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HYPERPOLARIZED ^{13}C MAGNETIC RESONANCE AND ITS USE IN METABOLIC ASSESSMENT OF CULTURED CELLS AND PERFUSED ORGANS

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Abstract

Diseased tissue is often characterized by abnormalities in intermediary metabolism. Observing these alterations *in situ* may lead to an improved understanding of pathological processes and novel ways to monitor these processes non-invasively in human patients. Although ^{13}C is a stable isotope safe for use in animal models of disease as well as human subjects, its utility as a metabolic tracer has largely been limited to *ex vivo* analyses employing analytical techniques like mass spectrometry or nuclear magnetic resonance spectroscopy. Neither of these techniques is suitable for non-invasive metabolic monitoring, and the low abundance and poor gyromagnetic ratio of conventional ^{13}C make it a poor nucleus for imaging. However, the recent advent of hyperpolarization methods, particularly dynamic nuclear polarization (DNP), make it possible to enhance the spin polarization state of ^{13}C by many orders of magnitude, resulting in a temporary amplification of the signal sufficient for monitoring kinetics of enzyme-catalyzed reactions in living tissue through magnetic resonance spectroscopy or magnetic resonance imaging. Here we review DNP techniques to monitor metabolism in cultured cells, perfused hearts, and perfused livers, focusing on our experiences with hyperpolarized $[1-^{13}\text{C}]\text{pyruvate}$. We present detailed approaches to optimize the DNP procedure, streamline biological sample preparation, and maximize detection of specific metabolic activities. We also discuss practical aspects in the choice of metabolic substrates for hyperpolarization studies, and outline some of the current technical and conceptual challenges in the field, including efforts to use hyperpolarization to quantify metabolic rates *in vivo*.

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1. Introduction – Importance of developing methods to observe metabolic flux in disease states

Metabolism is at the root of essentially all physiological processes (DeBerardinis and Thompson, 2012). The production and expenditure of energy, storage and breakdown of macromolecules, disposal of waste, and many other processes are subserved by thousands of enzymatic reactions at work in human cells. Recent work has produced insights that greatly extend the influence of metabolism and metabolites to include such seemingly disparate processes as signal transduction, post-translational modification of proteins, and epigenetic effects on gene expression (Choudhary et al., 2014; Kaelin and McKnight, 2013; Ward and Thompson, 2012). These observations further emphasize the principle that metabolism is inexorably intertwined with cellular function and tissue homeostasis. In short, normal tissue function cannot occur unless metabolism is properly regulated.

For most of the past century, metabolism research has been dominated by studies in organs like the liver, one of whose major functions is to maintain metabolic homeostasis for the entire body, and the skeletal muscle, heart and brain, whose normal function involves energetically-demanding processes. However, many other tissues are equally dependent on a broad complement of metabolic activities. As an example, an emerging theme in cell biology research is the importance of acute metabolic changes for enabling physiological cell growth and proliferation (Metallo and Vander Heiden, 2013; Plas and Thompson, 2005). Because growth and proliferation are viewed as metabolically demanding in terms of the need for energy and macromolecular synthesis, there has been interest in understanding how the signals that stimulate punctuated bursts of cell proliferation engage the metabolic network to satisfy these demands. T-cell activation is one of many processes now known to require several specific metabolic activities to support cell growth and proliferation (Gerriets et al., 2014).

Given the many links between metabolism and cellular function, it is unsurprising that most diseases feature abnormal metabolism at the cellular level (DeBerardinis and Thompson, 2012). Of the hundreds of monogenic diseases caused by mutations in single enzymes (the so-called “inborn errors of metabolism”), a high fraction affect the liver, heart, muscle and brain. Many other monogenic metabolic diseases involve poorly-defined effects on growth, either because of systemic metabolic imbalances or perhaps effects on specific populations of cells whose function is required for normal growth. Importantly, common diseases also involve altered metabolism. Among the most common causes of death in the United States, heart disease, cancer, stroke, and diabetes can all be viewed as involving altered metabolism at the level of the cell and/or organ. Thus, we need better tools to understand metabolic regulation in diseased tissues. Preferably, some of these tools will support metabolic analysis in intact tissue.

Metabolic dysregulation is a prominent and clinically-relevant feature of cancer biology. As early as the 1920s, Otto Warburg demonstrated that malignant cells have a propensity to take up excess amounts of glucose and convert it into lactate, even when oxygen availability was sufficient to oxidize glucose completely to carbon dioxide (Warburg, 1956b). The successful use of ^{18}F -fluoro-2-deoxyglucose (FDG) as a radiotracer for positron emission

tomography (PET) studies in cancer patients has validated the clinical importance of glucose uptake in human tumors (Gallamini et al., 2014). FDG-PET is commonly used to image the distribution of malignant tissue and to monitor the effects of therapy. Other imaging techniques have also been employed to observe altered metabolic states in tumors. Proton magnetic resonance spectroscopy (^1H MRS) enables the detection and quantitation of abundant metabolites, some of which have prognostic or diagnostic value. To date, this technique has been used most extensively in brain tumors and other central nervous system diseases (Oz et al., 2014). An example of a recent technical development is the use of ^1H MRS to detect 2-hydroxyglutarate, an “oncometabolite” produced by mutant forms of the metabolic enzymes isocitrate dehydrogenase-1 and -2 in brain tumors (Andronesi et al., 2012; Choi et al., 2012; Elkhaled et al., 2012; Pope et al., 2012). After a considerable amount of research over the past decade, metabolic reprogramming as a consequence of tumorigenic mutations in oncogenes or tumor suppressor genes is now considered to be one of the major biological hallmarks of cancer (Hanahan and Weinberg, 2011).

Stable isotope tracers like ^{13}C are widely used to investigate metabolism in living systems. Transfer of ^{13}C from a parent molecule (e.g. glucose) into downstream metabolites reports the activity of metabolic pathways, providing an important complement to measurements of steady-state metabolite levels. Because ^{13}C is a non-radioactive tracer that is effectively monitored using analytical techniques like nuclear magnetic resonance or mass spectrometry, administering ^{13}C to animals or human subjects is safe. A typical experiment involves the administration of one or more isotope-labeled nutrients to the subject, followed by periodic or end-point acquisition of tissues of interest (e.g. blood, urine, tumor), extraction of informative metabolites, and analysis of isotope enrichment patterns to infer metabolic pathway activity. This type of approach has been used successfully in mice and humans to probe metabolic alterations in cancer and other metabolic disorders (Busch et al., 2007; Maher et al., 2012; Marin-Valencia et al., 2012; Sunny et al., 2011; Ying et al., 2012; Yuneva et al., 2012).

Each of these methods has only a limited ability to report on the metabolic network. Stable isotope approaches are invasive and destructive; that is, they require tissue sampling and metabolite extraction in order to obtain information about metabolism. In addition, metabolic activity is inferred from ^{13}C enrichment rather than by observing metabolism directly through an imaging technique. On the other hand, while PET supports direct, non-invasive detection of a labeled metabolic probe, the resulting information is limited to anatomic localization of probe uptake and accumulation, with essentially no information about downstream metabolic activities. ^1H MRS quantifies metabolite pools but provides no information about flux, and is somewhat limited by the narrow chemical shift range of the ^1H spectrum.

