Published in final edited form as:

Metab Eng. 2012 March ; 14(2): 150–161. doi:10.1016/j.ymben.2011.12.005.

Selection of Tracers for ^{13}C -Metabolic Flux Analysis using Elementary Metabolite Units (EMU) Basis Vector Methodology

Scott B. Crown and Maciek R. Antoniewicz*

Department of Chemical Engineering, Metabolic Engineering and Systems Biology Laboratory, University of Delaware, Newark DE 19716, USA

Abstract

Metabolic flux analysis (MFA) is a powerful technique for elucidating *in vivo* fluxes in microbial and mammalian systems. A key step in ^{13}C -MFA is the selection of an appropriate isotopic tracer to observe fluxes in a proposed network model. Despite the importance of MFA in metabolic engineering and beyond, current approaches for tracer experiment design are still largely based on trial-and-error. The lack of a rational methodology for selecting isotopic tracers prevents MFA from achieving its full potential. Here, we introduce a new technique for tracer experiment design based on the concept of elementary metabolite unit (EMU) basis vectors. We demonstrate that any metabolite in a network model can be expressed as a linear combination of so-called EMU basis vectors, where the corresponding coefficients indicate the fractional contribution of the EMU basis vector to the product metabolite. The strength of this approach is the decoupling of substrate labeling, i.e. the EMU basis vectors, from the dependence on free fluxes, i.e. the coefficients. In this work, we demonstrate that flux observability inherently depends on the number of independent EMU basis vectors and the sensitivities of coefficients with respect to free fluxes. Specifically, the number of independent EMU basis vectors places hard limits on how many free fluxes can be determined in a model. This constraint is used as a guide for selecting feasible substrate labeling. In three example models, we demonstrate that by maximizing the number of independent EMU basis vectors the observability of a system is improved. Inspection of sensitivities of coefficients with respect to free fluxes provides additional constraints for proper selection of tracers. The present contribution provides a fresh perspective on an important topic in metabolic engineering, and gives practical guidelines and design principles for *a priori* selection of isotopic tracers for ^{13}C -MFA studies.

Keywords

Metabolic fluxes; isotopomers; isotopic tracers; metabolic network model; statistical analysis; flux observability

*corresponding author: Maciek R. Antoniewicz, Department of Chemical Engineering, University of Delaware, 150 Academy St, Newark, DE 19716, Tel.: 302-831-8960, Fax.: 302-831-1048, mranton@udel.edu.

1. INTRODUCTION

Metabolic fluxes are important parameters in elucidating cell physiology, whether for the reengineering of a cellular phenotype or evaluating the mechanisms of human disease (Kelleher, 2004; Moxley et al., 2009; Stephanopoulos, 1999). Currently, the use of stable-isotope (e.g. ^{13}C) tracers combined with measurements of isotopic labeling represents the state-of-the-art in flux determination (Sauer, 2006; Zamboni et al., 2009). After intense research and development over the past two decades, ^{13}C -based metabolic flux analysis (MFA) has reached a level of relative maturity and is widely used to probe fluxes in microbial and mammalian systems (Ahn and Antoniewicz, 2011; Leighty and Antoniewicz, 2011). The reconstruction of a comprehensive and accurate flux map depends largely on selecting an appropriate ^{13}C -tracer and obtaining as much information as possible about the amount and distribution of labeled atoms in metabolic products (Chang et al., 2008; Crown et al., 2011; Metallo et al., 2009). Given the importance of ^{13}C -MFA in metabolic engineering and beyond (Reed et al., 2010), it is surprising however that there are still no fundamental techniques for rational selection of tracers for ^{13}C -MFA studies.

In tracer experiments, metabolic conversion of labeled substrates generates molecules with distinct labeling patterns (i.e. isotopomers) that can be measured by nuclear magnetic resonance (NMR) (Szyperski, 1995), mass spectrometry (MS) (Antoniewicz et al., 2007a; Antoniewicz et al., 2011; Wittmann, 2002), or tandem mass spectrometry (MS/MS) (Choi and Antoniewicz, 2011; Jeffrey et al., 2002). Isotopic enrichments of metabolites are strongly dependent on the specific ^{13}C -labeling pattern of the substrates and network fluxes. Efficient computational methods have been developed for relating metabolite labeling patterns to network fluxes (Antoniewicz et al., 2007b). These mathematical models allow simulation of isotopic enrichments for all metabolites in a network for steady-state flux conditions. In ^{13}C -MFA the inverse problem is solved, namely fluxes are determined from isotopomer data using a least-squares parameter estimation approach (Antoniewicz et al., 2006). Implicit to the application of MFA is the need to determine not only flux values, but also confidence intervals of fluxes. Due to the inherent complexity of metabolic networks, however, it is not always possible to resolve all fluxes in a model with high fidelity (Metallo et al., 2009). To improve the resolution of metabolic fluxes, the researcher has influence over two key factors:

- 1) *Experimental measurements*: the choice of which external rates, metabolite pools, and intracellular metabolites are measured, and the specific isotopomer measurement technique to be used, e.g. NMR, MS, or MS/MS.
- 2) *Substrate labeling*: the choice of which substrate(s) are ^{13}C -labeled, and the specific labeling patterns of the substrates, e.g. $[1,2-^{13}\text{C}]$ glucose, $[\text{U}-^{13}\text{C}]$ glucose, etc.

Increasing the number of redundant measurements and improving the quality of measurements results in narrower confidence intervals of fluxes (Antoniewicz et al., 2006). Ultimately however, improving measurement precision can only improve flux observability of a network model to a limited extent. Thus, even if the measurements are highly accurate and precise, if a poor choice is made for the substrate labeling, certain fluxes may never be

observable (Metallo et al., 2009). As such, the network substrate plays a key role in flux observability. The choice of the tracer determines, for example, which isotopomers of metabolites can be formed in a network. In addition, the tracer selection influences the sensitivity of isotopomer measurements to changes in fluxes. Hence, when designing tracer experiments, careful thought should be applied not only to the measurements to be taken, but also to the choice of substrate labeling.

The goal of this work is to provide a new, rational framework for optimal tracer selection for ^{13}C -MFA, given a set of isotopic measurements. Certain efforts have been made to systematically select tracers for ^{13}C -MFA. Mollney et al. (Mollney et al., 1999) evaluated various glucose tracers for analysis of *C. glutamicum* metabolism using simulated NMR and GC-MS measurements, and selected the optimal glucose mixture based on a D-optimality design criterion. Similarly, Arauzo-Bravo et al. (Arauzo-Bravo and Shimizu, 2003) selected glucose tracers for use in *Synechocystis* sp. PCC6803 and Yang and Heinzle (Yang et al., 2006) designed a glucose tracer mixture for quantifying *C. glutamicum* metabolism with CO_2 as the sole experimental measurement. Metallo et al. (Metallo et al., 2009) evaluated a wide selection of glucose and glutamine tracers for the metabolism of a cancer cell line by examining the resulting nonlinear flux confidence intervals. Metallo identified optimal tracers for subnetworks of metabolism (i.e. glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle) and determined that $[1,2-^{13}\text{C}]$ glucose was the best tracer for the overall network.

