) \cdot b \\ &\text{C}: && C \cdot \frac{d\,c(t)}{dt} = v_1 \cdot b - v_2 \cdot c \\ &\text{D}: && D \cdot \frac{d\,d(t)}{dt} = w_1 \cdot b - w_2 \cdot d \\ &\text{E}: && E \cdot \frac{d\,e(t)}{dt} = v_2 \cdot c + w_2 \cdot d - o \cdot e \end{aligned} \tag{2}$$

subject to the initial settings

$$b(0) = c(0) = d(0) = e(0) = 0$$

that reflect the unlabeled system state at $t$=0 (see Wiechert and Nöh 2005 for more details).

For the entry pool B, it is easily possible to give an explicit solution of the equations, namely

$$b(t) = 1 - \exp\left(-\frac{upt}{B} \cdot t\right) \tag{3}$$

Having the corresponding measurements for pool B available (see unfilled squared markers in Fig. 2 for pools B, C, and D), it is immediately possible to determine the flux-to-pool size ratio $upt/B$ because it is the initial slope of $b(t)$ (cf. Eq. 3). However, without knowing either the pool size $B$ or the flux $upt$, none of the two quantities can be determined. Since the substrate uptake $upt$ is an external flux, it is typically measurable from bioreactor balances. Thus, an estimate of the pool size $B$ becomes available by using labeling data.

One step further downstream in the network the question arises which sort of data is required to determine the flux branch point at pool B, i.e., the absolute flux values $v_1$ and $w_1$. The explicit solution of the labeling enrichment in pool C can be calculated as

$$c(t) = \frac{C \cdot upt \cdot \left(\exp\left(-\frac{v_1}{C} \cdot t\right) - 1\right) + B \cdot v_1 \cdot \left(1 - \exp\left(-\frac{upt}{B} \cdot t\right)\right)}{B \cdot v_1 - C \cdot upt} \tag{4}$$

Under the assumption that besides the pool size $C$ also the labeling enrichment $c(t)$ is measured for at least a single time point in the transient labeling regime, i.e., $0<t<\infty$, Eq. 4 has exactly one unknown, namely $v_1$, and can therefore be solved to determine the rate $v_1$ by application of a numerical nonlinear equation solver. Notably, measurements taken at either $t$=0 or for $t=\infty$ contain no information. Clearly, with $v_1$, the flux value $w_1$ becomes directly accessible from the stoichiometric balances. Similarly, the labeling pattern of D is calculated by

$$d(t) = \frac{D \cdot upt \cdot \left(\exp\left(-\frac{r}{D} \cdot t\right) - 1\right) + B \cdot r \cdot \left(1 - \exp\left(-\frac{upt}{B} \cdot t\right)\right)}{B \cdot r - D \cdot upt} \tag{5}$$

with $r=upt-v_1$.

Having the measurements for the pool size $B$, the fluxes $upt$ and $v_1$, as well as the labeling pattern $d(t)$ for $0<t<\infty$ at hand, the pool size of pool D is the only unknown and, thus, can be determined from the measurements. Note that in the case where neither the pool size $C$ nor $D$ is measured, it is impossible to resolve the flux branch. The calculation scheme can be further continued in order to derive an expression for the labeling dynamics $e(t)$. Moreover, it becomes clear why in larger-scale networks a substantial part of the metabolic pool sizes have to be quantified, besides the labeling patterns, in order to identify the fluxes (Nöh and Wiechert 2006).

The example reveals important differences between the stationary and non-stationary case:

1. The stationary case assumes measurements at a time when labeling fractions show almost no change as indicated by a filled squared marker in Fig. 2. Consequently, the left-hand side of Eq. 2 can be replaced by zero and the equations become independent of the pool sizes. This explains why pool size measurements are not needed for classical stationary $^{13}$C-MFA.
2. In this simple example with only monocarbonic molecules, stationary measurements carry no information at all. After some time, every single pool will contain 100% labeled material, irrespective of the fluxes. Mathematically, this is reflected by the fact that the differential equations are redundant with the stoichiometric balances given in Eq. 1. Hence, no information about fluxes is contained in the labeling.

Concluding, the example demonstrates that the isotopic non-stationary method has the potential to gain more information on metabolic fluxes than its stationary counterpart. The computational cost, of course, is high, because instead of stationary balance equations, differential equations are to be solved.

