



HHS Public Access

Author manuscript

Metab Eng. Author manuscript; available in PMC 2019 April 19.

Published in final edited form as:

Metab Eng. 2013 March ; 16: 21–32. doi:10.1016/j.ymben.2012.11.010.

Parallel labeling experiments and metabolic flux analysis: past, present and future methodologies

Scott B. Crown and Maciek R. Antoniewicz

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

Abstract

Radioactive and stable isotopes have been applied for decades to elucidate metabolic pathways and quantify carbon flow in cellular systems using mass and isotope balancing approaches. Isotope-labeling experiments can be conducted as a single tracer experiment, or as parallel labeling experiments. In the latter case, several experiments are performed under identical conditions except for the choice of substrate labeling. In this review, we highlight robust approaches for probing metabolism and addressing metabolically related questions through parallel labeling experiments. In the first part, we provide a brief historical perspective on parallel labeling experiments, from the early metabolic studies when radioisotopes were predominant to present-day applications based on stable-isotopes. We also elaborate on important technical and theoretical advances that have facilitated the transition from radioisotopes to stable-isotopes. In the second part of the review, we focus on parallel labeling experiments for ^{13}C -metabolic flux analysis (^{13}C -MFA). Parallel experiments offer several advantages that include: tailoring experiments to resolve specific fluxes with high precision; reducing the length of labeling experiments by introducing multiple entry-points of isotopes; validating biochemical network models; and improving the performance of ^{13}C -MFA in systems where the number of measurements is limited. We conclude by discussing some challenges facing the use of parallel labeling experiments for ^{13}C -MFA and highlight the need to address issues related to biological variability, data integration, and rational tracer selection.

Keywords

Metabolism; isotopic tracers; stable isotopes; metabolic network model; tracer experiment design

1. INTRODUCTION

Cellular metabolism provides cells with the necessary precursors, energy (ATP) and electrons (NADH, NADPH) needed for cell growth, maintenance, and other essential cellular functions. The nature of metabolic pathways and their central role in cellular life have been the focus of research since the early 20th century (Kohler, 1977). Much of the initial work focused on elucidating the structure of metabolic pathways, whereas recent

[§]Corresponding author: Maciek R. Antoniewicz, Department of Chemical and Biomolecular Engineering, University of Delaware, 150 Academy St, Newark, DE 19716, Tel.: 302-831-8960, Fax.: 302-831-1048, mranon@udel.edu.

work addressed quantitative analysis of mass flow in metabolic systems through metabolic flux analysis (MFA) (Leighty and Antoniewicz, 2011; Toya et al., 2011). The research has contributed to important advances in a wide spectrum of applications, including understanding disease phenotypes (e.g. diabetes, cancer) (DeBerardinis et al., 2008; Jensen et al., 2008; Kelleher, 2001; Kelleher, 2004); development of noninvasive methods for probing metabolism using blood and urine analysis (Burgess et al., 2003; Yang et al., 1993; Yang et al., 1996); and reengineering cellular phenotypes to produce valuable products from renewable resources through metabolic engineering (Becker and Wittmann, 2012; Boghigian et al., 2010; de Jong et al., 2012; Iwatani et al., 2008; Lee et al., 2012; Niklas et al., 2010).

Central to the study of metabolic pathways, both in the 20th and 21st century is the use of isotopic tracers (Bier, 1987; Klein and Heinzle, 2012; Matwiyoff and Ott, 1973). An isotopic tracer is a molecule in which a specific atom, or atoms, has been replaced with a different isotope. The isotope can be either radioactive (e.g. ^{14}C , ^3H), or stable (e.g. ^{13}C , ^2H , ^{18}O , ^{15}N). Isotopic tracers have been applied for a variety of purposes in metabolic pathway analysis. A straightforward application of isotopic tracers is to probe whether a given pathway exists and how active it is, for example, by measuring the accumulation of isotopic labeling in a metabolic end-product of the pathway. Tracers can also provide insights into the structure of pathways, stereochemistry of enzymatic reactions, presence of converging pathways, and relative activity of competing pathways (Crown et al., 2011; de Graaf and Venema, 2008; Kruger et al., 2012; Quek et al., 2010).

Whether a tracer experiment is conducted with radioactive or stable isotopes, the experimental process shares a number of key steps. First, an isotopic tracer is introduced to the biological system, which can be as simple as a single organism culture, e.g. *E. coli* batch culture, or as complex as a human patient. Depending on the organism investigated, the tracer experiment may be performed *in vitro*, as in the perfusion of tissues isolated from animals; or *in vivo*, as in the direct incorporation of tracers into a complete functioning organism. Secondly, sufficient incubation time is allowed such that incorporation of the tracer into cellular metabolism can proceed. The third step is the isolation of the labeled metabolites. In the case of microbes and cultured cells, intracellular and extracellular metabolites can be obtained by extraction (Garcia et al., 2008). For *in vivo* studies (e.g. with humans), noninvasive methods must be used and this is commonly done through blood or urine analyses (Burgess et al., 2003; Yang et al., 1993; Yang et al., 1996). The next step is the measurement of labeling that has been incorporated into the metabolites of interest. In the case of radioactive tracers, the total radioactivity of a metabolite is assayed, or in some cases, the specific activity of metabolite carbons (^{14}C) or hydrogens (^3H) (Wolfe, 2004). For stable-isotope tracers, the incorporation can be detected using techniques such as nuclear magnetic resonance (NMR) (Schleucher et al., 1998; Szyperski, 1995), mass spectrometry (MS) (Antoniewicz et al., 2007a; Antoniewicz et al., 2011), and tandem mass spectrometry (MS/MS) (Antoniewicz, 2013; Choi et al., 2012; Jeffrey et al., 2002). The final step involves interpretation of the enrichment data, which can be purely qualitative, such as whether certain metabolites incorporate tracer atoms, or more quantitative, such as model-based analysis.

Isotopic experiments can be divided into two major categories: i) single labeling experiments, and ii) parallel labeling experiments (Fig. 1). For a single labeling experiment design, only one experiment is conducted, which can involve the use of a single labeled substrate (e.g. [1,2-¹³C]glucose), a mixture of tracers of the same compound (e.g. a mixture of [1-¹³C]glucose and [U-¹³C]glucose), or multiple labeled substrates (e.g. [U-¹³C]glucose and [U-¹³C]glutamine). For a parallel labeling experiment design, two or more tracer experiments are conducted in parallel. In each experiment a different tracer set is used. As shown in Fig. 2, parallel labeling experiments can be performed in a multitude of configurations. Typically, parallel experiments are started from the same seed culture to minimize biological variability (Ahn and Antoniewicz, 2013; Antoniewicz, 2006).

The goal of this review article is to highlight parallel labeling approaches for probing metabolism and addressing metabolically related questions. We are particularly interested in these studies because of the synergy of complementary information that can be obtained through the use of parallel labeling experiments. In the first part of the review, we provide a brief historical perspective. We discuss research from early 20th century when mainly radioisotopes were used, and then highlight major technical and computational advances that ushered in the use of stable-isotopes as the predominant technique for probing cellular metabolism. In the second part, we discuss the use of parallel labeling experiments in the context of metabolic flux analysis, focusing mainly on ¹³C-MFA. We discuss the advantages of using parallel labeling experiments in comparison to a single tracer experiment design, and describe methodologies that can be used for optimal selection of isotopic tracers. Finally, we highlight recent advances in integrative flux analysis of parallel labeling experiments, and conclude by discussing potential challenges and limitations of parallel labeling experiments for ¹³C-MFA.

2. HISTORICAL PERSPECTIVE: FROM RADIOISOTOPES TO STABLE-ISOTOPES

The use of isotopic tracers to study cellular metabolism has its roots in the 1930s. The seminal studies by Schoenheimer and Rittenberg marked the beginning of modern biochemistry (Kohler, 1977; Schoenheimer and Rittenberg, 1938; Schoenheimer and Rittenberg, 1940). Radioisotopes (e.g. ³H, ¹⁴C) were used intensively to catalog many of the metabolic pathways that are well-known today (Wolfe, 1984; Wolfe, 2004). Applications of radioactive tracers predominated metabolism research until the 1980s, when stable-isotopes became a feasible alternative due to advances in NMR and MS technologies and computational methods (Bier, 1987). Presently, stable-isotope experiments account for the majority of metabolic and flux studies. There are several practical benefits of using stable-isotopes, including the lack of radioactive emission and the ease of detection by MS and NMR (Des Rosiers et al., 2004). In the first part of this section, we highlight the use of radioisotope experiments for metabolism research. Next, we outline major technological and theoretical advances that allowed stable-isotopes to come to the forefront. Finally, we elaborate on the important role that stable-isotopes currently play for advancing our knowledge of metabolism, with a focus on parallel labeling experiments.

2.1. Radioisotopes and parallel labeling experiments

The value of using radioisotopes to study metabolism was first recognized by Hevesy who performed the first biological isotope-tracer studies (Hevesy, 1923). Because of the simplicity of measuring radioisotopes by scintillation counting and the relative ease of their production, i.e., relative to the challenges encountered in concentrating stable-isotopes in the early 20th century, radioactive isotopes were applied intensively in the decades that followed for biological research. A typical radioisotope experiment involved dosing cells with a small amount of a radioactive substrate and measuring the radioactivity in cellular metabolites and end-products. Activity measurements were often restricted to metabolites that were abundant in the cell and were easily isolated from other cellular components, such as glucose, lipid glycerol, fatty acids, lactate, and CO₂ (Jeffrey et al., 1991). Radioactivity was generally measured as total activity for the entire molecule, although in some cases the activity of individual carbon atoms or hydrogen atoms could be obtained as well, e.g. after chemical or enzymatic degradation of metabolites.

Radioisotope experiments have commonly been conducted with two or more isotopic tracers in parallel experiments, where each radioactive tracer specifically targeted a particular pathway. Differences in the radioactivity between experiments provided insight into a pathway's structure and activity. One such application, for example, addressed how pentose sugar phosphates were assembled into hexose phosphates. Horecker et al. (Horecker et al., 1954) applied [1-¹⁴C]ribose-5-phosphate and [2,3-¹⁴C]pentose-5-phosphates to rat liver tissues and measured specific activities of hexose-6-phosphate carbons, eventually elucidating the structure of the non-oxidative pentose phosphate pathway. Parallel radioisotope experiments were also applied to determine the structure, precursors and activity of other pathways in central and peripheral metabolism, including pentose phosphate pathway (Katz and Wood, 1960; Katz and Wood, 1963; Wang et al., 1956), gluconeogenesis (Hostetler et al., 1969; Mullhofer et al., 1977; White et al., 1968), anaplerosis (D'Adamo and Haft, 1965; Landau et al., 1960), fatty acid metabolism (Antony and Landau, 1968; Brown et al., 1956; Kam et al., 1978; Robinson and Williamson, 1977), and mevalonate shunt (Brady et al., 1982b; Edmond and Popjak, 1974; Weinstock et al., 1984). In addition, parallel experiments were applied to challenge assumptions regarding equilibration of hexose-6-phosphate pools (Katz and Rognstad, 1969; Landau and Bartsch, 1966; Landau et al., 1964), to investigate the magnitude of exchange fluxes (Katz et al., 1966; Landau and Bartsch, 1966), and examine CO₂ isotopic equilibration and compartmentation (Marsolaïs et al., 1987). In addition to parallel experiments, different isotope-types (e.g. ³H, ¹⁴C-labeled glucose) were sometimes used, for example, to determine hydrogen exchange by isomerization reactions (Katz and Rognstad, 1966; Katz and Rognstad, 1969).