Methods to image ^{13}C would be powerful tools to probe disease-associated metabolic changes, because they would enable direct visualization of metabolic activity and in principle could be repeated multiple times in the same subject. The wide chemical shift range of the ^{13}C NMR spectrum means that a very large number of individual carbon positions from intermediary metabolites could be monitored simultaneously. The major challenges associated with carbon imaging involve the low in vivo abundance and poor

gyromagnetic ratio of the ^{13}C nucleus; these factors, combined with the low level of spin polarization, result in a very small signal detectable by magnetic resonance. However, using a process termed hyperpolarization, the spin polarization can be greatly enhanced, thereby enhancing the magnetic resonance signal (Ardenkjaer-Larsen et al., 2003; Kurhanewicz et al., 2011). Hyperpolarization techniques temporarily redistribute the population of energy levels of ^{13}C nuclei into a non-equilibrium state. This state is highly unstable, with a T_1 varying with the chemical environment of the nucleus but typically lasting less than 60 seconds. However, the signal gain during this period of time can routinely exceed 10,000 fold, leading to a massive if transient increase in MR signal (Ardenkjaer-Larsen et al., 2003). This gain provides sufficient signal to introduce hyperpolarized materials into a biological system, observe the labeled nucleus and – if metabolism of the substrate is rapid enough – observe real-time transfer of the hyperpolarized nucleus to new molecules through enzyme-catalyzed metabolic reactions.

A large and growing number of metabolites from central carbon metabolism have been hyperpolarized for real-time metabolic studies (Gallagher et al., 2008a; Gallagher et al., 2008b; Gallagher et al., 2009; Golman et al., 2006; Moreno et al., 2014a; Rodrigues et al., 2014). However, $[1-^{13}\text{C}]$ pyruvate has received the most attention to date. Carbon-1 of pyruvate has an unusually long T_1 (approximately 45 seconds), providing ample opportunity to observe the transfer of this carbon to metabolites of pyruvate. Furthermore, pyruvate is positioned at the interface between anaerobic and aerobic metabolism, a key metabolic node. Pyruvate can be reduced to lactate, transaminated to alanine, carboxylated to oxaloacetate, or decarboxylated to acetyl-CoA, and in the right tissue, all of these reactions can occur rapidly enough to observe by NMR (Figure 1). Hyperpolarized $[1-^{13}\text{C}]$ pyruvate has been used extensively in mouse models of cancer, because the rapid interconversion of pyruvate and lactate is readily detected by NMR and has been demonstrated to differentiate benign from malignant tissue (Golman et al., 2006; Hu et al., 2011). Hyperpolarized $[1-^{13}\text{C}]$ pyruvate has also been used in human studies to detect malignant tissue in the prostate, where its integration with magnetic resonance imaging has great potential as a clinical tool to diagnose and monitor cancer in patients (Nelson et al., 2013).

In addition to its use for in vivo studies, hyperpolarized $[1-^{13}\text{C}]$ pyruvate has been used in a number of other applications, including cell culture studies and perfused organs (Harrison et al., 2012; Merritt et al., 2011; Merritt et al., 2007; Moreno et al., 2014a; Yang et al., 2014a; Yang et al., 2014b). These studies capitalize on the extremely fine temporal resolution afforded by hyperpolarization (up to one data point per second) to directly observe, and in some cases quantify, flux through one or more metabolic reactions. Here we discuss our experiences using hyperpolarization as an analytical approach to understand metabolism in ex vivo systems. We describe methods to achieve high levels of polarization in $[1-^{13}\text{C}]$ pyruvate using dynamic nuclear polarization (DNP); maximize the efficiency and information yield of cell culture experiments; and analyze metabolism in the perfused heart and liver. We also discuss future directions and challenges for hyperpolarization, including roles for substrates other than pyruvate, and prospects for deriving quantitative flux information from in vivo experiments.

2. Hyperpolarization Methods and Sample Preparation

2.1 Hyperpolarization via dissolution dynamic nuclear polarization

Among different techniques of resolving the problem of low-sensitivity of NMR signals, hyperpolarization via dissolution dynamic nuclear polarization (DNP) is regarded as one of the most versatile and effective to increase the amplitude of NMR signals (Ardenkjaer-Larsen et al., 2003). The underlying principle of DNP is the transfer of high electron spin polarization to the nuclear spins via microwaves, hence creating a larger spin population difference in the Zeeman energy levels of the system, resulting in significant enhancements of NMR signal. Originally used in particle and nuclear physics, the microwave-driven polarization transfer process in non-conducting solids occurs at cryogenic temperatures close to 1 K and at magnetic fields greater than 1 T (Abragam and Goldman, 1978). The NMR signal-amplifying power of DNP has recently been harnessed for biomedical and metabolic research via rapid dissolution which converts the frozen polarized ^{13}C sample at cryogenic temperature into hyperpolarized ^{13}C liquid solution at physiologically tolerable temperatures (Ardenkjaer-Larsen et al., 2003). In this case, the lifetime of the hyperpolarized ^{13}C NMR signal is dictated by the ^{13}C spin-lattice relaxation time T_1 .

2.2 ^{13}C DNP sample components and preparation

Optimized DNP sample preparation is crucial to the success of the hyperpolarized ^{13}C NMR or MRI experiments because it can significantly affect the maximum ^{13}C NMR signal enhancement levels. Typically, a ^{13}C DNP sample is a solution with 10–100 μL volume consisting of:

- a. *^{13}C -enriched substrates.* With the superb ^{13}C NMR signal sensitivity afforded by dissolution DNP, real-time assessment of cellular metabolism is now feasible. Some of the commonly used ^{13}C -enriched biomolecules for metabolic research via hyperpolarized ^{13}C NMR are ^{13}C glucose, ^{13}C pyruvate, ^{13}C acetate, ^{13}C glutamine, ^{13}C fumarate, ^{13}C ascorbic acid and numerous others (Keshari and Wilson, 2014). Most of these ^{13}C -enriched compounds are in powder form and thus need to be dissolved in a glassing matrix prior to DNP. In special cases, neat liquids such as ^{13}C pyruvic acid are self-glassing and thus, one only needs to add and mix trace amounts of free radicals to complete the DNP sample preparation. As mentioned above, $[1-^{13}\text{C}]$ pyruvate is the most commonly used agent, in part because the position of ^{13}C on the carbonyl is associated with a fairly long T_1 relaxation time. The long T_1 of this carbon is related to the fact that its nearest intramolecular contact with protons is with pyruvate's methyl group (carbon 3). Long ^{13}C T_1 s are highly advantageous for DNP because they translate to a long lifetime of the hyperpolarized ^{13}C NMR signal, and therefore provide greater opportunity to observe metabolic activities. The intrinsic biochemistry of intermediary metabolites drastically limits the number of compounds suitable for hyperpolarization studies, because the presence of protons directly bonded to the carbon nucleus reduces the T_1 to 2 to 10 seconds. A list of some of the commonly used ^{13}C -enriched compounds and their corresponding liquid-state T_1 relaxation times is given in Table 1.