Although the simple example demonstrates some major features of INST $^{13}$C-MFA, it is too much simplified to obtain an idea of the general case. Major challenges of INST $^{13}$C-MFA in practice are:

1. Metabolic networks are usually more entangled and comprehensive. Central carbon metabolism, which is

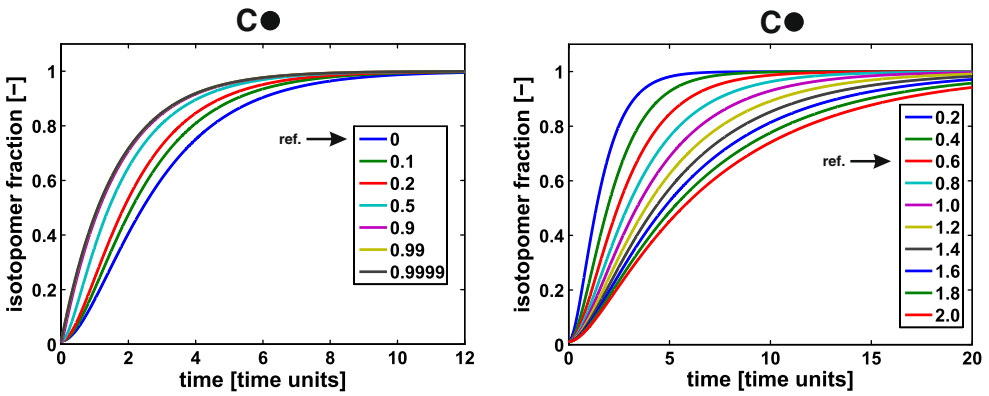


**Fig. 3** Influence of flux reversibility (*left*) and pool sizes (*right*) on the labeling enrichment of pool C for the illustrative example shown in Fig. 1. Flux reversibility ranges on a scale between 0, i.e., no exchange at all, to fast exchange (0.9999). The reference solution is indicated by an *arrow*

focused in nearly all published flux analysis studies so far, consists of up to 200 metabolites and reaction steps (Suthers et al. 2007; Ravikirthi et al. 2011). Recently, a genome-scale reconstruction of *E. coli* with more than 2,000 reactions has been automatically supplemented with atom mappings (Ravikirthi et al. 2011).

2. In general, metabolites have more than just a single carbon atom. This leads to an enormous number of different labeling fractions per metabolite and requires the general notation of isotopomers and isotopomer fractions which is not explained in this review (see Wiechert et al. 1999; Wiechert and Wurzel 2001). The only fact, the reader should keep in mind, is that for a metabolite with $n$ carbon atoms $2^n$, balances have to be formulated.
3. Realistic network topologies do not only contain sequences of reactions and branches but also reaction cycles. If cycles are present, labeling dynamics is much more complex than in the simple case (Klapa et al. 1999; Park et al. 1999). Special cases of cycles are bidirectional reaction steps with a cycle operating between two neighbored metabolite nodes, like kinase–phoshatase reaction pairs, e.g., the glycolytic reactions *fructose-bisphosphatase* and *phosphofructokinase* in *E. coli*. Figure 3 illustrates how a bidirectional flux $v_1$ will influence the behavior of the labeling enrichment of $c$ over time. Notably, variation of the reaction's reversibility yields a similar outcome as a variation of the pool size $C$.
4. It is impossible to give explicit solution formulas for the fluxes as a function of the measured labeling enrichment. Hence, an iterative parameter fitting procedure is needed to estimate fluxes from measured data. Due to highly nonlinear relations between fluxes and label patterns (cf. Eqs. 3, 4, and 5), only computationally costly global search strategies are suited for global flux identification.

## New applications for $^{13}$C-based flux analysis

The most striking difference between the classical isotopic stationary MFA method and the non-stationary approach is the very short time duration needed for the carbon labeling experiment. Recently, ILEs for *C. glutamicum* and *E. coli* demonstrated that it takes no longer than only a few seconds until labeled material is observed in all pools of glycolysis and a bit later also in the citric acid cycle (Noack et al. 2010; Nöh et al. 2007). Figure 4 illustrates the rapid increase of labeling in the glucose-6-phosphate pool of both microorganisms. Likewise, the very recent study of Hasunuma et al. (2010) for tobacco leaves showed a rapid metabolization of $^{13}CO_2$ within a similar time frame. Thus, for these microbes and plants, it is of high importance to take as many samples as possible in the first seconds after the switch to labeled material. This also underlines that advanced sampling and quenching devices are needed for this purpose.

In the cases shown in Fig. 4, it was possible to design highly informative experiments (Noack et al. 2010; Nöh et al. 2007) that produced meaningful metabolic flux distributions for the central metabolic pathways of both organisms. At the same time, another isotopic non-stationary chemostat experiment was reported in Schaub et al. (2008) for *E. coli* K-12 grown under aerobic, glucose-limited conditions at a dilution rate of 0.1 $h^{-1}$ (a 5-fold lower rate than in the study in Nöh et al. (2007) for the same organism). In this experiment, less samples were taken in the isotopic transient phase, meaning that the sampling intervals in these experiments were chosen quite large ($t$=91, 226, and 346 s). Moreover, samples within the isotopic stationary phase are taken and evaluated together with the non-stationary samples. With the combined information, it was possible to estimate reasonable fluxes.

