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Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks

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Abstract

The rapid emergence of metabolomics has enabled system-wide measurements of metabolites in various organisms. However, advances in the mechanistic understanding of metabolic networks remain limited, as most metabolomics studies cannot routinely provide accurate metabolite identification, absolute quantification and flux measurement. Stable isotope labeling offers opportunities to overcome these limitations. Here we describe some current approaches to stable isotope-labeled metabolomics and provide examples of the significant impact that these studies have had on our understanding of cellular metabolism. Furthermore, we discuss recently developed software solutions for the analysis of stable isotope-labeled metabolomics data and propose the bioinformatics solutions that will pave the way for the broader application and optimal interpretation of system-scale labeling studies in metabolomics.

Metabolomics is a rapidly growing field of postgenomic biology focusing on system-wide studies of metabolite levels and transformations in biological samples. Recent advances in modern high-throughput bioanalytical platforms, in combination with rapidly improving computational capabilities for data analysis and interpretation, and the free availability of numerous organism-specific metabolite databases, make it possible to annotate and quantify hundreds of metabolites in a single experiment. The resulting metabolite profiles provide a

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highly informative snapshot of an organism's physiology and are widely used both in fundamental biology and in clinical research.

A major benefit of metabolomics is the unbiased approach and the resulting ability to generate and test hypotheses based on the behavior of the whole biological system [1]. While it is not possible to detect every metabolite in a system, untargeted studies involve large-scale detection of a wide range of structurally diverse metabolite features and offer semiquantitative information about metabolite abundance. These untargeted studies can generate hypotheses about novel or important metabolites and pathways, but generally require follow-up targeted studies to confirm metabolite identities and accurately measure metabolite concentrations [2].

A major limitation of many metabolomics studies is the lack of dynamic information to allow interpretation of data in the context of metabolic fluxes [3, 4]. While metabolomics may demonstrate an increased abundance of a certain metabolite under specific conditions, it cannot determine whether this is the result of increased flux from a synthesizing enzyme, decreased flux towards a consuming enzyme, or alteration in transport of the metabolite into or out of the cell or between various compartments within the cell. Furthermore, many **metabolic pathways** are interconnected, and metabolite levels are often maintained by a number of metabolic processes, which cannot be adequately disentangled by measurement of steady-state metabolite concentrations alone [3]. Metabolomics studies are primarily performed with either NMR or MS-based analytical platforms. NMR is inherently quantitative and nondestructive, offering the potential to obtain real-time metabolite concentrations from live cells. However, MS-based approaches are more commonly used for metabolomics due to its higher sensitivity and detection of a much larger range of metabolites [5]. Isotope labeling offers advantages for both NMR and MS-based metabolomics studies; however, in this review we will focus on LC–MS-based applications of isotope labeling to metabolomics.

Isotope labeling has been used for many years to study metabolic fluxes and determine the structure of metabolic pathways and networks. They were vital in the early days of biochemical pathway analysis when biochemists first began to trace the conversion of one chemical to another following incorporation of heavy atoms from precursor substrates into different metabolic products. The establishment of MS-based analytical tools has enabled the use of stable (non-radioactive) isotopes for this purpose, as the mass spectrometer can reliably separate isotopically labeled compounds based on mass difference. **Stable isotopes** have been used with MS for several decades by pharmacologists and toxicologists as internal standard (IS) to enable absolute quantification of drugs and metabolites. Recent advances in metabolomics with ultra-high-resolution MS have led to the establishment of stable isotope tracer-based metabolomics approaches to quantification, identification and pathway analyses [1]. The technologies are now available to allow global unbiased assessments of **metabolic flux** in biological systems and enable the unambiguous tracing of heavy elements through complex metabolic networks [3, 6]. In the context of systems biology, this provides ample opportunities for reconstructing and validating both stoichiometric and dynamic computational models of metabolism. Furthermore, metabolic networks deduced by isotope-labeling techniques can be used as scaffolds for integrating

and interpreting multiple postgenomics datasets, such as transcriptomics and proteomics profiles.

Today, use of isotopologous variants of common metabolites is facilitating advances in three main areas of metabolomics research: metabolite identification; metabolite quantification; and pathway discovery and flux analysis.

Stable isotopes in MS

Stable isotopes have the same number of protons as common elements, and consequently share the same physicochemical properties, but they differ in mass due to a difference in the number of neutrons. Among biochemically relevant elements, carbon, hydrogen, nitrogen, oxygen and sulfur all have two or more stable isotopes with measurable abundance in nature. The natural abundance of stable isotopes is often exploited in label-free metabolomics studies to assist metabolite identification, in combination with accurate mass, to determine the molecular formula [7]. For example, carbon is found predominantly as the light isotope, ^{12}C (98.89% abundance), but also in the form of a heavy stable isotope, ^{13}C (1.11%), with an additional neutron, in addition to trace amounts of a radioactive heavy isotope, ^{14}C. **Isotopologs**, that is metabolites containing stable isotopes and their unlabeled counterparts, have the same chemical formula and structure and hence generally behave identically during chromatographic separation (an exception being deuterated compounds that can differ from their common hydrogen containing counterparts in chromatographic properties [8]). However, in a mass spectrometer isotopologs can be readily differentiated by mass (*m/z*). Therefore, the mass spectrum for an unlabeled metabolite contains the major monoisotopic peak, in addition to low abundance peaks representing all combinations of the naturally abundant isotopes. For example, a metabolite with four carbons (e.g., aspartate; $C_4H_7NO_4$) naturally possesses a ^{13}C peak 1.00335 Da higher in mass, and at roughly 4% abundance (4 × 1.11%), compared with the monoisotopic (^{12}C) peak (Figure 1) . The exact abundance of each isotopic peak can be calculated based on the binomial distribution, that is the natural abundance of single-carbon-labeled aspartate is $4 \times ([1.11\%]^1) \times ([100\text{-}1.11\%]^{4\text{-}1}) = 4.3\%$, and unlabeled U-$^{12}C$-aspartate is $(100\text{-}1.11\%)^4 = 95.7\%$. Additional peaks representing the natural H, O and N isotopes, and peaks for molecules containing more than one heavy atom, are also present at low abundance. Ultra-high resolution Fourier transform MS is required to resolve all of these peaks, while unit resolution mass spectrometers will produce a single peak for isotopologs with the same nominal mass, necessitating additional mathematical deconvolution for accurate isotope quantification. Stable isotope-labeling studies introduce heavy isotopes of common elements (e.g., ^{13}C, ^{15}N, ^{2}H, ^{18}O and ^{34}S), resulting in metabolites that produce co-eluting LC–MS peaks of greater mass. For example, aspartate produced from a U-^{13}C-labeled carbon source would have a mass of 138.0582 in positive mode ESI-MS, 4.0134 Da (4 × 1.00335) higher than the monoisotopic peak corresponding to the U-^{12}C containing compound at 134.0448 Da. It is important to consider natural isotope abundances when quantifying metabolite isotopologs from stable isotope-labeling experiments, particularly for low resolution MS, large molecules or derivatized analytes (e.g., in GC–MS); however, the natural abundance is insignificant for small molecules when more than two heavy atoms are incorporated and analyzed on high resolution LC–MS.

