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NMR-Based Stable Isotope Resolved Metabolomics in Systems Biochemistry

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Abstract

Metabolism is the basic activity of live cells, and monitoring the metabolic state provides a dynamic picture of the cells or tissues, and how they respond to external changes, for in disease or treatment with drugs. NMR is an extremely versatile analytical tool that can be applied to a wide range of biochemical problems. Despite its modest sensitivity its versatility make it an ideal tool for analyzing biochemical dynamics both in vitro and in vivo, especially when coupled with its isotope editing capabilities, from which isotope distributions can be readily determined. These are critical for any analyses of flux in live organisms. This review focuses on the utility of NMR spectroscopy in metabolomics, with an emphasis on NMR applications in stable isotope-enriched tracer research for elucidating biochemical pathways and networks with examples from nucleotide biochemistry. The knowledge gained from this area of research provides a ready link to genomic, epigenomic, transcriptomic, and proteomic information to achieve systems biochemical understanding of living cells and organisms.

Keywords

Isotope editing; Stable Isotope Resolved Metabolomics (SIRM); isotopomer distribution analysis

1. Introduction

NMR spectroscopy is a very powerful and versatile tool for elucidating the structure and conformation of both small and macro molecules in the solution or solid state [3–11] (and this issue). In addition, NMR methods provide detailed information about molecular and internal dynamics over a wide range or timescales by determining rotational and translational diffusion coefficients and internal motions of molecules [13]. Furthermore, NMR can be applied to chemical dynamics, for studying mechanism as well as flux measurements both in vitro and in vivo [3, 14, 15].

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A critical feature of NMR for molecular structural and quantitative analysis is its isotope-selective detection and sensitivity of nuclear spin properties to the intra- and inter-molecular environment, as well as to the robust and quantitative nature of NMR measurements. These advantages have made NMR an early choice for metabolite profiling efforts [16–19] and an excellent partner for mass spectrometry (MS)-based metabolite profiling [20]. For example, NMR analysis provides crucial structural parameters including functional groups, covalent linkages, and non-covalent interactions including stereochemistry, which are difficult to acquire by MS methods.

Metabolomics has become a vibrant field for elucidating the functional biochemistry of diverse organisms and model systems [12] and their responses to altered conditions and pathologies [21–30]. Although various mass spectrometry platforms are commonly used in metabolomics studies [31–37], the unique capabilities of NMR provide several advantages including isotope-selective editing of complex mixtures, detailed positional isotopomer analysis for enriched metabolites, *de novo* structure determination of unknown metabolites (both unenriched and enriched), accurate quantification without the need for standards, and in situ analysis of pathway dynamics from cells to whole organisms [2, 3, 6, 38, 39].

This review focuses on the utility of NMR spectroscopy in metabolomics, with an emphasis on NMR applications in stable isotope-enriched tracer research for elucidating biochemical pathways and networks such as those shown in Figures 1 and 2. The knowledge gained from this area of research provides a ready link to genomic, epigenomic, transcriptomic, and proteomic information to achieve systems biochemical understanding of living cells and organisms. Although many such basic pathways are often assumed, the techniques can in fact be used to determine the differences in tissue specific metabolism as well as discover unexpected metabolic transformations [40–43] that are not necessarily readily annotated from genomics. It is likely that in the fields of diverse speciation in the fungal and microbial worlds, many metabolic pathways remain to be discovered and characterized. Furthermore, with judicious use of stable isotope tracers (see below), it is possible in some instances to infer compartmentation even when the same metabolites are present in more than one [3, 44–46].

2. Stable Isotope Resolved Metabolomics (SIRM)

Although NMR is intrinsically an accurate quantitative method such that steady state concentrations of metabolites can be reliably determined [3], changes in amounts of metabolites only tell part of the metabolic story. This is because not only are most intracellular metabolites maintained at fairly narrow concentration ranges (homeostasis) but also metabolic pathways are highly interconnected in a complex network of reactions. For example the central highways of carbohydrate metabolism, glycolysis and the pentose phosphate pathway and the Krebs cycle are not simple linear sequences of transformation, but many of the intermediates are also involved in numerous other pathways. Thus, glucose-6-phosphate, the first committed intermediate of glycolysis, has several possible fates including (i) transformation to fructose-6-phosphate by phosphoglucose isomerase (glycolysis), (ii) diversion into the oxidative branch of the pentose phosphate pathway and (iii) in glycogen synthesis after conversion to glucose-1-phosphate. This intermediate is also