Since then, some other INST $^{13}$C-MFA experiments were reported which are listed in Table 1 along with the major objectives, characteristics, and performance parameters. All these experiments illustrate that INST $^{13}$C-MFA works very

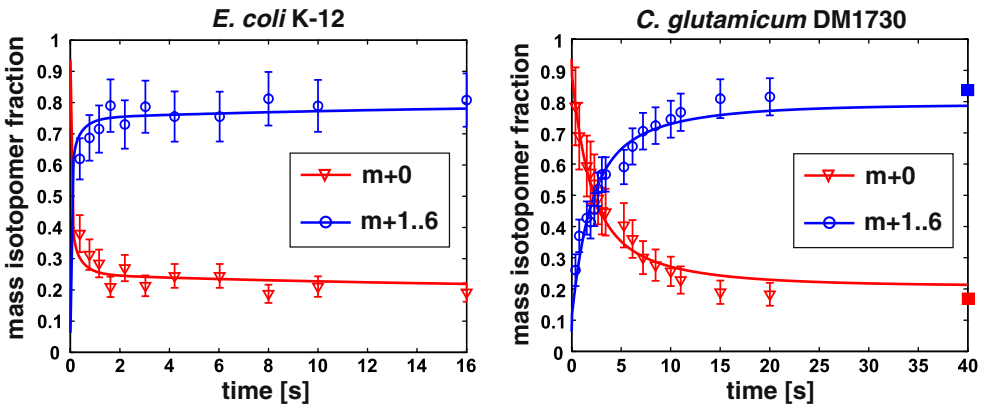


**Fig. 4** Fast labeling incorporation in glucose-6 phosphate originating from labeled glucose in the microorganisms *E. coli* K-12 and *C. glutamicum* DM 1730 (data taken from Noack et al. (2010) and Nöh et al. (2007)). m+0 represents the unlabeled glucose and m+1..6 the $^{13}$C-containing mass isotopomers

well although the evaluation effort for the laborious experiments is quite high and the whole procedure is far from being a routine application yet. This effort, however, is justified by a broad scope of new applications.

## Industrially relevant biotechnological fields of application

As a precondition for all MFA-based modeling approaches, metabolic fluxes must be approximately constant over at least the ILE's duration. If the organism is only experiencing slow changes in metabolic fluxes, observable at the time scale of hours, then the metabolic quasi-stationary assumption for a few minutes is usually justified (Wiechert and Nöh 2005). Nevertheless, the time required to reach the isotopic (pseudo-) steady-state severely hampers transferability and practical applicability of classical $^{13}$C-MFA to industrially relevant fermentation conditions and production strains. Although a premature termination of the ILE is possible, the extrapolation of steady-state values might lead to biased flux estimates (Wiechert and Nöh 2005). In contrast, the shortened labeling time window paves the way for the utilization of INST $^{13}$C-MFA in transient process phases of industrially relevant batch, repetitive batch, and fed-batch cultures. Moreover, any organism that would not keep metabolically stationary long enough to reach the isotopic steady state during labeling may be investigated with the non-stationary method. Similar arguments hold for slowly or non-growing organisms where the stationary $^{13}$C-MFA approach faces inherent problems (Maier et al. 2009; Sauer and Zamboni 2008). As a side effect, labeling costs for informative tracer substrates can be reduced due to the abbreviated labeling periods.

Another interesting case is growth on a single carbon source like $CO_2$ for plants (Allen et al. 2009; Schwender 2008), or methanol for *Pichia pastoris* (Charoenrat et al. 2005). As was shown by means of the simple example, in all these cases, the label equilibrium is solely determined by the percentage of labeled carbon present in the substrate. Thus, the isotopic stationary method will not yield any information about fluxes. Clearly, this reasoning likewise holds for all substrates that have only one stable isotope, e.g., for future hydrogen labeling experiments. The situation changes when the time-resolved labeling enrichment is available by non-stationary ILEs.

Tracer-based metabolomics has emerged as a valuable tool in systems biology for the exploration and analysis of the active biochemical pathways of a living cell (Lee et al. 2010). A potential future field of application in industrially relevant research is the investigation of non-model organisms for which functional annotation is not yet accomplished, and as a result, only rudimentary knowledge about the metabolic network structure is available. Hiller et al. (2010) developed a non-targeted MS-based technique using stable isotope tracers to identify labeled metabolites and active pathways involved in the glutaminolysis metabolism of carcinoma cell lines. In contrast to MFA methodologies, a priori knowledge of neither a reaction network nor a compound library is required. A similar approach trades under the name "isotopologue profiling", e.g., applied to investigate the nutrition of *Legionella pneumophila* (Eylert et al. 2010). Model-based INST $^{13}$C-MFA in combination with these quantitative non-targeted metabolomics approaches provides a unique opportunity to generate direct insight into the carbon network and its operation modes.

## Towards an enhanced spatial resolution of metabolic networks

An attractive perspective for INST $^{13}$C-MFA is the investigation of single pathways and intracellular processes that influence label distributions on the long time scale. These are related to large intracellular pools such as, for instance, storage metabolites or proteins that are subject to turnover (Grotkjaer et al. 2004; van Winden et al. 2005). For example, experimental observations with fully labeled

tracer demonstrated a permanent inflow of unlabeled material that hampers glycolysis or pentose phosphate pathway (PPP) metabolites from becoming fully labeled (Aboka et al. 2009). As illustrated with the simple example in Fig. 5a, b, these findings can be explained by a (slow) back-flux from an initially unlabeled and later on partly labeled material from pools interacting with the central metabolic intermediates. Such effects can hardly be analyzed in a purely stationary approach. In contrast, overlooking or neglecting those effects may lead to a significant bias in stationary flux estimates.