Experimental designs for stable isotope labeling

Stable isotope labeling can be applied to metabolomics and fluxomics studies of a variety of biological systems, including microbial, animal, plant and human studies [9-12]. The experimental design should be optimized depending on the sample characteristics and the purpose of the study. In all cases, the most basic and important determinants are the preparation of the samples and the choice of label for the system under investigation.

The selection of labeled nutrient is dependent on the study hypothesis and the known metabolic pathways in the organism of interest [13]. For example, hypothesis-free metabolomics studies that require extensive metabolite labeling utilize fully labeled carbon sources, such as U-^{13}C-glucose. Alternatively, for detailed analysis of central carbon metabolism it may be more appropriate to use ^{13}C-1,2-glucose to allow delineation of metabolites arising from the glycolytic and pentose phosphate pathways. Other common stable isotope tracers include: U-^{13}C-glutamine to measure TCA cycle anaplerosis, U-^{13}C,^{15}N-glutamine to detect pathways for carbon and nitrogen assimilation, or ^{13}C-bicarbonate to monitor CO_2 incorporation from the atmosphere. The cost of labeled pre cursors is an important consideration, particularly for studies of large organisms. A more cost-effective labeling strategy for animal studies involves partial labeling of metabolites by supplementing drinking water with D_2O [10].

Another aspect specific to stable isotope-labeling studies is the integration of sufficient quantities of heavy isotopologs into the metabolome to enable detection in MS analysis. The kinetics of nutrient assimilation should be considered for studies that require steady-state labeling. In particular, secondary metabolites and many macromolecules (including lipids) may take several cell cycles to reach steady-state labeling, while labeling of central carbon metabolites may occur within seconds. Accurate kinetic studies require an ‘instantaneous’ replacement of carbon source with the labeled nutrient, followed by rapid quenching and extraction of metabolites at defined time points [14]. As the rapid change in carbon source is technically challenging, especially for cells in suspension culture, an alternative approach is to add additional labeled nutrient to the existing growth medium. An alternative is to grow microbes on labeled substrates creating a saturated heavy isotope-labeled metabolome to which cheaper, more readily available nonheavy isotopologs are added to follow their distribution into the metabolome [15]. In each case the resulting metabolic perturbation is likely to impact the rates of nutrient assimilation compared with cells at steady state [16]. A chemostat methodology, whereby the unlabeled nutrient is replaced by the labeled nutrient at a predefined rate, allows for rapid kinetic studies under controlled conditions. This approach requires additional mathematical adjustment to interpret observed labeling kinetics in the context of the rate of infusion [17]. Recent efforts to incorporate isotopically nonstationary ^{13}C metabolic flux analysis allow the study of incorporation of isotopes into metabolites in a system at metabolic steady state but before isotope incorporation has reached steady state – this allows enhanced probing of the distribution of fluxes through a system [18].

Sample extraction procedures vary according to the sample type. The most important considerations in all cases are the rapid quenching of metabolic enzymes, and the recovery

and stability of metabolites during extraction and storage. Sources of contamination should be minimized, as nonbiological peaks detected by MS can interfere with the detected labeling patterns. Non-metabolism-related artefact signals can be detected if pure growth medium is analyzed along with the labeled and unlabeled samples. This allows discrimination of true biological mono-isotopic and isotopolog signals from metabolites and chemical contaminants in the medium. Using different ratios of carbon isotopes in control and experimental samples can also be used to differentiate artefacts (unlabeled) and true metabolites (labeled) [19]. Solvent blanks, authentic standards for identification or quantification, and pooled quality controls to monitor signal reproducibility are also recommended [20].

Representative applications for stable isotopes in metabolomics

Metabolite identification

Detection and reliable identification of structurally diverse chemicals without *a priori* knowledge is the fundamental requirement of untargeted metabolomics analysis. The numerous challenges and limiting factors in this task are well documented [2,21,22], the most notable being the inability to accurately assign metabolite identity to most peaks within the dataset. Stable isotope labeling has several advantages with respect to metabolite structural elucidation. Determination of the elemental composition is a major strategy for the putative identification of metabolites in MS-based metabolomics. However, even with high mass accuracy, a detected mass-to-charge (*m/z*) signal can arise from one of several different molecular formulae within the accuracy limit of the mass spectrometer. Using heavy atom labeling allows elemental composition to be determined [23]. For example, feeding the organism of interest uniformly ^{13}C-labeled nutrients can be used to determine the number of carbon atoms by measurement of the mass shift between labeled and unlabeled metabolites [24]. Similarly sources of nitrogen or sulfur carrying heavy isotopes of these atoms can be added to confirm the presence of these atoms in a target formula [25,26].