the entry point for galactose metabolism via the Leloir pathway [47, 48]. Similarly, in addition to being an intermediate in glycolysis, fructose-6-phosphate is the entry point to the hexosamine pathway, dihydroxyacetonephosphate (DHAP) is the entry point for production of glycerol-3-phosphate for glycerolipid synthesis, and 3-phosphoglycerate is the precursor of serine and glycine (Figure 1). The product of glycolysis, pyruvate, also has numerous fates, including reduction to lactate (lactic fermentation by lactate dehydrogenase, LDH), transamination with L-glutamate to alanine (via alanine amino transferase, ALT), oxidative decarboxylation to acetyl CoA (via pyruvate dehydrogenase, PDH) and ATP-dependent carboxylation to oxaloacetate (via pyruvate carboxylase, PC). The Krebs cycle receives carbon input not only from pyruvate, but also from amino acids and fatty acid oxidation. The degradation of the carbon skeleton of most amino acids is via the Krebs cycle (especially the liver) [12], and some cell types oxidize glutamine for energetic and anabolic purposes via the Krebs cycle [49–52]. The steady state concentration of the intermediates therefore depends on numerous inputs and outputs. To determine the sources and fates of the atoms that make up metabolites needs tracers. Commonly used stable isotope tracers are given in Table 1. These are commercially available compounds that probe different aspects of metabolism.

Spin topology and isotopomers-spectral editing

A solution to sorting out the complexity is to use NMR-active stable isotope tracers, especially ^{13}C and ^{15}N , and in some cases also ^2H . These tracers when incorporated into source compounds such as glucose, glutamine and others (cf. Table 1), generally have minimal influence on metabolic rates as primary isotope effects are small, and bond-breaking steps are not always wholly rate limiting in enzyme catalyzed reactions [65]. Isotope effects with perdeuterated substrates, however, have been observed [3, 66], indicating that some caution may be needed when comparing these substrates with the natural ones.

The metabolic transformations of stable isotope enriched compounds can be readily determined by mass spectrometry, which is routinely carried out in tracer studies [31, 67–70]. As MS measures mass, only the number of enriched atoms can be counted; without extensive fragmentation, the positional information is not readily available. In contrast, NMR has some unique capabilities including isotope editing (i.e. selection for molecules containing ^{13}C , ^{15}N or ^{31}P for example), and direct determination of the positional enrichment either by direct integration or from the patterns generated by scalar coupling [2, 3, 39]. Furthermore, as the stable isotopes are fully compatible with live cells and organisms, it is possible to measure metabolic processes in vivo [71] [72, 73] [74] [63] [3, 64] and see below.

Spin topology of metabolites

A 5-carbon metabolite such as Glu has 32 carbon isotopomers, and if ^{15}N is included, there are 64 isotopomers. As Glu is a hub metabolite that is involved in a large number of metabolic transformations, a significant number of these isotopomers may be present, and a quantitative analysis of the isotopomer distribution is needed to assess which pathways are active. For example, if uniformly ^{13}C -enriched glucose ($[\text{U-}^{13}\text{C}]$ -glucose) is the ^{13}C source

in cells, then specific ^{13}C isotopomers will be formed just from two turns of the Krebs cycle with pyruvate carboxylase input (Fig 2A). With $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{-Gln}$, different isotopomer distributions are produced, including the incorporation of ^{15}N into Asp and Ala by transamination of Glu (Fig. 2B).

Spectral simplification by isotope and editing and chemoselection

Owing to the large number of metabolites and narrow frequency range, 1D proton NMR spectra of cell and tissue extracts are highly overlapping in many regions, especially in the (phospho)sugars from 3.5 to 5 ppm, which is exacerbated by the presence of satellites from ^{13}C . There are two major routes to simplifying spectra while at the same time enabling extraction of specific information about metabolites and their function groups. The first is spectral editing using the metabolically incorporated stable isotope tracer. The editing then suppresses all protons that are not interacting with the isotope, so that the spectral display comprises resonances of atoms of metabolites that are part of the metabolic network accessed by the source. A simple approach is to use the HSQC experiment, which selects for protons directly attached to carbon or nitrogen, and in the case of nitrogen is useful for long range couplings (2–3 bonds) where ^{13}C is absent such as in nucleotides [2, 75]. There are many variants of these experiments that can select for individual labeled atoms, adjacent atoms (e.g. HCCH experiments) and for doubly enriched experiments, where either triple resonance can be used such as HCN or HNC experiments [3], or the influence of the passive coupling of one isotope in a 2D experiments is detected.