For quantitative studies of metabolism, empirical equations relating specific fluxes, e.g. fluxes at specific branch points, to activity measurements or ¹⁴CO₂ yields were derived using mass balances. The oxidative pentose phosphate pathway (oxPPP) and pyruvate carboxylase/pyruvate dehydrogenase (PC/PDH) fluxes have been among the most heavily investigated. As an example, triose-phosphate activities and ¹⁴CO₂ yields measured from parallel experiments with [1-¹⁴C]glucose or [6-¹⁴C]glucose were used to estimate the oxPPP flux (Katz and Wood, 1960; Katz and Wood, 1963; Landau et al., 1964). Similarly, ¹⁴CO₂

yields have been applied to parallel experiments with [1-¹⁴C] + [2-¹⁴C]acetate (Goebel et al., 1982; Kelleher, 1985; Strisower et al., 1952; Weinman et al., 1957), or [1,4-¹⁴C] + [2,3-¹⁴C]succinate (Kelleher and Bryan, 1985) to determine the flux split between PC and PDH reactions. In other studies, parallel experiments were used to relate fluxes in subnetworks to specific activities of metabolites such as glucose, lipid glycerol, lactate, glutamate, and ¹⁴CO₂. As an example, Rognstad and Katz (Rognstad and Katz, 1972) utilized [2-¹⁴C] + [3-¹⁴C]pyruvate/lactate to estimate gluconeogenic and TCA cycle fluxes in kidney slides; and Borowitz et al. (Borowitz et al., 1977) used a variety of glucose, fructose, ribose, and glycerol tracers to determine changes in the glycolytic flux in *Tetrahymena*.

Throughout the decades, radioisotope experiments have significantly contributed to the overall understanding of metabolism (Table 1). However, radioisotope experiments also suffer from several key limitations (Jeffrey et al., 1991). First, there is the practical limitation on the number of measurements that can be obtained from these experiments; and second, elaborate metabolite isolation protocols are often needed in these studies. As a consequence, radioactive experiments often rely on a few abundant and easily measured metabolites for analysis of metabolism. In addition, computational interpretation of data often requires numerous assumptions, some of which are known to be poor approximations, e.g. the assumption that ¹⁴CO₂ is only evolved from reactions in the TCA cycle; or that complete isotopic equilibrium exists between certain metabolites in glycolysis and other pathways. It is also important to note that many models relating specific activities to fluxes involved equations that included empirical parameters, some of which were not rigorously derived. As a result, the information obtained from radioactive experiments was confined to estimating only a handful of fluxes, rather than gaining a comprehensive understanding of metabolism. Despite these caveats, radioisotope experiments are still used presently for a variety of purposes, including the measurement of substrate uptake and product excretion rates (Allen et al., 2009; Alonso et al., 2011), and investigation of *in vivo* glucose and lipid dynamics (Jensen et al., 2001a; Jensen et al., 2001b; Previs et al., 2000).

2.2. Technological advances allowing widespread use of stable-isotopes

To quantify metabolism on a more global level than could be done with radioisotope studies, metabolic flux analysis (MFA) was developed based on stable-isotope experiments (Vallino and Stephanopoulos, 1993; Zupke and Stephanopoulos, 1995). The transition from radioisotopes to stable-isotopes was possible in a large part due to major advances in stable-isotope measurement techniques, including NMR and MS, and breakthroughs in computational capabilities and modeling approaches (Fig. 3). In the 1980s, stable-isotope techniques rapidly replaced radioisotope approaches as they were easier to conduct, allowed multiple measurements of isotopic enrichment from a single experiment, were safer to perform, and provided more informative data for flux determination (Bier, 1987; Matthews and Bier, 1983). In addition, several studies compared results from radioisotope and stable-isotope experiments and found only negligible differences between radioactivity assays and ¹³C-NMR (Cohen et al., 1981) and MS results (Strong et al., 1983).

For stable-isotope studies, quantification of individual positional isotopomers is ideally desired (Christensen and Nielsen, 1999). A positional isotopomer is defined as a molecule with a specific labeling pattern of its atoms. For a metabolite with n atoms, there are 2^n possible positional isotopomers. Knowledge of the complete positional isotopomer distribution provides the complete labeling state of a metabolite. Early NMR techniques, such as ^1H -NMR and ^{13}C -NMR, could not resolve all positional isotopomers (Chance et al., 1983; Cohen, 1983; Malloy et al., 1988; Walker et al., 1982). Proton NMR (^1H -NMR) only provided information regarding the fractional enrichments of carbons, while ^{13}C -NMR measured multiplet structures that contained only partial labeling information. With the introduction of 2D- ^{13}C , ^1H -COSY NMR (Szyperski, 1995), more detailed positional isotopomer information could be obtained; however, elucidation of the complete isotopomer distribution of molecules remains a challenge even today.

In the case of MS, multiple fragments of a molecule must be measured to obtain positional labeling information. As an example, with GC-MS analysis of multiple fragments it is possible to determine the complete positional isotopomer distribution of a few small molecules (Dauner and Sauer, 2000). In practice, MS methods measure mass isotopomers, which are isotopomers of molecules with the same mass-to-charge ratio (m/z). Through analysis of multiple MS fragments for a given metabolite the amount of labeling information can be increased. For example, Di Donato et al. (Di Donato et al., 1993) was able to obtain 24 of 32 positional isotopomers of glutamate, and Katz et al. (Katz et al., 1993) had similar success with other metabolites. With the introduction of tandem mass spectrometry, measuring complete positional isotopomer distributions is possible for larger molecules, as was recently demonstrated by Choi et al. for aspartate (Choi and Antoniewicz, 2011; Choi et al., 2012).

3. CURRENT APPROACHES BASED ON STABLE-ISOTOPES

3.1. Pathway structure elucidation

Stable isotopes, like their radioactive counterparts, have played an important role in elucidating metabolism. In many cases, parallel labeling experiments have provided valuable information to elucidate the structure and function of metabolic pathways (Table 2). With careful selection of isotopic tracers and quantitative isotopomer measurements, the activity of pathways can be determined and inconsistencies in a hypothesized network can be identified. Stable-isotope techniques have been applied to a variety of biological systems, for example, to quantify glucose metabolism via Embden-Meyerhof-Parnas (EMP), Entner Doudoroff (ED), and oxPPP (Blank et al., 2005; Fuhrer et al., 2005); to validate amino acid biosynthesis pathways, especially for organisms where multiple alternative routes are known to exist (Feng et al., 2009; Tang et al., 2010; Tang et al., 2009); to elucidate the exact stereochemistry of enzymatic reactions, e.g. *Re*- vs. *Si*-citrate synthase (Amador-Noguez et al., 2010; Feng et al., 2009; Tang et al., 2009); and to demonstrate bifurcation of TCA cycle in several microbes (Amador-Noguez et al., 2010; Olszewski et al., 2010). Through careful selection of isotopic tracers, several studies have also provided valuable information on the reversibility of reactions. For example, ^{13}C -glutamate tracers were used by various investigators to quantify the exchange flux of isocitrate dehydrogenase and reductive

carboxylation flux in the TCA cycle (Des Rosiers et al., 1994; Metallo et al., 2012; Yoo et al., 2004). Lastly, parallel tracer investigations have been applied to determine the structure of metabolic pathways outside of central metabolism, including the biosynthesis of isoprenoids (Schwender et al., 1996), polyketides (Simpson, 1998), polyalkanoates (Anderson and Dawes, 1990), and shikimate metabolites (Dewick, 1995).

3.2. Empirical models and multiple isotopes

Empirical models relating NMR and MS data to carbon fluxes were popular in the 1980s and 1990s for data analysis, i.e. before the emergence of ^{13}C -MFA. These models were often extensions of previously developed methods for analysis of radioisotope labeling studies. The models were often limited to a handful of measurements, such as NMR fine structures of glutamate, mass isotopomer distributions of TCA cycle intermediates, and deuterium enrichments of glucose; and were often based on many simplifying assumptions to make the calculations manageable. Although relatively simple compared to current ^{13}C -MFA approaches, empirical modeling efforts led to several useful applications, for example, to quantify the relative utilization of competing substrates, measure fatty acid oxidation, study hepatic glucose production, quantify TCA cycle fluxes, and determine anaplerotic fluxes with a variety of pyruvate, acetate, lactate, glucose, glutamine and fatty acid tracers (Comte et al., 1997a; Comte et al., 1997b; Jones et al., 1993; Malloy et al., 1988; Malloy et al., 1990a; Malloy et al., 1990b; Sherry et al., 1988).

Since parallel labeling experiments are infeasible for studies involving living subjects, labeling experiments were also conducted using simultaneous infusions of multiple tracers and different isotopes. For example, to investigate glucose production in humans a tracer bolus of [1,6- ^{13}C]glucose, $^2\text{H}_2\text{O}$, and [U- ^{13}C]propionate tracers had been used by Jones et al. (Jones et al., 2001), as well as several other combinations of tracers and isotopes (Chandramouli et al., 1997; Chevalier et al., 2006; Jin et al., 2005). Similarly, whole-body energy balance was routinely investigated using a doubly-labeled water approach, in which $^2\text{H}_2\text{O}$ and H_2^{18}O were simultaneously injected into living subjects as a bolus and the differences in elimination rates of ^2H and ^{18}O from body water were monitored in time (Bederman et al., 2006; Brunengraber et al., 2002).

3.3. Emergence of ^{13}C -metabolic flux analysis

Currently, ^{13}C -MFA studies are the preferred method for obtaining detailed metabolic fluxes in microbial, mammalian and plant systems (Iwatani et al., 2008; Kruger et al., 2012; Quek et al., 2010). Current approaches are based on rigorous isotopomer balancing techniques that were established in mid-1990s (Schmidt et al., 1997; Zupke and Stephanopoulos, 1994). In the past decade, quantitative evaluation of metabolic fluxes using ^{13}C -MFA has become a key activity in many research fields, including in metabolic engineering, systems biology and biomedical investigations (Kelleher, 2001; Moxley et al., 2009). The emergence of ^{13}C -MFA as the preferred analysis method was spearheaded by the development of advanced computational approaches to comprehensively simulate all isotopomers in network models (see Fig. 3). With the isotopomer framework in place (Schmidt et al., 1997), fluxes could be finally determined for large-scale systems that encompassed most of central metabolism, including reversible reactions, as well as amino acids metabolism and fatty acid

biosynthesis. The widespread use of ^{13}C -MFA studies was also fueled by the development of easy-to-use programs for ^{13}C -MFA such as 13CFLUX (Wiechert et al., 2001), OpenFlux (Quek et al., 2009), and Metran (Yoo et al., 2008).

The development of metabolic flux ratio analysis (Metafor) offered an alternative method to analyze mass spectrometry data and quantify fluxes, including from parallel labeling experiments (Fischer and Sauer, 2003). Metafor analysis essentially decouples amino acid mass isotopomers into the mass isotopomers of precursor metabolites, which are then used to determine flux ratios at specific convergence points in metabolism. Commonly, Metafor is conducted with parallel experiments using $[1-^{13}\text{C}]$ glucose and a mixture of $[U-^{13}\text{C}]$ /natural glucose (Fischer and Sauer, 2003; Fischer et al., 2004). Compared to ^{13}C -MFA approaches, however, Metafor analysis has several drawbacks. First, the method relies on manually derived expressions that are specific to certain network models and tracers, which significantly limits which biological systems can be investigated. In addition, because not all MS and NMR data can be utilized for flux analysis, fluxes are determined with the same precision as with rigorous ^{13}C -MFA methods.

4. PARALLEL LABELING EXPERIMENTS

In the past few years, the use of parallel labeling experiments (PLE's) for ^{13}C -MFA has increased significantly (Table 2). The main advantage of PLE's is the potential to generate complementary labeling information that can dramatically improve the resolution of fluxes in complex systems (Allen et al., 2009; Alonso et al., 2010; Alonso et al., 2007; Leighty and Antoniewicz, 2012; Schwender et al., 2006). Comprehensive integration of data from PLE's is accomplished by concurrently fitting measurements from multiple experiments to a single flux model (Antoniewicz, 2006). This type of integrated ^{13}C -MFA approach returns, in the ideal case, a single set of fluxes that satisfies all isotopomer measurements from all parallel experiments.

4.1. Advantages of parallel labeling experiments

As we discussed in the previous sections, the use of PLE's is a well-established concept in metabolism research. Thus far, most studies have focused on semi-quantitative data analysis. Recently, however, several integrative methods were described for comprehensive data analysis and ^{13}C -MFA using parallel experiments (Antoniewicz, 2006; Leighty and Antoniewicz, 2012; Schwender et al., 2006). These developments represent a major step forward in ^{13}C -MFA field. In this section, we discuss several advantages of using PLE's for ^{13}C -MFA compared to a single-tracer-experiment approach: i) tailoring ^{13}C -experiments to resolve specific fluxes with high precision, ii) reducing the length of labeling experiments by introducing multiple entry-points of isotopes, iii) validating biochemical network models, and iv) improving performance of ^{13}C -MFA in systems where the number of measurements is limited.

