Review

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Microbes and Metabolism

TARDIS-based microbial metabolomics: time and relative differences in systems

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Metabolomics can play a particularly important role in elucidating novel anabolic and catabolic pathways in bacteria and fungi, and in understanding the dynamics of metabolism. In these approaches, an isotopically labelled substrate, with an artificially high abundance of isotopic label, is fed to the microorganism under study. The products become isotopically labelled, and can be measured using a combination of mass spectrometry and nuclear magnetic resonance spectroscopy. This mass isotopomer analysis is referred to as time and relative differences in systems (TARDIS)-based analysis, as it measures and quantifies the temporal sequential emergence of these labelled products. In this review, we cover this topic from an experimental point of view in relation to the study of metabolism, and summarise how the application of radioactive and stable isotopes is being used in pathway elucidation and metabolic flux determination (fluxomics).

An introduction to metabolomics and the application of isotopes to study metabolism

The terms 'metabolomics' and the synonymous 'metabonomics' are relatively young terms coined in 1998 [1] and 1999 [2] respectively, which refer to the holistic and quantitative analysis of the small molecules, metabolites, present within a biological system. These metabolites (the complete collection being defined as the metabolome) typically have molecular weights less than 1000 Da, and are therefore small compared with proteins, transcripts and genes. As with the other '-omics,' the etymology of these terms combines the suffix '-ome', referring to the complete analysis of the prefix derived from the Greek word *metabole* (meaning change), which in this case refers to metabolism. *Metabole* in the present context is of course entirely appropriate, given the highly dynamic nature of the metabolome, relative to the somewhat static nature of the genome of the organism under study. Even in steady-state conditions, in which metabolite concentrations and metabolic flux are constant, dynamic processes are in operation because the flow of carbon into and out of metabolite pools is in constant operation (i.e. metabolite molecules are constantly being consumed and synthesised). The application of isotopes, both radioactive (e.g. ^{14}C and ^{32}P) and stable (e.g. ^{13}C), in the study of metabolism will be reviewed here, with an emphasis on experimental protocols and applications, including the application of isotopes to: (i) discover novel metabolite pathways and (ii) quantify flux within metabolic networks.

Post-genomic approaches are widely employed within the life sciences, and the majority are used in a so-called 'knowledge discovery' or inductive process [3]. In metabolomic studies, these processes investigate the many small metabolites that are extracted from cells or are isolated from the medium surrounding cells in culture, defined as the exometabolome or metabolic footprint [4]. The integration of appropriate and robust sample collection and preparation methods with a range of analytical techniques and univariate and multivariate data analysis tools allows the inference of specific differences related to biological questions [5]. The intracellular metabolome is highly dynamic in nature, with marked changes in metabolite concentrations and fluxes being measured in timescales of seconds or minutes. This is observed during perturbations to the system, as metabolite concentrations and fluxes are constant in steady-state conditions. Appropriate 'quenching' methods are used to arrest intracellular metabolism instantaneously (or at least as fast as is possible) to acquire a representative snapshot of the metabolome [6–8]. Quenching is followed by the fracture of the cellular membrane(s) to release the intracellular metabolome, and suitable extraction methods are used before analysis [8,9]. Analytical instruments such as gas or liquid chromatography (GC or LC) typically linked to mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and optical or vibrational spectroscopies are used in a holistic manner to investigate large collections of metabolites [10].

These knowledge-discovery studies investigate the system at a particular snapshot in time, especially targeted to

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intracellular metabolism. However, metabolism can operate in a dynamic manner, and approaches to quantify metabolic processes can provide complementary data and knowledge about carbon or nitrogen flow through metabolic networks. In metabolomics, investigating this dynamic nature can involve the measurement of the flux of an element (typically carbon) through the biological system, or the measurement of changes in metabolite anabolism or catabolism. In this review flux is defined as the quantitative rate of production or consumption of an entity (metabolite or element) in a metabolic network. This flux is directly related to the reaction rates of enzymes that in general have not been calculated by enzyme kinetics. The common approach to perform this is to use stable isotopes (e.g. ^{13}C and ^{15}N). These are naturally present at low abundances (^{13}C at 1.1% compared with ^{12}C, ^{15}N at 0.4% compared with ^{14}N), but are introduced to the biological system at artificially high abundances (e.g. 100% ^{13}C or ^{15}N), and their dynamic distribution through the metabolic network is subsequently qualitatively and quantitatively monitored. This is known as mass isotopomer analysis, with a mass isotopomer defined as two isomers of a single metabolite differing only in the position of isotopes.