Some of these isotopomers can be readily detected using TOCSY, which shows quite different cross-peak patterns for the protonated carbons of different isotopomers [65, 76]. It is possible to determine ^{13}C -enrichment in carboxylate groups using HCCO experiments and discriminate molecules that are singly or multiply labeled and whether there are adjacent ^{13}C atoms using HCCH via the $^1J_{\text{CC}}$ of 40–50 Hz [2, 3, 39, 77]. Similarly, where multiple labels such as ^{15}N and ^{13}C are used, it is possible to discern the position and enrichment of one label when editing for another. For example, phosphorylated compounds can be selected by HSQC using the $^3J_{\text{PH}}$ coupling, which is typically a few Hz. Such an HSQC spectrum of a mixture will select all of the (sufficiently abundant) phosphorylated compounds including the nucleotides [78] greatly simplifying the NMR spectrum. Taking the example of AMP or UMP from cells exposed to $[\text{U-}^{13}\text{C}]\text{-glucose}$, then the ribose subunit will also be ^{13}C -enriched, and the $\text{H5}'/\text{H}'''$ resonances will be split by the (passive) $^1J_{\text{CH}} \approx 126$ Hz. If the HSQC-TOCSY experiment is carried out, then other positions in the ribose ring will also be detected. The ratio of the satellite intensities to that of the central ^{12}C peak provides an estimate of the fractional enrichment at that position. Similarly experiments can be carried out that utilize two stable isotopes simultaneously (multiplexing), for example ^{15}N and ^{13}C . We have used this approach to determine the positional ^{13}C enrichment in γ -amino butyrate in rice coleoptiles grown in the presence both ^{15}N nitrate and ^{13}C acetate [65].

Chemoselection combines selection of metabolites in a mixture that are derivatized according to their functional group. If the derivatizing agent contains a stable isotope, then in those metabolites that have a particular derivatizable functional group will be observed in

the NMR experiment. Gowda et al. [79] have developed the use of ^{15}N -cholamine which reacts with free carboxylates in the presence of a catalyst forming a peptide bond. As $^1\text{J}_{\text{NH}}$ is around 91 Hz, the peptide unit is readily observed using $^1\text{H}\{^{15}\text{N}\}$ -HSQC, and a large number of resonances can be detected in complex cell extracts. Similarly, we have developed ^{15}N aminoxy reagents that react specifically with aldehydes and ketone, but not carboxylates [38]. This reagent and ^{15}N -cholamine are now available from the Metabolomics workbench (<http://www.metabolomicsworkbench.org/standards/index.php>). This includes the reducing sugars, which are otherwise difficult to assign by NMR because of the spectral overlap. Depending on the compound the spectra are edited using the $^2\text{J}_{\text{NH}}$ or $^3\text{J}_{\text{NH}}$ couplings, which are in the 2–3 Hz range. Although small, the long T_2 in metabolites makes a long-range HSQC experiment sufficiently sensitive, and can be combined with TOCSY to obtain more information about the spin connectivity, and thus assignments, in the target metabolites. In both cases, only the target metabolite resonances are observed in the ^{15}N -edited spectra. Other functional groups can be detected using appropriate reagents, including amino groups and sulfhydryl groups [80].

Considerations of analytical accuracy and precision

The absolute accuracy of NMR is very high if sufficient care is taken over the experimental conditions [3]. These include the sample quality, uniform excitation and relaxation periods, and the method of integration or resonances. As modern NMR spectrometers have a very high linear dynamic range (typically $10^5:1$), only one internal standard is needed to determine the number of molecules present in the sample. In 1D ^1H NMR it is common to use a peak deconvolution in most metabolomics applications, the relaxation delays are set long compared with most T_1 values, so that correction for incomplete relaxation is small, and generally simple. With care, absolute concentrations can be determined with high accuracy (better than 5%, subject to certain caveats discussed in section 3 below) and precision (CoV of <1–2 %) which is far lower than any biological variance (reviewed in [3]).

