**In Vivo Metabolic Imaging of [1-13C]Pyruvate-d₃ Hyperpolarized By Reversible Exchange With Parahydrogen**


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**Abstract**

Metabolic magnetic resonance imaging (MRI) using hyperpolarized (HP) pyruvate has shown promise as a non-invasive technique for diagnosing, staging, and monitoring response to treatment in cancer and other diseases. The clinically established method for producing HP pyruvate is dynamic nuclear polarization; however, it is rather expensive and slow. Here, we
demonstrate fast (6 min), low-cost production of HP \([1^{13}\text{C}]\text{pyruvate-d}_3\) in aqueous solution using Signal Amplification By Reversible Exchange (SABRE), and in vivo metabolic MRI. The injected solution was sterile, non-toxic, pH neutral and contained \(\approx 30\) mM \([1^{13}\text{C}]\text{pyruvate-d}_3\) polarized to \(\approx 11\%\) (residual 250 mM methanol and 20 \(\mu\)M catalyst). It was obtained by rapid solvent evaporation and metal filtering. The procedure was well tolerated by all four mice studied here. This achievement is a significant step of making HP MRI available to a wider community. Fast, low-cost, and high-throughput parahydrogen-hyperpolarization has become a viable alternative for metabolic MRI of living organisms.

**Introduction**

Non-invasive imaging techniques such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are widely used in oncology for characterizing tumors before treatment, therapy planning, monitoring tumor progression, evaluating treatment response, and follow-up. Most clinical guidelines rely on morphological criteria such as size and location of the tumor, e.g. RECIST criteria.\(^1\) However, many effective therapies, including promising immunotherapies, do not manifest early morphological changes such as tumor size reduction, even if the patient responds well to treatment.\(^2\) The current advances in molecular tumor biology, genetics, and imaging technology have led to a major paradigm shift in oncology to utilize molecular imaging as a superior diagnostic and prognostic marker of cancer staging, response to treatment and survival. \([^{18}\text{F}]\text{Fluorodeoxyglucose (FDG) Positron Emission Tomography (FDG PET)}\) is the most widely clinically applied molecular imaging technique that reports on aberrant energy metabolism, which is a hallmark of many cancers. Despite limitations (including long examination times >1h and the use of ionizing radiation), this radioactive tracer has revolutionized treatment and management of many cancers. Its success relies on the FDG uptake, which is elevated in proliferating tumors. However, PET is not able to distinguish between metabolic products. Non-radioactive molecular contrast agents providing deeper insights in aberrant metabolism with faster scan time are needed. Hyperpolarized (HP) \([1^{13}\text{C}]\text{pyruvate MRI}\) has shown great promise
for non-invasive, non-ionizing molecular imaging, e.g. for tumor grading or therapy response assessment.\[^{3-8}\] Such molecular MRI scan takes about one minute and employs no ionizing radiation. As a result, it has been embraced as a next-generation molecular imaging modality. Similarly to FDG, pyruvate is a key metabolite at the cross-section of cellular energy pathways. The high acquisition speed of HP pyruvate MRI builds on the ability to visualize (and distinguish) the conversion of metabolites into downstream products \textit{in vivo}, which mitigates the requirement for contrast agent clearance from the surrounding tissues. Since the first demonstration in human 2011, HP \[^{1-13}\text{C}]\text{pyruvate} MRI has made a remarkable progress, and this technique is currently being investigated in over 30 clinical trials according to clinicaltrials.gov. Moreover, HP \[^{125}\text{Xe}\] gas has been recently FDA approved for ventilation lung imaging. Following this landmark regulatory decision, it is anticipated that HP \[^{1-13}\text{C}]\text{pyruvate} may also soon be approved for the use in oncology.

The success of HP \[^{1-13}\text{C}]\text{pyruvate} MRI was enabled by dissolution Dynamic Nuclear Polarization (dDNP), which is the leading technique for producing HP pyruvate.\[^{9-11}\] However, clinical-scale dDNP instrumentation is expensive (>\$2M) and requires about one hour to produce a bolus of HP \[^{1-13}\text{C}]\text{pyruvate}. Therefore, faster, more cost-effective, and simpler technologies are needed to improve access of the biomedical community to this promising molecular contrast agent.