In addition to the determination of the molecular formula, isotope labeling can assist with the structural identification of metabolites. In many cases a molecular formula is not sufficient to accurately identify metabolites, as multiple isomers exist in biology for most known metabolites. Labeling with predicted metabolic precursors [11,27] or partial labeling with generic carbon sources [6,28], often aids the identification of metabolites based on prior knowledge of biochemical pathways. Alternatively, labeling can assist the interpretation of MS fragmentation spectra from electron ionization MS, MS/MS or MS^n[15,25].

The extensive application of untargeted metabolomics in the last decade has revealed many unidentified metabolites in biological systems, and it is expected that stable isotope labeling will play an important role in identifying many of these novel metabolites in the coming years. Some recent examples of this approach include the discovery of a new fumigaclavine secondary metabolite in *Aspergillus fumigatus* [29], and eight novel deoxynivalenol derivatives in wheat [11].

Absolute metabolite quantification

LC–MS-based metabolomics studies offer the broadest scope of detection for small-molecule metabolites in biological systems. However, although the MS-based relative quantification of metabolites gives us useful information, absolute quantification in MS-based metabolomics is often impacted by ion suppression or enhancement from co-eluting compounds in the sample matrix [30]. This ion suppression leads to a high dependency of metabolite response on the sample matrix, and impacts the relationship between the LC–MS response (peak area) and true intracellular metabolite concentrations, resulting in a nonlinear and matrix-dependent calibration curve [31].

A full understanding of cellular responses and metabolic flux requires both qualitative and quantitative information, including absolute metabolite concentrations across the metabolic network, which impacts metabolic reaction rates. In order to overcome the matrix-dependent response of MS, heavy atom-labeled isotopologs of metabolites can be used as IS. Absolute quantification using stable isotope labeling, however, is limited by the availability and cost of ^{13}C-labeled authentic standards. To overcome this limitation, fully labeled metabolite extracts from a cellular system can be generated as an alternative to spiking individual authentic standards. In this experimental design, a large batch of cells is grown in the presence of a fully labeled stable isotope carbon source. Ideally these cells should come from the same species or tissue as the samples of interest to ensure all relevant metabolites are present; however, providing a fully labeled carbon source is typically only possible in cells adapted to a simple fully defined medium such as *Escherichia coli* or *Saccharomyces cerevisiae*. In this way, many hundreds of metabolites can be fully labeled (a caveat being where atmospheric carbon dioxide provides unlabeled atoms to the system) providing an ideal source of labeled IS. For more complex organisms it may be necessary to use a heterologous source of isotope-labeled extract, such as the commercially available uniformly labeled algal extract [32]. A fixed amount of the labeled extract is then spiked into the study samples, which allows absolute quantification of the metabolites of interest by reference to a calibration curve of unlabeled authentic standards containing the labeled IS extract (Figure 2) [33-37].

An alternative method for absolute quantification of intracellular metabolites without constructing an external calibration curve has been developed. In this method, a model organism was grown in U-^{13}C-labeled carbon source medium and extracted in organic solvent spiked with known concentrations of unlabeled IS. Absolute concentrations were calculated based on the ratio of labeled intracellular metabolites to unlabeled IS and were corrected with reference to the intracellular volume of the extracted cells [38]. The benefit of this isotope-dilution approach to quantification was demonstrated by measurement of the absolute concentrations of more than 100 metabolites in *E. coli*. These data revealed high concentrations of many metabolites, significantly above the concentration required for half maximum reaction rate (K_m) value of their consuming enzymes. Notably, a number of lower glycolytic metabolites were present at concentrations close to their respective K_m, indicating substrate-dependent modulation of flux is important for these reversible central pathways [33].

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Absolute metabolite quantification is not essential in many metabolomics studies involving comparison of two or more experimental conditions. Nevertheless, complete stable isotope labeling allows accurate comparative quantification without the limitations associated with matrix effects. Analogous to the SILAC approach in proteomics, the unlabeled and labeled samples can be mixed and analyzed simultaneously, avoiding the potential for differential ion suppression [19,25,39].

An alternative approach to absolute quantification for LC–MS metabolomics is post-extraction derivatization with differentially labeled derivatizing agents (analogous to ICAT methods in proteomics). This offers the advantage of generating isotopic IS for all metabolites of interest and is not confined to organisms that can be grown in fully defined media in cell culture. The limitations of this approach are the increased requirements for sample preparation, the limited suitability of derivatization reagents for a diverse range of metabolites, and the introduction of chemical complexity that precludes high-throughput metabolite identification based on accurate mass. Nevertheless, several isotopically labeled derivatization reagents have been demonstrated for applications in metabolomics including: DiART [40] and methylation [8] for amines, dansylation for amines and phenols [41], and *p*-dimethylaminophenacylation for organic acids [42].

Pathway discovery & metabolic flux analysis

Role of metabolites in regulation of metabolic flux

The application of stable isotope labeling provides important information about metabolic flux that could not be demonstrated by classical label-free metabolomics studies. A number of important recent discoveries have demonstrated the power of stable isotope labeled metabolomics to advance our understanding of cellular metabolism.