Because of the extended time needed to record 2D NMR spectra, it is customary to reduce the recycle time close to the mean T_1 value of the analytes to maximize sensitivity with 90° excitation pulses. This substantially distorts the intensities for spins having different T_1 values. Also, in HSQC experiments recorded at high magnetic field strengths, uneven excitation occurs across the spectrum, in part owing to a shift-dependent variation of $^1\text{J}_{\text{CH}}$ values (from ca. >200 Hz for aromatic C-H pairs to ca. 125 Hz for methyl groups). For absolute quantification, this requires additional corrections, such as suggested by [81].

2D experiments, however, are more generally used to define the sites of enrichment (“isotopomer analysis”), as well as the degree of enrichment at each site. The main issue then is the differential relaxation rate constants for protons attached to ^{12}C versus those bonded to ^{13}C . Because the fractional enrichment is calculated as a ratio of peak intensities (e.g. $\text{volume}(^{13}\text{C})/[\text{volume}(^{12}\text{C})+\text{volume}(^{13}\text{C})]$), such that even with relatively short recycle times, the quantitative accuracy and precision of the fractional ^{13}C levels is high in TOCSY, as described [65, 76].

3. Metabolic pathway reconstruction

In order to proliferate, cells make the precursors of the components of biomass including nucleotides, lipids and some amino acids via endergonic anabolism. Furthermore, during proliferation, in order to maintain homeostasis, the metabolites must also be synthesized or obtained from the external medium. Complex metabolites such as the nucleotides (and nucleotide sugars) need multiple pathways to supply the carbon and nitrogen that comprise the product, and these processes are in fact complex metabolic networks of pathways [75].

Nucleotide synthesis

A large part of metabolism is mediated and enabled by nucleotide co-factors. Anabolic metabolism is endergonic, and for the most part is driven by the coupled hydrolysis of nucleoside triphosphates. ATP is the most widely used energy currency, but GTP, UTP and CTP are also used in specific contexts [75]. Furthermore in addition to driving thermodynamically unfavorable reactions, the nucleotides are also used to increase the chemical reactivity of otherwise unreactive metabolic subunits, by forming covalent linkages. This includes activation of acetate as AcCoA, a universal acetyl donor, and the methyl as S-Adenosyl Methionine (SAM), as well as in protein biosynthesis (cf. aminoadenylation), UDP-hexoses for glycosylation and complex carbohydrate synthesis, and CDP-choline for lipid synthesis among others. The nucleoside triphosphates are also the direct substrates for nucleic acid biosynthesis.

The redox dinucleotides, including NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ are needed for catabolic oxidation (NAD^+) and anabolic reduction (NADPH) as well as other functions such as regulation by ADP ribosylation (NAD^+). The nucleotides are ubiquitous in metabolism, and unsurprisingly their concentrations are very tightly regulated [75].

(i) Free nucleotide and RNA synthesis—The concentration of ATP, ADP and AMP and their ratios regulate the function of live cells, as these nucleotides are used to maintain the non-equilibrium state of the cell in maintain membrane potentials via ion gradients coupled to ATP hydrolysis, protein turnover, and anabolic metabolism which is highly endergonic. Most of this is determined by phosphorylation at the substrate level and by mitochondrial oxidative phosphorylation. In addition, ATP along with GTP, UTP and CTP are needed as substrate for RNA and DNA biosynthesis, which remove the nucleotides from the free pools. This cells that are proliferating, or shift to a state of increased protein synthesis, must up regulate the de novo synthesis of the nucleotides to produce the macromolecules. The synthesis of the nucleotides is highly regulated [75]. Purine synthesis is cytoplasmic and the base is built directly on the ribose-phosphate subunit deriving from the pentose phosphate pathway, whereas the pyrimidine bases are built separately using both cytoplasmic and mitochondrial reactions, and then condensed onto the activate ribose phosphate (PRPP) as Uracil. SIRM studies can distinguish between the ribose subunit synthesis and the base synthesis. The ribose subunits in the free nucleotide pool can be detected by TOCSY and HSQC owing to the characteristic chemical shifts and spin topology [82], from which the ^{13}C isotopomer distribution can be determined [20, 65, 76]. In most cases, this arises from glucose, unless there is significant gluconeogenesis,

