Advancements in Pulsed Stable Isotope-Resolved Metabolomics

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Abstract

The understanding of biochemical processes of metabolism is gained through the measurement of the concentration of intermediates and the rate of metabolite conversion. However, the measurement of metabolite concentrations does not give a full representation of this dynamic system. To understand the kinetics of metabolism, the system must be described and quantified in terms of metabolite flow as a function of time. In order to measure the metabolite flow, or more precisely the metabolic flux through a biological system, substrates of the cell are labelled with stable isotopes. The usage of these substrates by the cell leads to the incorporation of the isotopes into downstream intermediates.

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The most important metabolic pathways are encompassed in the central carbon metabolism (CCM). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), the central carbon metabolism "is the most basic aspect of life". It includes all metabolites and enzymatic reactions within: glycolysis and gluconeogenesis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), amino acids and nucleotide metabolic pathways. Some molecules are at the crossroad of metabolic pathways, interconnecting diverse metabolic and therefore functional outcomes. Labelling these nodal metabolites and analysing their isotopic composition allows the precise determination of the metabolic flow within the biochemical networks that they are in.

Application of stable isotope labelled substrates allows the measurement of metabolic flux through a biochemical pathway. The rapid turnover of metabolites in pathways requires pulse-feeding cells with a labelled substrate. This method allows for the determination of different cell states. For example, the action of a drug from immediate impact until the compensatory response of the metabolic system (cell, organs, organisms). Pulsed labelling is an elegant way to analyse the action of small molecules and drugs and enables the analysis of regulatory metabolic processes in short time scales.

1   Introduction: A Brief History of Isotopic Labelling

The use of isotope labelled molecules in metabolic research began within the first decades of the last century. In the early days of metabolism research, radioactive isotopes were used to investigate the metabolic flow in bacteria, plants and animals. In order to investigate the structure of biochemical pathways and specifically metabolic cycles, e.g. tricarboxylic acid cycle, reverse tricarboxylic acid cycle or Calvin-Benson cycle, isotopes were applied in a time resolved manner. Time resolution allowed for the elucidation of the consecutive order of chemical reactions within the investigated pathways. The use of radioactive isotopes was of paramount importance to decipher the flow of carbon within cells and organisms.

In 1910, English chemist Frederick Soddy observed that "elements of different atomic weights may possess identical (chemical) properties" and therefore belong to the same position in the periodic table. This included not only radioactive elements but also stable isotopes of atoms, i.e. atoms with the same number of protons but different numbers of neutrons in their nucleus. We can refer to these elements as "hot" or "cold" isotopes of an atom in dependency of their radio- or non-radioactivity. The presence of radioactive isotopes was discovered by black spots occurring on photosensitive emulsions, as the decaying radioactive element

produced traceable radiation. This phenomenon gave rise to years of research into the isotopes of the periodic table and their many uses in a broad range of analytical fields, including: chemistry, geology, biology, physics and medicine (Wilikinson 2018).

Georg Charles de Hevesy was a Hungarian radio-chemist and Nobel Prize in Chemistry laureate, recognised in 1943 for his achievements in the development of radioactive tracers in the study of metabolism in animals. Hevesy is considered the first to use radioisotopes to measure metabolic flux in biological systems. One such (sadly unpublished) experiment of Hevesy and Rutherford describes the practices of a shrewd landlady in 1911, Manchester. Hevesy was convinced that his landlady was recycling food, much to the denial of such practice. In order to thwart the thrifty landlady, Hevesy "spiked" a portion of leftover meat with a tiny amount of a radioactive material. A few days later he brought an electroscope to the table to demonstrate to the indignant landlady that the food served that day was radioactive. Hevesy is thought to have enjoyed fresh hot meals as a result of his radioactive tracing experiment (Myers 1996). Hevesy continued his endeavours into the usage of tracers in biological systems. In 1934, Hevesy and Hofer first used tracers in medicine by using an enriched stable isotope to determine the rate of elimination of water from the human body. Hevesy and Hofer drank dilute deuterated water and assayed the isotopic dilution of the deuterium in their urine. From their results they concluded the average time a water molecule spent in their bodies was 13 ± 1.5 days (Hevesy and Hofer 1934).