In contrast to prokaryotic systems, eukaryotes are subject to subcellular *compartmentation* of metabolism, a phenomenon that complicates $^{13}$C-MFA significantly. Not only duplication of metabolites or whole reaction pathways is well known from animal, fungal, and plant systems but also segregations like in hyphae-building fungi leading to highly complex metabolic networks. General reviews of $^{13}$C-MFA in plants, mammalian cells, and fungi are given in Allen et al. (2009) and Niklas et al. (2010). In Masakapalli et al. (2010), it is shown that stationary $^{13}$C-MFA was not capable to discriminate between three alternative models differing in the compartmentation of the PPP by using $^{13}$C-NMR data from multiple steady-state labeling experiments in plants. The INST $^{13}$C-MFA method has the potential to provide valuable information about inter-compartmental exchange fluxes if it is possible to dissect more abundant compounds (e.g., by NMR) that are involved in several reactions in different compartments (cf. Fig. 5c).

A controversy discussed phenomenon is the so-called *metabolic channeling* (Agius and Sherratt 1997; Kholodenko

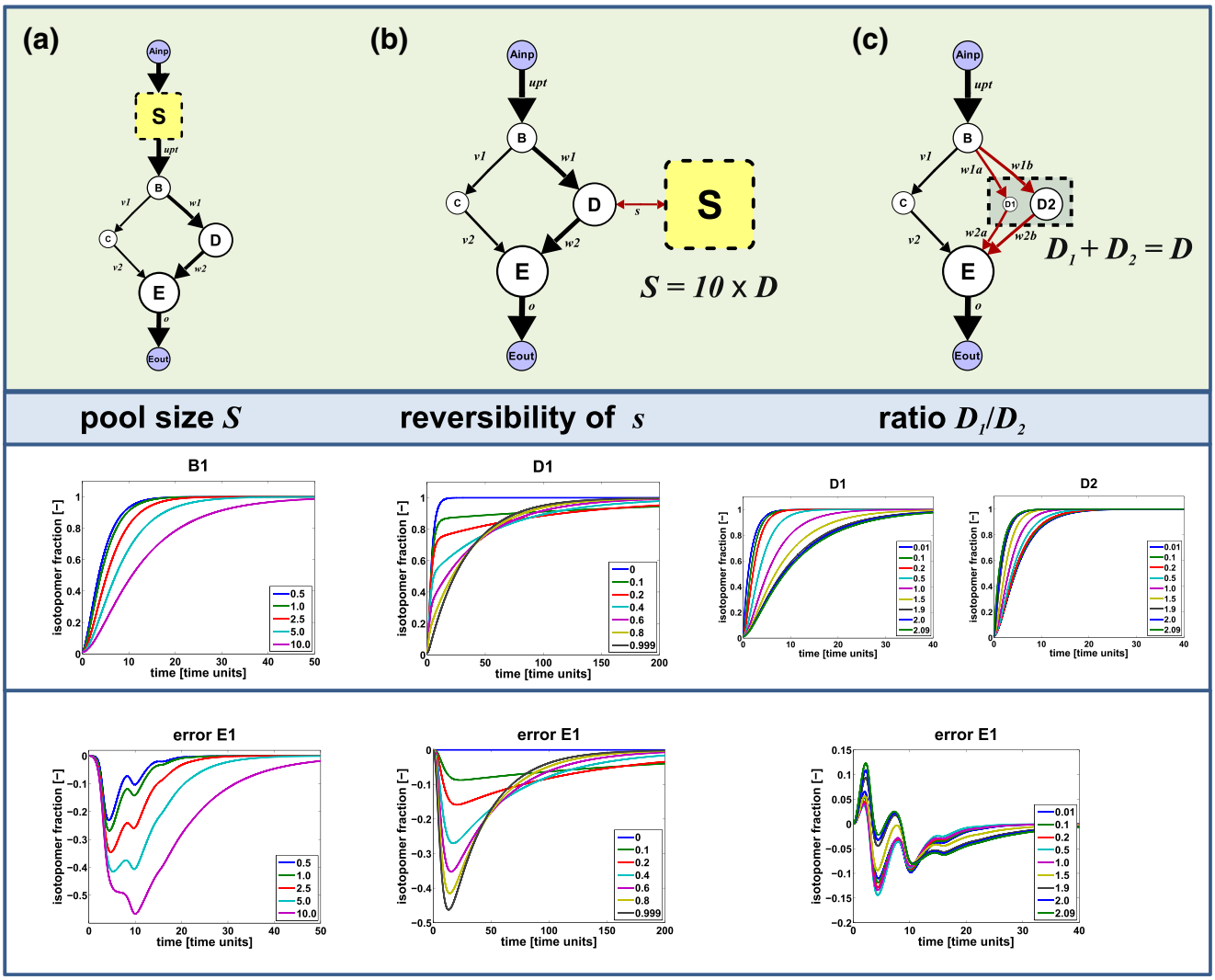


**Fig. 5** Influencing factors of labeling demonstrated on a simple network example: storage pool upstream (**a**) and downstream (**b**) in the network; effect of compartmentation (**c**). *First row*: network; *second row*: varied parameters; *third row*: exemplary labeling enrichment; *last row*: error in labeling enrichment of E induced by the variation with respect to the reference solution shown in Fig. 1