when ^{13}C can be detected from glucogenic precursors such as lactate and some amino acids [83, 84]. Similarly, the H6 and H5 of the pyrimidine rings shows characteristic TOCSY and chemical shift patterns, and reveal the source of the carbons, which may be both glucose (via glycolysis and the Krebs cycle) or glutamine via glutaminolysis and the Krebs cycle (Figure 2)[82]. Similarly, the incorporation of ^{15}N from Gln into the purine rings of nucleotides can be determined using the substantial long range ^{15}N - ^1H scalar couplings to H8 and H2 (Figure 3) [2, 85]. This shows that as expected the Gln amido nitrogens were incorporated into the N3 and N9 positions, but also the N1 derived from the ^{15}N Gln. This can be accounted for by the activity of AST which transaminates Glu (the product of glutaminase) with OAA to make Asp, the nitrogen donor for N1 [75] (cf. Fig. 2B).

(ii) Redox dinucleotide NAD, NADH, NADP, NADPH quantification and synthesis/turnover—Generally the total concentration of $\text{NADH} + \text{NAD}^+$ in mammalian cells is of the order 0.5–1 mM, with the free NAD^+ concentration 700–1500 times higher than NADH [86], though the ratios may vary substantially in different compartments such as the cytosol and mitochondria. These ratios have been determined in vivo using the pyruvate/lactate redox couple and by NMR using DNP [86]. In contrast, the total $\text{NADPH} + \text{NADP}^+$ is about 10 fold lower than $\text{NADH} + \text{NAD}^+$, and the ratio of $\text{NADP}^+/\text{NADPH}$ has been variously estimated as 0.02 to 2 in mammalian cells [70, 87, 88] to >20 in yeast (free concentrations) or 1 (total concentrations) [89]. The source of the hydride varies according to cell type, and can also be followed using deuterated substrates [70, 87]

In cell extracts, the amounts of NAD^+ and NADP^+ can be readily determined by NMR, as the nicotinamide resonances are well resolved, and those of NAD and NADP can be easily integrated (Figure 4A). The nicotinamide resonances of the reduced forms however overlap with many other resonances [90]. Recently [1] et al. have shown that the adenine C8H resonance of ATP, ADP, AMP, NAD^+ , NADH, NADP^+ and NADH are resolved in extracts (Fig 4B), permitting determination of the absolute amount of each nucleotide. In mouse cardiac muscle, for which the extraction process was optimized, after converting ng/mg tissue to mmol/kg tissue the ratios of ATP to ADP and AMP were ca. 7.5:1 and 10:1 respectively, while the $\text{NAD}^+/\text{NADH} = 2.8$, $\text{NADPH}/\text{NADP}^+ \approx 1.6$. Furthermore, the NAD^+/NADH ratio implies a lactate/pyruvate ratio of ca. 1300 at pH 7.4, which is much higher than is generally observed either in extracts or in vivo [59, 86, 91]. This method does however open up the possibility of using NMR to determine the rate of NAD(P)^+ reduction using deuterated substrates [70, 87].

The discrepancy between amounts and concentration ratios in vivo and those determined by extraction may be attributed to two main causes. First some of the nucleotides are unstable, and their integrity depends on the details of the extraction protocol, especially for the reduced nucleotides. The second is a different consequence of the extraction protocol. Many extraction techniques measure the total nucleotides as the various dehydrogenases are denatured during extraction, and release the bound nucleotides. Furthermore, the ratio of the free nucleotides versus the ratio of the bound nucleotides is in general not equal, because of differential affinity of the dehydrogenases for NAD^+ and NADH, often an more than an order of magnitude [92]. In contrast, NMR of live cells gives estimates of amounts of common metabolites in the free state, that are significantly different from those obtained by

denaturing extraction protocols, or by mass spectrometry, which explicitly requires a deproteinizing extraction prior to analysis [93]. Such differences attributable to binding of metabolites to proteins have also been observed for untreated plasma or serum [94, 95].