The well-defined pathways of central carbon metabolism, including glycolysis, pentose phosphate pathway, TCA cycle and related pathways, are most commonly studied by isotope-labeling approaches. The measurement of central carbon flux during metabolic perturbation by genetic (e.g., gene knockout) or exogenous (e.g., drug treatment) factors provides novel information about the regulation of pathways and their roles in growth and disease. Understanding how individual metabolites regulate fluxes through the network is central to our appreciation of regulated systems.

For example, in a recent study, the central carbon metabolism of colon cancer cells was determined with U-^{13}C-glucose labeling to investigate glycolysis in cells expressing the PKM2 isoform of pyruvate kinase [43]. Stable isotope incorporation into phosphoenolpyruvate and pyruvate enabled measurement of pyruvate kinase activity levels, and it was possible to demonstrate allosteric activation of PKM2 by serine. Relative levels of isotope incorporation into related amino acids and TCA cycle intermediates demonstrated that PKM2 has a key regulatory role as ascertained by genetic silencing (shRNA knockdown) and adding serine to stimulate allosteric modulation [43]. An example of *in vivo* labeling of human lung cancers with U-^{13}C-glucose revealed isotopic enrichment in a number of central carbon metabolites compared with noncancerous tissue [12], with enhanced production of glucose-derived ^{13}C-1,2,3-aspartate and ^{13}C-2,3-glutamate

demonstrating increased activity of pyruvate carboxylase in tumor tissue. Upregulation of pyruvate carboxylase was subsequently confirmed at the mRNA and protein level, offering a potential target against which to intervene in cancer chemotherapy. Similar non-targeted approaches tracing the appearance of heavy atoms in metabolites derived from labeled precursors have also been described for GC–MS studies in a lung carcinoma cell lines [44].

The regulation of metabolism is a central part of systems biology and labeling of *E. coli* with U-^{13}C-glucose and related carbon sources has recently enabled significant advances in our understanding of metabolic flux regulation at the transcriptional [45], post-transcriptional [46] and allosteric levels [47]. A novel regulatory mechanism and overflow metabolism were identified in *E. coli* pyrimidine metabolism by ^{15}N-orotate labeling [9]. The incorporation of labeled nitrogen into pyrimidine nucleotides enabled measurement of *de novo* pyrimidine synthesis and revealed a new regulatory mechanism for pyrimidine homeostasis. ^{15}N-orotate labeling, in combination with genetic mutants, confirmed the previously known feedback inhibition of carbamoyl phosphate synthetase by UMP, and of aspartate transcarbamoylase by UTP and CTP. In addition, a novel product inhibition mechanism acting on UMP kinase was shown to maintain UTP and CTP homeostasis with excess UMP eliminated by a previously unidentified uridine phosphatase activity [9].

Novel pathway discovery

The ability to discover new metabolic pathways by following the distribution of heavy atoms from isotopologs through the metabolic network has already been exploited during the classical period of biochemical pathway identification (although those studies were mostly based on radioactive isotopes, which enabled easier detection before the development of highly sensitive MS instrumentation suitable for metabolomics). Untargeted metabolomics studies routinely detect putative metabolite signals that were not anticipated according to the pre-existing models of metabolic networks in specific organisms. Stable isotope labeling enables confirmation of the biosynthetic nature of novel metabolites [24-26], and can also delineate the active metabolic pathways responsible for the production of novel or known metabolites.

Recent examples of pathway identification include a stable isotope-labeled metabolomics study of a mixed community of extremophilic microorganisms, where ^{15}N-ammonium sulfate was used to identify endogenous production of unexpected metabolites in an acidic, metal-rich environment [48]. Seeking interdependencies between members of environmental communities is of particular interest, moving beyond single organism systems. Taurine and hydroxyectoine production was identified, and is thought to be involved in protection from osmotic stress. Proteomic and genomic evidence then allowed identification of the microorganisms most likely to be responsible for the synthesis of these metabolites. While *Leptospirillum* group II bacteria are capable of producing hydroxyectoine, the complete enzymatic pathway for taurine production was not identified in any of the bacterial species inferred to be present in the biofilm. The genome of the dominant fungal species present in the biofilm, *Acidomyces richmondensis*, however, contains enzymes responsible for partial synthesis of taurine, and it is proposed that the remaining oxidative steps are catalyzed nonenzymatically in this iron-rich environment, although it would be of interest to determine

the degree to which metabolite precursor sharing occurs within communities of this type [48].

In another study of unique microbial metabolism, 1-^{13}C-acetate labeling of the methylotroph *Methylobacterium extorquens* AM1 demonstrated activity of the ethylmalonyl–CoA pathway for production of glyoxylate [28]. Bacteria that lack isocitrate lyase require an alternative pathway to regenerate glyoxylate from acetyl-CoA. Two alternative pathways were previously proposed to account for this activity, the glyoxylate regeneration cycle, and the ethylmalonyl–CoA pathway. Detection of ^{13}C-labeling in intermediates of the ethylmalonyl–CoA pathway, and the isotopomer profile of propionyl–CoA, were consistent with activity of the ethylmalonyl–CoA pathway rather than the glyoxylate regeneration cycle and further analysis of the positional isotopomers of glycine following ^{13}C-methanol labeling, confirmed the metabolic network model containing the ethylmalonyl–CoA pathway [28].

In parasites too, metabolic profiling has revealed hitherto unknown pathways. In the apicomplexan parasite *Toxoplasma gondii* significant accumulation of the unexpected metabolite, GABA was found. The enzymes responsible for the GABA shunt were subsequently identified bioinformatically and confirmed by genetic knockout in combination with U-^{13}C-glutamine labeling [27].

Even in well studied organisms like *S. cerevisiae*, new pathways continue to emerge. Metabolomics analysis of a yeast knockout mutant (Δ*YKR043c*) revealed altered levels of novel seven- and eight-carbon mono- and diphosphorylated sugars. Subsequent labeling with U-^{13}C-glucose confirmed the identity of these metabolites and, in combination with biochemical and genetic studies, identified the gene as sedoheptulose-1,7-bisphosphatase and described a novel riboneogenesis pathway [49].