et al. 1996; Ovadi and Srere 2000), the tunneling of intermediates over several reaction steps. As one experimental approach to prove channeling, isotopic tracers have been used in Shearer et al. (2005), although in a more qualitative than quantitative way. INST $^{13}$C-MFA might provide a unique opportunity to generate new insights in vivo. If channeling happens, labeled tracer will appear in downstream metabolites faster than it can be explained by related enzyme kinetic models (Welch and Easterby 1994). In Nöh et al. (2007) and Noack et al. (2010), no such indications for channeling were found.

## Rapid sampling, sample processing, and biochemical analytics

The rapid inactivation of cellular metabolism is indispensible for a representative snapshot of the in vivo cellular state at sampling time (cf. Fig. 4). Optimally, this process has to be at least one order of magnitude faster than the turnover time, i.e., the ratio of in vivo pool sizes and metabolic rates, of the intermediates. Typical turnover times are in the order of seconds and for several central metabolites even below the estimated value for G6P (Heijnen 2010). Therefore, the major challenges to carry out high quality INST $^{13}$C-MFA are:

1. Withdrawal of representative samples must be rapid but without disturbing steady-state conditions in the reactor.
2. Enzymatic activity must be instantaneously stopped to avoid post-sampling artifacts. In addition to the previous point, the reliable quenching capability defines an upper limit for the sampling volume.
3. Extraction of intracellular metabolites must be quantitative (which is not required in case of classical $^{13}$C-MFA).
4. Measurement devices must be highly sensitive to allow the quantification of labeling patterns in intracellular intermediates from samples in nanomolar concentration ranges (Luo et al. 2007).

All these points address recent challenges of quantitative metabolomics, which stresses the close relation between this *omics* technology and INST $^{13}$C-MFA. In the following sections, we summarize some conceptual landmarks and current questions. More details can be found in several specialized papers cited below.

### Process sampling interface

Many different automatic rapid sampling devices that enable immediate cell inactivation have been developed since the 1990s as surveyed in Schädel and Franco-Lara (2009) and van Gulik (2010). Rapid sampling devices have been developed for stimulus response experiments where a microbial culture is exposed to a sudden stimulus, e.g., by the addition of a glucose pulse. The response to the perturbation is monitored and data are used for kinetic modeling approaches (see also "Beyond INST $^{13}$C-MFA" section). However, not all of these sampling devices can be utilized for INST $^{13}$C-MFA because their operation mode needs to maintain the metabolic steady state. As common practice for ILEs today, the cells are isotopically but not metabolically perturbed inside the bioreactor by switching from unlabeled substrate to an isotope-labeled one at a defined time point. Automatic sample withdrawal follows a pre-calculated sampling time protocol (Lange et al. 2001; Schaefer et al. 1999; Theobald et al. 1993). In particular, to lever $^{13}$C-MFA in an industrial scale, the sensor reactor approach was developed that enabled labeling experiments in a small scale by a reactor driven in parallel to the production reactor (Drysch et al. 2003; El Massaoudi et al. 2003; Grönke 2010).

### "Freezing" and extracting the metabolic state

The discussion of appropriate quenching protocols is controversial and far from being generally solved (Bolten et al. 2007; Taymaz-Nikerel et al. 2009; Villas-Boas and Bruheim 2007; Wittmann et al. 2004). It should be only pointed out that one of the most popular quenching method, i.e., exposing the cells to a −40°C to −50°C cold methanol–water mixture, faces the phenomenon that intracellular metabolites may "leak out" of the cell. If the loss is not properly quantified, this cellular leakage leads to severely underestimated metabolic pool size observations (Wittmann et al. 2004). An organism-specific optimization of quenching solutions and the temperature gradients the cells are exposed to seem to diminish this effect but cannot eliminate it completely (e.g., Link et al. (2008)). "Differential methods" correcting the amount of metabolites in the whole broth with the measurements of metabolites in cell-free aliquots by fast filtration are more generally applicable (Cao-Hoang et al. 2008; Taymaz-Nikerel et al. 2009; Canelas et al. 2008). Hence, leakage correction is particularly difficult if metabolites of interest are found in the culture supernatant during the ILE.

The tight interplay of the organism under investigation, physico-chemical properties of targeted metabolite classes as well as the installed analytic platform have led to a vast amount of lab-specific extraction protocols (see, for example, Canelas et al. (2009) and references therein for yeast). A lack of quality measures and rigorous inter-laboratory comparison studies hampers the development of more universal methods.