Despite these exhaustive efforts for specific organisms, each of these studies relied on an experimentally determined flux map as a starting reference point for the selection of tracers. This raises the question: given a set of isotopic measurements, does the choice of the optimal tracer depend on the specific reference flux map? In other words, for a given network with an unknown flux map, is it possible to identify optimal tracers to elucidate fluxes in the model? Furthermore, in the case where there is not an optimal tracer, what flux information can be obtained from an experiment for a chosen substrate labeling? In an attempt to address these questions and move beyond empirical and largely trial-and-error based tracer selection procedure we developed a new methodology based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007b). The EMU method was originally developed as a technique to simplify isotopomer calculations by determining the minimal amount of labeling information required to simulate isotopomers of metabolites. In this study, we evaluated if the EMU methodology could also be applied for rational design of tracers. Here, we demonstrate that EMU decomposition of a metabolic network model allows all metabolites in the model to be expressed as a linear combination of so-called EMU basis vectors, regardless of the complexity of the system. By expressing isotopomer measurements as a linear combination of substrate EMUs we established a new framework for evaluating the information content of isotopic measurements and provide rational design principles for optimal selection of tracers for ^{13}C -MFA studies.

2. METHODS

2.1. Nomenclature

The tracer experiment design framework presented here is built using mass isotopomer distributions (MIDs) of EMUs as state variables (Antoniewicz et al., 2007b). An EMU is defined as a specific subset of a metabolite's atoms. EMU size is defined as the number of atoms comprising a particular EMU. We use a subscript notation to denote atoms present in an EMU. For example, A_{234} indicates that the EMU is comprised of atoms 2, 3, and 4 of metabolite A. Convolution of EMUs is denoted by " $\times$ ", for example $A_{12} \times A_3$. We use a superscript notation to indicate convolutions of the same EMUs, for example $A_{12} \times A_{12} = (A_{12})^2$. Furthermore, a subscript notation (with ones and zeros) is used to denote the labeling patterns of isotopomers. For example, A_{1100} indicates that metabolite A has four atoms and that atoms 1 and 2 are labeled and atoms 3 and 4 are unlabeled. A MID is a vector that contains the fractional abundances of each mass isotopomer of an EMU, i.e. $[M+0, M+1, \dots, M+n]$ for an EMU of size n .

2.2. EMU decomposition

EMU decomposition of the metabolic network models was accomplished using Metran software (Yoo et al., 2008). The resulting EMU networks were decoupled into separate and smaller sub-networks using the technique described by Young et al. (Young et al., 2008), and simplified using the technique described by Antoniewicz et al. (Antoniewicz et al., 2007b). Algebraic solutions to EMU models were derived using Maple 14 (Waterloo Maple Inc.). Sensitivities of EMU basis vector coefficients to free fluxes were calculated using finite differences.

2.3. Metabolic flux analysis

Metabolic flux analyses were performed using Metran software (Yoo et al., 2008), which is built on the EMU framework. In short, fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the simulated and model-predicted MIDs using non-linear least-squares regression (Antoniewicz et al., 2006). In all cases, flux estimation was repeated at least ten times starting with random initial values for all fluxes to find a global solution. At convergence, standard deviations and 95% confidence intervals for all fluxes were calculated using the parameter continuation technique described by Antoniewicz et al. (Antoniewicz et al., 2006). This technique is based on evaluating the profile of SSR as a function of one flux, while the values for the remaining fluxes are optimized. The 95% confidence interval for an evaluated flux corresponds to flux values for which SSR increased by less than 3.84 (Antoniewicz et al., 2006). A flux was considered non-observable if the 95% confidence interval was larger than the estimated flux value.

3. RESULTS AND DISCUSSION

3.1. A simple motivating example

Selection of isotopic tracers for ^{13}C -MFA is not a trivial task, even in simple systems. Consider, for example, the network model shown in Fig. 1. The stoichiometry and atom transitions are given in Table 1. In this system, metabolite A is the substrate, metabolite E is

the product, and the intermediate metabolites B, C and D are assumed to be at metabolic and isotopic steady state. This network has two free fluxes, namely the substrate consumption rate v_1 and the flux of the reversible cleavage/condensation reaction v_4 . We would like to determine flux v_4 based solely on the measurement of the MID of E and a known uptake rate of A. Three key questions that we would like to answer are: (1) which labeling pattern(s) of A can be used to resolve flux v_4 ? (2) Are there certain tracers that will never allow flux v_4 to be observed? (3) Is there a rational criterion that can be used *a priori* to identify good tracers from poor tracers?

Even though the network model is simple, the answers to these questions are not obvious. To answer them we used simulated data, assuming the fluxes shown in Fig. 1, and performed MFA to estimate fluxes in the model and determine 95% confidence intervals of fluxes, for all possible labeling patterns of A. Since metabolite A has three atoms, there are 8 ($=2^3$) possible isotopomers of A. We performed MFA with all 8 pure tracers of A and evaluated the confidence interval of flux v_4 . Surprisingly, none of the pure tracers allowed flux v_4 to be observed. The 95% confidence interval for flux v_4 always ranged from zero to infinity. In other words, all of these tracers would be poor choices for the labeling of A. In addition to pure tracers, mixtures of tracers can be used. Next, we evaluated all possible equimolar binary (50/50) mixtures of isotopomers of A. There are 28 unique isotopomer combinations, as illustrated in Fig. 2. Of the 28 binary mixtures, only 10 resulted in non-infinite confidence intervals for v_4 .

This trial-and-error procedure for selection of tracers using an assumed reference flux map reflects current state-of-the-art in tracer experiment design. While it does provide answers to the first two questions, i.e. which are the potentially good and poor tracers, it doesn't provide any insight into why certain tracers and combinations of tracers are more appropriate than others. For example, do the 10 feasible tracer combinations share a common feature that allows flux v_4 to be determined? To address this question we applied the EMU methodology.

3.2. EMU basis vectors framework

The network in Fig. 1 was decomposed into its respective EMU networks as shown in Fig. 3A. The size 3 EMU network demonstrates that the network product, E_{123} , can be formed via two EMU pathways: one pathway from A_{123} via reactions v_1 and v_2 , and a second pathway from D_{12} and C_1 via reactions v_4 and v_2 . EMUs D_{12} and C_1 can be further traced to A_{12} and A_3 , respectively. Fig 3B shows the combined EMU model. For this simple system, it was possible to derive an analytical solution to the EMU network model:

$$E_{123} = \frac{v_1}{v_1 + v_4} \cdot A_{123} + \frac{v_4}{v_1 + v_4} \cdot (A_{12} \times A_3) \quad (1)$$

This equation clearly illustrates that E_{123} can be derived from either A_{123} , or the condensation of A_{12} and A_3 . We define A_{123} and $A_{12} \times A_3$ as the EMU basis vectors for this model. An EMU basis vector can be viewed as a unique way for assembling substrate EMUs to form the product. By definition, EMU basis vectors have the same EMU size as the product. Ultimately, one can interpret the MID of the product as a linear combination of

EMU basis vector MIDs, where the coefficients are solely a function of free fluxes. The coefficients quantify the fractional contribution of each EMU basis vector to the product. Thus, by definition, the sum of the coefficients must equal one. In matrix notation, Eq. 1 becomes:

$$E_{123} = [A_{123} \ (A_{12} \times A_3)] \cdot \begin{bmatrix} \frac{v_1}{v_1 + v_4} \\ \frac{v_4}{v_1 + v_4} \end{bmatrix} \quad (2)$$

In general, the solution to any EMU network model can be expressed as:

$$x = BV \cdot c(v) \quad (3)$$

Where, x is the MID of the simulated product, BV is a matrix whose columns are the EMU basis vector MIDs and c is a contribution vector that is only a function of free fluxes. The rank of the BV matrix indicates how many EMU basis vectors are linearly independent. Depending on the choice of substrate labeling several EMU basis vectors may become linearly dependent and this will affect flux observability, as is discussed next.

