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Methods and advances in metabolic flux analysis: a mini-review

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Abstract Metabolic flux analysis (MFA) is one of the pillars of metabolic engineering. Over the past three decades, it has been widely used to quantify intracellular metabolic fluxes in both native (wild type) and engineered biological systems. Through MFA, changes in metabolic pathway fluxes are quantified that result from genetic and/or environmental interventions. This information, in turn, provides insights into the regulation of metabolic pathways and may suggest new targets for further metabolic engineering of the strains. In this mini-review, we discuss and classify the various methods of MFA that have been developed, which include stoichiometric MFA, ^{13}C metabolic flux analysis, isotopic non-stationary ^{13}C metabolic flux analysis, dynamic metabolic flux analysis, and ^{13}C dynamic metabolic flux analysis. For each method, we discuss key advantages and limitations and conclude by highlighting important recent advances in flux analysis approaches.

Keywords Metabolism · Isotopic labeling · Metabolic network model · Flux quantification

Abbreviations

MFA	Metabolic flux analysis
DMFA	Dynamic metabolic flux analysis
^{13}C-MFA	^{13}C-metabolic flux analysis
^{13}C-DMFA	^{13}C dynamic metabolic flux analysis
^{13}C-NMFA	Isotopic non-stationary ^{13}C-metabolic flux analysis
SSR	Variance-weighted sum of squared residuals

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Introduction

Metabolic flux analysis (MFA) has been a cornerstone of metabolic engineering since the inception of the field [70, 75]. In the past three decades, various flux analysis techniques have been developed and applied to elucidate intracellular metabolism in a wide range of biological systems, ranging from simple microbes such as *Escherichia coli* [44] to more complex eukaryotic systems such as *Saccharomyces cerevisiae* [49], as wells as plants [32, 37, 46, 54, 67], mammalian cells [29, 50, 63, 89], thermophiles [71, 74], obligate anaerobes [13], and other cellular systems [1, 53, 88, 94]. MFA studies were initially based on balancing fluxes around intracellular metabolites within an assumed network stoichiometry, the so-called stoichiometric MFA [77], where external rate measurements such as glucose uptake rate, growth rate, and CO_2 evolution rate provided constraints to determine intracellular fluxes. In the past decade, however, a number of more advanced MFA techniques have emerged for determining metabolic fluxes with improved accuracy and precision. In this mini-review, we provide an overview of the different MFA methods that have been developed and briefly highlight the advantages and limitations of each technique.

Methods in metabolic flux analysis

Figure 1 shows the classifications of the various MFA methods that have been developed thus far. The three main distinguishing characteristics between the different MFA

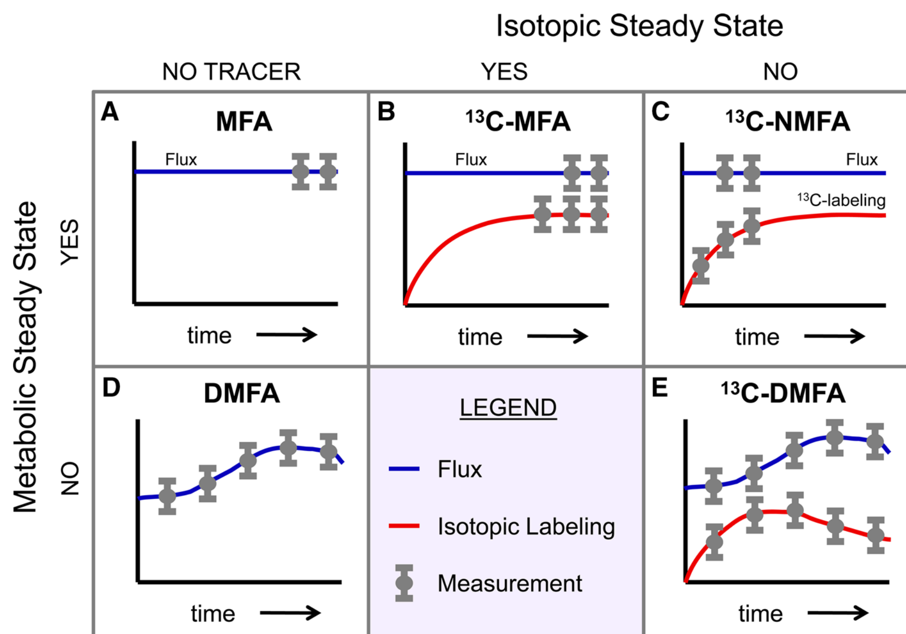

Fig. 1 Classification of different methods for metabolic flux analysis. The main distinguishing characteristics between the different metabolic flux analysis methods are whether stable-isotope tracers (such as ^{13}C) are applied, and whether metabolic steady state is assumed. **a** MFA at metabolic steady state (without isotopic tracers); **b** ^{13}C-MFA at metabolic and isotopic steady state; **c** ^{13}C-NMFA at metabolic steady state and isotopic non-steady state; **d** DMFA at metabolic non-steady state (without isotopic tracers); and **e** ^{13}C-DMFA at metabolic and isotopic non-steady state