The simplest way of measuring the total flux through a microbial system is to measure the input and exit fluxes by analysis of the exometabolome. For example, to define total carbon flux in a microbial culture with a single carbon source (e.g. glucose), the rate of consumption of glucose and the rate of production of metabolites and biomass (a combination of glycogen, proteins, lipids and other metabolites) as observed in the exometabolome can be measured (an example is provided for yeast in [11]). These exometabolome measurements can be used to constrain flux balance analysis (FBA) of genome-scale models [12], and have been used to demonstrate differences in genetically related strains of yeast [13]. However, this approach is somewhat limited in determining specific metabolite or metabolite pathway-related changes in the intracellular metabolome, such as the replenishment of intermediates in anabolic reactions or reversible reactions, hence the exometabolome is not sufficient to resolve biosynthetic fluxes.

Developments in metabolomics [10,14–19] have advanced to measurements of metabolite concentrations within the cells, although typically only as a snapshot of metabolism at the point of sampling. Further technical advances have occurred in the measurement of the dynamic flow of metabolites through metabolic networks in a cell, and through the application of stable isotopes, defined as 'fluxomics'. Although it predates metabolomics, fluxomics is increasingly being used in the study of metabolite pathways and metabolic and elemental flux [20].

Mechanisms for metabolite pathway and network elucidation

The application of stable isotopes to determine flux has developed over the past 15 years. Preceding developments focused on the elucidation of biochemical pathways, and as part of this process some studies used radioisotopic (e.g. ^{14}C or ^{32}P) metabolic tracers. Although metabolomics is considered an emerging field, it does of course have its basis in some of the work from the giants in biochemistry such as Sir Hans Krebs, the Nobel Laureate of 1953, who discovered the tricarboxylic acid (TCA), or eponymous Krebs cycle [21]. In his Nobel Lecture, delivered on 11 December 1953, (http://nobelprize.org/nobel_prizes/medicine/laureates/1953/krebs-lecture.pdf), Krebs detailed his exciting findings, and mentioned several times that since his initial work he and others had used radioisotopes to show conclusively the fate of carbon atoms from specific metabolite intermediates such as ^{14}C-labelled acetate [22]. These experiments allowed many biochemical pathways to be elucidated and confirmed in animals, plants and, of course, microbes.

There have been many instances in which ^{14}C labelled substrates were fed to bacteria to discover novel pathways. In the pioneering work of Quayle and colleagues, this radioactive labelling of metabolite intermediates allowed the elucidation of specific pathways in methylotrophs to be discovered. Methylotrophs grow on C_1 compounds such as methanol and formaldehyde as the sole source of carbon. Quayle was able to identify the ribulose monophosphate cyclic route for formaldehyde oxidation in bacteria [23], a pathway that 'borrowed' enzymes from the Entner–Doudoroff pathway to fix formaldehyde. Using similar approaches, he was also able to discover the assimilation of additional C_1 compounds by the serine pathway, which differs from other formaldehyde-assimilation pathways in that its intermediates are carboxylic acids and amino acids rather than carbohydrates [24,25].

In the above examples, the fate of ^{14}C-labelled substrates was discovered using the radioactive nature of the immediate and subsequent products from these intermediates. The sequential emergence of these products could be measured in a time series (temporally), and in terms of carbon flow, the relative differences in these metabolites could be used to elucidate novel anabolic and catabolic pathways. Hence even back in the first-half of the 20th century (well before Dr Who!), time and relative differences in systems (TARDIS)-based metabolite pathway elucidation was being conducted. Radioactive isotopes have now been replaced with stable isotopes in tracer experiments, offering reduced health and safety concerns for the researcher, and easier handling and application in the laboratory. Recent examples of pathway elucidation have been observed [26], and novel approaches are being developed to perform 'tracer-based metabolomics' which have been reviewed elsewhere [27].