- b. *Glassing solvents.*** The choice of glassing solvents or matrix depends mainly on the criterion of maximum solubility of the ^{13}C substrates. Equally important is the requirement that these solutions form amorphous or non-crystalline solids when subjected to cryogenic temperatures (Kurdzesau et al., 2008; Lumata et al., 2011a). Amorphous conditions are also required to evenly distribute the ^{13}C nuclear spins and the free radical electrons across the sample volumes. Common examples of glassing matrices used for ^{13}C DNP are solutions composite of two solvents mixed in 1:1 vol/vol ratio such as glycerol/water, DMSO/water, ethanol/water, methanol/water, sulfolane/DMSO, ethyl acetate/DMSO, among others. The volume components may be adjusted when higher solubility of a hydrophilic ^{13}C substrate is needed; for example, increasing the ratio of water:glycerol to 4:1 has been used to accommodate some substrates (Lumata et al., 2011a). Concentrations of ^{13}C -enriched biomolecules in glassing matrices are typically on the order of a few moles/liter.
- c. *Free radicals.*** The next step is the addition of trace amounts of free radicals to the ^{13}C substrate solutions. The following stable, organic free radicals were proven to be effective polarizing agents for dissolution DNP (Lumata et al., 2012a; Lumata et al., 2011d; Lumata et al., 2013c) as well as their experimentally-determined optimal concentrations for DNP: tris{8-carboxyl-2,2,6,6-benzo(1,2-d:4,5-d)-bis(1,3)dithiole-4-yl)methyl sodium salt, (trityl OX063, 15 mM); 1,3-bisdiphenylene-2-phenylallyl (BDPA, 20 mM or 40 mM); 2,6-di-tert-butyl- α -(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy (galvinoxyl, 20 mM); 2,2-diphenyl-1-picrylhydrazyl (DPPH, 40 mM); and 4-Oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (4-oxo-TEMPO, 30–50 mM). Of these five free radicals, the most commonly used are the water-soluble polarizing agents trityl OX063 and TEMPO, since most ^{13}C -enriched substrates used in DNP are hydrophilic. The other three free radicals – BDPA, galvinoxyl and DPPH – can be dissolved in special solvents such as sulfolane/DMSO and sulfolane/ethyl acetate. Generally, the narrow electron spin resonance (ESR) linewidth free radicals trityl OX063 and BDPA were shown to be better polarizing agents for DNP of low-gamma nuclei such as ^{13}C spins (Lumata et al., 2014; Lumata et al., 2012b). This is due to the fact that their ESR linewidths matches the ^{13}C Larmor frequencies, leading to more efficient hyperpolarization.

A typical sample preparation procedure is outlined below:

- Weigh out the desired amounts of ^{13}C substrate and free radical using analytical balance. Use 1.5 mL microcentrifuge tubes as containers.
- Prepare the glassing solvent solution. Dissolve the ^{13}C substrate in the glassing solvents using a vortex mixer.
- Transfer the ^{13}C substrate solution into the tube containing the free radical. Use the vortex mixer to dissolve the free radical in solution. In some cases, a sonicator bath may be used to completely dissolve the substrate and/or free radical (Lumata et al., 2012c; Lumata et al., 2011d).

2.3 Other DNP sample optimization tips

2.3.1 Gadolinium doping—Inclusion of trace amounts (1–2 mM) of Gd^{3+} complexes such as Gd-DOTA into the ^{13}C DNP sample was shown to increase the ^{13}C DNP-enhanced polarization level by as much as 300% in the commercial 3.35 T HyperSense polarizer (Lumata et al., 2011a); Lumata et al. (2012d). However, this sample preparation practice is so far only recommended for ^{13}C DNP samples that are doped with trityl OX063 free radical. Inclusion of the Gd-complex in ^{13}C samples doped with the other free radicals led to negligible increases in ^{13}C polarization levels. In addition to Gd^{3+} complexes, other lanthanides such as Holmium-DOTA have shown similar beneficial effects on trityl OX063-doped ^{13}C samples. This DNP-enhancing phenomenon is ascribed to the shortening the T_1 relaxation time of the free radical electrons, leading to a more efficient DNP (Lumata et al., 2013a).

2.3.2 Deuteration of the glassing matrix: Do's and Don'ts—Using deuterated glassing solvents could be beneficial in some cases leading to large increases in hyperpolarized ^{13}C NMR signal. In other instances, however, this method may negatively impact the ^{13}C DNP-NMR signal enhancement (Lumata et al., 2013b). For example:

- *Do's: ^{13}C DNP samples doped with large ESR-linewidth free radicals.* Use deuterated glassing solvents for ^{13}C samples doped with large ESR linewidth free radicals galvinoxyl, DPPH and TEMPO. This sample preparation method was shown to double or triple the DNP-enhanced ^{13}C NMR signal in the frozen sample state. The idea is that deuterons in the glassing matrix are lesser heat loads than protons due to their smaller magnetic moments; thus, the former would lead to more efficient ^{13}C hyperpolarization (Kurdzesau et al., 2008; Lumata et al., 2013b).
- *Don'ts: ^{13}C DNP samples doped with narrow-ESR linewidth free radicals.* Do not use deuterated glassing solvents for ^{13}C samples doped with narrow ESR linewidth free radicals such as trityl OX063 and BDPA. Doing so would lead to decreases in ^{13}C DNP levels by as much as 50%. Protons do not effectively couple to the electron dipolar system of trityl and BDPA, rendering them effectively absent from the DNP process. With glassing matrix deuteration, the narrow ESR linewidth free radical electrons have to polarize not only ^{13}C spins but also the deuterons, leading to less efficient ^{13}C DNP (Goertz, 2004; Lumata et al., 2013b).

2.3.3 ^{13}C enrichment of the glassing matrix—This technique is employed to expedite the ^{13}C hyperpolarization process while the sample is inside the polarizer at cryogenic temperatures. For a typical ^{13}C DNP sample such as sodium [$1\text{-}^{13}\text{C}$]pyruvate in 1:1 vol/vol DMSO:water doped with trityl OX063, the microwave irradiation time needed to achieve the maximum DNP-enhanced ^{13}C polarization is about 2–3 hours. By using ^{13}C -enriched DMSO in the glassing matrix, the hyperpolarization time can be 2–3 times faster, thus saving time and cost of cryogenics. This phenomenon can be explained by a faster nuclear spin diffusion process as ^{13}C inter-nuclear distance is decreased with the increasing ^{13}C concentration in the system. This is analogous to a domino effect in terms of nuclear polarization transfer (Lumata et al., 2011b).

2.3.4 Frozen pellets—This sample preparation practice is especially useful for preparing large DNP sample volumes where amorphous formation of the frozen sample is sometimes compromised. Normally, small aliquots (10–100 μL) of ^{13}C DNP samples are pipetted into a sample cup (made of polyetheretherketone (PEEK) or Teflon) with approximately 200 μL volume capacity. To ensure glass formation, these small sample aliquots are flash-frozen by dipping the sample cup into liquid nitrogen (LN_2). Difficulties arise with large samples (e.g. 200 μL) because of the time necessary to freeze the entire volume. This may allow a fraction of the sample to crystallize. Small droplets of the sample may be prepared by pipetting out small volumes ($\sim 10 \mu\text{L}$) of ^{13}C sample solution into a styrofoam container with LN_2 . These small volumes are flash-frozen immediately, then transferred using plastic tweezers into the sample cup, which is also kept in LN_2 . Once filled with frozen droplets, the sample cup is immediately inserted into the polarizer at cryogenic temperature.

2.4 Operational steps of ^{13}C hyperpolarizer

2.4.1 HyperSense commercial polarizer—The HyperSense polarizer (Oxford Instruments, UK) is a highly automated commercial polarizer that operates at 3.35 T and at 1–1.6 K with a W-band (94 GHz) microwave source. The following are the experimental steps that we typically follow to polarize ^{13}C DNP samples:

- Prepare cool-down and filling of liquid Helium (LHe) into the cryostat sample space of the polarizer. This can be done by clicking “cooldown” on the software displayed on the PC. This step will turn on the rotary vane pump connected to the polarizer as well as adjust the needle valve opening for LHe. Once the LHe level in the polarizer reaches 65% and the temperature is close to 1.4 K, the insert sample button on the PC is activated.
- Pipette out the desired ^{13}C DNP sample volume into the cup (typically 10–100 μL). Flash-freeze the sample cup into LN_2 . Attach the sample cup snugly at the bottom of the insertion stick. When ready, press “insert sample” on the computer.
- The HyperSense polarizer will then automatically block the pump connection to the polarizer and overpressure the sample space of the cryostat close to 1 Atmosphere. The upper entry of cryostat will then open for sample insertion. Open the upper polarizer door for sample insertion.
- Quickly insert the sample stick into the bottom of the cryostat. Dislodge the sample cup inside the cryostat immediately by simultaneously holding the outer tube and pulling the inner tube of the sample insertion wand.
- Close the polarizer door immediately and click “finish” on the computer. Allow the polarizer to get back to the base temperature for a couple of minutes.
- Click “polarize” to start the ^{13}C hyperpolarization process. This will turn on the microwave source to irradiate the sample at 3.35 T and 1.4 K. Make sure that the optimum microwave irradiation frequency for ^{13}C with the relevant free radical is established before the experiment. It is also recommended to monitor the ^{13}C solid-state polarization buildup curve of the ^{13}C DNP sample for documentation purposes and to ensure that the sample is being properly hyperpolarized.