4.2. Tailoring ^{13}C -experiments to resolve specific fluxes with high precision

One of the factors contributing to a poor resolution of fluxes when using a single tracer experiment is the structure of the network model itself. The network dictates the flow of

^{13}C -atoms throughout the system and the labeling patterns that can be produced. For many biological systems, however, it is difficult to select one tracer that allows good flux observability for the entire network. PLE's can offer an alternative solution to this problem.

It has been well known that for realistic network models there is rarely one optimal tracer that can simultaneously resolve all key fluxes with high precision (Crown et al., 2012; Metallo et al., 2009; Schellenberger et al., 2012). Studies have shown that different parts of central metabolism require different ^{13}C -tracers. As an example, Metallo et al. (Metallo et al., 2009) identified optimal glucose and glutamine tracers for subnetworks of the mammalian network model with GC-MS measurements. It was determined that [1,2- ^{13}C]glucose was optimal for flux estimation of glycolysis and pentose phosphate pathway fluxes, while [U- ^{13}C]glutamine was found to be ideal for TCA cycle fluxes. In a similar study, we recently optimized glucose tracers for estimating oxPPP and PC fluxes in mammalian cells with lactate as the only measured metabolite (Crown et al., 2012). We found that [2,3,4,5,6- ^{13}C]glucose was optimal for resolving oxPPP flux and [3,4- ^{13}C]glucose was optimal for estimating PC flux. In addition to resolving net fluxes in a model, resolution of exchange fluxes can often require very specific labeling of tracers (Follstad and Stephanopoulos, 1998; Metallo et al., 2009). Similarly, resolution of metabolic cycles in eukaryotic cells (e.g. cross-compartmental pyruvate cycling) is challenging when using only a single tracer experiment (Wahrheit et al., 2011; Zamboni, 2011).

In general, the PLE approach provides the researcher with more design flexibility. Separate experiments can be performed in parallel with carefully selected tracers to accurately quantify specific fluxes of interest. As an example, for the mammalian network model, two experiments could be run in parallel using the two optimal tracers identified by Metallo et al. (Metallo et al., 2009). The labeling data from each experiment could then be integrated to achieve improved flux resolution. In simulation studies we have found that when multiple ^{13}C -labeled substrates are used for ^{13}C -MFA, it is often more advantageous to run the experiments in parallel rather than using all tracers in a single experiment. For example, introducing [1,2- ^{13}C]glucose and [U- ^{13}C]glutamine simultaneously in a single experiment produces less informative labeling data than if two separate experiments are performed with [1,2- ^{13}C]glucose and [U- ^{13}C]glutamine independently.

We believe that there are two major factors contributing to improved flux precision for parallel experiments. First, the increased number of measurements (i.e. twice the number of measurements for two PLE's compared to a single tracer experiment) can result in an improvement of the flux precision. Second, and perhaps more importantly, parallel experiments provide complementary information for each other, which may aid in improved flux precision. For example, two tracers may provide poor flux precision when used in single tracer experiments, but may excel when analyzed in a parallel experimental design. The resulting improvement or "tracer synergy" distinguishes parallel labeling experiments from single tracer experiments; however, presently these synergistic effects are not well understood and should be further investigated.

4.3. Reducing length of labeling experiments by introducing multiple entry-points of isotopes

Another application of PLE's is to reduce the labeling time needed to achieve isotopic steady-state, which can greatly simplify the modeling work needed for ^{13}C -MFA. As an example, in mammalian cells isotopic steady-state is often not obtained for all relevant metabolites in a practical time frame (Ahn and Antoniewicz, 2011). Although it is possible to apply isotopic non-stationary ^{13}C -MFA to calculate fluxes, this is still a challenging approach that requires significant experimental and computational effort (Young et al., 2008). As a result, isotopic non-stationary ^{13}C -MFA is still not widely used in the metabolic engineering community. An alternative PLE approach would consist of conducting experiments in parallel with carefully selected entry-points for ^{13}C -tracers such that isotopic steady state can be achieved quickly for the relevant metabolites. We have found, for example, that in CHO cells glycolysis and pentose phosphate pathway metabolites rapidly approach isotopic steady-state when glucose tracers are used, and very fast labeling dynamics are observed for TCA metabolites when glutamine tracers are used (Ahn and Antoniewicz, 2013). Using the PLE strategy, two experiments can be run in parallel with glucose and glutamine tracers, thus allowing for isotopic steady-state to be reached for all relevant metabolites within a short time frame. Using this approach, metabolic fluxes can then be calculated using stationary ^{13}C -MFA methods instead of isotopic non-stationary ^{13}C -MFA (Ahn and Antoniewicz, 2013).

4.4. Validating biochemical network models

A central premise of ^{13}C -MFA is the assumption that the network model used for flux calculations is complete, i.e., that the model accounts for all significant enzymatic and transport reactions occurring in the biological system. In the current post-genomic era, experimental validation of metabolic network models is an increasingly important activity, as genome-scale models are constructed for newly sequenced organisms at an ever increasing pace, including for many poorly characterized microbes (Kim et al., 2012). These automatically generated models are likely to contain many errors, e.g. missing reactions and incorrect annotations. The PLE methodology is ideally suited for quantitative dissection of complex metabolic systems and may be especially instrumental for validating metabolic network models and quantifying metabolic phenotypes for less well studied organisms. We believe that PLE's can have a significant impact on improving basic biology understanding in the coming years.

A key advantage of PLE's for model validation is that these experiments provide significantly more independent measurements that place more stringent constraints on the network model assumptions. A recent study from our group demonstrates the utility of PLE's for model validation. Here, *E. coli* was grown in parallel cultures on varying mixtures of natural glucose and $[\text{U-}^{13}\text{C}]$ glucose (0, 20, 40, 60, 80, 100%) (Leighty and Antoniewicz, 2012). A common assumption when performing ^{13}C -MFA in microbial systems is that CO_2 incorporated via carboxylation reactions has the same labeling as CO_2 that is generated inside the cells via decarboxylation reactions. However, we demonstrated that ^{13}C -MFA produced inconsistent flux results with this common assumption. On the other hand, an updated model that accounted for external CO_2 incorporation into central metabolism, i.e.

air naturally contains about 0.04% unlabeled CO₂, produced statistically acceptable fits for parallel labeling experiments with good flux agreement.

4.5. Improving performance of ¹³C-MFA in systems where the number of measurements is limited

PLE's also offer significant advantages for resolving fluxes in systems where the number of measurements is low due to limitations of the measurement technique, or due to limited number of accessible metabolites. In these circumstances the number of free fluxes may be equal or greater than the number of independent measurements, and the system may be poorly resolved, or not observable at all, when using a single tracer experiment strategy. In these cases, PLE's can be used to increase the number of independent measurements. As such, flux resolution can be improved and biological systems that were previously poorly determined, or non-observable, may become fully observable. The application of PLE's for increasing the number of redundant measurements in poorly resolved systems has been demonstrated already in several studies.

For example, PLE's were used to determine fluxes in *Corynebacteria* from measured mass isotopomers of extracellular products (Kiefer et al., 2004; Wittmann and Heinzle, 2001a; Wittmann and Heinzle, 2002; Wittmann et al., 2004). Given that a single experiment did not provide enough constraints to calculate all fluxes, parallel experiments with [1-¹³C]glucose and a mixture of [U-¹³C]/natural glucose were conducted. PLE's were also conducted with ¹³C-glucose and ¹³C-glutamine tracers to determine fluxes in mammalian cells using mass isotopomer measurements of secreted lactate as the only measured metabolite (Henry and Durocher, 2011; Niklas et al., 2011; Strigun et al., 2012). As a third example, Yang et al. (Yang et al., 2006) proposed a ¹³C-respirometry flux analysis methodology to analyze the metabolism of *Corynebacterium glutamicum* using only ¹³C-enrichment data from evolved CO₂. To make the system observable, three experiments were conducted in parallel, with [1-¹³C]glucose, [6-¹³C]glucose and [1,6-¹³C]glucose.

5. FUTURE OUTLOOK

5.1. Selection of ¹³C-tracers for parallel labeling experiments

Parallel labeling experiments require careful selection of tracers to take full advantage of the additional experimental effort required. The significance of a judicious selection of tracers for flux analysis has been known since the early years of ¹³C-MFA, i.e. even for single tracer experiments (Follstad and Stephanopoulos, 1998; Mollney et al., 1999; Wittmann and Heinzle, 2001b). The choice of the isotopic tracer determines which isotopomers of a metabolite can be formed in a network and the sensitivities of isotopic measurements with respect to flux changes, which are directly related to the precision with which fluxes can be determined. Despite the importance of proper tracer selection, this step is still often overlooked or given very little thought.

One of the predominant methods for tracer selection and experiment design is based on a grid-search type of approach. The process involves calculating the parameter covariance matrix for various isotopic tracers of interest. For these calculations an assumed

measurement set, a set of fluxes, and an assumed network model are needed. To compare between different tracers and determine which tracer is optimal for a given system, the D-optimality criterion is commonly used (Mollney et al., 1999). The D-optimality criterion is related to the covariance matrix of the free fluxes and provides a measure of single parameter confidence intervals and correlations between the estimated parameters. A relative information score for each tracer is then determined from the D-optimality criterion, given an assumed reference tracer experiment. The tracer scheme that produces the highest information score is then selected as the optimal tracer (Arauzo-Bravo and Shimizu, 2003; Noh and Wiechert, 2006; Yang et al., 2006).

The use of the covariance matrix for tracer experiment design relies on the inherent assumption that the underlying non-linear isotopomer balances can be approximated by linearization near the optimal solution. Metallo et al. (Metallo et al., 2009) introduced another method using a precision scoring system, which captures the true nonlinearity behavior of flux analysis systems. The precision scoring method requires the calculation of nonlinear confidence intervals for free fluxes (Antoniewicz et al., 2006). The method computes a score for each flux, based on the upper and lower bounds for the confidence intervals, and using a flux weighting parameter. If a flux has a score of zero, it is unidentifiable; if the score is one, the flux is optimally identifiable. The precision score is the sum of the scores for each flux. Similar to the grid-search method, precision scores are compared for various tracers of interest and the tracer that has the highest score is selected as optimal. Walther et al. (Walther et al., 2012) recently proposed a genetic algorithm for tracer design that takes advantage of the precision scoring system in the evaluation of optimal tracers.

Recently, we introduced an alternative approach for tracer selection, which does not rely on trial-and-error strategies for identifying optimal tracers (Crown et al., 2012; Crown and Antoniewicz, 2012). The framework is based on the elementary metabolite units (EMU) framework developed by Antoniewicz et al. (Antoniewicz et al., 2007b). We have demonstrated that every metabolite in a network can be decomposed into a linear combination of so-called EMU basis vectors (EMU-BV), where each EMU-BV represents a unique way of assembling substrate EMU's into the measured metabolites. The corresponding coefficients quantify the weights of each EMU-BV to the measurement. The methodology decouples the dependencies between isotopic labeling (i.e. EMU-BV's) and free fluxes (i.e. coefficients) in a network model. To select optimal tracers, we conducted sensitivity analyses on the EMU-BV coefficients with respect to free fluxes. Through efficient grouping of coefficient sensitivities we were able to propose rational selection criteria in a number of example systems. Using this methodology we were able to identify novel tracers that drastically improved flux resolution in comparison to previous studies (Henry and Durocher, 2011). We believe that the EMU-BV method can be valuable for selecting optimal tracers for PLE's. For example, applying this method in parallel for key fluxes of interest in a model can allow optimal tracers to be tailored for different sections of the system. In the end, detailed flux maps can be determined with high precision by integrating the labeling data from the individual parallel experiments.