Potential for network-wide pathway elucidation

The power of stable isotope-labeled metabolite profiling to determine the architecture of metabolic pathways has been clearly demonstrated, providing direct information about novel metabolic routes [9,27,28,49]. However, to date, these studies have been limited to the targeted profiling of known or anticipated metabolic pathways, commonly focusing on the extensively studied area of central carbon metabolism. Several tools have also been developed for network-wide pathway elucidation by the combination of untargeted (or semitargeted) metabolomics with stable isotope labeling [6]. This approach provides the potential to discover novel metabolic pathways, and reveals important information about active pathways for organisms that have multiple possible carbon sources. This approach was previously limited by the difficulties associated with simultaneous identification of hundreds of metabolites, and the detection and quantification of all relevant isotopolog signals. Recent developments in metabolomics technology include methods for widely-targeted metabolomics studies, which accurately identify and quantify hundreds of known metabolites [50], and advances in putative metabolite identification from high-resolution LC–MS-based untargeted studies, based on accurate mass-derived formula determination and predictive tools for retention time [51] or MS/MS spectra [52]. Furthermore, software solutions to routinely extract isotopolog abundances on a large scale are now available (see

below). Biochemical interpretation of these data still requires a significant level of manual curation, but the growing availability of atom-resolved metabolic networks, where the source of specific atoms in each molecule can be traced from precursor substrates, offers the potential for computational approaches to biological inference and hypothesis generation based on network-wide isotope-labeled metabolomics data.

Extensive information about pathway flux can be gained by collection of time-course data, to trace incorporation of metabolites, or by utilization of partially labeled, or mixed labeled/unlabeled isotopic precursors. In a proof-of-concept study, the addition of 50% U-^{13}C-glucose to the procyclic form of the protozoan parasite, *Trypanosoma brucei*, revealed labeling patterns that allowed assignment of the biosynthetic routes for many metabolites. This included confirmation of succinate production via the glycosomal fermentation pathway, rather than the TCA cycle, and incorporation of pentose phosphate cycle intermediates into the novel metabolite octulose phosphate [6].

An alternative system-wide application of isotope labeling is the SiDMAP approach to isotope enriched metabolome ('isotopolome')-wide association studies, which aim to detect relationships between known phenotypes and isotope labeling of metabolites [53]. In this approach, the isotope profile of a number of defined metabolic end-products is measured to indicate the flux through known pathways [54]. While this approach does not capture the extent of cellular metabolism, and cannot identify unanticipated metabolites or pathways, it provides a rapid and accessible means to measure flux through a number of central pathways in response to genetic, environmental or pharmacological perturbation [53].

Challenges of stable isotope-labeled metabolomics data analysis

A plethora of computational tools are available to analyze the datasets created by MS-based metabolomics studies [2], including widely used open source software for LC–MS, such as MZmine [101], mzMatch [102], Ideom [103] and XCMS [104], and commercial software, such as SIEVE [105], MassHunter [106], Progenesis CoMet [107] and MarkerLynx [108]. These programs are designed for identifying and quantifying metabolites of interest in data gathered from nonisotopically labeled data (some can be used to manually extract isotope-labeling information); however, they lack features that are critical for a successful global analysis of data from stable isotope-labeled metabolomics studies.

The specific analytical approach to each stable isotope-labeled metabolomics study is dependent on the aim of the study. For example, LC–MS data for metabolite identification from an untargeted study would be analyzed differently to flux data from a targeted study of central carbon metabolism. Nevertheless, all stable isotopelabeled metabolomics studies share the common requirement to extract accurate isotopolog intensities from the raw data and present this data in a format suitable for biological interpretation. The following features would be desirable in a comprehensive computational solution for the systematic analysis of data derived from heavy isotope labeling experiments:

- Visualization options for unlabeled and labeled chromatograms for rapid visual comparison and assessment of peak shape and relative intensity across samples. The generation of supporting information, such as diagnostic plots of the observed

labeling patterns, needs to be efficient enough for a quick exploration of the large amount of compounds detected in untargeted isotope profiling;

- Consideration of all of the biologically relevant stable isotopes (^{13}C, ^{2}H, ^{15}N, ^{18}O and ^{34}S) and different numbers of heavy atoms (e.g., the analytical considerations associated with U-^{13}C-glucose labeling differ considerably from ^{13}C-bicarbonate). Furthermore, the software must be able to handle all permutations of label incorporation in nutrients labeled with multiple isotopes (e.g., glutamine labeled with ^{13}C and ^{15}N);
- Output that facilitates downstream statistical and modeling analyses and can be easily integrated with existing software.

Currently available tools that are specifically designed to analyze labeled MS data (Table 1) include CAMERA [55], MetExtract [56], MAVEN [57] and mzMatch-ISO [58]. CAM-ERA is an R tool that is specifically designed for the annotation and evaluation of mass spectral features including isotope peaks, adducts and fragments that co-elute from a chromatographic column. Although the software provides some basic visualization of the light and heavy isotopolog chromatograms, rapid differentiation and relative quantification of isotope patterns is not currently possible with this software. Similar limitations with visualization apply to MetExtract, which has a comprehensive user interface that enables custom parameters to be defined. MetExtract employs a brute force method to extract peaks from mass spectra rather than exploiting other well-established peak-picking algorithms. MAVEN has a highly intuitive interface for exploring and validating metabolomics data rapidly and reliably. It has robust and easily comprehensible plots to differentiate the labeling patterns between replicates and sample groups within an experiment. The pathway visualizer, and isotopic flux animator in this software offer automated inference into biological events detected within a study, making it an excellent tool for the analysis of isotope labeling studies in systems biology. Unfortunately, custom algorithm development and data integration that would allow the software's extension require expertise in system level programming language, C++. mzMatch-ISO is an open-source software that combines all of the desired features listed previously and has been employed recently in a number of targeted and untargeted labeling experiments [6,43]. It is derived from the mzMatch.R suite of metabolomics analysis tools [59], providing access to automated data analysis through a well-defined pipeline [60]. It uses the XCMS centWave [61] peak-picking algorithm augmented by an algorithm that fills missing signals from raw mzXML [62] data to provide precise quantification of all isotopologs. Further-more, the processing pipeline includes several filters that remove noise and signals of low intensity. These filters not only provide cleaner data in untargeted isotope profiling, but also reduce the time and effort required for downstream statistical analysis and interpretation.