The accounts of Hevesy are far more than comical anecdotes. They provide an understandable example for the use of radioisotopes in analytical chemistry. Through the development of mass spectrometry, stable isotopes replaced radioisotopes in tracing experiments. In mass spectrometry, a metabolite can be identified by the mass spectrum of its fragments, i.e. the intensity of the fragment's peaks at a defined mass-to-charge (m/z) ratio. The additional neutron in the atomic nucleus of isotopes makes the atom 1 Da heavier and increases the m/z ratio. The incorporation of isotopes into a metabolite changes the atomic composition of this intermediate and induces a shift in the respective fragment's mass spectrum. Specifically, stable isotopes of carbon (^{13}C), nitrogen (^{15}N), oxygen (^{18}O) and hydrogen (^{2}H) can be introduced into organic compounds. By tracing the incorporation of the isotope, the metabolic fate of these compounds within biological systems can be characterised (Wilikinson 2018). The incorporation of these isotopes can be resolved in molecules such as sugars, amino acids or nucleotides. This method of isotope labelling was used to describe the effect of oncogenes on cancer cell metabolism. For example, Le et al. applied ^{13}C-Glucose and $^{13}C,^{15}N$-Glutamine to trace glucose and glutamine metabolism when the MYC oncogene was induced in P493 cancer cells. The authors showed that glutamine plays an essential role in the cells' proliferation and survival, highlighting targets of glutamine metabolism for cancer therapy (Le et al. 2012). The usage of isotope labelling provides insights into the dynamics and kinetics of metabolism, as a function of time and cell state.

2   Time- and Isotope-Resolved Metabolomics

2.1   Stable Isotope-Resolved Metabolomics

In order to measure the metabolite flow, or more precisely the metabolic flux through a biological system, substrates of the system are labelled with stable isotopes. These substrates may be, glucose or glutamine which provide carbon and nitrogen to the central metabolic pathways (Fig. 1). The usage of these substrates by the cell leads to the incorporation of the isotopes into downstream intermediates. After isotopically labelling a biological system and extracting the cellular metabolites, mass spectrometry is then employed to analyse the number of heavy atoms incorporated (isotopologues) and their positions (isotopomers) in detected metabolites (Bruntz et al. 2017). Isotopologues can be identified by increasing mass shifts, as every heavy atom incorporated rises the m/z ratio by one. Isotopomers, on the other hand, can be distinguished by the heavy atom incorporation visible in different fragments
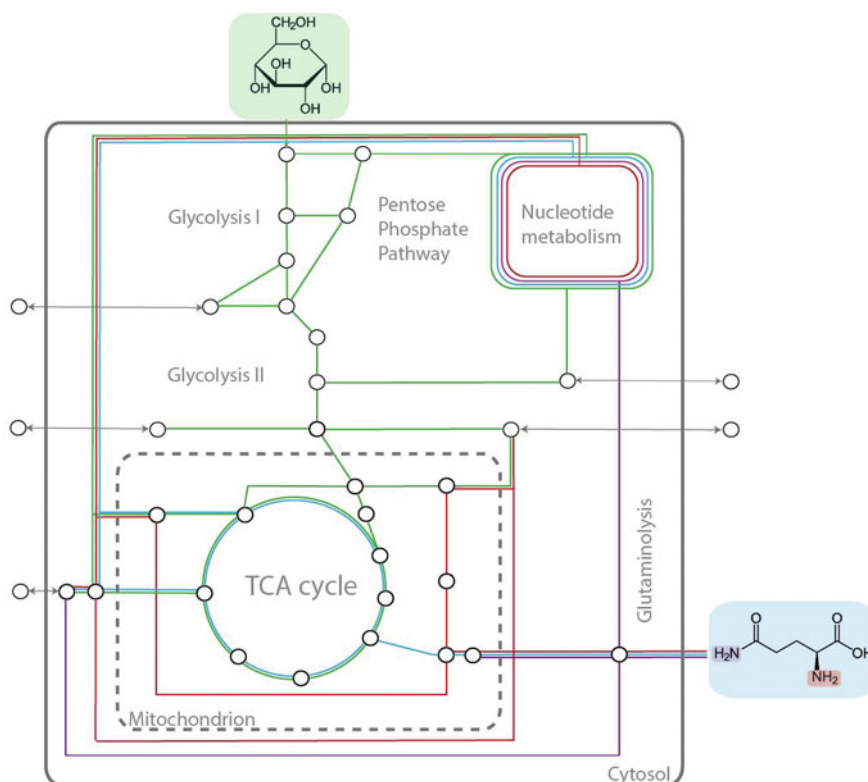
Fig. 1 Scheme of central metabolic pathways indicating carbon and nitrogen flow. The scheme displays main central metabolic pathways: glycolysis, pentose phosphate pathway, nucleotide metabolism, tricarboxylic acid cycle and glutaminolysis. The different colours depict the contribution of the distinct carbon and nitrogen atoms stemming from glucose or glutamine, respectively

of the same metabolite. In summary, the usage of isotope labelled substrates allows for the follow-up of interconnected anabolic and catabolic pathways. The tracing of isotope labels in metabolites, and the atomic position in which the label resides, is referred to as stable isotope-resolved metabolomics (SIRM).