5.2. Potential disadvantages of parallel labeling experiments

Despite the many benefits of PLE's, there are also some potential drawbacks as compared to single tracer experiments. The first and most transparent caveat is the increased amount of time and resources required to conduct PLE's, given that two or more experiments must be performed in parallel. The total number of parallel experiments will depend on the complexity of the system and the required flux precision, but it is reasonable to expect anywhere from 2 to 6 experiments in parallel. With each additional experiment the cost of the study increases. Furthermore, the amount of time and resources required for sampling, labeling measurements, and data processing also increases with each additional experiment. Ultimately, it is at the researcher's discretion to decide as to whether the increased flux resolution is worth the additional cost, time and resources required as compared to a single tracer experiment.

Another potential disadvantage of PLE's is related to inconsistencies that may be observed due to biological variability. Before addressing this aspect, it is important to understand the sources of error in a typical ^{13}C -MFA experiment. In general, the majority of random errors result from metabolite concentration measurements (e.g. HPLC data for calculating external rates) and ^{13}C -labeling measurements (e.g. GC-MS, NMR data). In addition, systematic errors may also be present due to the specific analytical and sampling techniques used, e.g. for culture sampling, cell harvesting, extraction of the metabolites, sample pre-treatment, etc. The flux analysis process inherently assumes that the largest source of error is due to random measurement errors. When experiments are run in parallel, these sources of error are still present; however, there is the additional concern of biological variability. The key question is: is the relative contribution of biological variability to the total error small or large? In cases of simple systems, such as microbial batch or continuous cultures, this may not be a major concern as these experiments are highly reproducible (Leighty and Antoniewicz, 2012). It is expected that the random errors from measurements will remain the dominant source of error. However, for more complex systems, such as mammalian cultures and extended fed-batch fermentations (Ahn and Antoniewicz, 2012; Antoniewicz et al., 2007c), errors due to biological variability may not be negligible. If the experiment-to-experiment errors become the largest contributor to the total error, the benefits of running parallel labeling experiments may be negated. The last potential caveat of PLE's is whether current ^{13}C -MFA software packages are capable of successfully integrating ^{13}C -labeling data from multiple experiments for comprehensive flux analysis. Without such techniques in place, analyzing and interpreting data from PLE's may be difficult for laboratories that do not have in-house experience.

Overall, we do not see the disadvantages raised in this section as damning to the PLE methodology. It is important, however, that researchers are aware of these limitations so that proper means of action can be taken to attenuate the potential impact. The increased time and resources required for parallel experiments place an impetus on the development of efficient analytical procedures and cost-effective ^{13}C -tracer selections. In the end, the quality of information accrued from PLE's is expected to outweigh the cost-benefit compared to a single tracer experiment. Studies focusing on optimizing cell culture performance can also mitigate the effects of biological variability. Lastly, we believe that software limitations for

analysis of PLE's are only a short-term concern, since new algorithms for PLE's can be relatively easily incorporated into existing programs to allow rigorous and integrated analysis of multiple data sets.

5.3. Future directions

There are several aspects of PLE's that require additional attention going forward. As we described in this review, when conducting tracer experiments the researcher has control over the choice of isotopic tracers and the measurement set. Unfortunately, there is still little understanding regarding the interplay between the choice of tracer and measurement set. Currently, methods are available where, given a measurement, an optimal tracer can be determined for a set of fluxes of interest (Crown et al., 2012). Similarly, given a tracer, the minimal measurements set can also be determined (Chang et al., 2008). However, the question regarding which tracers and which measurements are optimal for a given network is still considered in isolation, rather than in conjunction with each other. In the case of PLE's, another level of complexity is added, as the investigator can also control how many experiments are performed in parallel. The interconnections and relationships between the tracer selection, measurement choice, and number of parallel experiments is an important question to address. For example, consider a hypothetical case where several experiments are conducted in parallel, but the flux resolution still needs to be improved. What is the appropriate course of action: should more isotopic measurements be collected? Should an additional experiment be performed in parallel? Should one of the experiments involve a different tracer? Similarly, is it better to have fewer experiments in parallel with plentiful ^{13}C -enrichment measurements, or is it better to have many experiments with more scarce labeling data? These questions highlight the importance of a rational decision-making process when approaching PLE design. Currently, this thought process is still underdeveloped.

To address these issues going forward, an emphasis on efficient methods for optimal tracer selection is essential. Due to the number of variables involved in tracer experiment design, i.e. single or multiple tracer substrates, the composition of tracer mixtures, and number of parallel experiments, the number of possible experiment designs can increase exponentially. Current experiment design methodologies that focus on trial-and-error simulations to determine optimal tracers will be increasingly inefficient for PLE's due to the near-infinite number of possibilities. A rational and systematic method for approaching tracer selection is therefore needed as a precursor to simulation-based studies. The premise of a rational framework is to efficiently reduce the experiment design space to a feasible and tractable subset, which can then be further evaluated using simulation based approaches. Ideally, the methods should also account for the impact of measurement error and biological variability on the expected outcomes.

6. CONCLUSIONS

In this paper, we have reviewed the use of parallel labeling experiments for metabolism research, both in the context of a historical perspective and current applications in ^{13}C -MFA. Parallel labeling experiments offer significant advantages in comparison to traditional single

tracer experiments. However, there are also several challenges that must be addressed for its successful implementation. As we described above, we believe the advantages of parallel labeling experiments will ultimately outweigh the challenges that lie ahead. If anything, the challenges facing parallel labeling experiments should only reinforce the need for careful planning of experiments and development of improved analytical, experimental and computational approaches. Future studies should focus on addressing the issues outlined in this review, as well as identifying additional unforeseen difficulties. In the end, we believe that parallel labeling experiments are a powerful approach for probing metabolism with the potential to become the predominant technique used for high-resolution ^{13}C -MFA.

ABBREVIATIONS

ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas
EMU	elementary metabolite unit
EMU-BV	elementary metabolite unit basis vector
GC-MS	gas chromatography-mass spectrometry
MFA	metabolic flux analysis
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance
oxPPP	oxidative pentose phosphate pathway
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PLE	parallel labeling experiment

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, 2012 Towards dynamic metabolic flux analysis in CHO cell cultures. *Biotechnol J.* 7, 61–74. [PubMed: 22102428]
- Ahn WS, Antoniewicz MR, 2013 Parallel labeling experiments with [1,2- ^{13}C]glucose and [U- ^{13}C]glutamine provide new insights into CHO cell metabolism. *Metab Eng.* doi: 10.1016/j.ymben.2012.10.001.
- Allen DK, Ohlrogge JB, Shachar-Hill Y, 2009 The role of light in soybean seed filling metabolism. *Plant J.* 58, 220–34. [PubMed: 19077167]
- Alonso AP, Dale VL, Shachar-Hill Y, 2010 Understanding fatty acid synthesis in developing maize embryos using metabolic flux analysis. *Metab Eng.* 12, 488–97. [PubMed: 20406696]

- Alonso AP, Raymond P, Hernould M, Rondeau-Mouro C, de Graaf A, Chourey P, Lahaye M, Shachar-Hill Y, Rolin D, Dieuaide-Noubhani M, 2007 A metabolic flux analysis to study the role of sucrose synthase in the regulation of the carbon partitioning in central metabolism in maize root tips. *Metab Eng.* 9, 419–32. [PubMed: 17869563]
- Alonso AP, Val DL, Shachar-Hill Y, 2011 Central metabolic fluxes in the endosperm of developing maize seeds and their implications for metabolic engineering. *Metabolic Engineering.* 13, 96–107. [PubMed: 20969971]
- Amador-Noguez D, Feng XJ, Fan J, Roquet N, Rabitz H, Rabinowitz JD, 2010 Systems-level metabolic flux profiling elucidates a complete, bifurcated tricarboxylic acid cycle in *Clostridium acetobutylicum*. *J Bacteriol.* 192, 4452–61. [PubMed: 20622067]
- Anderson AJ, Dawes EA, 1990 Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiol Rev.* 54, 450–472. [PubMed: 2087222]
- Antoniewicz MR, Comprehensive analysis of metabolic pathways through the combined use of multiple isotopic tracers. Ph.D. Thesis. Massachusetts Institute of Technology, 2006.
- Antoniewicz MR, 2013 Tandem mass spectrometry for measuring stable-isotope labeling. *Curr Opin Biotechnol.* doi: 10.1016/j.copbio.2012.10.011.
- 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]
- Antony GJ, Landau BR, 1968 Relative contributions of alpha-, beta-, and omega-oxidative pathways to in vitro fatty acid oxidation in rat liver. *J Lipid Res.* 9, 267–9. [PubMed: 5640506]
- 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]
- Becker J, Wittmann C, 2012 Bio-based production of chemicals, materials and fuels -*Corynebacterium glutamicum* as versatile cell factory. *Curr Opin Biotechnol.* 23, 631–40. [PubMed: 22138494]
- Bederman IR, Dufner DA, Alexander JC, Previs SF, 2006 Novel application of the “doubly labeled” water method: measuring CO₂ production and the tissue-specific dynamics of lipid and protein in vivo. *Am J Physiol Endocrinol Metab.* 290, E1048–56. [PubMed: 16368786]
- Bier DM, 1987 The use of stable isotopes in metabolic investigation. *Bailliere’s clinical endocrinology and metabolism.* 1, 817–36. [PubMed: 3330431]
- Blank LM, Lehmbeck F, Sauer U, 2005 Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS Yeast Res.* 5, 545–58. [PubMed: 15780654]
- Boghigian BA, Seth G, Kiss R, Pfeifer BA, 2010 Metabolic flux analysis and pharmaceutical production. *Metab Eng.* 12, 81–95. [PubMed: 19861167]
- Borowitz MJ, Stein RB, Blum JJ, 1977 Quantitative analysis of the change of metabolite fluxes along the pentose phosphate and glycolytic pathways in *Tetrahymena* in response to carbohydrates. *J Biol Chem.* 252, 1589–605. [PubMed: 402368]
- Brady PS, Scofield RF, Ohgaku S, Schumann WC, Bartsch GE, Margolis JM, Kumaran K, Horvat A, Mann S, Landau BR, 1982a Pathways of acetoacetate’s formation in liver and kidney. *J Biol Chem.* 257, 9290–3. [PubMed: 6809735]