- Wait until the ^{13}C solid-state polarization buildup curve reaches a plateau or maximum value. The ^{13}C buildup curves are typically exponential with a waiting time of 1–3 hrs.
- Once the ^{13}C DNP sample reaches the maximum solid-state polarization, click the dissolution button on the computer.
- Open the polarizer door. Inject 4 mL of water or other approved solvent into the top of the automated dissolution wand. Close the upper valve of the dissolution wand.
- Align the bottom of the dissolution wand with the center opening of the cryostat. Close the polarizer door.
- Click “Start dissolution” for the process to start. The water or solvent in the small cylinder container of the dissolution wand will begin to heat and pressurize. This process takes about 3.5 minutes before the dissolution process begins.
- Once the temperature reaches 200 °C and pressure is at 10 bars, the microwave source is turned off, the pump is blocked from the polarizer, and the sample space of the cryostat is over-pressured with He gas.
- The dissolution wand is automatically inserted in the cryostat, acquiring the sample cup and injecting superheated water or solvent into the sample via high pressure He gas.
- A diluted liquid solution containing hyperpolarized ^{13}C biomolecules comes out of the polarizer via a PTFE or Teflon tubing. In 8–10 s, approximately 4 mL of hyperpolarized liquid will be collected into a beaker. This hyperpolarized liquid is then ready for immediate administration into cells, perfused organs or in vivo into living subjects for dynamic assessment of metabolism.

Other notes

- The frequency of microwave irradiation varies among different radicals and is dependent on the magnetic field. The optimal frequency of irradiation must be determined for each radical experimentally.
- The polarization times are specific to the sample being hyperpolarized. The glassing matrix and presence or absence of Gd^{3+} also influence the polarization time. For new analytes, several experiments optimizing the sample conditions such as glassing matrix and radical concentration should be performed to improve the highest possible solid state enhancement in the shortest possible time.

2.4.2 Homebuilt DNP hyperpolarizer—The general operational steps for polarizing ^{13}C samples in our homebuilt 129 GHz DNP hyperpolarizer (Lumata et al., 2014) are very similar to that of the automated HyperSense commercial polarizer, although ours is operated manually. The sample cup in the homebuilt polarizer is optimized to handle up to 600 μL of ^{13}C DNP sample volume. This is advantageous for hyperpolarized ^{13}C NMR or MRI studies of animals larger than small rodents, due to requirement of higher concentration and/or volume of hyperpolarized liquids.

3. Dynamic Assessment of Metabolism in Cells

While a major advantage of hyperpolarized ^{13}C studies is its ability to enable non-invasive monitoring of metabolism in intact tissues, there are several reasons to perform hyperpolarization experiments in cultured cell models as well. First, hyperpolarized substrates allow kinetic assessment of discrete metabolic reactions on a time scale that would be impractical using other methods. Second, cancer cells or other cell lines may provide convenient and affordable systems to assess candidate hyperpolarized substrates prior to deploying these molecules into more cumbersome biological models, including perfused organs and live animals.

The methods described below outline approaches to observe metabolism of hyperpolarized molecules in fresh suspensions of adherent cancer cells. We have demonstrated that some of the metabolic rates observed using hyperpolarized $[1-^{13}\text{C}]$ pyruvate were maintained during short periods of suspension (Yang et al., 2014a). It should be noted that bioreactor systems have also been used to observe metabolism of encapsulated adherent cells, providing an alternative to the simple suspension method outlined here (Keshari et al., 2010).

3.1 - Preparation of cultures for hyperpolarization experiments

3.1.1 Scale and quality of cells used

- As metabolic rates vary substantially among established cell lines, pilot experiments may be required to optimize conditions for each cell line. The procedure described here has been applied successfully to multiple rapidly proliferating, highly glycolytic cancer cell lines.
- In a typical *in vitro* analysis using hyperpolarized $[1-^{13}\text{C}]$ pyruvate, we use 40–80 million cells harvested during exponential growth. This has provided good reproducibility of metabolic rates among individual experiments.

3.1.2 Details of cell culture and harvesting

- Specific plating protocols should be optimized for each cell line, but plating 5×10^6 to 8×10^6 cells into each of several 150 mm-dish works well for most adherent cancer cell lines. These cultures can be expected to reach 80–90% confluency – the target for cell harvest – approximately 36–48 hr after plating.
- Harvest $75\text{--}150 \times 10^6$ cells from three to five 150-mm dishes by using trypsin-EDTA (0.05% for most cell lines) to disengage from the dish. Inactivate the trypsin by diluting in serum-containing culture medium, centrifuge to pellet the cells, then briefly rinse once in 10 mL pre-warmed PBS. Re-suspend the culture at a concentration of $50\text{--}100 \times 10^6$ cells/ml in fresh medium lacking glucose and pyruvate, but containing 10% dialyzed fetal calf serum, 4 mM glutamine, and standard concentrations of other amino acids.
- Time the preparation of the cell suspension to minimize the time in suspension prior to the hyperpolarization experiment, typically no more than 15 minutes. In the interim, keep the cells in a conical tube in a 37°C water bath with frequent mixing to protect against depletion of oxygen from the medium. Trypan blue staining can

be performed on a small aliquot of cells right before the hyperpolarization experiment to ensure high viability.

- In the moments preceding dissolution of the hyperpolarized material, transfer the cell suspension into a 10 mL syringe connected with a long teflon tube which goes through a free cap which will later be fitted to a 5-mm NMR tube (see Figure 2).

3.2 - Administration of hyperpolarized ^{13}C substrate and data acquisition

- Dissolve hyperpolarized samples using an automated process like the one described above. For cell-based experiments, we use 4 ml of 15.3 mM sodium bicarbonate at 190°C. Approximately 4 ml of hyperpolarized liquid (pH ~7) should be evacuated from the hyperpolarization chamber and collected in a beaker.
- Using a handheld p1000 pipettor, immediately transfer 0.2 mL of the hyperpolarized solution into the bottom of a 5-mm NMR tube.
- Affix the cap fitted with the Teflon tube connected to the syringe containing the cell suspension, as described above.
- Center the tube into a Varian 10-mm broadband probe tuned to ^{13}C in a 14.1-T magnet.
- Initiate acquisition of ^{13}C NMR spectra immediately after the NMR tube settles into the magnet.
- As the acquisition is initiated, transfer 0.8 mL of the cell suspension into the NMR tube using the syringe fitted with tubing. This produces a final volume of 1 mL, with 6 mM pyruvate and a cell density of $0.4\text{--}0.8 \times 10^8$ cells/mL. Note that the rationale for this method is to enable observation of the initial period of metabolism as the cells first encounter the hyperpolarized material, as the initial rate of ^{13}C transfer is highly informative.
- Maximizing reproducibility of transfer may require some modifications, particularly for cell lines that become viscous when suspended at the concentrations used here. Following the cell suspension with 0.2–0.5 mL of air bubbling may improve the homogenization of the mixture. Alternatively, the cell suspension can be mixed thoroughly and precisely with the hyperpolarized material in the NMR tube outside the magnet, although this requires some sacrifice of acquiring NMR spectra over the initial phase of ^{13}C transfer.
- At the end of the ^{13}C NMR acquisition (usually no more than 3–4 minutes after mixing the cells with hyperpolarized material), pellet the cells by centrifugation, save the culture medium, and flash freeze the pellet in liquid nitrogen. This enables complementary analysis using mass spectrometry or other methods to validate or aid in the interpretation of data acquired in the hyperpolarization experiment.