3.3. Analysis of example model using EMU basis vectors

To resolve flux v_4 , or in other words, to distinguish the direct pathway from the condensation pathway, the labeling of A should be chosen such that the MID of the first EMU basis vector A_{123} does not equal the MID of the second EMU basis vector $A_{12} \times A_3$. This is illustrated in Figure 4 for four substrate labeling choices: two pure tracers A_{100} and A_{111} , and two 50/50 binary mixtures A_{010}/A_{111} and A_{100}/A_{111} . For the pure tracers, the MIDs of A_{123} and $A_{12} \times A_3$ were equal and the confidence interval of v_4 was infinite. The same was true for the mixture A_{101}/A_{111} . Only when A_{123} and $A_{12} \times A_3$ had different MIDs, as was the case for the mixture A_{100}/A_{111} , could flux v_4 be resolved. When we compared MIDs of A_{123} and $A_{12} \times A_3$ for all 8 pure tracers and all 28 binary mixtures, a distinct pattern became apparent as depicted in Fig. 2. Only for the 10 cases where the EMU basis vectors had different MIDs was flux v_4 observable. Thus, in this example, there was a perfect correlation between flux observability and linear independence of EMU basis vector MIDs.

3.4. Observability of an extended network model

The previous example illustrated that when the EMU basis vectors were equal, the free flux in the model could not be resolved. In this section, we expand on the EMU basis vector methodology through investigation of an extended version of the example model. The network model in Fig. 5 includes three additional reactions. The stoichiometry and atom transitions are given in Table 2. In this network, metabolite A is the substrate, metabolites F, G, and H are network products, and intermediates B, C, D, and E are assumed to be at metabolic and isotopic steady state. This network has three free fluxes: the substrate

consumption rate v_1 , and fluxes v_2 and v_4 . Here, we would like to determine fluxes v_2 and v_4 based on the measurement of the MID of F and a known uptake rate of A.

First, we evaluated the observability of the model using simulated data, assuming the fluxes shown in Fig. 5. We performed MFA with all 8 pure tracers of A and evaluated 3×28 binary mixtures (25/75, 50/50, and 75/25) of isotopomers of A. The results are summarized in Fig. 6. As in the previous example, none of the pure tracers allowed fluxes v_2 and v_4 to be estimated. Of the 28 mixtures of A, only 4 mixtures allowed both fluxes to be observed in all cases: A_{000}/A_{011} , A_{100}/A_{111} , A_{010}/A_{001} , and A_{110}/A_{101} . Three other mixtures of A were also feasible, but only when the ratio of the two tracers was not 50/50 (A_{100}/A_{001} , A_{110}/A_{001} , and A_{011}/A_{110}). The seven feasible tracer choices were a subset of the 10 tracer combinations identified in the first example (Fig. 2).

3.5. Analysis of extended model using EMU basis vectors

Next, we evaluated the observability of the model using the EMU basis vector methodology, and compared the results to the numerical analysis above. The combined EMU decomposition of the model is shown in Fig. 7A. The main difference with the first model is the presence of a third EMU pathway to produce F_{123} via the condensation of A_{12} and A_1 . As a result, this network has three EMU basis vectors: A_{123} , $A_{12} \times A_3$, and $A_{12} \times A_1$ (Fig. 7B).

Ideally, we would like the three basis vectors to be independent (rank of 3) to maximize the observability of the model. Fig 6 summarizes the results of flux observability and EMU basis vector rank. Again, there was a perfect correlation between flux observability and the number of independent (i.e. rank of) EMU basis vectors. In all cases, when the EMU basis vectors were linearly independent (rank of 3) all fluxes were observable. This was the case for seven tracer combinations (as discussed above). For three of the seven tracer combinations, however, the rank was reduced to 2 and the system became non-observable when the mixture was equimolar. This arose for the tracer combination A_{100}/A_{001} , A_{001}/A_{110} and A_{110}/A_{011} . This can be explained by the fact that A_1 and A_3 were equal for equimolar mixtures, and as a result the EMU basis vectors $A_{12} \times A_3$ and $A_{12} \times A_1$ were identical. Similarly, we can explain why the remaining 3 tracer combinations from the first example did not work well in this extended model. For the tracer combinations A_{000}/A_{101} , A_{000}/A_{111} , and A_{010}/A_{111} the rank was always 2, regardless of the ratio of the tracers, since A_1 and A_3 were always equal for these tracer mixtures.

The correlation between the rank of EMU basis vectors and flux observability is also illustrated in Fig. 8, which shows representative confidence intervals of fluxes v_2 and v_4 for four tracer choices, with EMU basis vector ranks of 3, 2, 2 and 1, respectively. When all three EMU basis vectors were independent (Fig. 8A), both fluxes were resolved with narrow confidence intervals. When only two vectors were independent (Fig. 8B-C), both fluxes could not be observed. Interestingly, different confidence intervals were obtained depending on which two EMU basis vectors were dependent. When $A_{123} = A_{12} \times A_3$ (Fig 8B), flux v_2 could be resolved with high precision even though v_4 was non-observable. On the other hand, when $A_{12} \times A_3 = A_{12} \times A_1$ (Fig. 8C), neither flux could be resolved with high certainty. Only a lower limit could be established for flux v_2 and an upper limit for flux v_4 .

When all three EMU basis vectors were the same (Fig. 8D), no information was obtained regarding either flux.

3.6. Number of independent EMU basis vectors and sensitivities of coefficients

We investigated the connection between the number of independent EMU basis vectors and flux observability further. Thus far, the results from the two example models suggested that the number of linearly independent EMU basis vectors must be greater than the number of free fluxes in order to make the system observable, i.e. in the first example model a rank of 2 was required to observe 1 free flux, and in the extended network model a rank of 3 was required to observe 2 free fluxes. One may wonder why it is not possible to estimate two free fluxes in a model with two independent EMU basis vectors. To address this question we derived an analytical solution to the extended network model, shown here in matrix form:

$$F_{123} = \begin{bmatrix} A_{123} & (A_{12} \times A_3) & (A_{12} \times A_3) \end{bmatrix} \cdot \begin{bmatrix} c_1 \\ c_2 \\ c_3 \end{bmatrix} \quad (4)$$

with

$$c_1 = \frac{2 \cdot (1 - R) \cdot S}{1 + S}, c_2 = \frac{2 \cdot R \cdot S}{1 + S}, c_3 = \frac{1 - S}{1 + S} \quad (5)$$

And

$$R = \frac{v_4}{v_1 + v_4} \text{ and } S = \frac{v_2}{v_1} \quad (6)$$

The maximum rank of the EMU basis vector matrix is fixed by the number of EMU basis vectors (i.e. number of columns m) and the number of mass isotopomers (i.e. number of rows n):

$$\max(\text{rank}(BV)) = \min(m, n) \quad (7)$$

For the extended network model, the EMU basis vector matrix is an 4×3 matrix since there are four mass isotopomers of F_{123} (M+0, M+1, M+2, and M+3) and three EMU basis vectors. The maximum rank is therefore 3, and at most 3 coefficients can be estimated. However, because the sum of all coefficients must equal 1 (see section 3.2), only 2 coefficients can be independent. With 2 independent coefficients, at most 2 free fluxes can be determined. Thus, in general, the maximum number of free fluxes that can be determined in a system is: $\max(\text{rank}(BV)) - 1 = \min(m, n) - 1$.