In the above examples, isotopes of carbon were studied. These are most frequently used in pathway elucidation and flux measurements because of the presence of carbon in organic metabolites in biological systems. Carbon exists naturally in our environment as two stable isotopes (98.9% ^{12}C and 1.1% as ^{13}C) and a radioactive isotope (^{14}C). The above experiments of Krebs and Quayle measured the β^- emissions produced during the decay of ^{14}C. Nitrogen exists as two isotopes, ^{14}N and ^{15}N (their natural abundances are 99.6% and 0.4% respectively); neither of these are radioactive, thereby limiting the study of nitrogen metabolism in historic studies. The ^{13}C isotope of carbon has a nominal mass of 13 (accurate mass of 13.003355) and so can be easily differentiated using MS from the other

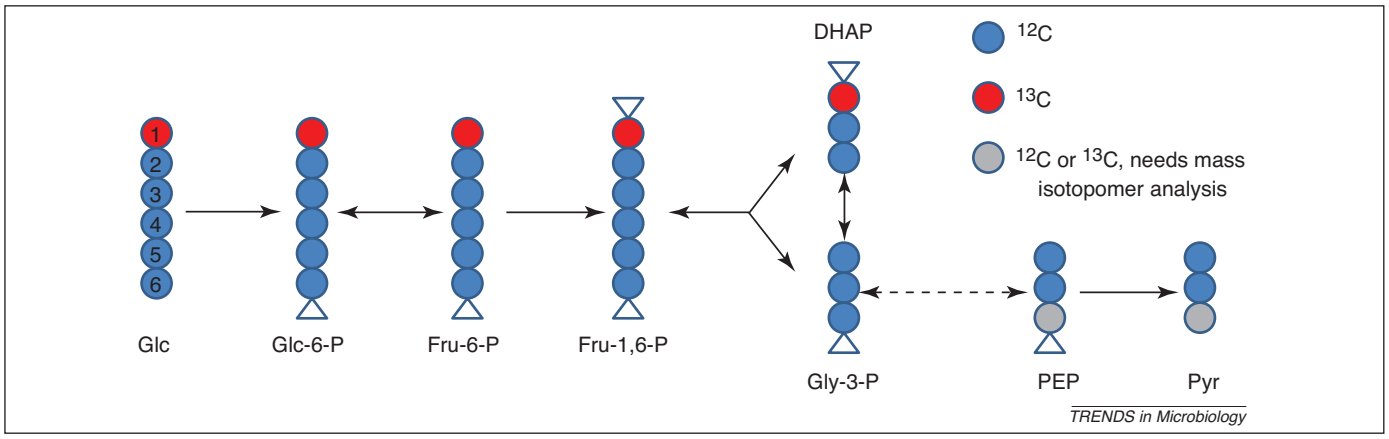

Figure 1. Incorporation of 1–$^{13}C_6$-D-glucose in glycolytic intermediates. This results in 50% 3–^{13}C-pyruvate which needs to be measured using mass isotopomer analysis. Glc, D-glucose; Glc-6-P, D-glucose-6-phosphate; Fru-6-P, D-fructose-6-phosphate; Fru-1,6-P, D-fructose-1,6-phosphate; DHAP, dihydroxyacetone phosphate; Gly-3-P, D-glyceroaldehyde-3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