3.3 Techniques to maximize detection of products

Our group has successfully combined hyperpolarization with two other techniques aimed to enhance ^{13}C NMR signals. These are described briefly below.

3.3.1 Selective pulses—Shaped pulses are especially helpful in cases where the NMR signals emanating from products of interest are small compared to the ^{13}C signal of the parent compound. For instance, in *in vitro* hyperpolarized ^{13}C NMR experiments on cancer cell suspensions, the LDH-catalyzed product $[1-^{13}\text{C}]$ lactate signal is dwarfed by the hyperpolarized ^{13}C signal from the parent compound, $[1-^{13}\text{C}]$ pyruvate. Signal from hyperpolarized bicarbonate arising from decarboxylation of $[1-^{13}\text{C}]$ pyruvate is typically much smaller than $[1-^{13}\text{C}]$ lactate. To enhance the ^{13}C lactate signal, we applied a Gaussian-shaped selective radiofrequency (RF) excitation pulse at the frequency where $[1-^{13}\text{C}]$ lactate is expected. For Varian or Agilent NMR spectrometers, we create the selective shaped pulses using the VNMRJ PBox software (Harrison et al., 2012). This shaped pulse will only excite $[1-^{13}\text{C}]$ lactate spins with no or minimal RF excitation effect on the $[1-^{13}\text{C}]$ pyruvate spins. In cases where there are two or more tiny NMR signals of metabolic products of interest, double- or multiple Gaussian selective pulses can be generated to simultaneously interrogate the kinetics of the metabolic products in real-time. An important consideration in using shaped pulses is that the NMR signal of the metabolic product should be located at a distinct distance from NMR signal of the parent compound, otherwise there will be substantial RF excitation of the ^{13}C spins from the parent compound. Moreover, the use of selective pulses prolongs the availability of hyperpolarized ^{13}C magnetization of the parent compound since the latter is not or only minimally excited by the selective shaped RF pulse.

3.3.2 Cryogenic probes—In addition to hyperpolarization, the invention of cryogenically-cooled probes (“cryoprobes”) for NMR is considered to be another important advancement in terms of sensitivity enhancement. This NMR technology is based on the fact that cooling the NMR coil and its tuning and matching components substantially reduces the random thermal motions of electrons termed as the Johnson-Nyquist noise. In addition, the preamplifier, filters and transceiver electronics are also cooled to reduce the noise originating from the electronics (Kovacs et al., 2005). The use of an NMR cryoprobe results in an immediate improvement of signal-to-noise ratio (SNR) by a factor of 3–5 relative to the SNR obtained in a conventional NMR probe. Unlike dissolution DNP, the improvement in NMR sensitivity produced by an NMR cryoprobe is persistent, provided that the cryogenic conditions of NMR electronics are maintained. We use a 10 mm ^1H - ^{13}C Bruker CryoProbe system (Bruker Biospin, Billerica, MA) installed in a 600 MHz NMR magnet. This Cryoprobe system is located adjacent to the commercial HyperSense polarizer and a homebuilt DNP polarizer. When hyperpolarization is combined with the cryoprobe, the high ^{13}C SNR that we normally obtain for ^{13}C DNP is further improved by a factor of 4. To our knowledge, combining these two NMR technologies provides the highest ^{13}C NMR sensitivity currently achievable. Consistent with this idea, we have been able to use a hyperpolarization/cryoprobe combination to observe time-resolved signals previously achievable only using selective pulses (Yang et al., 2014b). Furthermore, the cryoprobe allows straightforward studies of the perfused mouse heart, an organ of such small size that only the combination of hyperpolarization and the cryoprobe has produced published results (Purmal et al., 2014).

4. Dynamic Assessment of Metabolism in Perfused Organs

Perfused organs, particularly the heart and liver, have long been used as models to analyze intermediary metabolism, particularly with mass spectrometry and conventional ^{13}C NMR spectroscopy. We have applied hyperpolarization approaches to the perfused heart and liver from mice to observe dynamic metabolism of ^{13}C -labeled probes. Basic experimental setup for these experiments is described below.

4.1 Hyperpolarized ^{13}C NMR of Perfused Heart

4.1.1 Preparation of the perfused heart—Animals are anesthetized and a trans-abdominal incision is then performed to expose the thoracic cavity. The heart is removed from the chest cavity by excising the pulmonary artery, vena cavae and aorta. Animal death occurs within seconds by exsanguination. The blood vessels are cut as close to the heart as possible without causing any damage to the heart. The excised heart is transferred to ice-cold saline to rinse off the blood and arrest the beating heart. All subsequent procedures prior to the start of perfusion are carried out on ice-cold saline.

Fat deposits and other contaminant tissue on the heart are removed surgically. After sufficient cleaning, a plastic cannula is inserted into the aorta. To provide robust cannulation and avoid any leaks, a surgical suture is used to tie the tissue around the cannula. Care must be taken to avoid any rupture of the tissue during the insertion of the cannula and subsequent application of the suture.

Special concerns

- After excising the heart, it is important to transfer the heart to ice-cold saline rapidly to prevent ischemic injury.
- Insertion of the cannula into the aorta must be performed carefully so that no air bubbles are introduced into the heart. This can typically be accomplished by leaving the cannula submerged in the ice-cold saline as the heart is being cleaned.
- Leaving the heart in ice-cold saline more than a few minutes often results in poor function. The time taken between the excision of the heart and start of perfusion should be minimized as much as practically possible.

4.1.2 Preparation of the perfusion rig—A straightforward setup to perfuse the hearts in constant pressure Langendorff mode is to utilize a vertical column providing the appropriate hydrostatic pressure (see Figure 3). Although portable perfusion systems exist (with electronic pressure and flow rate control), the glass columns are more directly amenable to studying perfused hearts inside an NMR magnet. The perfusate of interest is maintained at a fixed height (in order to provide constant hydrostatic pressure) in the column using peristaltic pumps. Continuous supply of oxygen is maintained by aerating the perfusate constantly with a mixture of 95% oxygen/5% carbon dioxide while temperature is regulated using a heated water jacket. A simple balloon catheter is typically used as a valve to turn the flow of perfusate on or off in this setup.

Special concerns

- Depending on the perfusate, additional equipment may be needed. For example, a thin film oxygenator effectively oxygenates perfusates containing long chain fatty acids and bovine serum albumin. Bubbling gas directly into such a perfusate will introduce excessive foaming in the perfusion column.
- With the dissolution of CO₂, pH of the perfusate is likely to be altered slightly. Hence, pH should be adjusted prior to attaching the heart to the perfusion rig.

4.1.3 Heart perfusion & Setting up the NMR spectrometer—Once a bubble-free connection is established, perfusate flow is established by deflating the balloon catheter. The heart typically starts beating within a few seconds (typically less than 30). Heart function is monitored in either a continuous mode or at discrete intervals as the instrumentation permits. Heart function can be typically monitored by recording the heart rate and oxygen consumption.