methods are (1) whether metabolic steady-state is assumed for the system, or not; (2) whether stable-isotope tracers are applied, or not; and (3) whether isotopic steady-state is assumed for the system, or not.

Flux analysis at metabolic steady-state: MFA

The key to calculating metabolic fluxes in living cells with stationary (or stoichiometric) MFA (Fig. 1a) is to analyze the biological system as an integrated biochemical network model. MFA relies on balancing fluxes around intracellular metabolites within an assumed metabolic network model. The first step in the analysis is to express the biochemical network model as a stoichiometric matrix in which rows represent balanced intracellular metabolites and columns represent metabolic fluxes in the model. The stoichiometric model also includes a “biomass reaction” that describes the drain of precursor metabolites needed for cell growth, which is constructed based on the measured biomass composition [45]. By assuming metabolic (pseudo) steady-state for intracellular metabolites, metabolic fluxes (v) are constrained by the stoichiometry matrix (S):

$$S \times v = 0 \tag{1}$$

To estimate metabolic fluxes, the stoichiometric constraints are complemented with measured external metabolic rates (r), such as growth rate, substrate uptake, and product accumulation rates:

$$R \times v = r \tag{2}$$

The combined system of Eqs. 1 and 2 is then solved by least squares regression:

$$\min \text{SSR} = \sum (r - r_{\text{m}})^2/\sigma_{\text{r}}^2 \tag{3}$$

$$\text{s.t. } R \times v = r$$

$$S \times v = 0$$

Using this approach metabolic fluxes can be estimated in systems that are fully determined (i.e., containing all the necessary external rate measurements), and overdetermined (i.e., containing a redundant set of external rate measurements). The main advantage of MFA is that it is easy to apply and thus accessible to many researchers, since it only requires simple linear algebra and relies on relatively robust measurements of extracellular metabolites [52]. A limitation of MFA for analysis of many biological systems is, however, that the number of constraints (i.e., stoichiometric and rate measurements) is often insufficient to observe all important intracellular metabolic pathways. To make the system fully observable, additional assumptions are needed, for example, leaving out specific pathways that are assumed to carry little or no flux, or including cofactor balances (e.g., NADH, NADPH, and ATP balances) as additional constraints. However, the use of cofactor balances is generally not encouraged. The presence of isoenzymes with alternative cofactor specificities, e.g., NADH- and NADPH-dependent malic enzyme or isocitrate dehydrogenase, and uncertainties regarding transhydrogenase activity and other futile cycles renders cofactor balances often uninformative. In some studies, NADH and NADPH were lumped together [15, 59], and external rates of NH_3, CO_2, and O_2 were used