Parahydrogen-induced polarization (PHIP) techniques have emerged as promising candidates to address this need for efficient polarization methods.\[^{12-25}\] PHIP utilizes the nuclear spin singlet isomer of molecular hydrogen, parahydrogen (pH\(_2\)), which acts as a cost-efficient source of hyperpolarization. In 2009, a "non-hydrogenative" PHIP approach called Signal Amplification By Reversible Exchange (SABRE) has been pioneered by Duckett and co-workers.\[^{26}\] SABRE is based on the reversible, simultaneous chemical exchange of pH\(_2\) and the target to-be-hyperpolarized molecule to an iridium-based exchange complex, such that hyperpolarization derived from pH\(_2\) is transferred to a substrate nucleus.\[^{27-35}\] In 2019, Duckett \textit{et al.} demonstrated the production of HP \[^{1-13}\text{C}]\text{pyruvate-h}_3\) via SABRE by doping the catalyst with a dimethyl sulfoxide (DMSO) co-ligand to improve the binding properties of pyruvate acting as a bidentate ligand.\[^{36}\] This breakthrough has garnered significant interest in
utilizing SABRE HP pyruvate for metabolic imaging studies.\textsuperscript{[37–40]} However, the SABRE technique typically employs organic solvents such as methanol for the hyperpolarization process, rendering the produced HP [1-\textsuperscript{13}C]pyruvate too toxic for \textit{in vivo} administration. Moreover, as SABRE hyperpolarization requires iridium-based catalysts, removal of residual Ir (below 1 ppm or less) in the injectable solution is required for bio-compatible formulations.\textsuperscript{[40–42]} While efforts have been made to eliminate toxic solvent and Ir in SABRE hyperpolarization,\textsuperscript{[40]} no peer-reviewed \textit{in vivo} studies have been reported to date.

In the present paper, we address the SABRE translational challenge and demonstrate, for the first time, metabolic imaging with SABRE in healthy mice, particularly using SABRE-polarized pyruvate. These results were enabled by spin-lock induced crossing (SLIC) SABRE\textsuperscript{[43–47]} at microtesla fields,\textsuperscript{[48,49]} which allowed us to achieve up to \textasciitilde16\% \textsuperscript{13}C polarization of [1-\textsuperscript{13}C]pyruvate-d\textsubscript{3}. We achieved rapid purification by solvent evaporation and metal filtration, reducing methanol and catalyst below acute toxic levels. Tail-vein administration of the agent and chemical shift imaging of pyruvate metabolic conversion enabled metabolic mapping of downstream HP lactate and alanine, demonstrating the translational promise of this technique.

\textbf{Results}

\textbf{Efficient SABRE hyperpolarization:} We obtained mean \textsuperscript{13}C polarizations for [1-\textsuperscript{13}C]pyruvate-d\textsubscript{3} of 13.1\pm3.2\% (N=5) with a maximum of 16.3\% at the time of detection (5-8 s after production) in methanol-d\textsubscript{4} using the setup and protocol described in the Methods section (Fig. 1a and Fig. 1b). These values correspond to a signal enhancement of up to \textasciitilde190,000-fold compared to the polarization at thermal equilibrium at 1T, which is only 0.000086\%. The high polarization levels were made possible by the long relaxation times during the SLIC-SABRE polarization process of $T_{1\rho} = 113.3 \pm 0.3$ s (Fig. 1c), which allowed for effective polarization build up over a 5-minute period (see SI Fig. S5).\textsuperscript{[48]}

\textbf{Fast purification:} Encouraged by these high polarization levels, and inspired by a recent study by Ding \textit{et al.}\textsuperscript{[17]}, we investigated the removal of methanol from the samples by rapid solvent evaporation.
As methanol and water do not form an azeotrope and hence evaporate at separate boiling points, we added phosphate-buffered D$_2$O to the HP SABRE sample in an NMR tube and placed the mixture in a hot water bath while applying vacuum (≈1 mbar). Strikingly, we found that evacuating the sample for 15 seconds at 98°C reduced methanol from 24.75 M to 250 mM while retaining 30 mM of the pyruvate in the resulting ≈150 µL aqueous solution (Fig. 1d and Figs. S6 and S10). Here, we found that extended evacuation, although it seemed to increase the pyruvate concentration (SI Fig. S11), was not a viable option due to the loss of water and limited yield of resulting solution.