For NMR experiments, an attachment comprised of an NMR tube is connected to the bottom of the perfusion rig and is lowered into the NMR magnet for acquisition of spectra (Figure 3). By utilizing a sodium free flush, the effluent from the beating heart is continuously removed. The flush is accomplished by inserting independent tubing to the bottom of the NMR tube. A sodium free solution that is otherwise osmotically balanced is pumped into the region below the heart at a high rate to force the removal of the perfusate exiting the heart. In this setup, shimming can be accomplished by optimizing the linewidth of ²³Na signal from the heart itself. ²³Na linewidth of less than 12 Hz (corresponding to ¹³C linewidth of ~10 Hz) can be routinely obtained with mouse hearts at a field strength of 14.1 T.

Special concerns

- It is critical to ensure that the position of the heart in the NMR tube corresponds to the center of the RF coil of the NMR probe.
- Shimming the magnet prior to the experiment is optimal when carried out as a two-step procedure. In the first step, shimming of the magnet is accomplished using the strong ²³Na signal from both the perfusate and the heart. Once a satisfactory peak shape and width is achieved, the sodium free flush is utilized and further shimming is carried out directly using the sodium signal from the heart.
- Shimming on the ²³Na signal is often sensitive to abnormalities in the heart function. For example, irregular heart beat can be correlated with fluctuations in the random and rapid variations in the signal which can be correlated with the changes in heart rate (if measured continuously).

For the dissolution, the hyperpolarized sample is usually dissolved using 4 mL of heated phosphate buffered saline and transferred into a beaker where it mixes with a predetermined volume of perfusate. This sample is injected gently, close to the heart, using a catheter that is inserted into the cannula immediately above the heart. Pyruvate metabolism is observed by measuring a series of time resolved ¹³C spectra with small flip angle radiofrequency pulses. Alternatively, variable flip angle schemes may be utilized if necessary (Xing et al., 2013).

Special concerns

- Mixing with perfusate ensures that the osmolarity of the hyperpolarized sample is similar to that of the perfusate and that the sample is adequately oxygenated.
- The injection of the sample should be smooth and constant. Rapid injection can generate turbulence and uneven mixing of the injected substrate with the solution present in the perfusion rig proper.

4.2 Hyperpolarized ^{13}C NMR of Perfused Liver

4.2.1 Hepatectomy

- Heparinize the mouse with about 0.07mL Heparin injected intraperitoneally and anesthetize with 0.09 mL Ketamine/Xylocaine, also administered intraperitoneally.
- Weigh the mouse and transfer to a prep station. There will be blood and run-off perfusate. It helps to have a mini work table lined with plastic cling wrap.
- Tape the feet of the mouse to the cling wrap while allowing the head and shoulders to fall over the edge of the work table. This allows the liver to rest upwards on the tissue lining the thoracic cavity while the cannulation is performed.
- Pinch the skin above the abdomen with forceps. Lift up and make a wide incision along the midline of the abdomen, taking care not to puncture any organs.
- Fold the skin up over the sternum to expose the abdominal cavity. Slide the fat and intestines with your middle finger to the right and outside of the body cavity, exposing the portal vein.
- Create a path underneath the portal vein close to the liver with a pair of forceps. Feed two 3" pieces of 4-0 silk suture under the vein and begin a 'left-over-right' knot. Taking care not to close the knot, tie them down close to the vein and lay the ends of the piece closer to the head up between the arms.
- Cannulate the portal vein by inserting a 22GX1" Terumo Surflo IV Catheter. Use your left hand to move the fatty tissue and create tension on the vessel if needed. Take care not to advance the needle too far for risk of puncturing the vein. An assistant can pull on the two ends of suture closest to the head of the mouse to also create more tension for ease of catheter insertion.
- Once inside the vessel, hold the needle still and use your index finger to advance the catheter off of the needle further into the vein, about 1/4". Do not push the catheter as far as it will go inside the vessel as this may also puncture or create a stoppage of flow and induce ischemia.
- While holding the needle and catheter in place, have an assistant tie the suture closest to the head of the animal down as tight as possible ensuring that the vein doesn't slip off the end of the catheter. Ideally there will be about 5mm between the end of the catheter tip and first suture knot.
- Let the needle lay in place while you tie down the second suture. Double knot both sutures and cut off any excess suture material.

- Carefully remove the needle by holding the catheter hub in place and pulling the needle back. Blood should travel back into the hub. Very little to no blood should seep into the body cavity at this point.
- Displace any air in the hub with perfusate and insert your perfusion line while holding the hub firmly. Start your perfusion of the liver by starting the pump connected to the perfusate.
- Cut open the vena cava. The mouse should expire within seconds from exsanguination and the liver should flush all of the blood out and turn from a red/maroon color to an off-yellow/mustard or tannish color.
- Livers are very delicate. Minimal time should elapse from cannulation to starting the perfusion pump.
- Dissect out the liver by removing first the kidneys followed by intestines and the stomach/pancreas.
- Carefully cut underneath the liver near the spine to separate from connective tissue.
- Switch to the head of the mouse and have an assistant hold the perfusate line in place.
- Cradle the head between your left thumb resting on the chin of the mouse and your index finger curled underneath the shoulders. Lift gently and roll the liver back down over itself towards its natural position.
- Start cutting away the connective tissues, beginning with the thoracic cavity tissue. The liver will slide freely on the plastic cling wrap once more tissue gets cut away.
- As you cut more connective tissues away from the liver, use your left hand to slide the carcass out and away from the liver. Take care in keeping the liver and perfusion line as still as possible. Quick movements with the liver will damage it and induce ischemia.
- Transfer the liver to experimental set up. Use a small cup for transport and take care not to introduce air while moving the catheter hub from set up to set up.

Once the liver has been successfully attached to the perfusion rig, the experimental procedure mirrors the heart protocol. The perfused mouse liver is of course larger than the heart, and as such a minimum NMR tube size of ~18 mm must be used to accommodate the mouse liver in the NMR magnet. Due to its larger size, the use of a sodium free flush can be more problematic, as excessively high flow rates can cause the liver to move, making shimming extremely difficult. However, with the proper flow rate for the flush, the two step ^{23}Na shimming procedure used for the heart will produce similar results for the liver. In addition to oxygen consumption measurements, metabolic viability of the liver is also easily assessed by inspection. If the liver is turning white, it is likely not being perfused correctly and should be discarded.

5. Challenges and Future Directions

5.1. Hyperpolarized substrates in addition to [1-¹³C]pyruvate

Multiple qualities define the utility of new hyperpolarized substrates (Comment and Merritt, 2014). A primary condition is the solubility of the compound in the matrix which carries the radical used for the DNP process. If a solvent mixture is necessary for production of the combined substrate and radical, it must be thoroughly miscible, as partitioning of the sample into separate phases precludes the process of nuclear spin diffusion necessary for DNP to be effective throughout the sample (Lumata et al., 2011b). Connected to the sample preparation is a phenomenological observation that absolute enhancement of the sample must be high to allow kinetic data to be collected. A good rule of thumb is that enhancements of ~10,000 will provide an initial signal amplitude suitable for acquisition of time course data. Another primary limitation is the nuclear T₁ of the substrate and its metabolites, as discussed above. Interestingly, if metabolic flux is highly upregulated, as for glucose metabolism in cancer, even agents with short T₁s can yield important metabolic data and potentially biological insights; see the following discussion of hyperpolarized glucose as an imaging agent in cancer. It is most beneficial if the compound in question is either transported or rapidly diffuses into the same space where metabolism occurs. Finally, a multiplicity of metabolic fates will enhance the information yield of the experiment. For example, pyruvate can produce up to 4 different compounds in single enzyme-catalyzed reactions, enabling simultaneous analysis of multiple pathways, including some that are redox-dependent and some that are not.