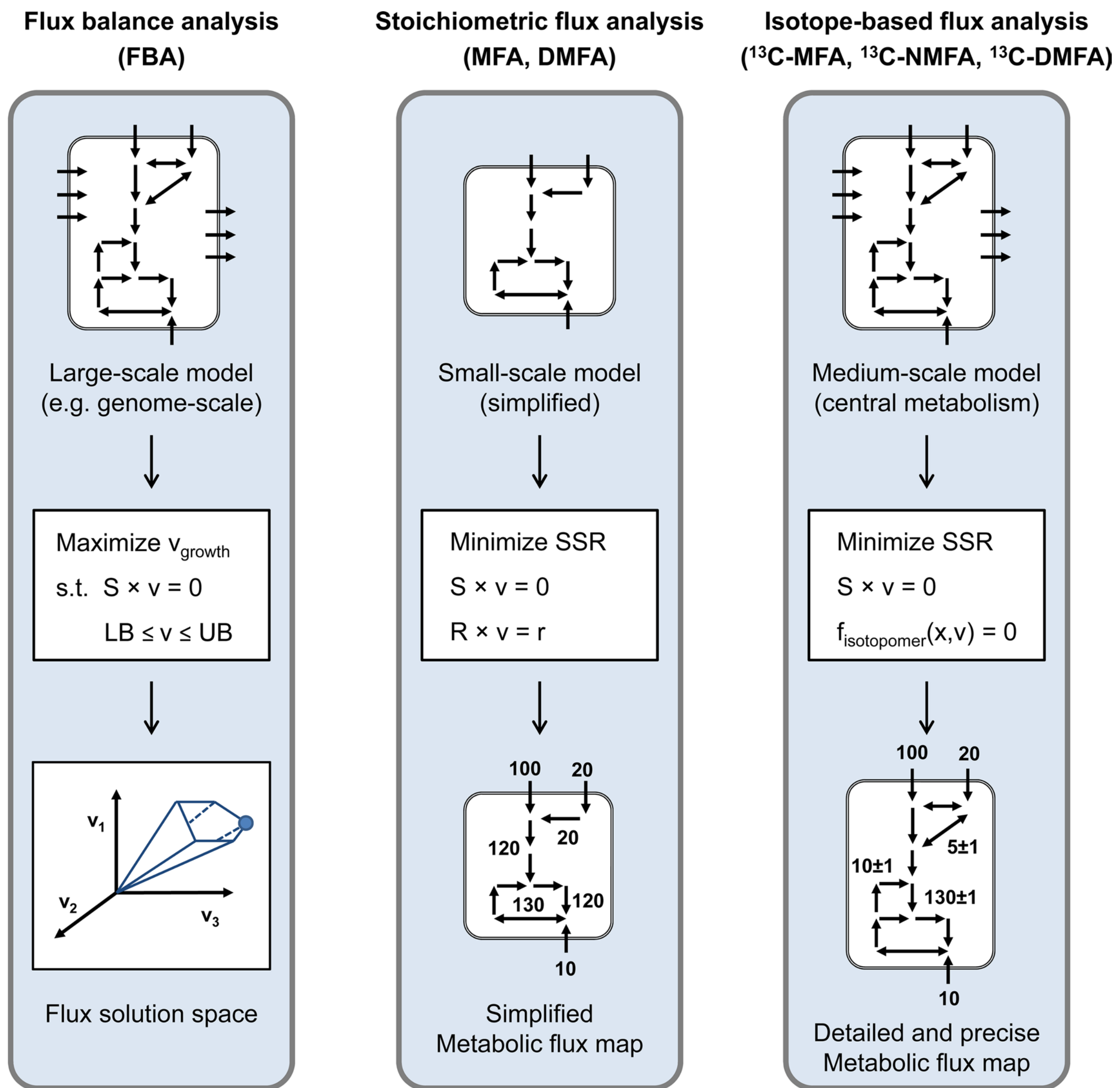

Fig. 2 Three classes of flux analysis approaches: (1) flux balance analysis (FBA) is an optimization-based approach that uses a large-scale model (e.g., genome-scale) and produces a flux solution space that satisfies an assumed cellular objective function (e.g., maximum cell growth). (2) Stoichiometric flux analysis is a set of data-driven approaches that use small-scale network models and external rate measurements to quantify fluxes in the simplified models. (3) Isotope-based flux analysis is a set of advanced data-driven approaches that use medium-scale network models and isotopic labeling measurements (e.g., GC-MS or NMR) to quantify highly precise metabolic fluxes in central carbon metabolism

as additional constraints to make the system observable [59, 96]. Alternatively, flux balance analysis (FBA) can be applied to quantify fluxes in underdetermined systems (Fig. 2) [60]. Here, in addition to applying constraints from measured extracellular rates, inequality constraints such as upper and lower bounds on fluxes are used, and an assumed biological objective is imposed on the model, for example, maximum growth rate or maximum ATP production [27]. In practice, however, FBA returns a large solution space consisting of many flux distributions that can all maximize the assumed cellular objective.