Cellular metabolism is a rapid, dynamic process, in which both anabolism and catabolism of metabolites produce energy, build macromolecules for biomass and generate intermediates involved in intra- and inter-cellular signalling. The understanding of biochemical processes of metabolism is inferred through the concentration of intermediates and the rate of metabolite conversion. However, the measurement of metabolite concentrations does not give a full representation of this dynamic system. In order to understand the kinetics of metabolism, it must be described and quantified in terms of metabolite flow as a function of time (Sauer 2006; Pietzke et al. 2014; Buescher et al. 2015; Jang et al. 2018).

2.2 Time Resolved Isotope Labelling Studies

Metabolism is a highly dynamic system in which biochemical reactions occur rapidly. This allows the cell to constantly meet its needs and adapt to changing stimuli and micro-environments. The time for which cells are exposed to a labelled substrate determines the amount of isotopic label incorporated into its metabolites. Supplying a cell with an isotope labelled substrate for extended periods of time will lead to complete usage and saturation of downstream pathways. This condition is referred to as isotopic steady state and solely reflects the usage of metabolic pathways in relation to each other, i.e. in which pathways the given substrate is involved in. In other words, if one applies the labelled substrate for such a period of time that the label incorporation has reached saturation, then the model of flux would be stationary (Fig. 2, left (II)).

To understand the flux of labelled metabolites, we aim to assess metabolism in a non-stationary state. In order to measure the metabolic activity, the time a labelled substrate is offered to a cell is limited. A labelling time is chosen where the label incorporation into intermediates of interest is in linear relationship to time (Fig. 2, left (I)). At this chosen labelling time, one can analyse the speed of a specific pathway. It is also possible to assess how different conditions the cell is exposed to affect the velocity of the pathway in use.

By avoiding saturating the system, information about pathway preference and directionality is provided, as discussed in Sect. 2.3. The reduction in labelling time can be implemented by a pulsed labelling-quenching strategy, referred to as pulsed stable isotope-resolved metabolomics (pSIRM).

We may take glycolysis as a metabolic pathway that can be analysed by a tracing experiment. $^{13}C_6$-Glc is used as a substrate for the glycolytic pathway. This labelled tracer contains ^{13}C at every carbon position on the glucose molecule. As glycolysis proceeds ^{13}C is incorporated into downstream metabolites (Fig. 2, right). The rate of label incorporation can be described in both functions of time and quantity of labelled metabolite. The rate of label incorporation, or indeed the metabolic rate,
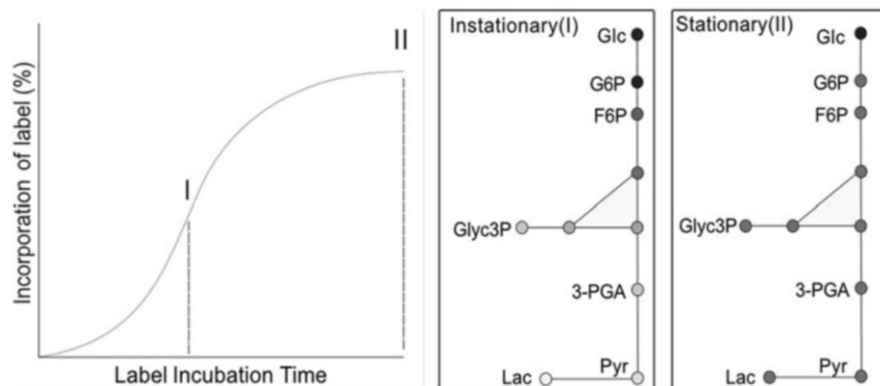
Fig. 2 Modelling the kinetics of metabolite labelling. As cells are incubated with a labelled substrate for a period of time, the quantity of label incorporated into metabolites increases. The rate of reaction, or the percentage of label in the metabolites relative to the unlabelled fraction, is dependent on the position of the metabolite in the pathway and the efficiency of associated enzymes. In order to understand the rate and kinetics of metabolic processes, we aim to observe labelled metabolites in the instationary phase (Fig. 2, left (I)). On the contrary, if all metabolites are saturated with label after a given period of time (Fig. 2, left (II)), then no kinetic information can be derived

of glycolysis is rapid. For instance, glucose-6-phosphate (G6P) – the primary step in glycolysis – is labelled to saturation in approximately 2–5 min (Pietzke et al. 2014). Longer labelling times are required for the label to reach saturation in metabolites downstream of G6P. Through a pulse-quench-harvest labelling strategy we can determine the rate of label incorporation into metabolites of the glycolytic pathway, as a function of the time cells are exposed to the labelled substrate. With this information we can determine how "glycolytic" a certain cell type is.