Another important consideration is the pool sizes of the metabolite derived from the hyperpolarized imaging agent. Pyruvate again has unique qualifications, as its circulating concentration is fairly low but the size of the alanine and lactate pools are ~3 and ~10 times as large. The signal amplitude in hyperpolarization experiments is intimately connected to the pool sizes. This phenomenon is exacerbated in the case of exchange. When exogenous lactate was added to cells studied with hyperpolarized pyruvate a nearly linear increase in hyperpolarized lactate signal intensity was observed (Day et al., 2007). The kinetics of isotope transport from the small intracellular pyruvate pool into the larger lactate pool is limited only by the amount of ¹³C label in the lactate pool that is subject to the reverse reaction. In cases where hyperpolarized [1-¹³C]lactate or [1-¹³C]alanine have been used, the resulting hyperpolarized [1-¹³C]pyruvate signal has been relatively small, confirming that fast enzyme kinetics is not sufficient for the production of a large hyperpolarized signal (Chen et al., 2008; Jensen et al., 2009). A large absolute number of HP nuclear spins associated with the detected metabolite is also necessary.

The preceding discussion explains the paucity of data collected with hyperpolarized lactate or alanine, though examples do exist (Bastiaansen et al., 2014). From the standpoint of a metabolic perturbation, lactate and alanine should both have fewer consequences than a bolus injection of the highly oxidized substrate pyruvate. However, due to the pool size effect, hyperpolarized [1-¹³C]lactate produces only a small amount of [1-¹³C]pyruvate and consequently a less intense hyperpolarized [¹³C]bicarbonate signal (Chen et al., 2008). Alanine is confronted with even more difficult circumstances, as its transport does not occur

through the high capacity monocarboxylate transporters (MCT) that enable lactate and pyruvate import, but is electrogenic in nature. The attendant lactate and pyruvate signals generated by injection of hyperpolarized alanine is therefore quite small.

5.1.1 Dihydroxyacetone—Recently, [2-¹³C]dihydroxyacetone (DHA) has been demonstrated for hyperpolarized imaging of the liver (Moreno et al., 2014b). DHA has many qualities similar to pyruvate that suggest it may be successfully used as an HP imaging agent. First, it polarizes to an outstanding level in a water-glycerol matrix, exceeding even the standard sample of trityl radical solubilized directly into pyruvic acid. Second, it has a long nuclear T₁, c.a. 32 seconds at 9.4 T, which facilitates its delivery and subsequent metabolism while a large signal remains. Third, DHA produces a variety of metabolic products after its initial phosphorylation to dihydroxyacetone phosphate (DHAP). Unfortunately, DHAP has a chemical shift that is unresolvable from the parent compound. DHA metabolism has not yet been observed in organs other than the liver, indicating that an as yet unidentified transporter may be responsible for its uptake. Alternatively, other tissues may lack the ability to phosphorylate DHA to DHAP, which would prevent its subsequent metabolism.

The initial report on DHA as a contrast agent produced new insights into glycolysis, glycogenolysis, and gluconeogenesis in the liver. The traditional model of regulation in the Embden-Meyerhof pathway identifies the 3 most important regulatory nodes as phosphoenolpyruvate carboxykinase/pyruvate kinase, fructose 1,6-bisphosphatase/6-phosphofructo-1-kinase, and glucose-6-phosphatase/glucokinase. It is difficult to observe kinetics at each separate point of control in an intact system. However, due to the chemical selectivity of MR, almost all the upstream (towards glucose) and downstream (towards pyruvate) metabolites can be detected with a 2 s time resolution using hyperpolarized [2-¹³C]DHA. In glycogenolytic versus gluconeogenic conditions, only pyruvate kinase produced an observable delay in the production of its product pyruvate and subsequent metabolites, while fructose-1,6-bisphosphatase and glucose-6-phosphatase did not. Prior to the introduction of hyperpolarization methods, insights like this were nearly impossible to generate, as the only option for generating kinetic measurements with this degree of time resolution would necessitate the serial, destructive acquisition of samples at each time point.

5.1.2 Glucose—Glucose itself is also a suitable hyperpolarized imaging agent, but only when deuteration of the ¹³C enriched carbon sites has been used to increase the T₁. The first hint of the power of hyperpolarized [U-¹³C, U-²H]glucose for studying metabolism was in the work of Meier, et. al., studying yeast metabolism (Meier et al., 2011). Nearly the entire glycolytic pathway from glucose to ethanol formation was observed. In addition, ¹³CO₂ and [¹³C]bicarbonate were detected and taken as a marker of pyruvate dehydrogenase flux. This implies that more than 12 separate enzymatic fluxes can be simultaneously observed. The same substrate was subsequently used to study tumor metabolism *in vivo* where lactate was the primary product observed (Rodrigues et al., 2013). In summary, the authors believe that hyperpolarization technology offers a tremendous opportunity to study enzyme kinetics in intact systems, increasing the likelihood that the insights generated could inform on metabolic control and flux in a variety of tissues and model systems.

5.2 Specific challenges associated with using HP substrates to quantify fluxes in vivo

One of the potentially impactful deliverables of hyperpolarization research is the development of methods to extract quantitative flux data from *in vivo* experiments, ultimately in human patients. Here we discuss some of the specific challenges associated with using hyperpolarization to definitively quantify metabolic fluxes. In the case of cell culture or isolated systems, this process is dramatically simplified because delivery of the hyperpolarized substrate and its initial concentration can be rigorously controlled. Knowledge of the substrate concentration is obviously a necessary precondition for absolute flux measurements, but extraction of simple rate constants might also provide metrics sensitive to metabolic changes. Estimates of rate constants or actual fluxes *in vivo* have been obtained by many labs using disparate methods. The first method introduced below has obvious counterparts in the biochemical literature. The other studies rely upon magnetic resonance phenomena to facilitate estimates of flux and exchange.

Michaelis-Menten kinetics are typically used to describe single substrate enzyme-catalyzed reactions. Seminal work by Zierhut, et. al., measured dose response curves for hyperpolarized $[1-^{13}\text{C}]$ pyruvate in mice and rats in normal tissue and in a model of prostate cancer (Zierhut et al., 2010). Using doses ranging from 50 to 725 $\mu\text{mol/kg}$, V_{max} and K_m values were measured using the appearance of hyperpolarized $[1-^{13}\text{C}]$ alanine and $[1-^{13}\text{C}]$ lactate. Tumors were shown to have larger k values (initial rates for forward flux) for lactate production as predicted by the Warburg description of aerobic glycolysis in cancer (Warburg, 1956a). Modeling of the kinetic curves was accomplished using equations that describe only flux from pyruvate into the lactate and alanine pools. While this is an important assumption, other experiments subsequently showed that due to the finite lifetime of the hyperpolarized substrate and its metabolites, the kinetic curves are largely insensitive to exchange when simple, constant repeat time, small flip angle experimental protocols are used for data collection (Harrison et al., 2012). The straightforward experimental design of this study produced estimates of values that are readily understood by the larger research community, and as such, this paper served an important place in communicating how HP substrates could give insights into *in vivo* kinetics.

Shortly after this study, Xu, et. al., reported an alternative method for producing estimates of K_m and V_{max} that used incrementally higher excitation angles for the MR experiment (Xu et al., 2011). Hyperpolarized nuclei are not in thermal equilibrium with surrounding spins in the system being studied. As such, the hyperpolarization state is in a constant state of decay back to the thermal equilibrium value. Also, the detection pulses used for observation of the magnetic resonance signal perturb the spin state in such a way that the hyperpolarization is not recoverable. This latter phenomena allows specific insights to be generated by starting with low flip angles and incrementing them to a maximum value of 90 degrees at each time point. Using this protocol, K_m and V_{max} can be estimated from a single injection of the hyperpolarized substrate. Intuitively, this experimental design can be explained by analogy to standard dose-response experiments. Since a 90-degree pulse consumes all the polarization in the region of interest, the subsequent delay before the protocol is repeated allows new hyperpolarized material to flow in. Since the imaging experiment is queued upon injection, each time point reflects the initial delivery and kinetics of metabolite

formation as a function of [pyruvate] in the observed tissue. Due to the specifics of the modified Michaelis-Menten equations used for this study, estimates of K_m are subject to effects derived from reverse flux from either alanine or lactate back to the exogenous pyruvate pool. However, quantification of the exchange is still problematic. Paired with the fast chemical shift imaging protocol used for detection of the metabolites, this progressive excitation method could have tremendous utility in cases where the researcher anticipates large differences in K_m and V_{max} , such as in malignant tissues.