Another important challenge is the rapid visualization of information from isotope labeled metabolomics data within the context of metabolic pathways and networks. Automatic mapping of labeled metabolites onto metabolic networks would provide an immediate view of the core flux map of an organism, and also provide an indication of the effect of perturbations on various aspects of metabolism [6]. Several tools provide facilities to assist this kind of visualization, including MetPA [63], MetExplore [64], iPath2 [65] and Pathos

[66]. All of these are web applications that can either infer metabolic networks (MetExplore) or map metabolites identified in a metabolomics study onto metabolic maps from KEGG [67] or MetaCyc [68]. However, none of these is specifically designed to make full use of the rich data provided by isotope-labeling studies, and several important challenges remain in this area. First and foremost, an important objective of many untargeted stable isotope-labeled metabolomics experiments is to facilitate tracing the route of individual atoms from a labeled nutrient source. Therefore, a specifically designed visualization tool for this type of data analysis should encapsulate and display such information. Although computationally trivial to implement, the major limiting factor for such a pathway visualization platform would be the underlying database. Instead of databases that map metabolite and reaction names, *in silico* atom-resolved databases of metabolic networks are required for this purpose. Some such databases for *E. coli* and a few other organisms exist in BioCyc and KEGG [69,70]; however, a software that uses these databases is not yet available.

An important feature of metabolomics data is that it can provide a snapshot of the most active pathways within an organism, which makes it an ideal source of data for the reconstruction of metabolic models and for validating the features of genome scale metabolic models. To do this, however, data from metabolomics experiments need to be exported in a form acceptable to popular metabolic modeling softwares such as the Cobra Tool Box [71], ScrumPy [72] and Copasi [73]. These pieces of software enable the application of various constraint-based pathway analysis and interrogation algorithms, such as enzyme subset analysis, elementary modes analysis [74,75] and flux balance analysis [76], to generate and test hypotheses regarding the biology of an organism. One challenge in achieving such comprehensive metabolic models is the presence of reactions in a network or pathway that could not be identified from the metabolomics data. Such gaps in reconstructed metabolic networks need to be filled using data from complete and accurate metabolic models from model repositories, such as the BioModels database [77], that host numerous curated and published databases.

Conclusion & future perspective

The integration of stable isotope labeling with metabolomics studies has been demonstrated in a number of biological systems and provides solutions to the major limitations of metabolomics: metabolite identification, quantification and flux analysis. Stable isotope labeling is expected to become a routine aspect of many metabolomics studies in the future, as the metabolomics field moves from a largely observational approach to a more detailed mechanistic investigation of cellular metabolism. Pioneering studies of stable isotope-labeled metabolomics have already discovered numerous novel metabolites, pathways and regulatory mechanisms [9,28,29,33,43]. The available tools for stable isotope-labeled metabolomics allow investigation of metabolic responses to various stimuli including pharmacological, environmental and genetic perturbations, which will inevitably lead to advances in our understanding of metabolic networks.

The incorporation of data from stable isotope-labeled metabolomics studies into computational flux models provides an exciting avenue to interpret metabolomics data. It is expected that gaps in our current knowledge of pathway connectivity will be closed as the

cycle of metabolic labeling and flux modeling informs the discovery of new metabolites, pathways and fluxes. Recent software advances allow the rapid extraction and quantification of isotopolog signals from MS-derived metabolomics data. The next essential step to enable the widespread application of labeling to network-wide isotopolog analysis is the development of user-friendly software to integrate experimental isotopolog data into genome-scale models of metabolism for visualization and predictive modeling.

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Key Terms

Metabolomics	Comprehensive measurement of metabolite abundances in a biological system.
Metabolic pathways	Series of biochemical reactions converting substrates (e.g., nutrients) into metabolic end-products. Definitions of the start and end points of metabolic pathways are often arbitrary, as most metabolic pathways are interconnected.
Stable isotopes	Any form of a chemical element that does not undergo radioactive decay, generally used to refer to heavy isotopes of common elements that have low abundance in nature (e.g., ^{13}C, ^{15}N, ^{2}H, ^{18}O and ^{34}S).
Metabolic flux	Rate of turnover of metabolites in a metabolic pathway.
Isotopologs	Two or more isotopic homologs of a molecule that differ only in their isotope composition and mass.

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Executive summary

Metabolite identification

- Stable isotope labeling aids in formula determination for identification of metabolites.

Absolute metabolite quantification

- Complete stable isotope labeling of an organism can provide metabolome-wide IS for absolute quantification.

Pathway discovery & metabolic flux analysis

- Stable isotope labeling enables flux measurements that allow the study of system-wide metabolic regulation.
- Novel metabolites and pathways can be discovered by application of stable isotope-labeled tracers.

Challenges of stable isotope-labeled metabolomics data analysis

- Recent software developments have begun to overcome the bottleneck in data analysis for stable isotope-labeled metabolomics.
- Further software solutions will be required to routinely interpret metabolome-wide stable isotope-labeling studies in a biological context.