Furthermore, we can study the effects on inhibitors on pathways by monitoring the relative changes in label incorporation upon inhibitor treatment. 3-Bromopyruvate (BrPyr), for example, is a strong glycolytic inhibitor. More specifically, BrPyr inhibits glycolysis at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction (Pietzke et al. 2014). In order to localise BrPyr action on GAPDH, cells were pre-incubated with BrPyr for 12 min followed for a tracing with $^{13}C_6$-Glc for 3 min to label glycolytic metabolites. They showed that the carbon flow through metabolites downstream of GAPDH was almost completely inhibited in BrPyr treated cells (Fig. 3). The concentration of 3PGA drops below the detection limit and the dramatic decrease in the label incorporation into pyruvate, lactate and citrate proved bromopyruvate's inhibitory activity in the lower glycolysis. In contrast fructose-1,6-bisphosphate (above the blockage) accumulates roughly fourfold, while at the same time the label incorporation decreases by a factor 4, so the total flow into this compound remains constant. This illustrates the connection of metabolite pool size and label incorporation and highlights the importance of considering both readouts simultaneously during a pSIRM experiment.
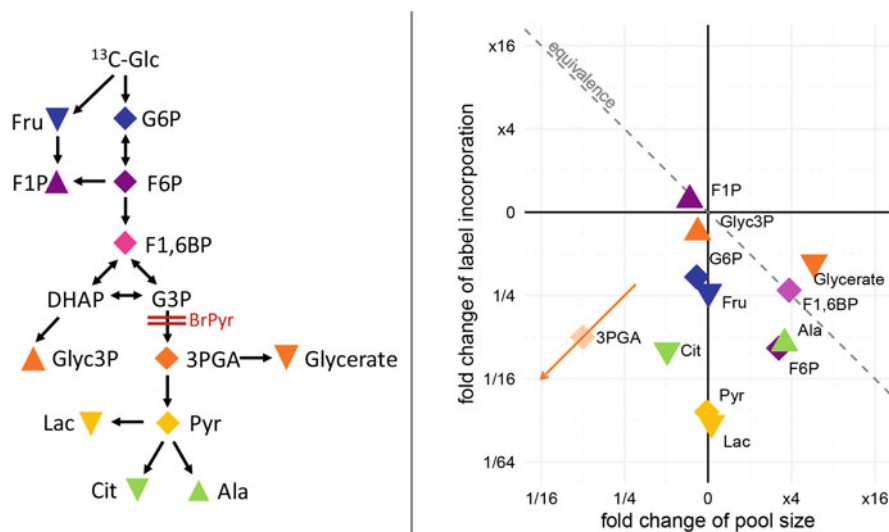
Fig. 3 pSIRM data in 2D. Each point represents one single metabolite of the CCM. On the x-axis fold-changes of metabolite concentrations and on the y-axis changes of isotope incorporation are shown. For this experiment cancer cells were incubated for a total time of 15 min with BrPyr and metabolites were measured using the pSIRM technology (see Pietzke et al. (2014)). The graph shows that in some metabolites a quantitative change in metabolite concentration can be observed. However, the effect on glycolytic inhibition can only be observed in the label incorporation of metabolites downstream of the GAPDH reaction

We have described the use of isotope labelled substrates for investigating the rate of reactions in metabolic pathways. One may also investigate the direction of metabolic pathways or deduce the relative dominance of a pathway by using substrates labelled at specific carbon positions. For example, the pentose phosphate pathway (PPP) – which links glycolysis to nucleotide synthesis – generates ribose-5-phosphate (R5P) from two reactions. The oxidative PPP utilises glucose-6-phosphate to produce 6-phosphogluconate which is de-carboxylated to ribulose-6-phosphate (Ru5P). In turn Ru5P is isomerised to R5P. The non-oxidative PPP cycles carbons from fructose-6-phosphate, glyceraldehyde-3-phosphate and erythrose-4-phosphate to produce xylulose-5-phosphate and R5P. This branch does not contain a de-carboxylation event and carbon number is maintained in the process. Therefore, we may employ a labelling strategy to decipher the ratio of the oxidative and non-oxidative branches by assessing the state of the de-carboxlyation event in the oxidative branch. By using 1,2-[^{13}C]-Glc as the labelling substrate we can measure the ratio of R5P present in the sample which contains a single (m/z + 1) or a double (m/z + 2) label. R5P which is derived from the oxidative PPP will only contain a single label as the labelled carbon is lost during the de-carboxylation event (Fig. 4).