isotopes of carbon, particularly from ^{12}C, whose accurate mass is 12.000000. By way of example, the nominal mass of glucose synthesised with ^{13}C in all carbon positions ([U-$^{13}C_6$]-D-glucose) is 186, compared with 180 for the abundant isotope of D-glucose (note that naturally produced glucose will have a mass isotope distribution in MS because of 1.1% of its carbons being naturally ^{13}C). Similarly, positional isotopically labelled glucose, labelled at the first two carbons ([1,2,-$^{13}C_2$]-D-glucose), will have a mass of 182, and again can be readily differentiated by MS. Thus, the above early TARDIS-based metabolite pathway-discovery experiments can now be repeated with ^{13}C- or ^{15}N- labelled substrates, and their metabolic fate followed over time within a particular system [28]. The differential labelling of source metabolites can be used to investigate different metabolic pathways. For example, [U-$^{13}C_6$]-D-glucose can be employed to study metabolism in general, whereas [1–$^{13}C_6$]-D-glucose can be used for studying glycolysis and TCA [29], and [1,2–$^{13}C_6$]-D-glucose or [1,6–$^{13}C_6$]-D-glucose can be used for studying the pentose phosphate metabolic pathway [30]. Other metabolites can also be used, including glutamine for the study of glutaminolysis in cancer cells [31]. An example of this pathway analysis is given for glycolysis (Figure 1), using positional isotopically labelled glucose at the C-1 position. In this example, the position of the isotope labelling is clear from glucose to fructose-1,6-phosphate. After these early stages of glycolysis, the enzyme fructose bisphosphate aldolase cleaves fructose 1,6-phosphate into dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (Gly-3-P). For this initial cleavage, the carbon positions are known (Figure 1). Only Gly-3-P proceeds through glycolysis to pyruvate (Pyr); however, as both products from the aldolase reaction are in equilibrium as a result of the enzyme triose phosphate isomerise, the exact isotopomers of the trioses beyond this reaction can generally only be elucidated by MS or NMR spectroscopy. As the major carbon flow is from glycolysis to TCA (or to acetate, ethanol or lactate during fermentation), the C-3 position on the subsequent metabolites between Gly-3-P and Pyr will on average be approximately 50% labelled.

Armed with a more tractable isotope-labelling route based on ^{13}C, recent microbial studies have now been conducted to discover novel metabolic pathways. One of the exciting prospects that has been highlighted by these demonstrations is that isotopomer-assisted pathway analysis can be used to identify not only novel metabolism but pathways in non-model species, which is very powerful for those organisms for which the biochemistry is poorly understood or for which a metabolic reconstruction from genome sequencing is not yet available. These recent pathway studies via TARDIS-based metabolite analysis have allowed characterisation of poorly understood metabolisms of environmental microbes, including *Sulfolobus* [32], *Dehalococcoides* [33] and many phototrophic bacterial species [34,35], allowing the discovery of novel metabolic routes, including a novel Entner–Doudoroff pathway [36], an amino acid synthesis route of isoleucine via a novel citramalate synthase in the reductive TCA cycle [37–39], and in-depth fluxomics of key carbon-fixation pathways [34].

Analytical technologies

MS and NMR spectroscopy are analytical technologies that are frequently used in isotope-based studies [30]. MS measures the mass (or more specifically the mass-to-charge ratio) of ionically charged metabolites. Different isotopes have different masses (as described above for carbon isotopes), and so can be easily distinguished and quantified, including when high-resolution mass spectrometers are employed, and this has been demonstrated within microbial systems [40–42]. MS, particularly when coupled with chromatographic techniques, provides highly selective and sensitive methods for the detection of a wide range of metabolites. Applications of gas chromatography–mass spectrometry (GC-MS) have included the quantification of the $^{13}C/^{12}C$ distribution in proteinogenic amino acids following stationary labelling of cultures (described below in more detail) [43]. Liquid chromatography–mass spectrometry (LC-MS) and the related capillary electrophoresis-MS have been used to determine the concentrations and $^{13}C/^{12}C$ distributions in metabolites in the study of *Escherichia coli* metabolism [44,45]. One of the rate-limiting steps in these studies is the analysis of these flux data, because of limited availability of software to process raw analytical data. However, advances in software are

being made, for example the Metabolomic Analysis and Visualization Engine (MAVEN) [46] and FiatFlux [19].