The analytical solution Eq. 4 provides additional insights into the selection of tracers for MFA, related to sensitivities of coefficients with respect to free fluxes. For tracer selections where the first two EMU basis vectors were equal, e.g. when $A_{123} = A_{12} \times A_3$, Eq. 4 simplifies to:

$$F_{123} = (c_1 + c_2) \cdot A_{123} + c_3 \cdot (A_{12} \times A_1) \quad (8)$$

In this case, the sum of c_1 and c_2 can be resolved, but not the individual coefficients. As a result, there are only two independent parameters (c_1+c_2 and c_3). The sensitivities of the coefficients to free fluxes are shown in Fig. 7C. It is clear that when $A_{123} = A_{12} \times A_3$, the sensitivity to v_4 is lost, i.e. $d(c_1+c_2)/dv_4 = 0$ and $dc_3/dv_4 = 0$, and as a result flux v_4 becomes non-observable. However, sensitivity of the coefficients to v_2 is retained, i.e. $d(c_1+c_2)/dv_2 = 1$ and $dc_3/dv_2 = -1$. This explains the result shown in Fig. 8B, where flux v_2 could be resolved but not flux v_4 .

3.7. Optimal ratio of tracers and D-optimality criterion

In addition to selecting which tracers are feasible for an experiment, a second important design parameter is the ratio of the tracers. As mentioned previously, three of the full rank tracer mixtures in the extended model had linearly dependent EMU basis vectors when the mixture was equimolar. To further explore the effect of the tracer composition, the 95% confidence intervals for fluxes v_2 and v_4 were plotted in Fig. 9 for two mixtures: A_{010}/A_{001} , which was always full rank (Fig. 9A); and A_{100}/A_{001} , which was full rank except for an equimolar composition (Fig. 9B). For the mixture A_{010}/A_{001} , the confidence interval of v_2 continually decreased as the fraction of A_{001} increased. For the mixture A_{100}/A_{001} , the confidence interval of v_2 decreased as the composition approached either of the pure tracers. For both tracer mixtures there was an asymptotic broadening of the confidence interval for flux v_4 as the composition approached pure tracers. For the mixture A_{100}/A_{001} , there was also a broadening of the confidence intervals as the composition approached an equimolar mixture. One technique for quantitatively selecting an optimal tracer mixture is through the use of the D-optimality criterion (Yang et al., 2005). Fig. 9C-D shows the normalized information index obtained from the D-optimality test. The maximum of the peaks corresponded to the optimal ratios of tracers. For the mixture A_{010}/A_{001} , the optimum was around 0.7, reflecting a trade-off between increasing v_2 confidence interval and decreasing v_4 confidence interval as the fraction of A_{001} increased above 0.5. As expected, for the A_{100}/A_{001} mixture, there were two optimal choices due to the singularity at 0.5. The optimal fractions of A_{001} were around 0.2 and 0.8, again reflecting the competing trade-off between the confidence intervals for fluxes v_2 and v_4 .

3.8. Tricarboxylic acid cycle example

The first two examples revealed a positive correlation between the EMU basis vector rank and flux observability and the importance of coefficient sensitivities. Specifically, we determined that an observable system must always have more independent EMU basis vectors than free fluxes. In both examples, this criterion was satisfied only when all EMU basis vectors were independent. In section 3.6, we also noted that the number of independent

EMU basis vectors is limited by the number of mass isotopomers. Since the number of mass isotopomers exceeded the number of EMU basis vectors in both examples, all EMU basis vectors could be independent. In more complex systems, however, the number of EMU basis vectors can be greater than the number of mass isotopomers, in which case not all EMU basis vectors will be independent. In this example we investigated how this affects flux observability, and how tracers should be selected in these more complex systems.

To address these questions, we considered a model of the tricarboxylic acid (TCA) cycle shown in Fig. 10. The stoichiometry and atom transitions for the network are given in Table 3. Aspartate and acetyl-coenzyme A (AcCoA) are the substrates in this system and glutamate and carbon dioxide are the products. This model has three free fluxes: citrate synthase flux v_1 , influx of aspartate v_8 , and the exchange flux of the reversible fumarase reaction v_7 . In this example, we would like to determine fluxes v_7 and v_8 , using only the MID of the network product glutamate and a fixed value for flux v_1 . First, we evaluated the observability of the model using simulated data, assuming the flux values shown in Fig. 10. For tracer selection, we only considered combinations of pure tracers of aspartate and AcCoA. In total there were 64 unique tracer combinations ($=2^4 \times 2^2$). The results are summarized in Fig. 11. Of the 64 tracer combinations, 32 allowed both free fluxes (v_7 and v_8) to be observed, 22 allowed only one flux (v_8) to be determined, and 10 tracer combinations did not provide any flux information.

The EMU decomposition for this network model was described in Antoniewicz et al. (Antoniewicz et al., 2007b). This system has 9 EMU basis vectors, shown in Fig. 12. Since there are two free fluxes in the model, there must be at least three independent EMU basis vectors to observe both fluxes. Analysis of the EMU basis vector rank and flux observability is shown in Fig. 11. Again, there was good agreement between the number of independent EMU basis vectors and flux observability. For all tracer combinations with ranks 3 or 4, either one or both fluxes were observable. For all tracer combinations with ranks 1 or 2, either none or only one flux was observable. These results are in line with the previous two example models. However, an interesting question arose for this system: why was the maximum observed rank only 4, considering that glutamate has 6 mass isotopomers ($M+0$, $M+1$, ..., $M+5$) and there are 9 EMU basis vectors? Also, why did certain tracer combinations with ranks of 3 and 4 not allow flux v_7 to be observed?

3.9. Number of independent EMU basis vectors for TCA cycle

To address the first question, we considered the individual EMU networks. The size 1 EMU network has three inputs: Asp_2 , Asp_3 , and $AcCoA_2$. However, since size 1 EMUs only have two mass isotopomers ($M+0$ and $M+1$), only two of the three inputs can be independent. The size 2 EMU network has four inputs: $Asp_2 \times AcCoA_2$, $Asp_3 \times AcCoA_2$, $AcCoA_2 \times AcCoA_2$, and Asp_{23} . Again, since size 2 EMUs only have three mass isotopomers ($M+0$, $M+1$ and $M+2$), at most three of the four inputs can be independent. Similarly, the size 3 EMU network has 9 inputs (Fig. 12), but is limited to 4 independent EMU basis vectors. The size 5 EMU network also has 9 EMU basis vectors. However, because the network product glutamate is produced via a single convolution between a balanced metabolite, OAC_{234} , and a network substrate, $AcCoA_{12}$ (which does not depend on fluxes), the number of

independent EMU basis vectors did not increase for the size 5 EMU network. As a result, the maximum number of independent EMU basis vectors for the entire system was 4, since OAC₂₃₄ only has 4 mass isotopomers.

This observation raises an important point regarding condensation reactions. Network substrates have fixed MIDs, i.e. independent of the fluxes in the system. Balanced metabolites, on the other hand, can be a function of several precursor MIDs (e.g. OAC₂ is a function of Asp₂, Asp₃, and AcCoA₂). The convolution of a balanced metabolite, OAC₂₃₄, with a network substrate, AcCoA₁₂, does not increase the number of independent basis vectors, because AcCoA₁₂ is common to all EMU basis vectors produced from the convolution. In contrast, convolutions of two or more balanced metabolites may result in the production of additional independent EMU basis vectors, as each balanced metabolite can depend on one or more substrate EMUs.