However, from the point of view of flux analysis and thermodynamics, it is more useful to determine the free concentrations. Consider the reaction catalyzed by lactate dehydrogenase (LDH):

The equilibrium constant for this reaction, K_{eq} , which can be measured in vitro, is:

$$K_{eq} = [\text{lactate}][\text{NAD}^+]/[\text{pyruvate}][\text{NADH}]10^{-pH} \quad (1B)$$

The concentrations refer to the free state, rather than the total (free plus bound).

The steady state reaction mechanism catalyzed by LDH can be approximated as a sequential ordered bi-bi enzyme mechanism with rapid quasi-equilibrium binding of substrates and coenzymes [96]. Ignoring the protons, the net flux, J , at steady state is given by:

$$J = k_{cat} \cdot [E]_t [S] \cdot [N] / [K_S K_N (1 + [S]/K_S (1 + [N]/K_N) + [P]/K_P (1 - [NH]/K_{NH}))] [1 - [P][NH]/[S][N]K_{eq}] \quad (2)$$

where $[S]$, $[N]$, $[P]$, $[NH]$ are the free concentrations of substrate pyruvate, NAD^+ , product lactate and NADH, $[E]_t$ is the total enzyme concentration, K_S , K_N , K_P , K_{NH} are the dissociation constants of the substrate from the enzyme and K_{eq} is the overall equilibrium constant. The functional form of the rate equation in terms of total concentrations of substrates and products requires knowing the concentrations of all of the bound forms of each, and leads to a far more complex rate equation. Furthermore, as there are several hundred dehydrogenases, this is unlikely to be determinable.

The term $[1 - [P][NH]/[S][N]K_{eq}]$ is the thermodynamic term that describes how close the reaction is to equilibrium, and also the direction of net flux, and is independent of the details of the enzyme mechanism. Thus for nucleotides, the thermodynamic driving force and reaction kinetics may not be correctly analyzed using total concentrations determined from extracts; rather the free concentrations, as potentially determined from in vivo NMR, may be more reliable.

If the reaction in Eq. (1) is at equilibrium, then

$$[\text{NAD}^+]/[\text{NADH}] = K_{\text{eq}} \cdot 10^{\text{pH}} \cdot [\text{pyruvate}]/[\text{lactate}] \quad (3)$$

The error in the ratio of $[\text{NAD}^+]/[\text{NADH}]$ is proportional to the errors in the ratio of $[\text{pyr}]/[\text{lac}]$, to the equilibrium constant and to the relevant intracellular pH. Furthermore, this is correct strictly only at equilibrium, where there is no net flux, only an exchange flux [97]. The value of K_{eq} has been estimated in vitro as $1\text{--}5 \times 10^{-5}$ in the pH range from 6.8 to 7.0 [92, 98, 99]. The cytoplasmic pH is generally about 7.4, though lower values close to 7 [86] and higher ones in active cancer cells may occur [100]. These can in fact be estimated by ^{31}P and ^{13}C NMR in cells, with an estimated error of <0.1 pH unit in carefully calibrated systems [101, 102]. Thus the nucleotide ratio may be ill determined according to the range of the equilibrium constants estimated in vitro, the uncertainty of the relevant pH, as well as the analytical imprecision in the concentration ratio of lac and pyr. This might amount to an uncertainty in the $[\text{NAD}^+]/[\text{NADH}]$ ratio as much as 3–4 fold. However, the in vivo ratio is estimated in the range 700–1500, so even a factor of 3–4 fold might reduce this ratio to $>200\text{--}500$, which remains much higher than the in vitro estimates of ≈ 3 (see above).

4. Conclusions and Future Directions

The high quantitative accuracy of NMR, the lack of a need for standards, the extensive isotope editing capabilities, direct isotopomer distribution analysis, structural identification and applicability to metabolic studies in vivo make NMR an extremely valuable analytical tool for tracer-based metabolic studies.

Extraction is a problem for nucleotide analyses in terms of absolute concentrations. The errors in absolute concentration determination however do not affect isotopomer distributions as these are calculated as ratios. The isotopomer distributions carry much more information about pathways than steady state concentrations, and may thus be more robust. As nucleotides typically bind numerous proteins, the methods of extraction distort the relative amounts (free versus bound), which also complicates flux analysis. In vivo methods including NMR and for NAD(P)H fluorescence methods might be profitably combined with isotopomer analysis in vivo and in extracts to analyze quantitatively cellular fluxes [103].