By contrast, NMR spectroscopy measures chemical shifts of nuclei of isotopes within atoms possessing an odd number of protons, an odd number of neutrons, or both. These nuclei exhibit mechanical spin phenomena, which are associated with angular momentum, and also possess a magnetic moment, which is measured using NMR spectroscopy. These measurements can play a powerful role in mass isotopomer analyses, and NMR has the advantage over MS in that the locations within the molecule of the ^{13}C isotope can more readily be elucidated [30,47]. Although gas-phase fragmentation MS experiments can also be employed to elucidate ^{13}C isotope position, NMR is more routinely used, even though it often lacks sufficient sensitivity. Although the coupling of chromatography and NMR is less frequent, improved selectivity and spectroscopic separation of metabolites can be provided if 2D NMR experiments are employed. Of course the combined application of MS and NMR can provide complementary information, as nicely demonstrated by Lane and colleagues [30]. The change in the $^{13}C/^{12}C$ isotope ratio in single or multiple positional carbons in a metabolite can also be determined. This can be used to define the positional carbon atom or atoms in a metabolite, which are labelled with an isotope (termed positional isotopomer distribution), and the level of isotope enrichment at each of these positions. This can allow the determination of the isotopic source through the metabolic network if a single metabolite is produced from multiple metabolic reactions. The ability not only to determine the change in isotope ratios (e.g. $^{13}C/^{12}C$) but also positional changes in metabolites provides great power for the study of metabolism, and can be used to study the interaction of multiple isotope sources simultaneously (e.g. ^{13}C and ^{15}N) [48].

The quantitative analysis of isotopes and their positions within metabolites is necessary to follow enzymatic-based metabolite transformations, particularly in undiscovered transformations, and both MS and NMR play important roles here. Although to our knowledge there are as yet no reports in the microbial literature, the recent findings that hyperpolarised metabolites can be used to follow chemotherapy in whole animals [49] could hold exciting prospects for assessing metabolite flux in real time within microbial systems.

Mass isotopomer analysis in microbial systems

Cultivation and measurement methods for mass isotopomer experiments

One of the first points to consider when studying microbial systems is the culture method. In flux-based experiments, the growth of the cultures is usually performed in defined minimal media with a single carbon source, to avoid transitions between multiple carbon sources. To measure fluxes accurately, the labelled substrate should be the primary supply of either carbon or nitrogen, otherwise additional isotope dilution will occur because of extraneous supplies of ^{12}C or ^{14}N. Both batch and continuous (steady-state) culture methods are used in fluxomic investigations to grow microorganisms (Figure 2). In the post-genomic era, there has been a recent resurgence in the application of continuous-culture methods because of the need for robust and reproducible samples to be collected [50,51]. A number of approaches can be used to produce steady-state cultures, the most common being a chemostat, in which the growth is performed at a reduced growth rate by the limitation of a particular nutrient (Figure 2b), thereby resulting in profiles characteristic of the nutrient-limited growth conditions. Auxostats provide an alternative method to produce pseudo-steady-state cultures without the induction of nutrient-limited conditions and at a growth rate equivalent to the maximum specific growth rate (μ_{max}). For example, in a permitistat (Figure 2c) [52], the electrical capacitance of the culture is measured by an internal probe, and a feedback loop regulates the media pump in response to the measured capacitance (biomass) of the culture. The biomass concentration is set to below the maximum biomass yield so that the growth rate of the culture is not constrained or limited by a particular nutrient. The growth rate of the culture is therefore equivalent to μ_{max}.

The application of mass isotopomer analysis can be used in either stationary or dynamic labelling experiments to investigate flux through metabolic pathways. The