Flux analysis at metabolic and isotopic steady state: ^{13}C-MFA

^{13}C-based metabolic flux analysis (^{13}C-MFA) is a more advanced technique for estimating metabolic fluxes in systems that are at metabolic steady state (Fig. 1b) [82, 93]. This technique makes use of ^{13}C-labeled tracers, combined with isotopomer balancing, metabolite balancing, and isotopic labeling measurements through techniques such as NMR [48, 72], mass spectrometry [9, 11, 12, 19, 26, 30, 35], and tandem mass spectrometry [7, 17, 18, 31, 34], to estimate fluxes. In a ^{13}C-MFA study, cells are cultured for

an extended period of time (typically >3 h) in the presence of a specifically labeled ^{13}C-tracer, e.g., [1,2-^{13}C]glucose [28], which results in the incorporation of ^{13}C-atoms into metabolic intermediates and metabolic products. If a proper selection of ^{13}C-tracer is made, then the measured ^{13}C-labeling distributions will be highly dependent on the relative values of intracellular metabolic fluxes [5]. Therefore, these ^{13}C-labeling measurements can be used as additional constraints to estimate fluxes. In ^{13}C-MFA, the following non-linear least squares regression problem is solved:

$$\min \text{SSR} = \sum (x - x_{\text{m}})^2/\sigma_x^2 + \sum (r - r_{\text{m}})^2/\sigma_{\text{r}}^2 \quad (4)$$

$$\text{s.t. } f_{\text{isotopomer model}}(v, x) = 0$$

$$R \times v = r$$

$$S \times v = 0$$

The objective of ^{13}C-MFA is therefore to find a set of feasible intracellular metabolic fluxes that minimizes the variance-weighted sum of squared residuals (SSR) between the measured and predicted ^{13}C-labeling measurements (x) and extracellular rate measurements (r). In ^{13}C-MFA, both metabolic steady state and isotopic steady state are assumed; that is, metabolic fluxes and isotopic labeling are assumed to be constant in time (Fig. 1b). The time needed to reach isotopic steady state in a system will depend on several factors, including relative metabolic activity of cells; which metabolite pools were measured; which substrate was used as the tracer; and the composition of the medium. For example, in mammalian cells, glycolytic metabolites may reach isotopic steady state within 3 h following the introduction of ^{13}C-labeled glucose, but TCA cycle metabolites may require >24 h to approach isotopic steady state [2, 47, 66, 92]. On the other hand, for [U-^{13}C] glutamine, TCA cycle metabolites typically reach isotopic steady state within 3 h, but it may take longer for glycolytic metabolites to approach isotopic steady state [4].

The main advantage of ^{13}C-MFA for quantifying fluxes is the fact that a large number of redundant measurements can be obtained for flux estimation. For example, using GC–MS one can easily obtain more than 50 mass isotopomer measurements to estimate on the order of ~5–20 unknown net and exchange fluxes in a model. The large number of redundant measurements greatly improves the accuracy and precision of estimated fluxes (Fig. 2). As such, the level of confidence in the flux results is much greater compared to stoichiometric MFA. Furthermore, significantly more complex metabolic network models can be investigated with ^{13}C-MFA. For example, it is possible to estimate parallel metabolic pathways (e.g., pentose phosphate pathway vs. glycolysis vs. Entner–Doudoroff pathway), cyclic pathways (e.g., pyruvate cycling between compartments), and bidirectional reversible fluxes [16, 65, 82]. Other important applications of ^{13}C-MFA include validation of metabolic network models and elucidation of reaction stereochemistries [14, 25, 39].

While ^{13}C-MFA is certainly more powerful for estimating in vivo metabolic fluxes than stoichiometric MFA, it is also more resource intensive, both experimentally and computationally. Solving large sets of non-linear isotopomer balances is not trivial, and the non-linear nature of the least squares regression problem requires iterative algorithms. In the past decade, several mathematical approaches have been developed to reduce the computational burden of ^{13}C-MFA. The first modeling framework for simulating intracellular ^{13}C-labeling was proposed by Zupke and Stephanopoulos based on atom mapping matrices [95]. In subsequent years, improved modeling approaches were introduced based on isotopomer balancing [64], cumomer balancing [83], bondomer balancing [69, 76], and most recently, elementary metabolite unit (EMU) balancing [10]. Currently, the EMU modeling approach is considered the most advanced and computationally most efficient method for simulating isotopic labeling distributions in metabolic network models. For example, it was shown that EMU simulations are several orders of magnitude more efficient than equivalent isotopomer and cumomer simulations without any loss of information [10]. For statistical analysis of flux results, confidence intervals of fluxes must be calculated using advanced statistical analysis methods that consider the inherent non-linearities in the isotopomer model [8], or alternatively, using computationally intensive Monte Carlo simulations [86]. Several powerful software packages have been developed in the past decade for ^{13}C-MFA based on the EMU modeling framework, including Metran [87], OpenFlux [62], INCA [90], and 13CFLUX2 [81].