3.10. Sensitivities of EMU basis vector coefficients

To address the question why certain tracer combinations with ranks of 3 and 4 did not allow the reversible flux v_7 to be observed we need to consider the sensitivities of EMU basis vector coefficients with respect to fluxes (as discussed in section 3.6). In sections 3.2 and 3.6, we demonstrated that any product can be expressed as a linear combination of EMU basis vectors, where the coefficients are only a function of free fluxes. In addition, we determined that the sum of all coefficients must equal 1:

$$c_1 + c_2 + \dots + c_n = 1 \quad (9)$$

Thus, by definition, the sum of the sensitivities of coefficients with respect to any flux must equal zero:

$$\frac{dc_1}{dv_i} + \frac{dc_2}{dv_i} + \dots + \frac{dc_n}{dv_i} = 0 \quad (10)$$

In other words, for any flux in the model, there must be at least one positive sensitivity and at least one negative sensitivity. As we discussed in section 3.6, when EMU basis vectors become linearly dependent, only the sum of the corresponding coefficients can be determined. In these cases, it is possible that the system becomes insensitive to one or more free fluxes. In effect, the positive and negative sensitivities may cancel each other out when certain EMU basis vectors become linearly dependent. The sensitivities for the 9 EMU basis vectors with respect to free fluxes v_7 and v_8 are shown in Fig. 12C. In the case of dc/dv_7 , there are three equal but opposite paired sensitivities. As a result, if an aspartate tracer is chosen such that $Asp_2 = Asp_3$ and $Asp_{123} = Asp_{234}$, each pair of sensitivities cancels out, and the functionality with respect to v_7 is lost (i.e. the glutamate measurement becomes insensitive to changes in flux v_7). Therefore, it will be impossible to estimate flux v_7 , even if the rank of the EMU basis vectors is 3 or 4. For example, this is the case for the tracer combinations $Asp_{1111} + AcCoA_{00}$ and $Asp_{0000} + AcCoA_{11}$, since $Asp_2 = Asp_3$ and $Asp_{123} =$

Asp₂₃₄. In summary, the two criteria that need to be satisfied to observe both free fluxes in the TCA cycle model are:

- 1) The EMU basis vector rank must be 3 or 4
- 2) Aspartate labeling must satisfy: Asp₂ Asp₃ or Asp₁₂₃ Asp₂₃₄

These criteria are consistent with the tracer selection rules that were established in the first two example models (section 3.6).

3.11. Importance of smaller size EMU networks

Analysis of the TCA cycle also underscores the importance of maximizing the number of independent EMU basis vectors at smaller size EMU networks. To illustrate this, we systematically examined the impact of tracer selection on size 1 EMUs and the overall flux observability. Since the size 1 EMU network has three inputs (Fig. 12), there are four possible relationships between the inputs for pure tracers: (1) Asp₂ = Asp₃ = AcCoA₂; (2) Asp₂ = AcCoA₂ Asp₃; (3) Asp₃ = AcCoA₂ Asp₂; or (4) Asp₂ = Asp₃ AcCoA₂.

In case (1), where Asp₂ = Asp₃ = AcCoA₂, i.e. either all labeled or all unlabeled, the size 2 EMU network will have a single mass isotopomer for OAC₂₃. For example, if Asp₂, Asp₃, and AcCoA₂ are unlabeled (and hence Asp₂₃ is unlabeled), OAC₂₃ will be unlabeled. As a result, the only way to diversify labeling in the EMU basis vectors is through the size 3 EMU network. The four contributions to the size 3 EMU network are OAC₂₃ × AcCoA₂, Asp₁₂₃, Asp₂₃₄, and AcCoA₁₂ × AcCoA₂. The contribution from OAC₂₃ × AcCoA₂ will be unlabeled. The other three EMU basis vectors can all generate M+1 mass isotopomers (i.e. if carbon 1 or 4 of aspartate is labeled, or the first carbon of AcCoA is labeled), but M+2 or higher mass isotopomers cannot be generated. Thus, it is clear that the maximum EMU basis vector rank will be two and the system will be non-observable, since at least three independent EMU basis vectors are needed.

Analysis of case (2), where Asp₂ = AcCoA₂ Asp₃, is schematically depicted in Fig. 13A. Assuming Asp₂ and AcCoA₂ are unlabeled (open circles), and Asp₃ is labeled (filled circle), OAC₂ has two possible mass isotopomers (M+0 and M+1), which is the maximum for a size 1 EMU. For size 2 EMU network, OAC₂₃ can be formed from Asp₂₃ or the condensation of OAC₂ × AcCoA₂. As shown in Fig. 13A, OAC₂₃ will have two mass isotopomers (M+0 and M+1). For a size 2 EMU network, this is one less than the maximum number of mass isotopomers. In the size 3 EMU network, OAC₂₃₄ will have M+0 and M+1 mass isotopomers, and depending on the labeling of Asp₁, Asp₄, and AcCoA₁, a third mass isotopomer (M+2) can be formed. Four independent EMU basis vectors can never be achieved for case (2), as there is no way to generate M+3. In more general terms, OAC₂₃₄ will have an EMU basis vector rank of 3 when Asp₃ = AcCoA₁, Asp₁ or Asp₄; otherwise, the system will be rank 2. Analogously, case (3) will have three independent vectors only when Asp₂ = AcCoA₁, Asp₁, or Asp₄. For any other tracer choice, the system will be rank 2.

For case (4), when Asp₂ = Asp₃ AcCoA₂ (schematically depicted in Fig. 13B), OAC₂ also has two mass isotopomers (M+0 and M+1). In the size 2 EMU network, OAC₂₃ is guaranteed three mass isotopomers, as Asp₂₃ (M+0) will always differ from OAC₂ ×

AcCoA₂ (combination of M+1 and M+2). Furthermore, the size 3 EMU network is guaranteed at least three different mass isotopomers (M+1, M+2, and M+3) from the convolution of $OAC_{23} \times AcCoA_2$. A fourth mass isotopomer (M+0) forms if the first or fourth carbon atom of Asp is unlabeled. More generally, case (4) will be rank 4 if $Asp_2 = Asp_1$ or Asp_4 ; and will be rank 3 otherwise.

These results highlight two major points about tracer choice and EMU basis vector rank (Fig 13C). First, diversity in the smaller size EMU networks is crucial to increasing the rank of the EMU basis vectors for the whole system. Since all higher size EMU networks are dependent on smaller size EMUs, an efficient way to increase variety at the larger network sizes is to diversify the smaller size EMU networks. Secondly, Fig. 13C illustrates that linear dependencies among size 1 EMUs fundamentally affect the EMU basis vector rank of the system. For case (1), the EMU basis vectors can never exceed a rank of 2. In cases (2) and (3), the highest rank that can be obtained is 3. In contrast, case (4) is guaranteed to have a rank of at least 3, and can achieve a maximum rank of 4.