In an effort to gain better insights into the exchange phenomena between pyruvate, lactate, and alanine as measured by hyperpolarized pyruvate, alternative MR protocols have been proposed. Larson, et. al., demonstrated that a stimulated echo acquisition mode (STEAM) excitation sequence could be tuned to make quantitative estimates of both forward and reverse flux *in vivo* while at the same time producing localized spectra (Swisher et al., 2013). The underlying phenomenon that allows exchange to be monitored is the frequency difference between pyruvate and its metabolites in the MR spectrum. This difference in frequency will manifest as a phase change in the spectra when the delays of the STEAM sequence are chosen appropriately. While the method is incredibly powerful, deconvolution of the effects of exchange between multiple pools is not straightforward.

Experimentally, a more challenging protocol that is based upon more traditional methods of measuring flux by MR is the inversion transfer method proposed by Kettunen et al. (Kettunen et al., 2010). Selective inversion of either pyruvate or lactate signals produces modulation of the exchanging metabolite that can be modeled according to the modified Bloch equations. This method relies upon mathematical fitting of the exchange curves generated from the time-dependent spectra. The magnetization inversion preparation phase of the sequence can be placed in front of any imaging sequence desired and is therefore at least as flexible as the method proposed by Xu. The primary drawback of these methods is insuring the inversion pulse is properly setup and calibrated. A potentially more robust method of extracting the same kinetic information about pyruvate-lactate exchange was proposed by the same group (Kennedy et al., 2012). In this case, $[U-^{2}H]$ lactate is the hyperpolarized substrate. Due to the exchange of protons that is inherent when lactate transits lactate dehydrogenase to for pyruvate, a simple spin echo method can be used to modulate the directly detected ^{13}C MR signals. The modulation again can be modeled using the Bloch equations, yielding accurate estimates of the rate constants describing the pyruvate-lactate equilibrium.

A fundamental challenge for the field of HP applications has been establishing methods for independently verifying the kinetic data observed. Steady state isotope tracer methods for measuring metabolic flux are extremely well established, and with proper experimental design they can be used to not only confirm HP results but also, when integrated with the HP data, produce a powerful tool for assessing global energy metabolism. The combination of HP and steady state tracer methods was first used to rigorously prove that HP $[^{13}C]$ bicarbonate produced by the heart arose exclusively from pyruvate dehydrogenase (PDH) flux (Merritt et al., 2007). By monitoring the ^{13}C enrichment of glutamate extracted from the perfused heart, it was established that the addition of a competing fatty acid to the perfusate effectively quenched pyruvate oxidation and hence the production of HP

[¹³C]bicarbonate by PDH flux. Further development of this approach led to the demonstration that HP [¹³C]bicarbonate production could be quantitatively modeled in cell culture (Yang et al., 2014a). This single absolute flux measurement could then be used to normalize a complete set of relative flux measurements obtained by tracer NMR methods. The result was a global, quantitative picture of Krebs cycle turnover. The juxtaposition of oxidative versus anaplerotic handling of pyruvate illustrated just how powerful this new method could be for establishing metabolic phenotypes in cancer cells. Future work marrying hyperpolarization with other quantitative methods should significantly enhance the interpretation of *in vivo* HP data as well as produce new insights into intermediary metabolism.

6. Conclusion

Hyperpolarization has vastly expanded the applicability of MR based methods for assessing metabolism due to the $\sim 10^4$ gain in sensitivity. Combined with the traditionally understood strengths of MR, i.e., the chemical selectivity and its applicability to living, functioning systems, powerful new insights into the kinetics of metabolic processes are being routinely generated. We believe that as the technology becomes more widely disseminated, it will find an increasing number of applications in basic science as well as in medical research.

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Figure 1. Pyruvate is a substrate for several reactions that may be monitored by hyperpolarized ^{13}C

The strengths of $[1-^{13}\text{C}]$ pyruvate as a probe for hyperpolarization experiments are demonstrated in this illustration. The ^{13}C nuclei are in black, with non-enriched carbons in white. Pyruvate exchanges rapidly with the large lactate and alanine pools via the enzymes lactate dehydrogenase (LDH) and alanine aminotransferase (ALT), respectively. These exchange reactions result in the production of $[1-^{13}\text{C}]$ lactate and $[1-^{13}\text{C}]$ alanine, both of which are readily visible in hyperpolarization experiments in many systems. Depending on the tissue type, it may also be possible to observe flux through pyruvate dehydrogenase

(PDH), which releases ^{13}C as bicarbonate and produces unlabeled acetyl-CoA (Ac-CoA). Ac-CoA may then enter the TCA cycle (TCAC). Pyruvate may also be carboxylated by pyruvate carboxylase (PC), generating labeled oxaloacetate (OAA). OAA may be transaminated to aspartate, condensed with Ac-CoA to produce citrate, or equilibrate with other 4-carbon TCAC intermediates (e.g. fumarate and malate, Fum/Mal). Thus, carbon-1 of pyruvate is positioned to probe multiple aspects of aerobic and anaerobic metabolism simultaneously.

Figure 2. Schematic of a highly efficient system to perform hyperpolarized ^{13}C NMR experiments in cultured cells

The cells must be rapidly harvested for the experiment without changing their metabolism. Mixing of the cells with the hyperpolarized agent is obviously essential. The preferred method is placing a small volume of the HP solution into the bottom of the tube and subsequently injecting a large volume of cells into it to cause turbulent mixing. Injecting a small volume of HP solution into a large volume of cells does not accomplish this goal. To record the initial kinetics, mixing of the cells and the imaging agent should take place inside the magnet with the experiment already queued.

Figure 3. Schematic of a perfused organ inside an NMR tube

In this case, the perfusion rig contains two separate cannula that supply perfusate for either the heart or the liver. The presence of two cannula (1 or 2, or both) allows switching of perfusates to maintain a constant concentration of substrate when the hyperpolarized media is injected through a catheter placed inside the cannula. In the case of the heart the developed pressure can be measured using tubing placed inside the aorta. The column height of the perfusate is set to provide the correct hydrostatic pressure for a heart. In the case of

the liver flow of the perfusate is set at 8 mL/min using a peristaltic pump distal from the MR magnet.

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Table 1

List of some of the ^{13}C -enriched compounds discussed in this text and their corresponding liquid-state ^{13}C spin-lattice T_1 relaxation times at 9.4 T and ambient temperature.

^{13}C -ENRICHED COMPOUND	^{13}C T_1 (s)	REFERENCES
[1- ^{13}C]pyruvate	42	(Lumata et al., 2011d)
[2- ^{13}C]dihydroxyacetone	32	(Moreno et al., 2014a)
[U- ^{13}C , U- ^2H]glucose	10	(Rodrigues et al., 2014)
[1,4- $^{13}\text{C}_2$]fumarate	24	(Gallagher et al., 2009)
[1- ^{13}C]glutamine	25	(Jensen et al., 2009)
[1- ^{13}C]ascorbic acid	16	(Bohndiek et al., 2011)
[1- ^{13}C]alanine	29	(Jensen et al., 2009)