Oxythiamine is an inhibitor of the non-oxidative PPP enzyme, transketolase (TKT). Boros et al. (1997) showed that oxythiamine inhibits Mia pancreatic adeno-carcinoma cell growth by 39%. By using 1,2-[^{13}C]-Glc labelling the authors
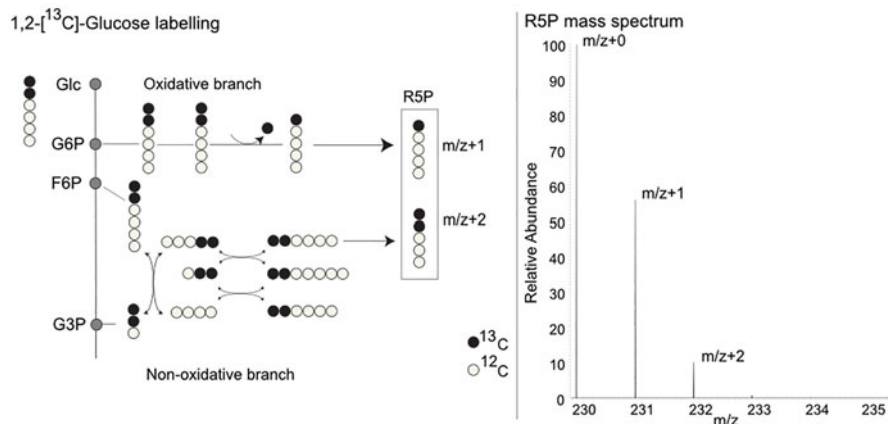
Fig. 4 Utilising 1,2-[^{13}C]-Glc in the analysis of the pentose phosphate pathway. The pentose phosphate pathway is comprised of the oxidative and non-oxidative branches. The oxidative branch observes a de-carboxylation event which removes a labelled carbon from glucose-6-phosphate. In the non-oxidative branch, labelled carbons are maintained as de-carboxylation does not occur. The ratio of single to double labelled R5P shows the relative dominance of the branches. One may observe the single or double labelled R5P in a mass spectrum generated by MS, by the relative abundance of R5P (m/z + 1) and R5P (m/z + 2). This is depicted in the simulated MS spectrum on the right

discovered that this tumour cell line generated R5P predominantly through transketolase and the non-oxidative PPP (85%). It was deduced that oxythiamine was an effective anti-tumour inhibitor due to the dependency of R5P synthesis, and downstream nucleotide synthesis, on the non-oxidative PPP (Boros et al. 1997). In summary, differential labelling of substrates permits not only the analysis of the kinetics of metabolism but also gives information on the relative dependencies of parallel reactions in different cell lines for targeted therapy development.

2.3   Isotope Tracing at the Crossroad of Metabolic Pathways

The most important metabolic pathways are encompassed in the central carbon metabolism (CCM). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), the central carbon metabolism "is the most basic aspect of life". It includes all enzymatic reactions within: glycolysis and gluconeogenesis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), amino acids and nucleotide metabolism pathways. Additionally, the CCM includes six known carbon fixation pathways (reductive pentose phosphate cycle (Calvin cycle), reductive citrate cycle, 3-hydroxypropionate bi-cycle, two variants of 4-hydroxybutyrate pathway and reductive acetyl-coenzyme A (CoA) pathway) as well as some pathways of methane metabolism, all not relevant in animal cells (Qiu 2013). Some molecules are at the crossroad of metabolic pathways,
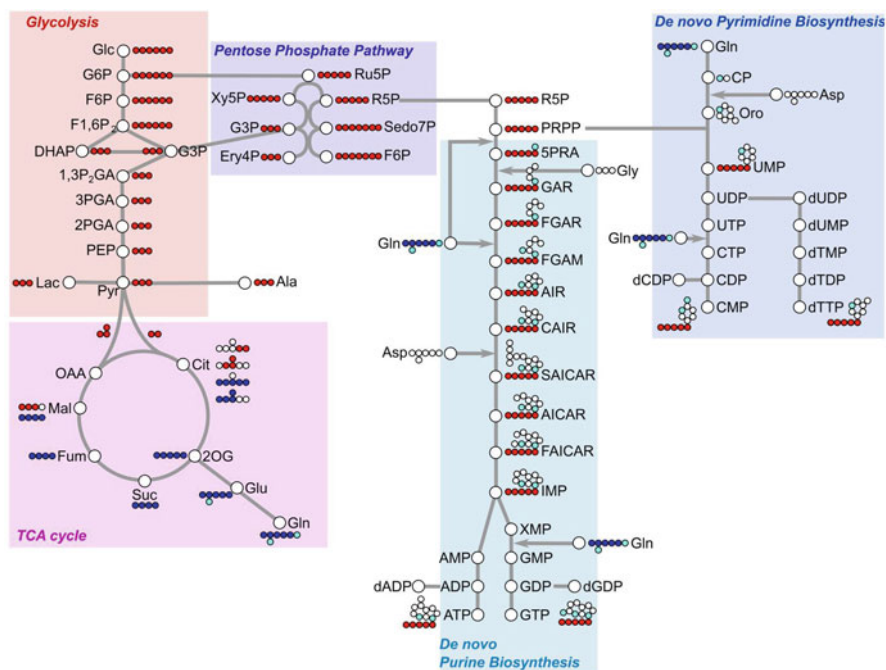
Fig. 5 ^{13}C and ^{15}N label integration into pathways of the central carbon metabolism. Pictogram of glycolysis, TCA cycle, pentose phosphate pathway, as well as de novo purine and pyrimidine biosynthesis. Red circles represent glucose-derived ^{13}C atoms. Blue represent the ^{13}C (dark blue) or ^{15}N (light blue) atoms derived from glutamine. Unlabelled C and N atoms are displayed in white