3.12. Selection of optimal tracer combinations for TCA cycle

In order to compare the technically feasible tracer selections and evaluate if the tracer experiment design is affected by the assumed reference flux map we employed D-optimality analysis, as described in section 3.7. For this analysis, we generated ~5000 random flux maps and evaluated flux observability for all tracer combinations and all flux maps. We observed that two distinct groupings of tracers arose (Fig 14A). One group of tracers produced an observable system in more than 80% of cases (these were the technically feasible tracer combinations), while a second group of tracers never produced an observable system. In other words, for the TCA cycle model the tracer experiment design did not depend strongly on the specific reference flux map used. While there was a distinct group of tracers that produced an observable system, the confidence intervals of fluxes varied significantly depending on which tracer set was used. Fig. 14B shows a comparison of five feasible tracer combinations that satisfied both criteria from the previous section. The relative information index from the D-optimality test varied by about 10-fold for the five tracer sets. In general, a larger value for the relative information index indicated that more flux information could be obtained from the tracer experiment (Yang et al., 2005). Fig. 14C displays the 95% confidence intervals for fluxes v_7 and v_8 for the five tracer combinations shown in 14B. In this example, the widths of the confidence intervals correlated well with the relative information index, i.e. tracer combinations with higher information indices produced smaller confidence intervals. Thus, the D-optimality criterion was found to be a good indicator of the precision for the estimated fluxes. Overall, the best tracer combination for the TCA cycle was $Asp_{1100} + AcCoA_{11}$, which corresponds to growing cells with [1,2-¹³C]aspartate and, for example, [U-¹³C]glucose as tracers. We also evaluated the optimal ratios of tracers, similar to the analysis in section 3.7. Flux observability for binary mixtures of either aspartate or acetyl-CoA tracers was investigated. The results with representative examples are shown in Supplementary Figure S1. The general conclusion from this analysis was that the pure aspartate and acetyl-CoA labeling schemes (shown in Fig. 11) were optimal for elucidation of the TCA cycle fluxes.

4. CONCLUSIONS

Metabolic flux analysis is currently the most reliable technique for measuring *in vivo* metabolic fluxes (Ahn and Antoniewicz, DOI: 10.1002/biot.201100052). The selection of an isotopic tracer or a mixture of tracers is a key decision point in ^{13}C -MFA. Thus far, design of tracer experiments has been empirical and focused mainly on a handful of tracers, such as $[1\text{-}^{13}\text{C}]\text{glucose}$ (~\$100/g), $[\text{U-}^{13}\text{C}]\text{glucose}$ (~\$200/g) and $[1,2\text{-}^{13}\text{C}]\text{glucose}$ (~\$800/g). The study by Metallo et al. (Metallo et al., 2009) was one of the first examples where other alternative tracers were considered, in that case for elucidating the metabolism of mammalian cells. Using simulation experiments and an assumed reference flux map, isotopic tracers were compared based on the predicted confidence intervals of fluxes. The work by Metallo et al. represents current state of knowledge regarding optimal selection of isotopic tracers for ^{13}C -MFA.

In this contribution, we introduced a new perspective on tracer experiment design for ^{13}C -MFA, as outlined in Fig. 15. Our methodology is based on the novel concept of EMU basis vectors. In contrast with previous trial-and-error methodologies, our framework for *a priori* tracer selection is based on fundamental concepts and criteria that arise directly from EMU decomposition of a metabolic network. We demonstrated that any metabolite in a network model can be described as a linear combination of so-called EMU basis vectors, where the corresponding coefficients indicate the fractional contribution of the EMU basis vector to the product metabolite. The strength of this approach is the decoupling of substrate labeling, i.e. the EMU basis vectors, from the dependence on free fluxes, i.e. the coefficients. In three example models with increasing model complexity we derived practical guidelines and design principles for *a priori* selection of isotopic tracers. We showed that flux observability depends inherently on the number of independent EMU basis vectors and the sensitivities of coefficients with respect to free fluxes. Specifically, we demonstrated that the rank of the EMU basis vectors must be greater than the number of free fluxes in order to resolve all the free fluxes in a network. When this criterion is not achieved, certain fluxes may still be estimated, but only when specific EMU basis vectors are linearly independent (Fig. 8B vs. 8C). These insights can be used as a guide for selecting feasible substrate labeling. Furthermore, inspection of coefficient sensitivities with respect to free fluxes provides additional constraints for a proper selection of tracers. This was especially useful for selecting tracers to elucidate exchange fluxes of reversible reactions. When several feasible labeling schemes existed, we implemented a D-optimality test. The D-optimality criterion correlated well with the observed flux confidence intervals and accounted for the propagation of measurement errors.

One practical guideline that we derived is the importance of maximizing the number of independent EMUs at smaller size EMU networks. Since all higher size EMU networks are dependent on smaller size EMUs, tracer selection should focus on maximizing the variation at the smallest size EMU networks. This concept explains, for example, why a mixture of $[\text{U-}^{13}\text{C}]\text{glucose}$ and unlabeled glucose is generally not an optimal tracer choice (Mollney et al., 1999). For this mixture the MIDs of the smallest size EMUs (e.g. size 1 EMUs) will always be equal, and thus the number of independent EMUs is not maximized. A more appropriate choice is, for example, a mixture of $[\text{U-}^{13}\text{C}]\text{glucose}$ and $[1\text{-}^{13}\text{C}]\text{glucose}$, as was

empirically found in several studies (Antoniewicz et al., 2007c; Mollney et al., 1999). Using our methodology it is clear that for this mixture there can be two independent EMUs of size 1, which is the maximum for a size 1 EMU network. In general, the use of uniformly labeled tracers without a positionally labeled counterpart is therefore not encouraged. The EMU basis vector framework also explains other cases that are known to be non-observable and poorly observable. For example, when no isotopic tracers are applied, or when all substrates are uniformly labeled, all EMU basis vectors will be equal and thus no flux information can be obtained from these experiments. It is also well known that MFA is more difficult using small molecules as tracers compared to using larger molecules as tracers. One extreme case is autotrophic growth with carbon dioxide as the sole carbon source. For autotrophic growth, the EMU basis vectors will always be equal (i.e. convolution of CO₂ carbon atoms) and thus no flux information will be obtained from steady state isotopomer measurements (Shastri and Morgan, 2007). The same concepts also explain why ¹³C-MFA with acetate tracers is inherently more challenging than MFA using glucose tracers (Feng et al.), i.e. it is more difficult to select tracers that generate independent EMU basis vectors starting with a C2-substrate than with a C6-substrate.

In summary, the results presented in this paper demonstrate that issues associated with optimal tracer experiment design and flux observability are not trivial and require a fundamental analysis. We have shown that EMU decomposition can provide valuable new insights into the selection of tracers for high resolution ¹³C-MFA, and that rational criteria can be developed to *a priori* distinguish good tracers from poor ones. At the same time, it is important to emphasize that flux resolution depends on both the tracer selection and the choice of measurements. This co-dependence is evident in the EMU basis vectors themselves. The tracer selection determines the MIDs of the basis vectors as well as the independence relationships. Further, the number of mass isotopomers in the measurements sets a hard limit on the maximum number of independent basis vectors, and hence the free flux observability. In this work, we explored the problem: given a measurement, which labeling strategy should be used to elucidate the most information regarding the fluxes. Going forward, however, the choice of labeling should be addressed in conjunction, rather than in isolation of, the set of measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by the NSF CAREER Award (CBET-1054120) to MRA and the NSF Graduate Fellowship to SBC.