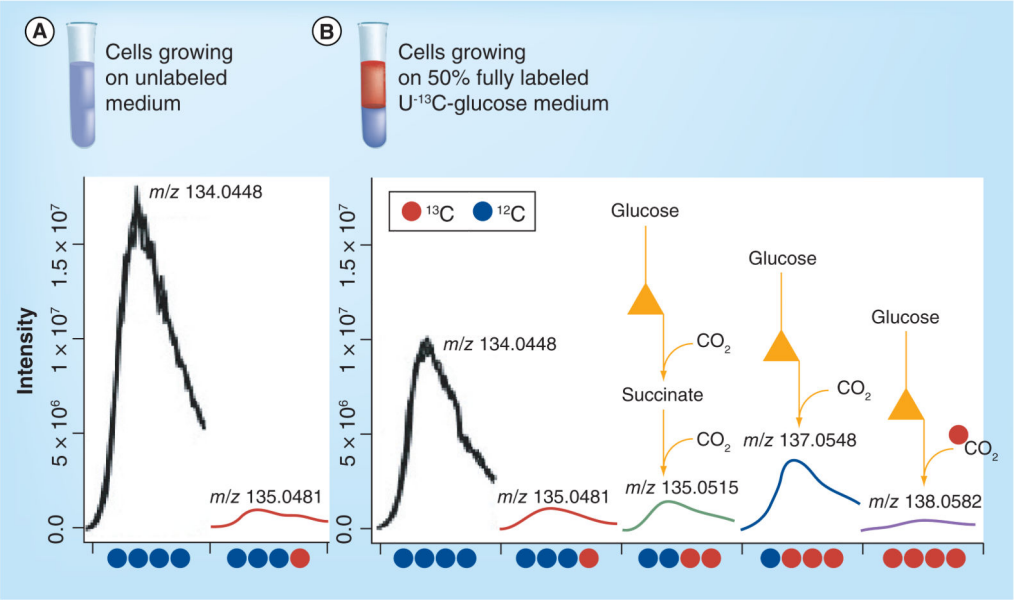

Figure 1. Isotopolog LC-MS signals for differentially labeled aspartate

Relative abundance of different isotopologs of aspartate in procyclic form *Trypanosoma brucei* growing on **(A)** unlabeled ^{12}C-glucose medium and **(B)** a 50:50 mix of labeled U-^{13}C-glucose and unlabeled U-^{12}C-glucose medium at steady state [6]. Isotopologs elute at the same retention time, but their masses differ by the difference in the mass of heavy and light carbon (1.00335 Da). Red circles show the number of labeled carbons that each isotope contains. Chromatograms show unlabeled (black), one (red; natural isotope abundance), two (green), three (blue) and four (purple) carbon-labeled isotopologs. These aspartate isotopologs are synthesized from the respective oxaloacetate (OXAC) isotopologs by transamination, and demonstrate active glycolysis and succinate fermentation pathways: the predominant three carbon labeled OXAC derives from phosphoenolpyruvate carboxykinase (PEPCK) activity on three-labeled (glycolysis-derived) phosphoenolpyruvate and unlabeled (atmospheric) CO_2. The two carbon-labeled isotopolog commonly indicates formation of two-labeled OXAC from the TCA cycle; however, TCA cycle activity is minimal in *T. brucei*, and in this case, two-labeled OXAC is derived from a reversible succinate fermentation pathway where the symmetrical structure of fumarate allows labeled carboxylic acid groups of dicarboxylic acid intermediates to be replaced by unlabeled (atmospheric) CO_2 through the reversible activity of PEPCK [78]. The low-abundance four carbon-labeled isotopolog derives from addition of labeled CO_2 to three-labeled phosphoenolpyruvate by PEPCK. The labeled CO_2 is generated biosynthetically from glucose by either the pentose phosphate pathway or by PEPCK in the reversible succinate pathway. Please see colour figure at: www.future-science.com/doi/full/10.4155/BIO.13.348