interconnecting diverse metabolic and therefore functional outcomes. Labelling these nodal metabolites and analysing their isotopic composition allows the precise determination of the metabolic flow within the biochemical networks that they are in (Fig. 5).

One example of such intersections is citrate. Glucose-derived pyruvate can be metabolised by pyruvate dehydrogenase to citrate or by pyruvate decarboxylase to oxaloacetate, which itself feeds into citrate during TCA cycling. On the other hand, glutamine can enter the TCA cycle via glutamic acid and 2-oxo-glutaric acid and feed into citrate via both, oxidative (classic) and reductive (reverse) TCA cycling. Via ATP-citrate lyase, citrate is further interconnected with fatty acid oxidation and fatty acid biosynthesis. When labelling with ^{13}C-glucose, ^{13}C-glutamine or ^{13}C-fatty acids one will be able to distinguish between different mass shifts in citrate, depending on the source of the ^{13}C-label as well as the directionality of TCA cycling. ^{13}C atoms derived from pyruvate dehydrogenase activity or fatty acid oxidation-derived acetyl-CoA will lead to an m/z + 2 mass shift in citrate, as two heavy carbon atoms are incorporated in the latter. On the contrary, an m/z + 3 mass shift is observed when pyruvate integrates into oxaloacetate and subsequently into citrate. Similarly, when providing cells with ^{13}C-glutamine, mass shifts of m/z + 4 and

m/z + 5 in citrate are the result of anaplerotic fuelling from glutamine into the TCA cycle and oxidative or reductive cycling, respectively.

Other examples of pathway interconnection are pyruvate and glutamine, as both can be metabolised into very different intermediates and play a role in distinct cellular functions. Pyruvate-derived ^{13}C-atoms can integrate, as described above, into citrate or oxaloacetate, but also into alanine or lactate. Thereby, metabolic flow through pyruvate merges amino acid metabolism, anaerobic glycolysis and TCA cycle. Glutamine, on the other hand, is an important nitrogen donor and is involved in amino acids metabolism and de novo biosynthesis of purine and pyrimidine nucleotides (Bayram et al. 2020). Additionally, via glutamic acid, the carbon backbone of glutamine feeds into TCA cycle, as well as polyamine and glutathione synthesis. The example of glutamine highlights the necessity of dual carbon and nitrogen labelling in order to greatly increase our understanding of pathway connectivity and metabolic fate. Combining [13]C- and ^{15}N-labelling allows the follow-up of glutamine usage, while distinguishing between the amino group, amido group and carbon backbone utilisation.

2.4 The Application of Ultra-High Resolution Mass Spectrometry Allows the Tracing of Different Isotopic Species

Using stable isotopes of different elements allows to determine the utilisation of two metabolic precursors (e.g. glucose and glutamine) in a single experiment simultaneously, or to follow the fate of different atoms from the same precursors (e.g. C and N from glutamine) into the downstream metabolic network.

For a long time such experiments have not been possible, since mass analysers with nominal mass resolution are not able to distinguish the mass increase due to the presence of different elemental isotopes. For example, the difference in the mass increase given by one ^{13}C or one ^{15}N is only 0.00632 Da (Fig. 6). According to the IUPAC definition (McNaught and Wilkinson 2008), the resolution needed to separate such mass difference for two molecules with nominal mass of 400 Da is around 60,000, far beyond the resolution of conventional quadrupole or ion trap analysers and barely reached by Time-of-Flight instruments. Only with the advent of (ultra) high resolution Fourier transform mass spectrometry mass analysers it became possible to analyse the isotopic fine structure of labelled compounds (Werner et al. 2008; Marshall and Hendrickson 2008).