On modern high field instruments equipped with cryoprobes, it is common to achieve SNR of 1500:1 on 2 mM sucrose in 90% H_2O . In a 5 mm Shigemi tube, this is about $0.6 \mu\text{mol}$ solute, implying that a 1D proton spectrum with $\text{SNR} > 10$ can be acquired on 0.4 nmol in around 10 minutes. At physiological salt concentration, this sensitivity decreases significantly (ca. 2 fold at 700 MHz), which can be partially offset using a 3 mm Shigemi tube.

Of particular interest for in vivo NMR studies there are methods that can enhance sensitivity thousands of fold including by Dynamic Nuclear Polarization or parahydrogen [104, 105][3, 14, 106–112] using a variety of commercially available polarizable substrates (cf Table 1) [3, 14]

Although much of metabolomics NMR is done using 1D acquisition [14, 113–115], the resolution in these experiments is relatively low, and isotopomer analysis may need higher dimensions, which can also make use of the isotope editing capabilities described above. However, using conventional sampling, even 2D methods with adequate digitization in the indirect dimension requires a large number of increments, making the experiment very lengthy which is not compatible with even medium throughput. For example, a conventionally sampled HSQC spectrum with resolution much better than the $^1J_{CC}$ coupling constants of 40–50 Hz, requires acquiring of the order 1000 fids (real + imaginary) at modest field strength (ca. 14.1 T), and with the need for signal averaging especially in weak samples, the time required for 16 transients per increment assuming a recycle time of 2 s is nearly 9 h. However, this can be reduced to <3 h with 33% sampling by nonuniform sampling (NUS), while ensuring that the resolution in F1 reaches the desired value. The time savings in 3D experiments can be even greater. For example, a conventionally sampled 3D spectrum with 64 increments in both indirect dimensions, and a recycle time of 1.5 sec, the experimental time with 16 scans/increment is about 27 h, with low resolutions in the indirect dimensions. The same experiment with 33% NUS in both indirect dimensions would need only 3 h with potentially higher resolution.

Furthermore, with dual receivers, it is possible to acquire parallel experiments with appropriate routing of coherences [116–119], which promises to speed up acquisition of multiple data sets significantly, making multidimensional NMR more routine, and expanding the capabilities of isotopomer analysis.

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Highlights

- The metabolic state provides a dynamic functional readout of live cells and tissues
- Stable Isotope Resolved Metabolomics (SIRM) permits tracing atoms through pathways
- Isotope editing by NMR enables selection of labeled metabolites in complex mixtures
- Flux measurements are possible both in vitro and in vivo using NMR-based SIRM

Figure 1. Biochemical network involving glucose metabolism

Glucose is oxidized to pyruvate (glycolysis). Several intermediates are involved in parallel and intersecting pathways: glycogen metabolism, pentose phosphate pathway, serine pathways, lipid biosynthesis and the Krebs Cycle. G6P: glucose-6-phosphate; F6P fructose-6-phosphate; F1,6BP fructose-1,6-bisphosphate; GAP glyceraldehyde-3-phosphate; DHAP dihydroxyacetone phosphate; 1,3bisPG 1,3-bisphosphoglycerate; 2PGA 2-phosphoglycerate; PEP phosphoenolpyruvate; Pyr pyruvate; OAA oxaloacetate; AcCoA acetyl CoA; Lac lactate; Ru5P ribose-5-phosphate; HK hexokinase; G6Pase glucose-6-phosphatase; PGI phosphoglucose isomerase; PFK1 phosphofructokinase 1; FBPase fructose 1,6 bisphosphatase; ALD aldolase; GAPDH glyceraldehyde-3-phosphate dehydrogenase; PGK phosphoglycerate kinase; PGM phosphoglycerate mutase; ENO enolase; PK pyruvate kinase; LDH lactate dehydrogenase; ALT alanine transaminase; PC pyruvate carboxylase; PDH pyruvate dehydrogenase; PPPox oxidative branch of the pentose phosphate pathway; PPPnx non-oxidative branch of the pentose phosphate pathway.