- Brady PS, Scofield RF, Schumann WC, Ohgaku S, Kumaran K, Margolis JM, Landau BR, 1982b The tracing of the pathway of mevalonate's metabolism to other than sterols. *J Biol Chem.* 257, 10742–6. [PubMed: 6809755]
- Brown GW Jr., Katz J, Chaikoff IL, 1956 The oxidative metabolic pattern of mouse hepatoma C954 as studied with C14-labeled acetates, propionate, octanoate, and glucose. *Cancer Res.* 16, 509–19. [PubMed: 13343123]
- Brunengraber DZ, McCabe BJ, Katanik J, Previs SF, 2002 Gas chromatography-mass spectrometry assay of the (18)O enrichment of water as trimethyl phosphate. *Anal Biochem.* 306, 278–82. [PubMed: 12123666]
- Burgess SC, Weis B, Jones JG, Smith E, Merritt ME, Margolis D, Dean Sherry A, Malloy CR, 2003 Noninvasive evaluation of liver metabolism by 2H and 13C NMR isotopomer analysis of human urine. *Anal Biochem.* 312, 228–34. [PubMed: 12531210]
- Chance EM, Seeholzer SH, Kobayashi K, Williamson JR, 1983 Mathematical analysis of isotope labeling in the citric acid cycle with applications to 13C NMR studies in perfused rat hearts. *J Biol Chem.* 258, 13785–94. [PubMed: 6643454]
- Chandramouli V, Ekberg K, Schumann WC, Kalhan SC, Wahren J, Landau BR, 1997 Quantifying gluconeogenesis during fasting. *Am J Physiol.* 273, E1209–15. [PubMed: 9435538]
- 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]
- Chevalier S, Burgess SC, Malloy CR, Gougeon R, Marliss EB, Morais JA, 2006 The greater contribution of gluconeogenesis to glucose production in obesity is related to increased whole-body protein catabolism. *Diabetes.* 55, 675–81. [PubMed: 16505230]
- Choi J, Antoniewicz MR, 2011 Tandem mass spectrometry: a novel approach for metabolic flux analysis. *Metab Eng.* 13, 225–33. [PubMed: 21134484]
- Choi J, Grossbach MT, Antoniewicz MR, 2012 Measuring complete isotopomer distribution of aspartate using gas chromatography/tandem mass spectrometry. *Anal Chem.* 84, 4628–32. [PubMed: 22510303]
- Christensen B, Nielsen J, 1999 Isotopomer analysis using GC-MS. *Metab Eng.* 1, 282–90. [PubMed: 10937821]
- Cohen SM, 1983 Simultaneous 13C and 31P NMR studies of perfused rat liver. Effects of insulin and glucagon and a 13C NMR assay of free Mg2+. *J Biol Chem.* 258, 14294–308. [PubMed: 6358220]
- Cohen SM, Rognstad R, Shulman RG, Katz J, 1981 A comparison of 13C nuclear magnetic resonance and 14C tracer studies of hepatic metabolism. *J Biol Chem.* 256, 3428–32. [PubMed: 7009612]
- Comte B, Vincent G, Bouchard B, Des Rosiers C, 1997a Probing the origin of acetyl-CoA and oxaloacetate entering the citric acid cycle from the 13C labeling of citrate released by perfused rat hearts. *J Biol Chem.* 272, 26117–24. [PubMed: 9334176]
- Comte B, Vincent G, Bouchard B, Jette M, Cordeau S, Rosiers CD, 1997b A 13C mass isotopomer study of anaplerotic pyruvate carboxylation in perfused rat hearts. *J Biol Chem.* 272, 26125–31. [PubMed: 9334177]
- Crown SB, Ahn WS, Antoniewicz MR, 2012 Rational design of 13C-labeling experiments for metabolic flux analysis in mammalian cells. *BMC Syst Biol.* 6, 43. [PubMed: 22591686]
- Crown SB, Antoniewicz MR, 2012 Selection of tracers for 13C-metabolic flux analysis using elementary metabolite units (EMU) basis vector methodology. *Metab Eng.* 14, 150–61. [PubMed: 22209989]
- 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]
- D'Adamo A Jr., Haft DE, 1965 An Alternate Pathway of Alpha-Ketoglutarate Catabolism in the Isolated, Perfused Rat Liver. I. Studies with DI-Glutamate-2- and -5-14c. *J Biol Chem.* 240, 613–7. [PubMed: 14275112]
- Dauner M, Sauer U, 2000 GC-MS analysis of amino acids rapidly provides rich information for isotopomer balancing. *Biotechnology progress.* 16, 642–9. [PubMed: 10933840]

- de Graaf AA, Venema K, 2008 Gaining insight into microbial physiology in the large intestine: a special role for stable isotopes. *Advances in microbial physiology*. 53, 73–168. [PubMed: 17707144]
- de Jong B, Siewers V, Nielsen J, 2012 Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. *Curr Opin Biotechnol*. 23, 624–30. [PubMed: 22169890]
- DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB, 2008 The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab*. 7, 11–20. [PubMed: 18177721]
- Des Rosiers C, Fernandez CA, David F, Brunengraber H, 1994 Reversibility of the mitochondrial isocitrate dehydrogenase reaction in the perfused rat liver. Evidence from isotopomer analysis of citric acid cycle intermediates. *J Biol Chem*. 269, 27179–82. [PubMed: 7961626]
- Des Rosiers C, Lloyd S, Comte B, Chatham JC, 2004 A critical perspective of the use of (13)C-isotopomer analysis by GCMS and NMR as applied to cardiac metabolism. *Metab Eng*. 6, 44–58. [PubMed: 14734255]
- Dewick PM, 1995 The Biosynthesis of Shikimate Metabolites. *Natural Product Reports*. 12, 579–607. [PubMed: 8559515]
- Di Donato L, Des Rosiers C, Montgomery JA, David F, Garneau M, Brunengraber H, 1993 Rates of gluconeogenesis and citric acid cycle in perfused livers, assessed from the mass spectrometric assay of the 13C labeling pattern of glutamate. *J Biol Chem*. 268, 4170–80. [PubMed: 8095046]
- Edmond J, Popjak G, 1974 Transfer of carbon atoms from mevalonate to n-fatty acids. *J Biol Chem*. 249, 66–71. [PubMed: 4809631]
- Feng X, Mouttaki H, Lin L, Huang R, Wu B, Hemme CL, He Z, Zhang B, Hicks LM, Xu J, Zhou J, Tang YJ, 2009 Characterization of the central metabolic pathways in *Thermoanaerobacter* sp. strain X514 via isotopomer-assisted metabolite analysis. *Appl Environ Microbiol*. 75, 5001–8. [PubMed: 19525270]
- Fischer E, Sauer U, 2003 Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. *Eur J Biochem*. 270, 880–91. [PubMed: 12603321]
- Fischer E, Zamboni N, Sauer U, 2004 High-throughput metabolic flux analysis based on gas chromatography-mass spectrometry derived 13C constraints. *Anal Biochem*. 325, 308–16. [PubMed: 14751266]
- Follstad BD, Stephanopoulos G, 1998 Effect of reversible reactions on isotope label redistribution--analysis of the pentose phosphate pathway. *Eur J Biochem*. 252, 360–71. [PubMed: 9546650]
- Fuhrer T, Fischer E, Sauer U, 2005 Experimental identification and quantification of glucose metabolism in seven bacterial species. *J Bacteriol*. 187, 1581–90. [PubMed: 15716428]
- Garcia DE, Baidoo EE, Benke PI, Pingitore F, Tang YJ, Villa S, Keasling JD, 2008 Separation and mass spectrometry in microbial metabolomics. *Current opinion in microbiology*. 11, 233–9. [PubMed: 18538626]
- Goebel R, Berman M, Foster D, 1982 Mathematical model for the distribution of isotopic carbon atoms through the tricarboxylic acid cycle. *Fed Proc*. 41, 96–103. [PubMed: 7056403]
- Henry O, Durocher Y, 2011 Enhanced glycoprotein production in HEK-293 cells expressing pyruvate carboxylase. *Metab Eng*. 13, 499–507. [PubMed: 21624497]
- Hevesy G, 1923 The Absorption and Translocation of Lead by Plants: A Contribution to the Application of the Method of Radioactive Indicators in the Investigation of the Change of Substance in Plants. *The Biochemical journal*. 17, 439–45. [PubMed: 16743235]
- Horecker BL, Gibbs M, Klenow H, Smyrniotis PZ, 1954 The mechanism of pentose phosphate conversion to hexose monophosphate. I. With a liver enzyme preparation. *J Biol Chem*. 207, 393–403. [PubMed: 13152115]
- Hostetler KY, Williams HR, Shreeve WW, Landau BR, 1969 Conversion of specifically 14 C-labeled lactate and pyruvate to glucose in man. *J Biol Chem*. 244, 2075–7. [PubMed: 5782000]
- Iwatani S, Yamada Y, Usuda Y, 2008 Metabolic flux analysis in biotechnology processes. *Biotechnol Lett*. 30, 791–9. [PubMed: 18224283]
- Jeffrey FM, Rajagopal A, Malloy CR, Sherry AD, 1991 13C-NMR: a simple yet comprehensive method for analysis of intermediary metabolism. *Trends Biochem Sci*. 16, 5–10. [PubMed: 2053137]

- Jeffrey FM, Roach JS, Storey CJ, Sherry AD, Malloy CR, 2002 ^{13}C isotopomer analysis of glutamate by tandem mass spectrometry. *Anal Biochem.* 300, 192–205. [PubMed: 11779111]
- Jensen MD, Chandramouli V, Schumann WC, Ekberg K, Previs SF, Gupta S, Landau BR, 2001a Sources of blood glycerol during fasting. *Am J Physiol Endocrinol Metab.* 281, E998–1004. [PubMed: 11595656]
- Jensen MD, Ekberg K, Landau BR, 2001b Lipid metabolism during fasting. *Am J Physiol Endocrinol Metab.* 281, E789–93. [PubMed: 11551856]
- Jensen MV, Joseph JW, Ronnebaum SM, Burgess SC, Sherry AD, Newgard CB, 2008 Metabolic cycling in control of glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab.* 295, E1287–97. [PubMed: 18728221]
- Jin ES, Burgess SC, Merritt ME, Sherry AD, Malloy CR, 2005 Differing mechanisms of hepatic glucose overproduction in triiodothyronine-treated rats vs. Zucker diabetic fatty rats by NMR analysis of plasma glucose. *Am J Physiol Endocrinol Metab.* 288, E654–62. [PubMed: 15562253]
- Jones JG, Sherry AD, Jeffrey FM, Storey CJ, Malloy CR, 1993 Sources of acetyl-CoA entering the tricarboxylic acid cycle as determined by analysis of succinate ^{13}C isotopomers. *Biochemistry.* 32, 12240–4. [PubMed: 8218301]
- Jones JG, Solomon MA, Cole SM, Sherry AD, Malloy CR, 2001 An integrated $(2)\text{H}$ and $(13)\text{C}$ NMR study of gluconeogenesis and TCA cycle flux in humans. *Am J Physiol Endocrinol Metab.* 281, E848–56. [PubMed: 11551863]
- Kam W, Kumaran K, Landau BR, 1978 Contribution of omega-oxidation to fatty acid oxidation by liver of rat and monkey. *J Lipid Res.* 19, 591–600. [PubMed: 97354]
- Katz J, Landau BR, Bartsch GE, 1966 The pentose cycle, triose phosphate isomerization, and lipogenesis in rat adipose tissue. *J Biol Chem.* 241, 727–40. [PubMed: 4379536]
- Katz J, Rognstad R, 1966 The metabolism of tritiated glucose by rat adipose tissue. *J Biol Chem.* 241, 3600–10. [PubMed: 4380669]
- Katz J, Rognstad R, 1969 The metabolism of glucose-2-T by adipose tissue. *J Biol Chem.* 244, 99–106. [PubMed: 5773293]
- Katz J, Wals P, Lee WN, 1993 Isotopomer studies of gluconeogenesis and the Krebs cycle with ^{13}C -labeled lactate. *J Biol Chem.* 268, 25509–21. [PubMed: 7902352]
- Katz J, Wood HG, 1960 The use of glucose- C^{14} for the evaluation of the pathways of glucose metabolism. *J Biol Chem.* 235, 2165–77. [PubMed: 14404802]
- Katz J, Wood HG, 1963 The use of C^{14}O_2 yields from glucose-1- and -6- C^{14} for the evaluation of the pathways of glucose metabolism. *J Biol Chem.* 238, 517–23. [PubMed: 13958489]
- Kelleher JK, 1985 Analysis of tricarboxylic acid cycle using $[^{14}\text{C}]$ citrate specific activity ratios. *Am J Physiol.* 248, E252–60. [PubMed: 3970196]
- Kelleher JK, 2001 Flux estimation using isotopic tracers: common ground for metabolic physiology and metabolic engineering. *Metab Eng.* 3, 100–10. [PubMed: 11289786]
- Kelleher JK, 2004 Probing metabolic pathways with isotopic tracers: insights from mammalian metabolic physiology. *Metab Eng.* 6, 1–5. [PubMed: 14734250]
- Kelleher JK, Bryan BM 3rd, 1985 A $^{14}\text{CO}_2$ ratios method for detecting pyruvate carboxylation. *Anal Biochem.* 151, 55–62. [PubMed: 3937461]
- Kelleher JK, Kharroubi AT, Aldaghlis TA, Shambat IB, Kennedy KA, Holleran AL, Masterson TM, 1994 Isotopomer spectral analysis of cholesterol synthesis: applications in human hepatoma cells. *Am J Physiol.* 266, E384–95. [PubMed: 8166258]
- Kharroubi AT, Masterson TM, Aldaghlis TA, Kennedy KA, Kelleher JK, 1992 Isotopomer spectral analysis of triglyceride fatty acid synthesis in 3T3-L1 cells. *Am J Physiol.* 263, E667–75. [PubMed: 1415685]
- Kiefer P, Heinzle E, Zelder O, Wittmann C, 2004 Comparative metabolic flux analysis of lysine-producing *Corynebacterium glutamicum* cultured on glucose or fructose. *Appl Environ Microbiol.* 70, 229–39. [PubMed: 14711646]
- Kim TY, Sohn SB, Kim YB, Kim WJ, Lee SY, 2012 Recent advances in reconstruction and applications of genome-scale metabolic models. *Curr Opin Biotechnol.* 23, 617–23. [PubMed: 22054827]