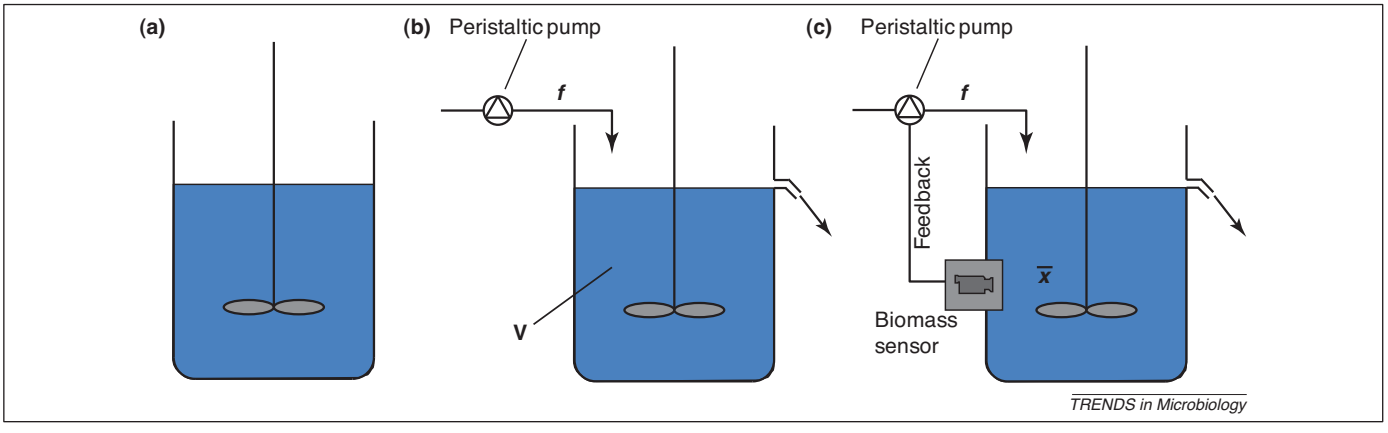

Figure 2. The main microbial culturing approaches used in fluxomic studies. **(a)** Batch culture: a non-steady-state culture with cells at different stages of their growth cycle. Growth rate changes with time in accordance with the classic growth curve, and available nutrient concentrations change with time. Metabolism operates in a non-steady state. **(b)** Chemostat culture: a growth rate-limiting substrate is added at a constant flow rate (f) in the inflowing medium. The volume (V) of the culture is kept constant by overflow of effluent through a side port. Metabolism operates in a steady state. **(c)** Turbidostat culture: a continuous flow culture in which biomass density is measured and controlled at a fixed value ($\bar{x}$) by automatic adjustment of the inflow rate (f) of the complete medium. Metabolism operates in a pseudo-steady-state.

stationary labelling of cultures is the most widely used approach to date. In this approach, an isotopically labelled carbon source is added to the culture until an isotopic steady state (the isotopic composition of the culture does not alter with time) is obtained. The proteinogenic amino acids are hydrolysed from the biomass, and analysed to reveal a labelling pattern indicative of the route by which the carbon source was metabolised in the metabolic network. For example, in *E. coli*, all amino acids are generated from the cell *de novo*, so inspection of the labelling patterns in the aromatic amino acids relative to the other amino acids provides an indication of carbon flow through the pentose phosphate pathway (PPP). The aromatic amino acids tyrosine, tryptophan and phenylalanine are all generated from the precursor erythrose-4-phosphate through the PPP. As many more metabolites than amino acids are measured, and even for amino acids they can be generated by several routes, it is of course more complex than this, therefore computational simulations estimate the intracellular fluxes based on the measured isotopic stationary data and extracellular fluxes. In the stationary labelling of cultures, a metabolic and isotopic steady state is required. A metabolic steady state is defined as when the metabolic fluxes do not vary with time (the sum of the fluxes into the metabolite pool is equal to the sum of the fluxes out of the pool), and is readily achievable in continuous-culture systems after a number of volume changes of the fixed working volume. Establishment of a steady state is defined as three [53] to five [54] volume changes of the culture. In batch cultures, the growth is time-dependent, thus intra- and exo-metabolome pools change during the time course of the experiment. In batch cultures, a pseudo-steady state is obtained thus the metabolic fluxes do not change with time relative to the biomass concentration.

An alternative approach in fluxomic experiments is to use dynamic (or non-stationary) analysis to investigate changes in the flux of microbial systems. Dynamic-flux experiments combine mass isotope data ($^{12}C/^{13}C$ or $^{14}N/^{15}N$ ratio data) and the quantitative analysis of metabolite concentrations [55]. Experiments to measure the dynamic flux in a system are experimentally more challenging to perform, and therefore fewer studies have been reported in the literature. Those reports that have been made on dynamic-flux analysis have generally involved bacteria that produce compounds of industrial importance [56,57]. Continuous cultures are an attractive growth option to investigate dynamic flux because the metabolite pools remain constant during the culture, thereby allowing a single (rather than time-consuming multiple) quantification of metabolite concentrations. Once a steady-state culture is established, the label is introduced to the culture at the same dilution rate (this must be performed to avoid perturbing the system; e.g. a pulse of carbon), and sampling is performed over a time course to track the incorporation of the label into the metabolites (examples are shown in Figure 3, which also highlights the different dynamics in carbon flow). The label is quickly incorporated into central metabolism metabolites, and the experiments are usually performed over one or two volume changes. To measure the dynamic changes in the isotopic labelling of metabolites, the culture must be rapidly sampled at a rate