Flux analysis at isotopic non-steady state: ^{13}C-NMFA

The assumption of isotopic steady state places limitations on the use of ^{13}C-MFA for analyzing the metabolism of certain biological systems. For example, in autotrophic systems, isotopic steady-state labeling measurements provide no flux information [91], while in mammalian cell cultures, the time required to reach isotopic steady state can be on the order of hours or even days [47, 51, 66, 92]. To address these issues, a new methodology termed isotopic non-stationary ^{13}C-MFA (^{13}C-NMFA) was developed (Fig. 1c) [84]. In ^{13}C-NMFA, metabolic fluxes are estimated at metabolic (pseudo) steady state, i.e., still assuming constant metabolic fluxes and constant metabolite pool sizes for intracellular metabolites, but allowing transient ^{13}C-labeling data [$x(t)$] and pool size measurements (C) to be used for flux quantification [85]:

$$\min \mathrm{SSR} = \sum (x(t) - x_{\mathrm{m}}(t))^2/\sigma_x^2 + \sum (r - r_{\mathrm{m}})^2/\sigma_{\mathrm{r}}^2 + \sum (C - C_{\mathrm{m}})^2/\sigma_{\mathrm{C}}^2 \quad (5)$$

$$\text{s.t. } C \times \mathrm{d}x/\mathrm{d}t = f_{\text{isotopomer model}}(v, x(t))$$

$$R \times v = r$$

$$S \times v = 0$$

As part of the parameter estimation procedure, ordinary differential equations (ODE) of isotopomer balances are numerically integrated to simulate isotopomer distributions as a function of time. The non-linear least squares regression techniques employed for parameter estimation in ^{13}C-NMFA are similar to the techniques used in ^{13}C-MFA. However, in addition to estimating metabolic fluxes, metabolite pool sizes are fitted in ^{13}C-NMFA to account for the observed labeling transients. The computational time for ^{13}C-NMFA is significantly higher than for ^{13}C-MFA. Fortunately, application of the EMU modeling framework has reduced the computational time by several orders of magnitude to less than an hour for a typical metabolic network model encompassing all of central carbon metabolism [90, 92].

Flux analysis at metabolic non-steady state: DMFA

All of the analysis methods described above rely on metabolic steady-state assumption, i.e. time-invariant metabolic fluxes. In recent years, several new techniques for so-called dynamic metabolic flux analysis (DMFA) have been developed [3, 41, 43, 56], which all focus on determining temporal transients in metabolic fluxes from time-course data (Fig. 1d). The objective of DMFA is to determine metabolic shifts in systems that are not at metabolic steady state from time-series of extracellular concentration [$c(t)$] and rate measurements [$r(t)$]:

$$\min \mathrm{SSR} = \sum (c(t) - c_m(t))^2/\sigma_c^2 + \sum (r(t) - r_m(t))^2/\sigma_r^2 \quad (6)$$

$$\text{s.t. } R \times v(t) = \mathrm{d}c/\mathrm{d}t$$

$$S \times v(t) = 0$$

DMFA methods generally assume that flux transients in cell culture are relatively slow, typically on the order of hours, compared to the time required to reach metabolic (pseudo) steady state for intracellular metabolism, which is typically on the order of seconds to minutes. With this assumption, DMFA analysis can be accomplished through the following three steps: (1) the experiment is divided into discrete time intervals; (2) average external rates are calculated for each time interval by taking derivatives of external concentration measurements; and (3) average fluxes are calculated for each time interval using classical MFA [6]. The results of these steady-state models, evaluated at multiple time points, are then combined to obtain a time profile of flux transients during the culture. An alternative to the first step is to apply data smoothing on extracellular measurements using splines [56], linear [61], or polynomial fitting [41], and then take derivatives of the smoothed data to increase temporal resolution of the estimated flux dynamics [56].

Recently, a new and improved approach for DMFA was introduced that directly fits the complete time-series data to quantify flux dynamics for the entire culture in a single step without the need for data pre-processing [38]. An advantage of this method is that it does not depend on manually selecting time intervals to describe flux transients. Instead, rigorous statistical criteria are used to automatically detect the level of dynamic information present in the data [6]. The key advantage of DMFA compared to stationary MFA is that it provides information on metabolic transient, which cannot be observed using classical MFA, with only modest additional experimental and computational effort. However, since DMFA is still based on stoichiometric metabolite balancing within an assumed (and typically simplified) metabolic model, DMFA carries the same limitations as MFA for resolving parallel pathways, cyclic pathways, and reversible reactions.