- Klein S, Heinze E, 2012 Isotope labeling experiments in metabolomics and fluxomics. Wiley interdisciplinary reviews. Systems biology and medicine. 4, 261–72. [PubMed: 22447740]
- Kohler RE, 1977 Rudolf Schoenheimer, isotopic tracers, and biochemistry in the 1930's. Hist Studies Phys Sci. 8, 257–298.
- Kruger NJ, Masakapalli SK, Ratcliffe RG, 2012 Strategies for investigating the plant metabolic network with steady-state metabolic flux analysis: lessons from an Arabidopsis cell culture and other systems. J Exp Bot. 63, 2309–23. [PubMed: 22140245]
- Landau BR, Ashmore J, Hastings AB, Zottu S, 1960 Studies on carbohydrate metabolism in rat liver slices. XV. Pyruvate and propionate metabolism and carbon dioxide fixation in rat liver slices in vitro. J Biol Chem. 235, 1856–8. [PubMed: 14413767]
- Landau BR, Bartsch GE, 1966 Estimations of pathway contributions to glucose metabolism and the transaldolase reactions. J Biol Chem. 241, 741–9. [PubMed: 5908138]
- Landau BR, Bartsch GE, Katz J, Wood HG, 1964 Estimation of Pathway Contributions to Glucose Metabolism and of the Rate of Isomerization of Hexose 6-Phosphate. J Biol Chem. 239, 686–96. [PubMed: 14154438]
- Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY, 2012 Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol. 8, 536–46. [PubMed: 22596205]
- 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]
- Leighty RW, Antoniewicz MR, 2012 Parallel labeling experiments with [U-(13C)]glucose validate E. coli metabolic network model for (13C) metabolic flux analysis. Metab Eng.
- Malloy CR, Sherry AD, Jeffrey FM, 1988 Evaluation of carbon flux and substrate selection through alternate pathways involving the citric acid cycle of the heart by 13C NMR spectroscopy. J Biol Chem. 263, 6964–71. [PubMed: 3284880]
- Malloy CR, Sherry AD, Jeffrey FM, 1990a Analysis of tricarboxylic acid cycle of the heart using 13C isotope isomers. Am J Physiol. 259, H987–95. [PubMed: 1975735]
- Malloy CR, Thompson JR, Jeffrey FM, Sherry AD, 1990b Contribution of exogenous substrates to acetyl coenzyme A: measurement by 13C NMR under non-steady-state conditions. Biochemistry. 29, 6756–61. [PubMed: 1975750]
- Marsolaïs C, Huot S, David F, Garneau M, Brunengraber H, 1987 Compartmentation of 14CO2 in the perfused rat liver. J Biol Chem. 262, 2604–7. [PubMed: 3102472]
- Matthews DE, Bier DM, 1983 Stable isotope methods for nutritional investigation. Annual review of nutrition. 3, 309–39.
- Matwiyoff NA, Ott DG, 1973 Stable isotope tracers in the life sciences and medicine. Science. 181, 1125–33. [PubMed: 4726440]
- Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, Jewell CM, Johnson ZR, Irvine DJ, Guarente L, Kelleher JK, Vander Heiden MG, Iliopoulos O, Stephanopoulos G, 2012 Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature. 481, 380–4.
- 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]
- Mullhofer G, Muller C, Von Stetten C, Gruber E, 1977 Carbon-14 tracer studies in the metabolism of isolated rat-liver parenchymal cells under conditions of gluconeogenesis from lactate and pyruvate. Eur J Biochem. 75, 331–41. [PubMed: 885133]
- Neese RA, Schwarz JM, Faix D, Turner S, Letscher A, Vu D, Hellerstein MK, 1995 Gluconeogenesis and intrahepatic triose phosphate flux in response to fasting or substrate loads. Application of the

- mass isotopomer distribution analysis technique with testing of assumptions and potential problems. *J Biol Chem.* 270, 14452–66. [PubMed: 7782307]
- Niklas J, Sandig V, Heinzle E, 2011 Metabolite channeling and compartmentation in the human cell line AGE1.HN determined by ¹³C labeling experiments and ¹³C metabolic flux analysis. *J Biosci Bioeng.* 112, 616–23. [PubMed: 21865082]
- Niklas J, Schneider K, Heinzle E, 2010 Metabolic flux analysis in eukaryotes. *Curr Opin Biotechnol.* 21, 63–9. [PubMed: 20163950]
- Noh K, Wiechert W, 2006 Experimental design principles for isotopically stationary ¹³C labeling experiments. *Biotechnol Bioeng.* 94, 234–51. [PubMed: 16598793]
- Olszewski KL, Mather MW, Morrissey JM, Garcia BA, Vaidya AB, Rabinowitz JD, Llinas M, 2010 Branched tricarboxylic acid metabolism in *Plasmodium falciparum*. *Nature.* 466, 774–8. [PubMed: 20686576]
- Previs SF, Withers DJ, Ren JM, White MF, Shulman GI, 2000 Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *Journal of Biological Chemistry.* 275, 38990–38994. [PubMed: 10995761]
- Quek LE, Dietmair S, Kromer JO, Nielsen LK, 2010 Metabolic flux analysis in mammalian cell culture. *Metab Eng.* 12, 161–71. [PubMed: 19833223]
- Quek LE, Wittmann C, Nielsen LK, Kromer JO, 2009 OpenFLUX: efficient modelling software for ¹³C-based metabolic flux analysis. *Microb Cell Fact.* 8, 25. [PubMed: 19409084]
- Robinson AM, Williamson DH, 1977 Control of glucose metabolism in isolated acini of the lactating mammary gland of the rat. The ability of glycerol to mimic some of the effects of insulin. *Biochem J.* 168, 465–74. [PubMed: 606248]
- Rognstad R, Katz J, 1972 Gluconeogenesis in the kidney cortex. Quantitative estimation of carbon flow. *J Biol Chem.* 247, 6047–54. [PubMed: 4651643]
- Schellenberger J, Zielinski DC, Choi W, Madireddi S, Portnoy V, Scott DA, Reed JL, Osterman AL, Palsson BO, 2012 Predicting outcomes of steady-state ¹³C isotope tracing experiments with Monte Carlo sampling. *BMC Syst Biol.* 6, 9. [PubMed: 22289253]
- Schleucher J, Vanderveer PJ, Sharkey TD, 1998 Export of carbon from chloroplasts at night. *Plant physiology.* 118, 1439–45. [PubMed: 9847119]
- Schmidt K, Carlsen M, Nielsen J, Villadsen J, 1997 Modeling isotopomer distributions in biochemical networks using isotopomer mapping matrices. *Biotechnol Bioeng.* 55, 831–40. [PubMed: 18636594]
- Schoenheimer R, Rittenberg D, 1938 The Application of Isotopes to the Study of Intermediary Metabolism. *Science.* 87, 221–6. [PubMed: 17770403]
- Schoenheimer R, Rittenberg D, 1940 The study of intermediary metabolism of animals with the aid of isotopes. *Physiological reviews.* 20, 218–248.
- Schwender J, Seemann M, Lichtenthaler HK, Rohmer M, 1996 Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. *Biochemical Journal.* 316, 73–80. [PubMed: 8645235]
- Schwender J, Shachar-Hill Y, Ohlrogge JB, 2006 Mitochondrial metabolism in developing embryos of *Brassica napus*. *J Biol Chem.* 281, 34040–7. [PubMed: 16971389]
- Sherry AD, Malloy CR, Roby RE, Rajagopal A, Jeffrey FM, 1988 Propionate metabolism in the rat heart by ¹³C n.m.r. spectroscopy. *Biochem J.* 254, 593–8. [PubMed: 3178775]
- Simpson TJ, 1998 Application of isotopic methods to secondary metabolic pathways. *Top Curr Chem.* 195, 1–48.
- Strigun A, Wahrheit J, Niklas J, Heinzle E, Noor F, 2012 Doxorubicin increases oxidative metabolism in HL-1 cardiomyocytes as shown by ¹³C metabolic flux analysis. *Toxicol Sci.* 125, 595–606. [PubMed: 22048646]
- Strisower EH, Kohler GD, Chaikoff IL, 1952 Incorporation of acetate carbon into glucose by liver slices from normal and alloxan-diabetic rats. *J Biol Chem.* 198, 115–26. [PubMed: 12999723]
- Strong JM, Anderson LW, Monks A, Chisena CA, Csyk RL, 1983 A ¹³C tracer method for quantitating de novo pyrimidine biosynthesis in vitro and in vivo. *Anal Biochem.* 132, 243–53. [PubMed: 6414331]

- Strong JM, Upton DK, Anderson LW, Monks A, Chisena CA, Cysyk RL, 1985 A novel approach to the analysis of mass spectrally assayed stable isotope-labeling experiments. *J Biol Chem.* 260, 4276–81. [PubMed: 3980477]
- 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]
- Tang KH, Feng X, Zhuang WQ, Alvarez-Cohen L, Blankenship RE, Tang YJ, 2010 Carbon flow of heliobacteria is related more to clostridia than to the green sulfur bacteria. *J Biol Chem.* 285, 35104–12. [PubMed: 20807773]
- Tang YJ, Yi S, Zhuang WQ, Zinder SH, Keasling JD, Alvarez-Cohen L, 2009 Investigation of carbon metabolism in “Dehalococcoides ethenogenes” strain 195 by use of isotopomer and transcriptomic analyses. *J Bacteriol.* 191, 5224–31. [PubMed: 19525347]
- Toya Y, Kono N, Arakawa K, Tomita M, 2011 Metabolic flux analysis and visualization. *Journal of proteome research.* 10, 3313–23. [PubMed: 21815690]
- Vallino JJ, Stephanopoulos G, 1993 Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. *Biotechnol Bioeng.* 41, 633–46. [PubMed: 18609599]
- Wahrheit J, Nicolae A, Heinzle E, 2011 Eukaryotic metabolism: measuring compartment fluxes. *Biotechnol J.* 6, 1071–85. [PubMed: 21910257]
- Walker TE, Han CH, Kollman VH, London RE, Matwiyoff NA, 1982 ¹³C nuclear magnetic resonance studies of the biosynthesis by *Microbacterium ammoniaphilum* of L-glutamate selectively enriched with carbon-13. *J Biol Chem.* 257, 1189–95. [PubMed: 6120165]
- Walther JL, Metallo CM, Zhang J, Stephanopoulos G, 2012 Optimization of ¹³C isotopic tracers for metabolic flux analysis in mammalian cells. *Metab Eng.* 14, 162–71. [PubMed: 22198197]
- Wang CH, Gregg CT, Forbusch IA, Christensen BE, Cheldelin VH, 1956 Carbohydrate metabolism in bakers’ yeast. I. Time course study of glucose utilization. *J Am Chem Soc.* 78, 1869–72.
- Weinman EO, Strisower EH, Chaikoff IL, 1957 Conversion of fatty acids to carbohydrate; application of isotopes to this problem and role of the Krebs cycle as a synthetic pathway. *Physiol Rev.* 37, 252–72. [PubMed: 13441426]
- Weinstock SB, Kopito RR, Endemann G, Tomera JF, Marinier E, Murray DM, Brunengraber H, 1984 The shunt pathway of mevalonate metabolism in the isolated perfused rat liver. *J Biol Chem.* 259, 8939–44. [PubMed: 6430893]
- White LW, Williams HR, Landau BR, 1968 Metabolism of pyruvate by rat adipose tissue in vitro. *Arch Biochem Biophys.* 126, 552–7. [PubMed: 5672514]
- Wiechert W, Mollney M, Petersen S, de Graaf AA, 2001 A universal framework for ¹³C metabolic flux analysis. *Metab Eng.* 3, 265–83. [PubMed: 11461148]
- Wittmann C, Heinzle E, 2001a Application of MALDI-TOF MS to lysine-producing *Corynebacterium glutamicum*: a novel approach for metabolic flux analysis. *Eur J Biochem.* 268, 2441–55. [PubMed: 11298764]
- Wittmann C, Heinzle E, 2001b Modeling and experimental design for metabolic flux analysis of lysine-producing *Corynebacteria* by mass spectrometry. *Metab Eng.* 3, 173–91. [PubMed: 11289793]
- Wittmann C, Heinzle E, 2002 Genealogy profiling through strain improvement by using metabolic network analysis: metabolic flux genealogy of several generations of lysine-producing *corynebacteria*. *Appl Environ Microbiol.* 68, 5843–59. [PubMed: 12450803]
- Wittmann C, Kim HM, Heinzle E, 2004 Metabolic network analysis of lysine producing *Corynebacterium glutamicum* at a miniaturized scale. *Biotechnol Bioeng.* 87, 1–6. [PubMed: 15211482]
- Wolfe RR, 1984 Tracers in metabolic research: radioisotope and stable isotope/mass spectrometry methods. *Laboratory and research methods in biology and medicine.* 9, 1–287. [PubMed: 6427541]
- Wolfe RR, 2004 *Isotope tracers in metabolic research: principles and practice of kinetic analysis.* Wiley-Liss.