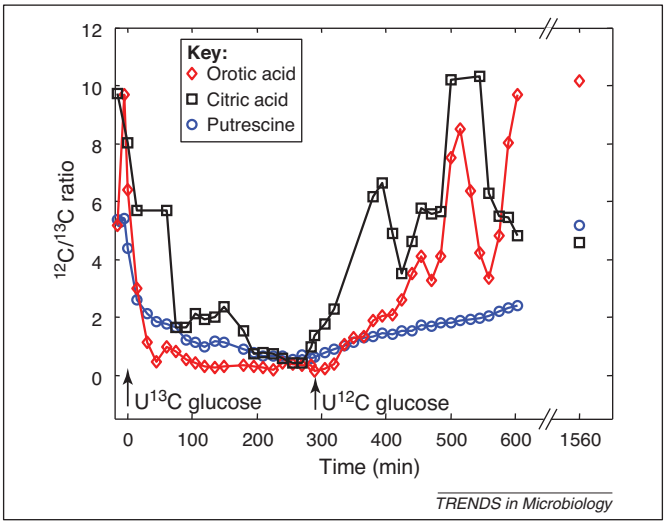

Figure 3. Example of dynamic labelling of three metabolites in glucose-limited chemostat cultures of *E. coli*.

faster than the turnover of the metabolites of interest. The turnover of metabolites in central metabolism is in the order of a few seconds [58], with intermediates of glycolysis reaching a steady state within seconds to a few minutes. By contrast, protein-bound amino acids take several hours to reach a steady state, hence the sampling procedure is less crucial in stationary flux measurements.

Measuring dynamic changes in intracellular metabolites requires the quenching of cultures to halt metabolism, and the complete recovery of the metabolite pool. A number of quenching solutions can be used to stop metabolism of the cultures [6–9]; however, the most popular approach is to use a pre-cooled organic solvent such as 60% methanol solution chilled to − 48 °C. In chemostat cultures, the residual concentration of the limiting nutrient (usually glucose and the labelled nutrient) is very low, thus rapid sampling and effective quenching of metabolism to inhibit residual enzyme activity is required to produce samples representative of the time point [59]. Rapid dynamic changes can be monitored with automated devices, allowing sampling every 5 seconds [60,61] or multiple (4–5) samples to be processed per second [62]. The leakage of metabolites during the quenching of metabolism is problematic in microbial cells, potentially resulting in underestimation of the metabolite pool. Various studies [6,7,63] have investigated approaches to prevent leakage of metabolites during the quenching procedure, including the use of different solvent solutions and buffers [6,7,63,64]. In addition, various methods are used to measure metabolite loss so that the quantification of the metabolites is not underestimated [8]. Extraction protocols are available to recover the metabolites from the cell biomass and accurately quantify the metabolite pool [8,9].

Use of isotopomers for fluxomics

Numerous studies have used stationary labelling to understand metabolic changes in microbial systems, including the effect of knockout mutations in the central metabolism of *E. coli* [65], shifts between oxidative and fermentative growth in *Saccharomyces cerevisiae* [66], and improvements in the production of lysine in *Corynebacterium glutacium* [67].

Proteins are very abundant in microbial systems and, as only 20 amino acids need to be measured, the analysis is relatively simple to perform compared with comprehensive metabolome-wide mass isotopomer analysis. Detailed protocols [68] and publicly available software packages (FiatFlux [19] and 13CFLUX [69]) are available to facilitate the novice user in the application of this tool. By contrast, dynamic-flux experiments are challenging to perform, and there are fewer published examples. Despite this, the usefulness of this approach has been demonstrated in chemostat growth of *E. coli* [55] to investigate the split ratio of glucose via the pentose phosphate and glycolysis pathways. Other examples include the dynamic incorporation of labelled ^{13}C in fed-batch cultures of *E. coli* [70], and nitrogen assimilation in batch cultures of *E. coli* [44]. In the studies by Yuan and colleagues, some of the experimental problems of investigating dynamic-flux changes were overcome by using a membrane-based growth method [71], which uses agar plates to the supply the nutrients (and label) to the cultures, thus experimental conditions are easily altered, and only small amounts of expensive labelled isotope are required. A combined quench and extraction method simplifies the quantification of the metabolite pool. One disadvantage of this approach to monitor dynamic changes is that the membrane culture is sacrificed at each time point, limiting its application to track time-course changes.