One implementation of (ultra) high resolution MS for isotope tracing is the direct infusion of the metabolites in an Fourier-transform ion cyclotron resonance mass spectrometer (Le et al. 2012; Yang et al. 2017). This approach offers excellent results in terms of achievable resolution and accuracy in the determination of the ratios between different isotopes, because the ion signal in the ion cyclotron resonance cell can be averaged for long times. However, in direct infusion highly abundant analytes can suppress the ionisation of low abundant ones (Han et al. 2008). In addition, in absence of another mean of separation, isomers cannot be distinguished because they have the same accurate m/z ratio, like G6P and F6P. MS/MS experiments can help
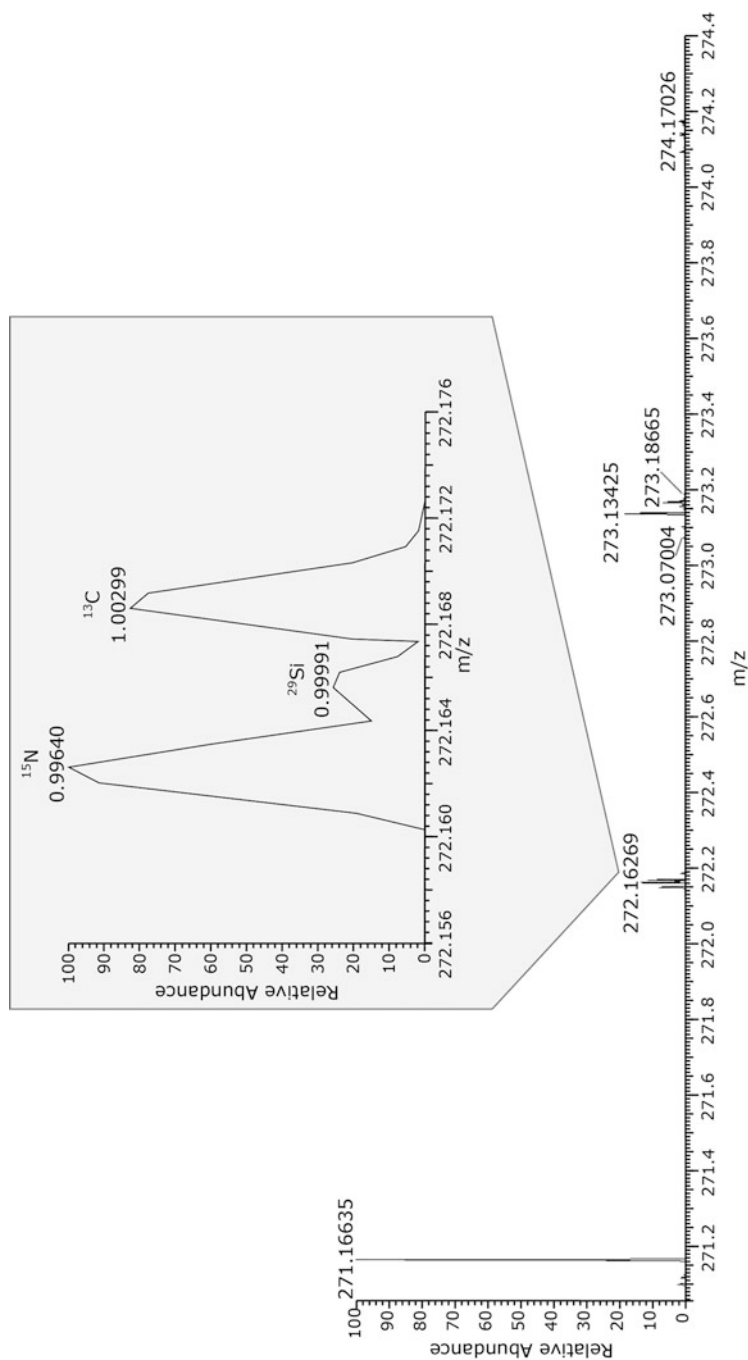
Fig. 6 MS spectrum of ^{15}N labelled Glutamine. The ion at m/z 271.16635 corresponds to the unlabelled fragment of TBDMS-derivatised glutamine with molecular formula C12H27ON2Si2. It contains 4 carbon and all nitrogen atoms of glutamine. Insert shows the magnification of the m/z + 1 peaks, revealing the separation of peaks due to the incorporation of ^{15}N or ^{13}C. The peak of ^{29}Si isotope, present in the derivatisation agent, is also resolved. The masses reported in the insert are the Δm relative to the monoisotopic peak

confirming or excluding the presence/absence of one or more isomers. Nevertheless, isomers often require different collision energies, making an accurate quantification difficult, if not impossible. Orbitrap mass analysers also can achieve high resolution with fast scanning rate (Makarov et al. 2006). With a scan rate up to 40 Hz (Kelstrup et al. 2018), this analyser is fast enough to be seamlessly hyphenated with GC or LC separation.