- Yang D, Beylot M, Agarwal KC, Soloviev MV, Brunengraber H, 1993 Assay of the human liver citric acid cycle probe phenylacetylglutamine and of phenylacetate in plasma by gas chromatography-mass spectrometry. *Anal Biochem.* 212, 277–82. [PubMed: 8368503]
- Yang D, Previs SF, Fernandez CA, Dugelay S, Soloviev MV, Hazey JW, Agarwal KC, Levine WC, David F, Rinaldo P, Beylot M, Brunengraber H, 1996 Noninvasive probing of citric acid cycle intermediates in primate liver with phenylacetylglutamine. *Am J Physiol.* 270, E882–9. [PubMed: 8967478]
- Yang TH, Wittmann C, Heinze E, 2006 Respiriometric ^{13}C 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]
- Yoo H, Stephanopoulos G, Kelleher JK, 2004 Quantifying carbon sources for de novo lipogenesis in wild-type and IRS-1 knockout brown adipocytes. *J Lipid Res.* 45, 1324–32. [PubMed: 15102881]
- 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, 2011 ^{13}C metabolic flux analysis in complex systems. *Curr Opin Biotechnol.* 22, 103–8. [PubMed: 20833526]
- Zupke C, Stephanopoulos G, 1994 Modeling of isotope distributions and intracellular fluxes in metabolic networks using atom mapping matrices. *Biotechnol Prog.* 10, 489–98.
- Zupke C, Stephanopoulos G, 1995 Intracellular flux analysis in hybridomas using mass balances and in vitro (^{13}C) nmr. *Biotechnol Bioeng.* 45, 292–303. [PubMed: 18623182]

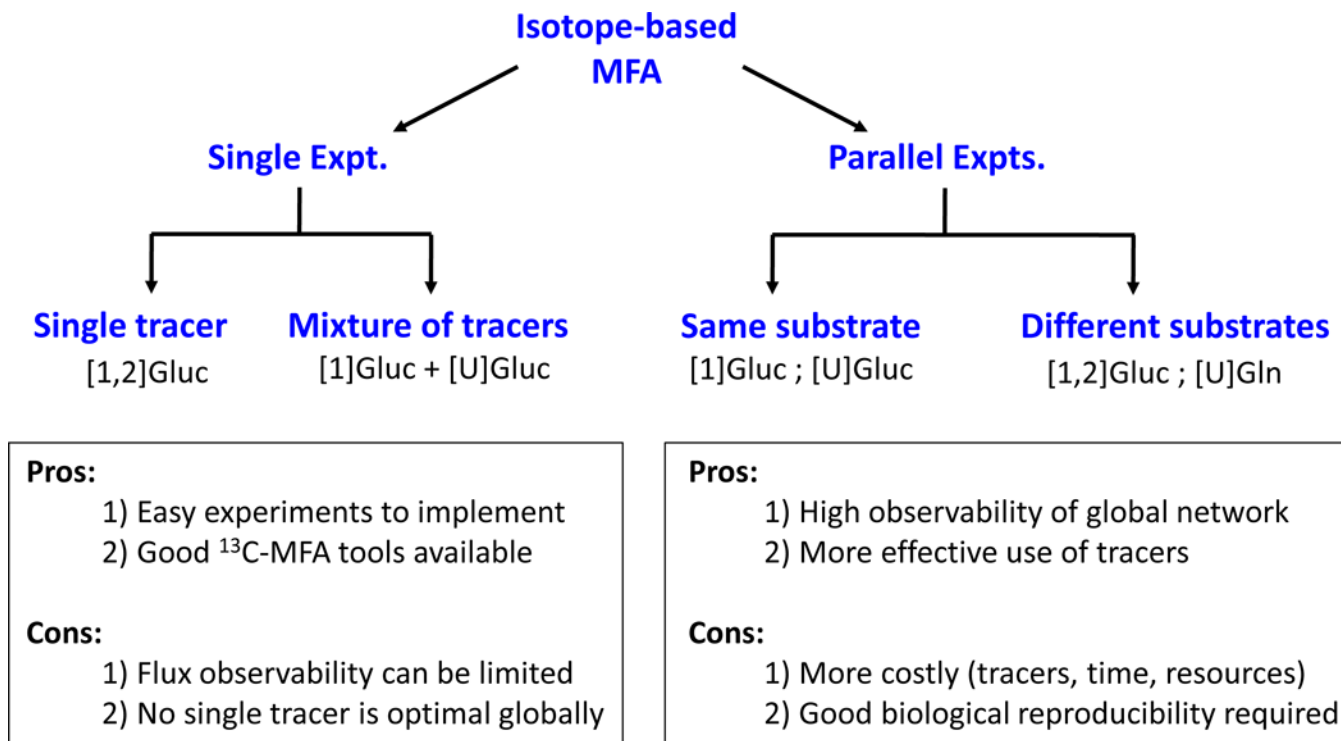
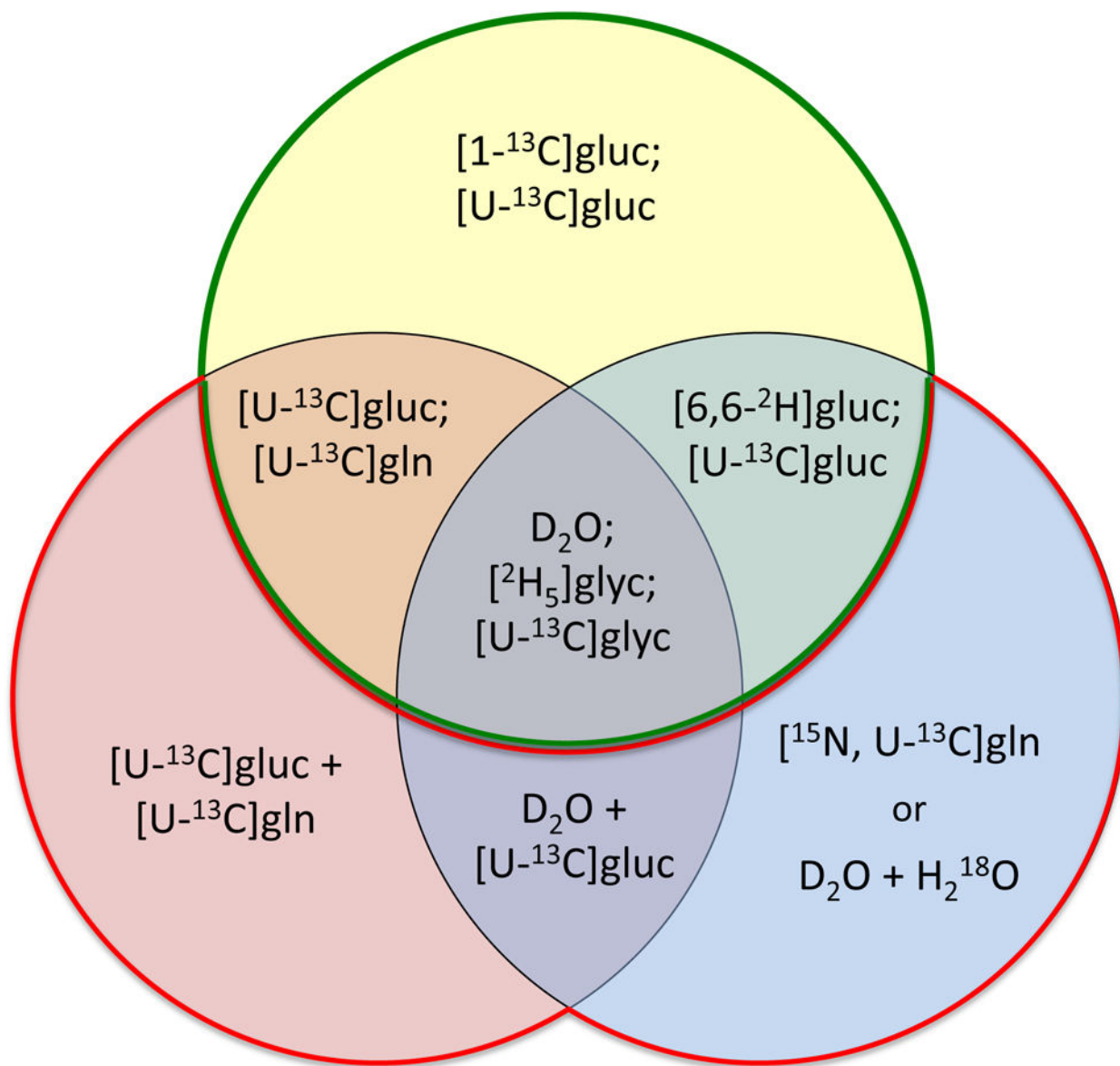


Figure 1. Experimental designs for single tracer experiments and parallel labeling experiments. Major advantages and disadvantages of both approaches are highlighted. [1]Gluc denotes [1-¹³C]glucose. [U]Gln denotes [U-¹³C]glutamine. A semicolon “;” denotes parallel experiments. A plus-sign “+” indicates multiple tracers used in a single experiment.

Parallel experiments



Multiple labeled substrates

Multiple isotope types

Figure 2.

Tracer experiment designs using parallel experiments, multiple labeled substrates, and multiple isotope types. A semicolon “;” denotes parallel experiments and a plus-sign “+” indicates multiple tracers used in a single experiment.

Technical Advances

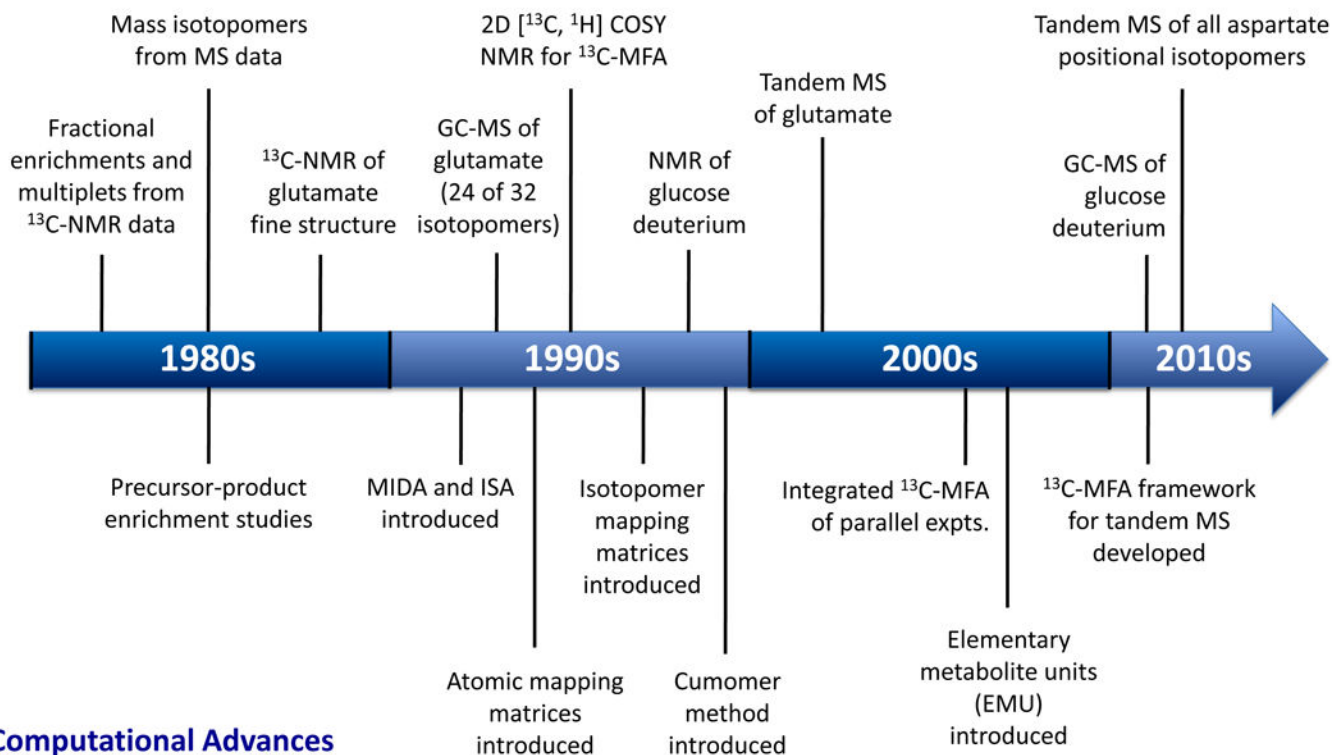


Figure 3. Timeline of important technical and computational advances that facilitated the transition from radioisotopes to stable-isotopes and further development of ¹³C-MFA.