In summary, although many flux studies are less reliant on immediate cessation of enzyme activity because they rely on pathway end-product analyses such as those from proteinogenic amino acids, metabolism does need to be quenched rapidly and in rapid succession to take suitable temporal snapshots of carbon and nitrogen flow through pathways and into metabolite networks in order to elucidate novel pathways.

Systems biology: data integration beyond mass isotopmer analyses

There are many instances in which whole genome sequences have led to metabolic reconstructions of microbes and as part of a systems biology approach, and this bottom-up modelling is appropriate for understanding intracellular metabolism. Fluxomics analyses can be used to enhance these models further by providing quantitative data about metabolite concentrations and alternative connections between pathways, and can be very useful for functional genomics investigations [72–74]. In addition, the integration of transcriptomics (and proteomics) data with metabolomics will further clarify how metabolism is regulated; this might be hierarchically controlled by allosteric regulations. A nice example of this is glycolysis in trypansosmes: it has been demonstrated that metabolic fluxes through this pathway are not regulated by gene expression only [75], thus providing a further rationale for integrating mRNA levels with metabolite concentrations.

Concluding remarks

Although based on rather old classic microbiological approaches, the application of isotopes in metabolomic studies of cellular microbial systems have advanced significantly in the past two decades, specifically with respect to analytical instrumentation, software and methods.

Box 1. Challenges and opportunities of fluxomics

Challenges

- Need to develop more rapid and robust sampling (to quench serial samples in the order of fractions of a seconds apart).
- Need to develop more sensitive techniques for population distributions.
- Can hyperpolarisable substrates allow NMR spectroscopy to deliver enough sensitivity to deliver real-time measurements?
- Need to apply fluxomic to currently poorly studied areas of metabolism (e.g. lipid metabolism).
- Financial costs of purchase or synthesis of isotopically labelled substrates.
- Ability to perform fluxomics in non-steady-state conditions.

Future opportunities

- Systematic investigations of genome-scale metabolic network reconstructions.
- Rational metabolic engineering of bacteria and fungi to overproduce molecules of interest (e.g. feedstocks for biochemical industries, biofuels, biodegradable plastics).

These advances have resulted in exciting discoveries relating to metabolism and regulation of biological systems [44,65,76,77]. Further developments and the more routine application of fluxomics (holistic metabolomics is currently being used far more widely) are expected in the years to come as the power of these TARDIS-based techniques are realised by a larger number of research groups. Indeed, exciting studies applied to mammalian systems are already being observed. These include the study of molecular pathophysiological mechanisms of diseases involving metabolism, including cancer [78,79]; the effect of lithium on glial–neuronal metabolism [77]; dynamic metabolism in rats [80]; and complex interactions of the human gut microflora metabolism [81].

With respect to microbial systems as detailed above, there are many instances in which TARDIS-based analyses have been used to elucidate novel pathways in non-model microorganisms, and to investigate fluxes and regulation within known metabolic processes. The specific areas focused on can be classified as central metabolism (e.g., glycolysis, TCA cycle and amino acid biosynthesis). However, other areas of metabolism are also hugely important in microbes, including lipid metabolism, and the expansion of applications of isotopomer analysis for pathway elucidation and flux analysis is essential. There are still many analytical challenges ahead (Box 1). Also emphasised are the opportunities that exist, perhaps the most pertinent of which is the need to design microbes for new synthetic biology processes, be they high-value products for the pharmaceutical industry, or biofuels, which are urgently needed as current fossil fuel supplies dwindle. The future integration of metabolomics and fluxomics is essential to derive biological information, not only in microbes but also in plants and mammals, which is required for the growing world population.

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