### Analysis of metabolic pool sizes and labeling patterns

Today, both pool size measurement and labeling pattern analysis are performed by state-of-the-art mass spectrome-

try (MS) devices. A high coverage of the metabolite spectrum as well as the ability to quantify the low abundance levels of intermediates is obtained with coupling the mass detector with a prior chromatographic separation or using time-of-flight analyzers with different ion sources (Garcia et al. 2008; Kusano et al. 2011; Ramautar et al. 2011; van der Werf et al. 2007). Major progress in the last years was achieved by the development of liquid chromatography (LC) MS technique and, in particular, tandem LC–MS (Bennett et al. 2009; Luo et al. 2007; van Winden et al. 2005). This technique allows the measurement with high coverage of central carbon intermediates, amino acids, nucleotides, and many more. Besides LC–MS/MS, recent progress in quantitative metabolomics has been made with capillary electrophoresis time-of-flight (CE-TOF) MS that has an even higher sensitivity than LC–MS (Ohashi et al. 2008; Toya et al. 2007).

In contrast to the pool sizes, measuring the absolute amount of label per metabolite is not required for flux determination. Thus, all techniques that are well established for the stationary method can be readily applied for the non-stationary approach. Besides LC–MS, gas chromatography (GC) MS and nuclear magnetic resonance (NMR) spectroscopy are routinely applied techniques, both targeting amino and organic acids. Recent work demonstrates the potential of high-resolution direct infusion Fourier transform ion cyclotron resonance (FT-ICR) MS and highly accurate gas chromatography-combustion isotope ratio (GC-C-IR) MS (Godin et al. 2007; Pingitore et al. 2007; Yuan et al. 2010).

It is well known that not only the number of measurements (pool sizes and labels) but also the type and quality of labeling information impact the identification of the fluxes (Isermann and Wiechert 2003; van Winden et al. 2001) (cf. Section "A simple example"). NMR, for instance, yields labeling information of single positions within a molecule, so-called positional enrichments. In contrast, MS-based technologies discriminate the labeled species by means of their masses, i.e., these techniques are oblivious to the fractional enrichment of specific atom positions. Inducing analyte fragmentation in the MS enables the view into carbon-containing metabolite segments (fragments) up to atomic resolution (Pingitore et al. 2007). Clearly, the simultaneous utilization of complementary analytical methods leads to highest resolution flux maps (Kleijn et al. 2007).

In the presence of compartments, metabolic networks become more comprehensive because inter-compartmental transport steps and enzyme localization have to be considered, too. Due to the disruptive extraction steps performed before the analysis, elucidation of compartment-specific metabolome data and label patterns is complicated. Thus, usually it is only possible to determine average measurements of the metabolites, also when they are located in different cellular compartments. This severely hampers flux analysis. A handful exceptions mainly from plant research apply in vivo NMR technology to resolve signals due to differences in pH, viscosity, or ionic strength to obtain amino acids or even labeling time courses (Aubert et al. 1998, 1999; Ratcliffe et al. 2001; Troufflard et al. 2007; Vogel et al. 1999).

For quantitative metabolomics, absence of isotope recovery standards, matrix effects in complex biological samples, and ion suppression issues are the current technical challenges to be solved (Jessome and Volmer 2006). Moreover, the development of automated, though reliable software is required to shorten manual analysis times coming along with the raw data evaluation (Brodsky et al. 2010).

## Computational tools for INST $^{13}$C-MFA

### Model equations: manual versus automated approaches

As mentioned before, the evaluation of ILEs is based on a mathematical model that describes the dynamics of the intracellular labeling states in dependency of the metabolic fluxes, network topology, and substrate labeling. In case of INST $^{13}$C-MFA, this results in a large differential equation system. Manual derivation of these model equations is a tedious and time-intensive process and trades under the term *kinetic flux profiling* (Yuan et al. 2008). For instance, application of kinetic profiling to describe infection of mammalian cells by human cytomegalovirus results in 69 differential equations (DE) for the central metabolic pathways (Munger et al. 2008). This surprisingly low number of DEs is due to the fact that only measured labeling patterns are considered in the modeling process which results in minimalistic systems to be solved.

Manual encoding of so many DEs, however, would lead to a high risk of typing errors. With each additional or changed reaction, tens of DEs are to be modified. This is, at the end, the limiting factor for systematic investigations with whole model ensembles or advanced optimal experimental design studies (Nöh et al. 2006; Nöh and Wiechert 2006; Möllney et al. 1999). Hence, a tool is needed to automatically generate all necessary equations from minimal information specified by the user. Several methods based on textual input formats (Pitkänen et al. 2008; Quek et al. 2009; Wiechert et al. 2001) or mapping matrices (Schmidt et al. 1997) have been proposed. Utilization of these tools for (INST) $^{13}$C-MFA leads to large nonlinear (differential) equation systems for all, theoretically possible labeled species, i.e., *isotopomers*. The systems dimension for central metabolism networks may have a dimension of 500–1,000 single equations. The numerical solution of these balance equations in the course of an

iterative parameter fitting procedure is a non-trivial computational task.