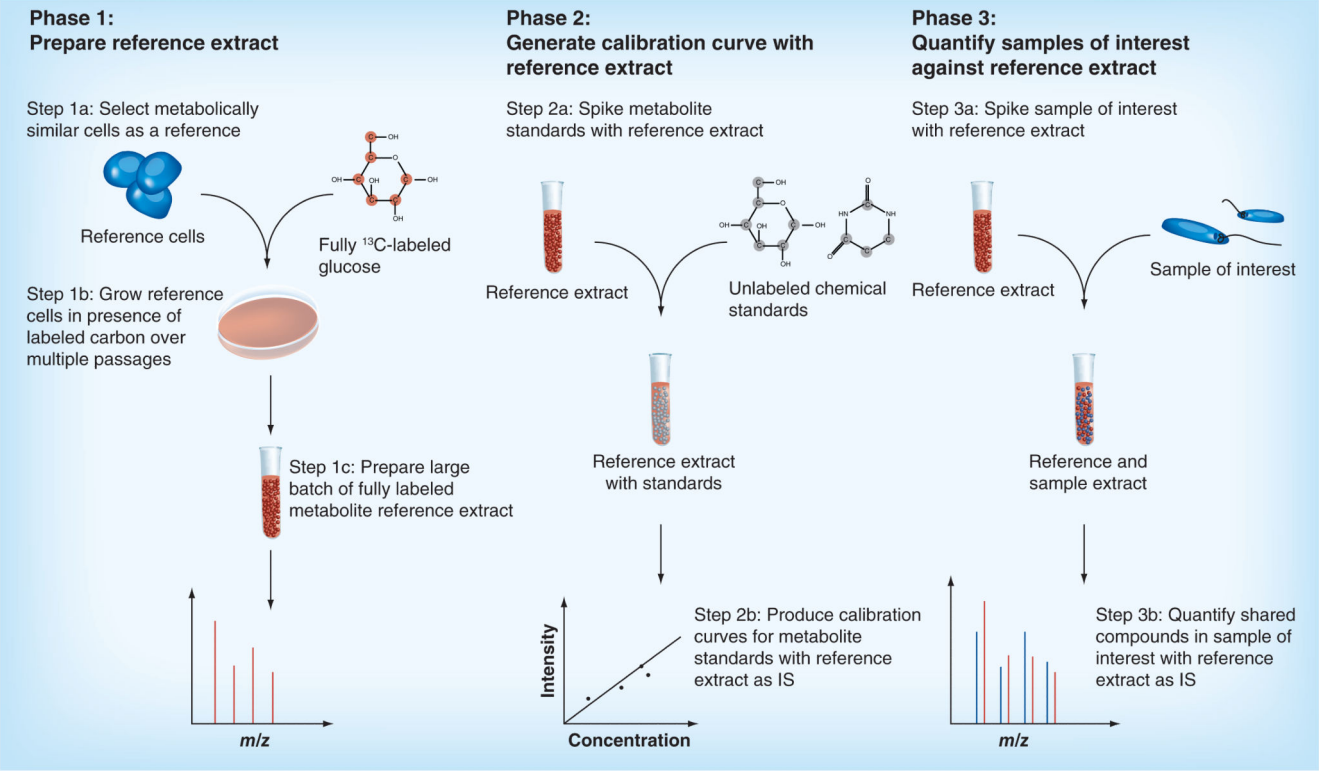

Figure 2. Methodology for absolute quantification using stable isotope labeling

Fully labeled metabolite extracts from a cellular system are used as an alternative to spiking expensive labeled standards for each individual metabolite [36]. See text for detailed explanation.

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Table 1
software for stable isotope-lab metabolomics data analysis

Software	Advantages	Disadvantages	Ref.
mzMatch-ISO	• Uses the standard XCMS peak-picking algorithm to pick peaks and can retrieve missing peaks from raw data • Written in the R statistical software. A single command with well-documented parameters is all that is required to run an analysis • Results include chromatograms and several plots that describe the labeling pattern within replicates and within an experiment • Can work on most biologically relevant isotopes – C, H, N, O and S • Can be used for both targeted and untargeted isotope profiling	• Has a command line interface • Has a steep learning curve	[58,102]
MAVEN	• Robust and easily comprehensible plots to differentiate the labeling patterns between replicates and sample groups within an experiment • The pathway visualizer and isotopic flux animator in this software offer automated inference into biological events detected within a study • A very robust user interface that is easy to understand and operate • Can be used as tool to quickly inspect the labeling pattern of a given metabolite	• Development of custom algorithms and data integration that would extend the capabilities of the software is challenging for a typical biologist	[57,109]
MetExtract	• Offers a very basic visualization of the monoisotopic and corresponding isotope peaks • Has a comprehensive user interface that enables custom parameters to be defined	• MetExtract employs a brute force method to extract peaks from mass spectra rather than exploiting other well-established peak-picking algorithms • The basic visualization of peaks offered is not sufficient in many applications	[56,110]
CAMERA	• Specifically designed for the annotation and evaluation of mass spectral features including isotope peaks, adducts and fragments that co-elute from a chromatographic column. • Written in the R statistical software and is open source. This offers plenty of scope for further extension	• The software provides only a basic visualization of the light and heavy isotopolog chromatograms • Rapid differentiation and relative quantification of isotope patterns is not currently possible with this software	[55,111]
IDEOM	• User-friendly and familiar interface in the form of Microsoft® Excel spreadsheets • Very easy to implement once the underlying software is installed • Results in the form of tables that can be easily exported for further statistical analyses	• Designed for the annotation and evaluation of mass spectral features including isotope peaks, adducts and fragments that co-elute from a chromatographic column • Cannot directly access raw data to retrieve missing peaks (requires mzMatch-ISO for this function)	[79,103]
iMS2Flux	• Provides a framework for automated isotopolog analysis for large datasets	• Additional software is required for the initial peak detection and for the final flux analysis	[80,112]

Software	Advantages	Disadvantages	Ref.
	• Focus on validation and correction of MS-derived data and output in format suitable for MFA	• Cannot directly access raw data when performing data checks	
FiatFlux†	• ^{13}C-MFA tool that has a convenient user interface • Facilitate FBA • GC–MS based tool	• Works only on ^{13}C-labeled data • Does not work on LC–MS data • Requires predefined steady-state stoichiometric model to predict flux • Cannot perform untargeted metabolomics and trace the route of labeled carbon atoms	[81,113]
^{13}C-Flux2†	• Aimed at providing a direct measure of flux in a system being investigated • Provide insights into metabolic pathway activity by comparing flux phenotypes under different environmental conditions and physiological states as well as for a variety of carbon sources • Has been used in numerous ^{13}C-MFA studies • Works on GC–MS, LC–MS and NMR data	• Requires a detailed steady-state stoichiometric model that encompasses the metabolism being studied • Command-line interface • Works only on ^{13}C labeled studies • Cannot perform untargeted metabolomics on labeled data	[82,114]
OpenFlux	• An attempt to make a flexible version of ^{13}C-Flux2 to perform steady-state ^{13}C MFA using mass isotopomer distribution data • Spreadsheet-based user interface	• Not applicable for targeted and/or untargeted metabolomics data analysis and isotope profiling	[83,115]

†These are metabolic-flux analysis tools that require a predefined stable steady-state stoichiometric model of the metabolism to determine the flux.
MFA: Metabolic flux analysis; FBA: Flux balance analysis.

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