ABBREVIATIONS

MFA	metabolic flux analysis
EMU	elementary metabolite unit
MS	mass spectrometry

MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance
MID	mass isotopomer distribution
TCA	tricarboxylic acid cycle
SSR	variance-weighted sum of squared residuals

REFERENCES

- Ahn WS, Antoniewicz MR, 2011 Metabolic flux analysis of CHO cells at growth and non-growth phases using isotopic tracers and mass spectrometry. *Metab Eng.* 13, 598–609. [PubMed: 21821143]
- Ahn WS, Antoniewicz MR, DOI: 10.1002/biot.201100052. Towards dynamic metabolic flux analysis in CHO cell cultures. *Biotechnol J.*
- Antoniewicz MR, Kelleher JK, Stephanopoulos G, 2006 Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements. *Metab Eng.* 8, 324–37. [PubMed: 16631402]
- Antoniewicz MR, Kelleher JK, Stephanopoulos G, 2007a Accurate assessment of amino acid mass isotopomer distributions for metabolic flux analysis. *Anal Chem.* 79, 7554–9. [PubMed: 17822305]
- Antoniewicz MR, Kelleher JK, Stephanopoulos G, 2007b Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metab Eng.* 9, 68–86. [PubMed: 17088092]
- Antoniewicz MR, Kelleher JK, Stephanopoulos G, 2011 Measuring deuterium enrichment of glucose hydrogen atoms by gas chromatography/mass spectrometry. *Anal Chem.* 83, 3211–6. [PubMed: 21413777]
- Antoniewicz MR, Kraynie DF, Laffend LA, Gonzalez-Lergier J, Kelleher JK, Stephanopoulos G, 2007c Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of *E. coli* producing 1,3-propanediol. *Metab Eng.* 9, 277–92. [PubMed: 17400499]
- Arauzo-Bravo MJ, Shimizu K, 2003 An improved method for statistical analysis of metabolic flux analysis using isotopomer mapping matrices with analytical expressions. *J Biotechnol.* 105, 117–33. [PubMed: 14511915]
- Chang Y, Suthers PF, Maranas CD, 2008 Identification of optimal measurement sets for complete flux elucidation in metabolic flux analysis experiments. *Biotechnol Bioeng.* 100, 1039–49. [PubMed: 18553391]
- Choi J, Antoniewicz MR, 2011 Tandem mass spectrometry: A novel approach for metabolic flux analysis. *Metab Eng.* 13, 225–33. [PubMed: 21134484]
- Crown SB, Indurthi DC, Ahn WS, Choi J, Papoutsakis ET, Antoniewicz MR, 2011 Resolving the TCA cycle and pentose-phosphate pathway of *Clostridium acetobutylicum* ATCC 824: Isotopomer analysis, in vitro activities and expression analysis. *Biotechnol J.* 6, 300–5. [PubMed: 21370473]
- Feng X, Tang KH, Blankenship RE, Tang YJ, Metabolic flux analysis of the mixotrophic metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*. *J Biol Chem.* 285, 39544–50. [PubMed: 20937805]
- Jeffrey FM, Roach JS, Storey CJ, Sherry AD, Malloy CR, 2002 ¹³C isotopomer analysis of glutamate by tandem mass spectrometry. *Anal Biochem.* 300, 192–205. [PubMed: 11779111]
- Kelleher JK, 2004 Probing metabolic pathways with isotopic tracers: insights from mammalian metabolic physiology. *Metab Eng.* 6, 1–5. [PubMed: 14734250]
- Leighty RW, Antoniewicz MR, 2011 Dynamic metabolic flux analysis (DMFA): A framework for determining fluxes at metabolic non-steady state. *Metab Eng.* 13, 745–55. [PubMed: 22001431]
- Metallo CM, Walther JL, Stephanopoulos G, 2009 Evaluation of ¹³C isotopic tracers for metabolic flux analysis in mammalian cells. *J Biotechnol.*

- Mollney M, Wiechert W, Kownatzki D, de Graaf AA, 1999 Bidirectional reaction steps in metabolic networks: IV. Optimal design of isotopomer labeling experiments. *Biotechnol Bioeng.* 66, 86–103. [PubMed: 10567067]
- Moxley JF, Jewett MC, Antoniewicz MR, Villas-Boas SG, Alper H, Wheeler RT, Tong L, Hinnebusch AG, Ideker T, Nielsen J, Stephanopoulos G, 2009 Linking high-resolution metabolic flux phenotypes and transcriptional regulation in yeast modulated by the global regulator Gcn4p. *Proc Natl Acad Sci U S A.* 106, 6477–82. [PubMed: 19346491]
- Reed JL, Senger RS, Antoniewicz MR, Young JD, 2010 Computational approaches in metabolic engineering. *J Biomed Biotechnol.* 2010, 207414. [PubMed: 21584279]
- Sauer U, 2006 Metabolic networks in motion: ¹³C-based flux analysis. *Mol Syst Biol.* 2, 62. [PubMed: 17102807]
- Shastri AA, Morgan JA, 2007 A transient isotopic labeling methodology for ¹³C metabolic flux analysis of photoautotrophic microorganisms. *Phytochemistry.* 68, 2302–12. [PubMed: 17524438]
- Stephanopoulos G, 1999 Metabolic fluxes and metabolic engineering. *Metab Eng.* 1, 1–11. [PubMed: 10935750]
- Szyperski T, 1995 Biosynthetically directed fractional ¹³C-labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism. *Eur J Biochem.* 232, 433–48. [PubMed: 7556192]
- Wittmann C, 2002 Metabolic flux analysis using mass spectrometry. *Adv Biochem Eng Biotechnol.* 74, 39–64. [PubMed: 11991183]
- Yang TH, Heinze E, Wittmann C, 2005 Theoretical aspects of ¹³C metabolic flux analysis with sole quantification of carbon dioxide labeling. *Comput Biol Chem.* 29, 121–33. [PubMed: 15833440]
- Yang TH, Wittmann C, Heinze E, 2006 Respirometric ¹³C flux analysis, Part I: design, construction and validation of a novel multiple reactor system using on-line membrane inlet mass spectrometry. *Metab Eng.* 8, 417–31. [PubMed: 16844397]
- Yoo H, Antoniewicz MR, Stephanopoulos G, Kelleher JK, 2008 Quantifying reductive carboxylation flux of glutamine to lipid in a brown adipocyte cell line. *J Biol Chem.* 283, 20621–7. [PubMed: 18364355]
- Young JD, Walther JL, Antoniewicz MR, Yoo H, Stephanopoulos G, 2008 An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol Bioeng.* 99, 686–99. [PubMed: 17787013]
- Zamboni N, Fendt SM, Ruhl M, Sauer U, 2009 (¹³C)-based metabolic flux analysis. *Nat Protoc.* 4, 878–92. [PubMed: 19478804]

Figure 1. Simple metabolic network model used to demonstrate the need for a more rational approach to tracer design. The assumed fluxes have arbitrary units.

Figure 2.

Analysis of flux observability and independence of EMU basis vectors in the example network model. Pure tracers are shown on the diagonal and binary mixtures of tracers are shown on the off-diagonal elements. Observability of flux v_4 correlated with the independence of the EMU basis vectors A_{123} and $A_{12} \times A_3$.

A EMU network size 1

EMU network size 2

EMU network size 3

B Combined EMU model **Figure 3.**

(A) Decoupled EMU reaction networks for the example network model. (B) Combined EMU reaction network showing the flow of isotopic labeling from the substrate A to the network product E. The two EMU basis vectors in the model were A_{123} and $A_{12} \times A_3$.