A modeling approach that is well suited especially in case of MS and positional measurement paradigms is the EMU approach (Antoniewicz et al. 2007; Young et al. 2008). Its application can substantially reduce the number of necessary equations by eliminating unnecessarily detailed information contained in the classical isotopomer fractions (Antoniewicz et al. 2007; Wiechert et al. 1999). The basic principle is, similar to kinetic flux profiling, to consider only those labeled species required to simulate the observed labeling patterns. The same concept is also applicable vice versa: only those isotopic forms have to be balanced that are reached by any substrate labeling. The joint application of both reduction approaches defines a set of "essential unknowns" the mathematical model has (Weitzel et al. 2007). These automated techniques pave the way for high-throughput evaluation of ILEs and even envision genome-scale $^{13}$C-MFA in the stationary case. The rigorous utilization and adaption for the non-stationary case is needed to provide informative insights in, e.g., lipid, penicillin, or cholesterol synthesis pathways (Kleijn 2007; Maier et al. 2009; Zamboni 2011).

### Evaluation of ILEs

Based on a computer representation of the underlying mathematical model, general, but also specialized algorithms can by applied for simulation, parameter fitting, statistical analysis, and experimental design. These algorithms are introduced and discussed in other papers (Antoniewicz et al. 2006; Nöh et al. 2006; Nöh and Wiechert 2006; Suthers et al. 2010; Yang et al. 2008) and will not be reviewed here in detail. Summarizing, the computational framework for INST $^{13}$C-MFA is settled and has been proven to work properly for several examples (cf. Table 1). However, at the present state, these tools are not publicly accessible. Reasons may be that they would require access to high performance workstations and computer clusters or are in a rather prototypical state and, thus, can be operated by only a handful of experts. In case of the classical stationary approach, some powerful tools are available (Cvijovic et al. 2010; Quek et al. 2009; Wiechert et al. 2001; Zamboni et al. 2005). For its non-stationary companion, this will certainly change in the next years.

## Time constants of isotope-labeling systems

The dynamics of labeling patterns in central carbon metabolism can be experimentally observed with a high coverage and temporal resolution (Hasunuma et al. 2010; Hofmann et al. 2008; Munger et al. 2008; Noack et al. 2010; Nöh et al. 2007; Schaub et al. 2008). This offers the direct evidence of the magnitude of time constants and stationarity times in metabolic systems. In particular, the INST $^{13}$C-MFA method offers the potential to verify or disprove the inherent assumption of stationary $^{13}$C-MFA that the isotopic labeling state is equilibrated.

### The diversity of stationarity times

Although the absolute stationarity time may be quite different between different experiments and organisms, it is commonly accepted that the labeling state of central metabolic intermediates becomes saturated considerably much faster compared to free and proteinogenic amino acids. More interestingly, in several studies (Hasunuma et al. 2010; Hofmann et al. 2008; Maier et al. 2008; Munger et al. 2008; Noack et al. 2010; Nöh et al. 2007; Schaub et al. 2008), it was consistently observed for microorganisms, plants, and mammalians that the "upper" metabolic pathways, i.e., glycolysis and PPP, reach a quasi-stationary state much faster than the tricarboxylic acid (TCA) cycle and its associated biosynthesis pathways. For instance, in *C. glutamicum* stationarity time ranges from 30 to 200 s in the glycolysis, whereas the values observed for the TCA cycle are one order of magnitude higher (Noack et al. 2010). In free amino acids, a wide range of stationarity times between 1,000 and 3,000 s was found. An even stronger separation of time scales was observed in Hasunuma et al. (2010). Table 1 gives a condensed overview.

Since the fluxes in glycolysis and TCA cycle are typically in the same order of magnitude, the time constants of the overall isotopic labeling enrichment are predominately determined by the pool sizes (Wiechert and Nöh 2005) (cf. Fig. 6). Here, the time constant of a metabolite pool can be estimated by the ratio between the total incoming flux to the pool and the pool size, equivalent to the inverse turnover rate (cf. Eq. 3).

### The pitfall of incomplete networks

Interestingly, even if in the course of a parameter fitting process, pool sizes were allowed to vary over wide ranges, the labeling data for some pools could still not be fully explained. In each case, the observed labeling dynamics were slower than the predicted one (Munger et al. 2008; Nöh et al. 2007; van Winden et al. 2005). This indicates that unlabeled material may be fed back to the central metabolic pathways from biomass compounds, storage metabolism, or lipid synthesis. The origin of this labeled material may be protein turnover feeding unlabeled amino acids back into the system, DNA/RNA turnover influencing the pentose-5-phosphate pools, or storage carbohydrates