3 Applications and Future Perspectives

In line with the general "OMICS" concept, metabolomics aims to measure all metabolic components of a biological system at once in a quantitative manner. Unfortunately that's not possible so far. The chemical space of biomolecules regarding polarity and size as well as their quantities – ranging from a few molecules up to millimolar concentrations – is hindering its simultaneous detection. One step further is the measurement of the dynamics of metabolism. The combination of stable isotope labelling and mass spectrometric detection made a big contribution in this regard. More and more methods allow the simultaneous measurement of metabolite concentrations and isotope incorporation (Pietzke et al. 2014). Such integrated methods are a prerequisite when metabolic dynamics in vivo or in tissues will be analysed in a clinical context. Often a consecutive sampling and parallel quantification in additional samples is not possible. The application of pSIRM together with the present knowledge of the biochemical network allows to define certain metabolic nodes that contain superior information of the usage of metabolic pathways, e.g. citrate. Future method development may focus on such nodes, allowing for more directed pSIRM applications with tailored isotopically labelled substrates.

3.1 Applications of pSIRM

In the last years, pSIRM has been applied to successfully analyse metabolic changes within short and defined windows, e.g. during differentiation of cells. Delp et al. found that immature and mature neurons rely on different fuels. Precursor cells were found to be mainly glycolytic and strongly dependent on glutamine. During differentiation, however, they lost their glutamine dependency while gaining flexibility in energy production (Delp et al. 2018). By analysing the glutamine-derived carbon utilisation in high salt-treated differentiating macrophages a specific down-regulation of the succinate to fumarate conversion could be revealed. This observation led to the identification of sodium as strong regulator of complex II activity (Geisberger et al. 2021).

pSIRM can also be applied in vivo as well as ex vivo in tissue slices or organs. By administering $^{13}C_6$-glucose into the peritoneum of a hepatocellular carcinoma mouse model, Berndt et al. described enhanced glycolytic rates in tumours compared to normal liver. These data contributed to the creation of individualised metabolic profiles of tumours and modelling predictions of the efficacy of drug therapies

(Berndt et al. 2020). Similarly, application of stable isotopes in situ to cancerous and non-cancerous lung tissue revealed higher activity of glycolysis and the Krebs cycle in the tumour tissue (Fan et al. 2009). In brain tissue slices from naked mole rats, e.g. labelling with $^{13}C_6$-fructose uncovered the ability of these animals to metabolise fructose as fuel for neuronal tissues under hypoxic conditions (Park et al. 2017).

Beside the application of stable isotope labelled organic compounds, inorganic compounds such as CO_2 can be used for labelling experiments. For example, Rohwer et al. used $^{13}CO_2$ to demonstrate the reductive carboxylation in gastric cancer cells (Rohwer et al. 2016). These are only few examples of the wide range of applications pSIRM; a very versatile technique to describe metabolism in function of quantity and time.

3.2 Perspective Towards Single Cells

The advent of single cell "OMICS" has allowed for the analysis of the architecture of heterogenous cell samples at single cell resolution. Currently, single cell proteomics, genomics and transcriptomics dominate the single cell technology landscape, while single cell metabolomics is still in its infancy. The discrepancy of single cell metabolomics is mainly due to the hardware of mass spectrometry being incompatible with single cell resolution. A mammalian cell contains roughly 1 pL of analytical volume. Due to this extremely small sample volume GC-MS methods often use around two million cells per analysis. To further add complexity, metabolism is highly dynamic – temporally and spatially – in tissue. Therefore, acquisition of single cells and their processing presents many challenges in the context of metabolic profiling.

Firstly, we will address the process of sampling single cells. The sorting of cells from a sample is essential to single cell analysis. Fluorescence-activated cell sorting (FACS) employs fluorescent labels to sort cells of specified origin. FACS may be coupled to mass spectrometry methods to assess the profiles of sorted cells. However, the FACS process may interfere with metabolic profiles and therefore FACS coupled with MS is more suited to proteomic analysis (Bandura et al. 2009). To minimise the sampling time methods such as matrix-assisted laser desorption/ionisation (MALDI) employ a laser to ablate cells and their metabolites in situ. Following laser ablation the ionised metabolites are analysed via MS. Advances towards single cell resolution have been reported. By using a combination of computational imaging techniques and nuclei staining, the points of laser ablation during MALDI-MS analysis can be inferred as metabolite acquisition from a single cell (Rappez et al. 2021). However, this method is not quantitative and is biased to high abundant metabolites and proteins.

Current technologies are aiming to sample single cells through microfluidic trapping coupled with acquisition of intracellular metabolites through micro capillaries. However, efforts to move to a microfluidic environment present their own complications, mainly due to maintaining homeostatic environment in a miniaturised platform (Ali et al. 2019).

In conclusion, as mass spectrometers become more sensitive coupled with inventive single cell trapping techniques, metabolomics will be a valid and useful addition to the single cell "OMICS" universe.

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