Figure 4.

Illustration of the importance of independence of EMU basis vectors for flux observability. Shown are the MIDs of EMU basis vectors and the associated confidence interval of flux v_4 for four labeling choices of substrate A: (left to right) pure tracer A_{100} ; pure tracer A_{111} ; 50/50 mixture of A_{101}/A_{111} ; and 50/50 mixture of A_{100}/A_{111} . Only the last mixture had different EMU basis vectors and flux v_4 was observable.

Figure 5.
Extended metabolic network model. The assumed fluxes have arbitrary units.

Figure 6.

Analysis of flux observability and EMU basis vector rank in the extended network model. Pure tracers are shown on the diagonal and binary mixtures of tracers are shown on the off-diagonal elements. The square colors denote the EMU basis vector rank (i.e. number of independent EMU basis vectors) and the circles correspond to flux observability. Observability of the free fluxes correlated with the independence of EMU basis vectors. Squares with upper and lower colored triangles denote cases where the EMU basis vector rank was different for 50/50 mixtures of tracers, i.e. compared to 25/75 and 75/25 mixtures.

Figure 7.

(A) Combined EMU reaction network for the extended model, showing the flow of isotopic labeling from substrate A to product E. (B) EMU basis vectors for the extended network model. (C) Coefficient sensitivities ($\times 10^2$) with respect to free fluxes, v_2 and v_4 . If A was chosen such that $A_{123} = A_{12} \times A_3$, functionality with respect to v_4 was lost.

Figure 8.

Effect of tracer selection on confidence intervals of fluxes in the extended network model. The minimized sum of squared residuals is plotted against the flux value for four choices of tracers, with different EMU basis vector dependence relationships: (A) all three EMU basis vectors are independent, [50/50 mixture of A_{010}/A_{001}]; (B) two vectors are independent, where $A_{123} = A_{12} \times A_3$, [50/50 mixture of A_{101}/A_{001}]; (C) two vectors are independent, where $A_1 = A_3$, [50/50 mixture of A_{000}/A_{111}]; (D) all three vectors are the same, [50/50 mixture of A_{010}/A_{101}].

Figure 9.

Effect of tracer composition on confidence intervals of fluxes and the normalized information index for binary mixtures of A_{010}/A_{001} (A, C) and A_{100}/A_{001} (B, D). The confidence interval plots (A, B) display a competing trade-off between the resolution of fluxes v_2 and v_4 . The overall optimal tracer composition was determined as the composition that maximized the normalized information index (C, D).

Figure 10.
Simplified model of the TCA cycle. The assumed fluxes have arbitrary units.

⊖

 Neither observable

⊕

 Only v_8 observable

⊕

 Both observable

Figure 11. Analysis of flux observability and EMU basis vector classification for the TCA cycle. Flux observability was evaluated for all combinations of aspartate tracers and acetyl-CoA tracers. The square colors denote the EMU basis vector rank, the circle colors correspond to whether v_7 functionality was lost due to tracer selection, and the symbol within the circle denotes the flux observability. Observability of both free fluxes in the model required an EMU basis vector rank of 3 or 4, and required that $\text{Asp}_2 = \text{Asp}_3$ and/or $\text{Asp}_{123} = \text{Asp}_{234}$.

Figure 13.

Examination of EMU basis rank principles for the TCA cycle model. Depicted is the flow of isotopic labeling through EMU size 1, 2, and 3 networks for two cases: (A) $Asp_2 = AcCoA_2$

Asp_3 and (B) $Asp_2 = Asp_3 \neq AcCoA_2$. Open circles denote unlabeled atoms and solid circles indicate labeled atoms. Gray circles denote atoms that can be either unlabeled or labeled. EMU combinations in the size 3 networks represent the possible EMU basis vectors for OAC_{234} . A maximum of four mass isotopomers can be produced for OAC_{234} and the choice of labeling of the gray atoms determines the independence relationships for EMU basis vectors. General statements regarding the independence relationships are shown in (C). For brevity, the size 5 EMU network was omitted since this network only had a single input ($OAC_{234} \times AcCoA_{12}$) and did not influence the overall EMU basis vector rank.

Figure 14.

Analysis of the effects of tracers and reference flux map on the confidence intervals of fluxes in the TCA cycle model. (A) Analysis of the effect of reference flux map. Flux observability was evaluated for each tracer combination using ~5000 flux maps. Two distinct groupings of tracers arose. One group of tracers produced an observable system in more than 80% of cases and a second group of tracers never produced an observable system. (B) Relative information index for five tracer combinations that were identified as feasible choices. The reference case was Asp₀₁₀₀/AcCoA₀₁ using the fluxes in Fig. 10. (C) Confidence intervals of free fluxes v_7 and v_8 for the five tracer combinations. The relative information index correlated well with the confidence intervals of fluxes.

Figure 15.

Schematic demonstrating the strategy for selecting isotopic tracers for ^{13}C -MFA using the EMU basis vector methodology.

Table 1:

Stoichiometry and atom transitions for the reactions in the example network model.

Reaction number	Reaction stoichiometry	Atom transitions
1	$A \rightarrow B$	$abc \rightarrow abc$
2	$B \rightarrow E$	$abc \rightarrow abc$
3 and 4	$B \leftrightarrow C + D$	$abc \leftrightarrow c + ab$

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2:

Stoichiometry and atom transitions for the reactions in the extended network model.

Reaction number	Reaction stoichiometry	Atom transitions
1	$A \rightarrow B$	$abc \rightarrow abc$
2	$B \rightarrow E$	$abc \rightarrow abc$
3 and 4	$B \leftrightarrow C + D$	$abc \leftrightarrow c + ab$
5	$B + D \rightarrow E + 2G$	$abc + de \rightarrow abd + c + e$
6	$E \rightarrow F$	$abc \rightarrow abc$
7	$C \rightarrow H$	$a \rightarrow a$

Table 3:

Stoichiometry and atom transitions for the reactions in the TCA cycle model.

Reaction number	Reaction stoichiometry	Carbon atom transitions
1	$\text{OAC} + \text{AcCoA} \rightarrow \text{Cit}$	$\text{abcd} + \text{ef} \rightarrow \text{dcbfea}$
2	$\text{Cit} \rightarrow \text{AKG} + \text{CO}_2$	$\text{abcdef} \rightarrow \text{abcde} + \text{f}$
3	$\text{AKG} \rightarrow \text{Glu}$	$\text{abcde} \rightarrow \text{abcde}$
4	$\text{AKG} \rightarrow \text{Suc} + \text{CO}_2$	$\text{abcde} \rightarrow \frac{1}{2} \text{bcde} + \frac{1}{2} \text{edcb} + \text{a}$
5	$\text{Suc} \rightarrow \text{Fum}$	$\frac{1}{2} \text{abcd} + \frac{1}{2} \text{dcba} \rightarrow \frac{1}{2} \text{abcd} + \frac{1}{2} \text{dcba}$
6	$\text{Fum} \rightarrow \text{OAC}$	$\frac{1}{2} \text{abcd} + \frac{1}{2} \text{dcba} \rightarrow \text{abcd}$
7	$\text{OAC} \rightarrow \text{Fum}$	$\text{abcd} \rightarrow \frac{1}{2} \text{abcd} + \frac{1}{2} \text{dcba}$
8	$\text{Asp} \rightarrow \text{OAC}$	$\text{abcd} \rightarrow \text{abcd}$