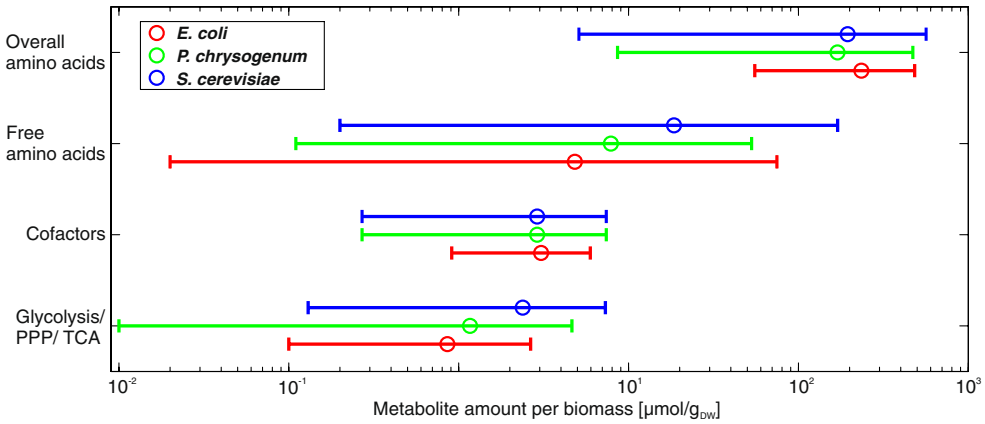


**Fig. 6** Pool sizes of intracellular metabolites in respect to cell dry weight correlated for different classes of metabolites. The minimal, maximal, and average values are plotted for each class. Data were taken from Heijnen (2010), Lange and Heijnen (2001), Nielsen (1997), and Pramanik and Keasling (1998)

like trehalose or glycogen in *Saccharomyces cerevisiae* (cf. Fig. 6) (Aboka et al. 2009; Nöh et al. 2007; van Winden et al. 2005). These are, however, hypotheses originating from observed data that still have to be validated experimentally. As mentioned before, a comprehensive quantification of the metabolites' pool sizes besides their labeling patterns within all pathways of interest is a mandatory prerequisite for reliable flux estimation. By integrating observed data over the whole labeling time span, INST $^{13}$C-MFA provides the opportunity to consistently estimate lower and upper bounds for storage pool sizes and the "diluting" fluxes feeding unlabeled material back to the central metabolism.

### Coupling transient and stationary phases

In Noack et al. (2010), the isotopic non-stationary and stationary methods are independently applied using data from one single experiment. Remarkably, resulting flux maps are not consistent. A closer investigation unveils that the flux distribution found by the stationary method lacks a viable phenotype. The unrealistic flux solution, however, can be avoided by integration of additional constraints shifting the solution in a physiologically more reasonable direction. In contrast, the non-stationary approach gives reasonable fluxes from the measurements. In a different approach, data from the stationary and the (end of the) non-stationary labeling regime are coincidentally evaluated and the result validated by data from some proteinogenic amino acids (Schaub et al. 2008). The resulting flux map is found to be in good agreement with the measurements.

The lessons learned show that quite different time scales are in fact present, and integrating more data gives more confidence to the final flux solution. This opens an option to couple the stationary and non-stationary approach and enforce a time scale separation: if it is possible to use knowledge of time constants to reveal "faster" and "slower" parts of the network these network parts may be separated. For the "faster" metabolic pathways, it may be feasible to justify the stationary approach, whereas for the "slower" ones, the non-stationary method is applied. For instance, the study of Maier et al. (2009) showed that glycolytic and PPP fluxes can be estimated with the stationary approach whereas cholesterol pathway fluxes were inferred with the non-stationary approach. Clearly, such approximate approaches strongly depend on the coupling of the rapidly metabolizing pathways to storage pools.

## Beyond INST $^{13}$C-MFA

Rapid sampling and quenching devices have not only been used for INST $^{13}$C-MFA but are originally developed to generate data for the estimation of kinetic parameters in mechanistic network models. In contrast to INST $^{13}$C-MFA, stimulus response experiments aim to perturb the cells' metabolism by introducing a sudden environmental change to deflect metabolism from its steady state (Oldiges and Takors 2005; Theobald et al. 1997; Visser et al. 2002). This leads to a rapid change of the intracellular metabolite concentrations which become observable in a subsecond scale. Mechanistic models are then set up for the enzymatic reactions, aiming to describe the dynamics. These models are traditionally based on quantitative rate laws or approximating kinetic formalisms. The derived models obtain quite a huge number of unknown parameters (up to 9 per reaction) that have to be estimated from the measured data. This poses a challenge on data quantity and quality. As it is recently reported, even large amounts of data from multiple experiments are not sufficient to estimate all kinetic parameters with reasonable statistical confidence (Wahl et al. 2006; Nikerel et al. 2006).

This is the offspring of a new idea which combines stimulus response experiments with ILEs. From an exper-

imental viewpoint, the realization is straightforward and, in principle, does not need any further equipment. However, the question is whether the additional consideration of labeling data really can help to improve the estimation of kinetic parameters. This question is addressed in Wahl et al. (2008), where an additional gain of information could be shown. However, it also turned out that without good knowledge of measurement precisions, the information surplus is not as high as expected. These results are one reason why the tremendous effort of doing a stimulus response experiment with measuring labeling patterns has still not been undertaken. Due to the encouraging theoretical results, it makes sense to start with the investigation of single metabolic paths like, for example, a biosynthesis pathway or lipid synthesis in order to bring the method into practice.

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