Flux analysis at metabolic and isotopic non-steady state: ^{13}C-DMFA

The logical extension to DMFA is to incorporate isotope labeling measurements to allow for the estimation of fluxes of cyclic pathways, parallel pathways, and reversible reactions. Methods for ^{13}C-DMFA (Fig. 1e), however, are still in their infancy and there is a clear need for further research and development in this area to make such methods more widely accessible and less cumbersome to implement. An illustrative example of ^{13}C-DMFA is the analysis of a fed-batch fermentation of *E. coli* overproducing 1,3-propanediol [12]. In that study, metabolic fluxes were determined at 20 time points during an *E. coli* fed-batch culture from analysis of ^{13}C-labeling dynamics of proteinogenic amino acids. To account for the transients in isotopic labeling of the substrate and proteinogenic amino acids two dilution parameters were introduced, termed D- and G-parameters, in analogy with the isotopomer spectral analysis (ISA) modeling framework [33]. Using this approach, detailed time-resolved metabolic flux maps were constructed and metabolic shifts could be identified during the fed-batch fermentation [12].

Recent advances in metabolic flux analysis

An important recent advance in metabolic flux analysis is the use of parallel labeling experiments combined with rigorous data integration to estimate highly precise metabolic fluxes in complex systems [22]. This methodology was recently termed COMPLETE-MFA [40], short for complementary parallel labeling experiments technique for metabolic flux analysis. Here, instead of performing a single ^{13}C-tracer experiment, as is the case in ^{13}C-MFA, multiple tracer experiments are performed in parallel. Different isotopic tracers are used in each parallel culture. Labeling data from all experiments are then combined and rigorously integrated to estimate a single flux map that describes all experiments. The advantage of COMPLETE-MFA is that it produces flux results that are significantly more precise than can be obtained with ^{13}C-MFA [40]. To date, the most extensive parallel labeling study performed has consisted of integrated analysis of 14 parallel labeling experiments in *E. coli* [24]. The COMPLETE-MFA methodology is currently viewed as the gold standard in ^{13}C-based flux estimation.

Conclusions

Metabolic flux analysis has proven to be a valuable tool in metabolic engineering. ^{13}C-MFA is now well-established and widely used for quantifying fluxes in many prokaryotic systems [23, 36, 73, 78]. However, ^{13}C-MFA of eukaryotic systems still remains challenging due to difficulties resulting from compartmentation of metabolites and metabolic reactions [68, 79]. In mammalian systems, analysis is further complicated by difficulties in maintaining metabolic steady state for extended periods of time and by the slow labeling dynamics. A key factor contributing to slow labeling dynamics in mammalian systems is the exchange of intra- and extracellular metabolites such as lactate and amino acids from the complex media [3]. Recently, detailed metabolic flux maps were established for CHO cells using both isotopic stationary ^{13}C-MFA and isotopic non-stationary ^{13}C-NMFA [2, 4]. Key advantages of ^{13}C-NMFA include shorter labeling times and reduced usage of expensive ^{13}C-tracers [57, 84]. However, ^{13}C-NMFA requires multiple measurements, i.e. time course of ^{13}C-labeling, and more advanced algorithms for parameter fitting and statistical analysis. Fortunately, dedicated software tools for ^{13}C-NMFA have recently become available and this is expected to accelerate the use of ^{13}C-NMFA as a powerful tool for flux analysis [58, 90].

Difficulties in flux analysis related to compartmentalization have not been properly addressed. Currently, there are no well-established methods to estimate compartment-specific fluxes in eukaryotic systems, and it is still an open question whether ^{13}C tracers provide the best strategy to resolve questions related to cellular compartmentation [42]. Without reliable extraction techniques for isolating compartment-specific metabolite pools and ^{13}C-labeling, it may be difficult to elucidate parallel pathways in multiple compartments using current methodologies [55, 79]. Novel computational approaches are also needed for optimal tracer experiment design [5, 20, 21, 80], and new analytical techniques to complement current data for determining fluxes in compartmentalized network models. Finally, improved techniques are needed to acquire dynamic flux maps. Several methods for DMFA have already been developed that model dynamic behavior and metabolic shifts in cell cultures based on analysis of dynamic extracellular metabolite data. To fully resolve intracellular metabolic flux maps under dynamically changing culture conditions, however, new ^{13}C-DMFA methods will be needed to fully integrate dynamic metabolite concentrations and ^{13}C-labeling data.

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Conflict of interest The authors declare that they have no conflict of interest.

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