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Figure 2. Glu isotopomers from Krebs cycling

Isotopomers created via Krebs cycle activity by: (A) $^{13}\text{C}_6$ glucose. Black dots are ^{12}C . $^{13}\text{C}_6$ glucose (red dots) produces $^{13}\text{C}_3$ pyruvate which can enter the Krebs cycle either via PDH (2 ^{13}C) or PCB (3 ^{13}C , shown as green dots), which give rise to different isotopomers of Krebs cycle intermediates and anabolic products such as uracil and its precursor Asp, distinguishable by NMR as $^{13}\text{C}_{1,2} + ^{13}\text{C}_{3,4}$ via PDH in the forward direction, and $^{13}\text{C}_{1,2,3}$ Asp via PCB (B) $^{13}\text{C}_5, ^{15}\text{N}_2$ Gln plus unlabeled glucose. Red dots are ^{13}C atoms from Gln, blue dots are the two nitrogen atoms. Open circles are ^{12}C . The anaplerotic input of fully labeled Gln produces the fully labeled Krebs cycle intermediates. Fully labeled OAA from Gln condenses with glucose-derived AcCoA to produce quadruple labeled citrate, which becomes via the Krebs cycle triply labeled α KG, and doubly labeled succinate. AST will transaminate OAA with Glu, transferring the amino nitrogen to Asp. The isotopomers produced evolve on further cycles, and differ with other inputs to the cycle. Isotopomer analysis is needed to sort out the resulting complex patterns.

Figure 3. ^{15}N incorporation from Glutamine in purine nucleotides

Spectral editing via $^1\text{H}\{^{15}\text{N}\}$ -HSQC detects ^{15}N -isotopomers of nucleotides. A549 cells were grown for 24 h in the presence of 5 mM unlabeled glucose and 4 mM $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -glutamine. The spectra of the polar extract were recorded at 14.1 T at 20 °C with acquisition times of 0.15 s in t_2 and 0.021 s in t_1 and a recycle time of 1.6 s. The value of the INEPT transfer delay was set to 10 ms.

A. 1D $^1\text{H}\{^{15}\text{N}\}$ -HSQC spectra at different time points. B. 2D HSQC shows editing of the complex proton spectrum. The long range ^1H - ^{15}N couplings shows utilization of both the amido (blue) and amino N of Gln. Adapted from ref. [2] “With permission of Springer”

Figure 4. Nucleotide analysis by ^1H NMR

^1H NMR spectra of polar extracts of cells or tissues.

A. ^1H NMR spectrum at 600 MHz of an extract of BEAS-2B cells (an immortalized bronchial epithelial cell line) showing the spectral region containing the resonances of the nicotinamide subunits of NAD^+ (N) and NADP^+ (NP). The spectrum was recorded at 288 K with an acquisition time of 2 s and a recycle time of 6 s (512 transients). The free induction decays were zero-filled once and apodized using an unshifted Gaussian function and a 05 Hz line broadening exponential.

B. ^1H NMR spectrum of an extract of mouse heart showing the Adenine nucleotide region (C8-H) resonances) of different nucleotides including oxidized and reduced forms of NAD^+ and NADP^+ . From [1]

Table 1

Commonly used stable isotope tracers

Tracer	Pathways Probed	References
[U- ¹³ C]-glucose	Glycolysis, PPP, TCA cycle, hexosamine, nucleotide, and lipid biosynthesis	[3, 53]
[¹³ C1,2]-glucose	Non-oxidative vs. oxidative PPP	[54, 55]
[¹³ C3,4]-glucose	PC anaplerosis	[56, 57]
¹³ C-1 glucose	Glucose metabolism in vivo	
[U- ¹³ C, ¹⁵ N]-glutamine	Glutaminolysis, nucleotide biosynthesis, TCA cycle, fatty acid biosynthesis, amino acid metabolism	[53, 58, 59]
[U- ¹³ C]-fatty acids e.g. octanoate	β-oxidation, fatty acid biosynthesis	[26, 60]
[¹³ C]-serine	Serine metabolism, one-carbon metabolism, lipid biosynthesis	[61]
[¹³ C]-glycerol	Lipid biosynthesis	[62]
¹³ C ₁ / ¹³ C ₂ pyr	LDH reaction/DNP	[63]
¹³ C ₆ , ² H ₇	Glycolysis/PPP DNP	[64]