Table 1.

Radioisotope studies with parallel labeling experiments

Organism	Tracers	Measurements	Major achievements	Reference
Rat liver slices	[1]lac; [2]lac	Gluc, ¹⁴ CO ₂ activity	Quantified isotopic dilution in TCA cycle	(Strisower et al., 1952)
Rat liver	[1]r5p; [2,3]p5p	Activity of h6p carbons	Determined structure of non-oxPPP	(Horecker et al., 1954)
Yeast	[1]; [2]; [6]; [U]gluc	¹⁴ CO ₂ activity	Estimated oxPPP, EMP and pyruvate fixation fluxes	(Wang et al., 1956)
n/a	[2]; [3]gluc	Activity of h6p carbons	Accounted for h6p recycling in estimation of oxPPP	(Katz and Wood, 1960)
Rat liver slices	[1]; [2]prop; [1]; [2]pyr; bicarb	Gluc, glycogen, FA, lact, ¹⁴ CO ₂ activities	Elucidated pyruvate and propionate conversion to glucose and label shuffling in TCA cycle	(Landau et al., 1960)
Rat adipose tissue	[1]; [6]gluc	Lipid, ¹⁴ CO ₂ activities	Evaluated methods for oxPPP estimation	(Katz and Wood, 1963)
Tissue slices	[1]; [6]gluc; [1]; [6]fruc	Glycerol, ¹⁴ CO ₂ yields	Estimated oxPPP flux and hexose isomerization	(Landau et al., 1964)
Isolated rat liver	[2]; [5]glu	Gluc, ¹⁴ CO ₂ , FA, glycogen activities	Identified metabolism of akg to citrate via reductive carboxylation	(D'Adamo and Haft, 1965)
Rat liver	[1]; [2]lac; [1]; [16]palm; [18]stear; [10]ole	Activity of glycogen carbons	Examined relative contribution of α, β, and ω-oxidation of FA	(Antony and Landau, 1968)
Rat adipose tissue	[1]; [2]; [3]pyr; bicarb	Glycogen, glyc, FA, lact, ¹⁴ CO ₂ activities	Probed pyruvate metabolism and glyceroneogenesis	(White et al., 1968)
Rat adipose tissue	[1- ³ H, ¹⁴ C]; [2- ³ H, ¹⁴ C]; [3- ³ H, ¹⁴ C]; [6- ³ H, ¹⁴ C]gluc	³ H in gluc, H ₂ O; ³ H: ¹⁴ C ratio in FA, glyc, lact	Investigated isotope discrimination and H-exchange by isomerization reactions	(Katz and Rognstad, 1969)
Kidney cortex slices	[2]; [3]lact; [2]; [3]pyr	Activity of gluc, lact, ala, glu carbons	Estimated TCA cycle and gluconeogenesis fluxes	(Rognstad and Katz, 1972)
<i>Tetrahymena</i>	Gluc, fruc, ribose, glyc tracers	Glycogen, glyc, RNA, ¹⁴ CO ₂ activities	Estimated glycolysis and PPP fluxes	(Borowitz et al., 1977)
Rat liver	[1]; [2]lact; [1]; [2]pyr; [1]oct; bicarb	Activity of carbons, fragments and molecules	Extensive study on various gluconeogenic precursors	(Mullhofer et al., 1977)
Rat liver and kidney	Variety of palm tracers	Activity of hydroxy-butyric acid carbons	Probed multiple pathways for acetoacetate formation	(Brady et al., 1982a)
Rat liver	[1,4]; [2,3]succinate	¹⁴ CO ₂ yields	Determined pyruvate carboxylation/decarboxylation fluxes into TCA cycle	(Kelleher and Bryan, 1985)

Notes: [1]gluc denotes [1-¹⁴C]gluc, unless stated otherwise. The semicolon “;” denotes parallel experiments.

Abbreviations: AA, amino acids; ac, acetate; acac, acetoacetate; akg, α-ketoglutarate; ala, alanine; asp, aspartate; bicarb, bicarbonate; CS, citrate synthase; FA, fatty acid; fruc, fructose; gln, glutamine; glu, glutamate; gluc, glucose; glyc, glycerol; h6p, hexose-6-phosphate; ile, isoleucine; ISA, isotopomer spectral analysis; lact, lactate; leu, leucine; lys, lysine; MIDA, mass isotopomer distribution analysis; MVA, mevalonate; oct, octanoate; ole, oleate; p5p, pentose-5-phosphate; palm, palmitate; prop, propionate; pyr, pyruvate; r5p, ribose-5-phosphate; stear, stearate; thr, threonine.

Table 2.

Stable-isotope studies with parallel labeling experiments

Organism	Tracers	Measurements	Major achievements	Reference
Isolated rat hepatocytes	$^{15}\text{NH}_4^+$; $[5-^{15}\text{N}]\text{gln}$	MS - uracil	Related mass isotopomers to precursor enrichments and pyrimidine biosynthesis	(Strong et al., 1985)
Rat hearts	$[3]\text{pyr}$; $[2]\text{lac}$; $[2]\text{ac}$ + $[3]\text{prop}$; $[1,2]\text{ac}$ + $[3]\text{pyr}$	NMR - glutamate	Analyzed ^{13}C -NMR spectra and modeled flux of TCA cycle	(Malloy et al., 1988)
3T3-L1 adipocytes	$[1]\text{ac}$; $[1,2]\text{acac}$	MS - fatty acids	First ISA study on fatty acid synthesis	(Kharroubi et al., 1992)
Human Hep-G2 cells	$[1]\text{ac}$; $[\text{U}]\text{acac}$; $[4,5]\text{MVA}$; $[1,2,3,4]\text{oct}$	MS - cholesterol	Examined cholesterol synthesis as a polymerization of MVA precursors	(Kelleher et al., 1994)
Sprague-Dawley rats	$[3]$; $[1]\text{lact}$; $[2]\text{glyc}$; $[6-^3\text{H}]\text{gluc}$	MS - glucose	First MIDA study on gluconeogenesis pathway	(Neese et al., 1995)
Rat hearts	$[1]\text{oct}$; $[\text{U}]\text{lact}$ + $[\text{U}]\text{pyr}$; $[\text{U}]\text{oct}$ + $[\text{U}]\text{lact}$ + $[\text{U}]\text{pyr}$; $[\text{U}]\text{glu}$; $[\text{U}]\text{ac}$	MS - citrate and its fragments	Estimated relative rates of PC/PDH/CS/FA oxidation	(Comte et al., 1997a)
<i>C. glutamicum</i>	$[1]\text{gluc}$; 40% $[\text{U}]\text{gluc}$	MS - ext. ala, lys, trehalose	Performed ^{13}C -MFA using parallel data sets and ratios of M+ x /M+0 isotopomers	(Wittmann and Heinzle, 2001a)
<i>E. coli</i>	$[1]\text{gluc}$; 20% $[\text{U}]\text{gluc}$	MS - biomass AA	Developed metabolic flux ratio analysis (MetaFor)	(Fischer and Sauer, 2003)
<i>E. coli</i>	$[1]\text{gluc}$; 20% $[\text{U}]\text{gluc}$	MS - biomass AA	Demonstrated improved flux observability with PLE's	(Fischer et al., 2004)
<i>C. glutamicum</i>	$[1]\text{gluc}$; 50% $[\text{U}]\text{gluc}$ or fruc	MS - ext. lys, trehalose	^{13}C -MFA with only external metabolite measurements	(Kiefer et al., 2004)
Brown adipocytes	$[\text{U}]\text{gluc}$; $[\text{U}]\text{gln}$; $[\text{U}]\text{ac}$; $[\text{U}]\text{acac}$	MS - fatty acids	Utilized ISA to quantify carbon sources for <i>de novo</i> lipogenesis	(Yoo et al., 2004)
Various bacteria	$[1]\text{gluc}$; 20% $[\text{U}]\text{gluc}$	MS - biomass AA	Quantified glucose metabolism via EMP/ED/oxPPP in several bacteria using MetaFor	(Fuhrer et al., 2005)
<i>B. napus</i>	$[1,2]\text{gluc}$ + $[\text{U}]\text{gluc}$; $[\text{U}]\text{ala}$; 50% $[\text{U}]\text{gln}$	MS - biomass AA, FA, gluc, glyc	Integrated ^{13}C -MFA: utilized $[^{15}\text{N}]\text{-tracers}$ to assess protein synthesis and turnover	(Schwender et al., 2006)
Isolated hepatocytes	$[^2\text{H}_3]$; $[\text{U}]\text{glyc}$; $^2\text{H}_2\text{O}$	MS - glucose	Integrated ^{13}C -MFA: quantified net and reversible fluxes in gluconeogenesis pathway	(Antoniewicz, 2006)
<i>C. glutamicum</i>	$[1]$; $[6]$; $[1,6]\text{gluc}$	MS - CO_2	^{13}C -MFA with CO_2 as the only labeling measurement	(Yang et al., 2006)
Maize root tips	$[1]$; $[2]\text{gluc}$	NMR - int. sugars, AA	Integrated ^{13}C -MFA: estimated fluxes from ^{13}C -enrichments	(Alonso et al., 2007)
Soybean seedlings	$[\text{U}]\text{gluc}$; $[\text{U}]\text{gln}$	MS - biomass AA, int. sugars, organic acids	Integrated ^{13}C -MFA: utilized two labeled substrates to improve flux resolution	(Allen et al., 2009)

Organism	Tracers	Measurements	Major achievements	Reference
<i>D. etherogenes</i>	[1]; [2]lac; bicarb	MS - biomass AA	Elucidated pathways for ile, leu, thr biosynthesis, and identified <i>Re</i> -CS	(Tang et al., 2009)
<i>C. acetobutylicum</i>	[U]gluc; [U]glu; [U]asp; others	MS - int. metabolites	Demonstrated bifurcated TCA cycle and identified <i>Re</i> -CS	(Amador-Noguez et al., 2010)
<i>P. falciparum</i>	[U]gluc; [U- ¹³ C, ¹⁵ N]asp; [U- ¹³ C, ¹⁵ N]gln	MS - int. metabolites	Demonstrated bifurcation of TCA cycle and reductive carboxylation	(Olszewski et al., 2010)
HEK-293	[1]; [6]; [U]gluc; [U]gln	MS - ext. lactate	¹³ C-MFA with lactate as the only labeling measurement	(Henry and Durocher, 2011)
<i>E. coli</i>	20, 40, 60, 80, 100% [U]gluc	MS - biomass AA	Integrated ¹³ C-MFA: identified exchange of CO ₂ and validated <i>E. coli</i> model for ¹³ C-MFA	(Leighty and Antoniewicz, 2012)

Notes: [1]gluc denotes [1-¹³C]gluc, unless stated otherwise. A semicolon “;” denotes parallel experiments, and plus-sign “+” denotes multiple tracers used in a single experiment. Abbreviations are same as in Table 1.