To reduce polarization losses during the purification procedure, which consisted of 15 seconds of vacuum and approximately 40 s of sample handling (including filtering the solution through a 1.2 µm microporous syringe filter), the whole procedure was conducted at an elevated magnetic field of ≈450 mT. Additionally, we retained the pyruvate in phosphate-buffered D$_2$O instead of phosphate-buffered H$_2$O, which features shorter lifetimes throughout the investigated fields (Fig. 1c). Using this approach, we reproducibly obtained 60 ± 7 % of the initial $^{13}$C polarization ($N = 5$), and the final ≈30 mM pyruvate in the extracted samples were polarized up to ≈11% (Fig. 1b). Note that producing freshly purified HP [1-$^{13}$C]pyruvate-d$_3$ with our protocol took only ≈ 6 min per dose.
Figure 1: SABRE hyperpolarization and purification of [1-13C]pyruvate-d3. 

a. 13C NMR spectra of [1-13C]pyruvate-d3 in methanol-d4 before (orange) and in pH-buffered D2O after purification (blue) compared with an external 13C-enriched reference solution (black) at 1 T. 

b. Reproducibility of hyperpolarization and purification investigated in 5 independent samples.

c. Longitudinal relaxation times $T_1$ and $T_1p$ in presence of the SLIC pulse at different fields in methanol-d4 (orange), phosphate-buffered D2O (blue), and phosphate-buffered H2O (light blue). *These data were acquired in thermally polarized samples, see SI.

d. Remaining concentrations of methanol and pyruvate after applying vacuum and a 98°C water bath for different amount of time to a 600 µL sample of SABRE solution (50 mM sodium pyruvate-h3 in 24.75 M methanol-OH) mixed with 600 µL phosphate-buffered D2O (see SI Fig. S6 and S10).
**Aqueous sample analysis:** After reducing the methanol content to 250 mM, the catalyst visibly precipitated from the solution. As an additional measure, the solution was passed through a 1.2 µm filter, effectively removing the precipitated catalyst and resulting in a clear solution (SI Fig. S8). Later in the *in vivo* experiments, we found these two visual inspections of the sample, one before and one after filtering, helpful to determine whether methanol and catalyst were successfully removed.

We used inductively coupled plasma optical emission spectroscopy (ICP-OES) to quantify the remaining iridium content in the samples, which showed successful reduction from 6 mM to 20 µM, i.e. to <0.5 ppm after purification. Directly before administration, we measured the temperature of the solutions to be 35±2°C.

We tested the purified solutions to evaluate if they are suitable for *in vivo* applications. Our analysis indicated that the resulting aqueous solutions were sterile (see SI Fig. S12) and had neutral pH and osmolarity (see SI Fig. S9). Cytotoxicity assays showed no adverse effects on cell viability when exposed to the purified SABRE pyruvate solutions, particularly at concentrations reached in the bloodstream after *in vivo* injection and even at a twofold higher concentration. The toxicity observed at a 10 times higher concentration was likely caused by the residual catalyst and high concentration of pyruvate (see SI Fig. S13).

**In vivo metabolic imaging:** To demonstrate that our purified SLIC-SABRE [1-13C]pyruvate-d3 is suitable for *in vivo* metabolic imaging, we performed first experiments on healthy mice using a dedicated mouse quadrature resonator and a 7 T preclinical MRI system (Bruker, Ettlingen, Germany). To obtain high 13C signal *in vivo*, we decided to use an HP sample only if polarization prior purification was ≥15% and if the purified sample volume was ≈150 µL (production time ≈6 min per dose). 15 s after starting the bolus injection at a dose of up to 5 µL/g of an HP pyruvate solution through the tail vein, we performed chemical shift imaging (CSI) from a 3-mm axial slice covering the kidneys and parts of the liver and gut (Fig. 2). Strong HP 13C signal was observed, 13C spectra and metabolic maps were
The $^{13}$C signal of pyruvate was found mainly in the aorta and vena cava. While in the kidney mostly lactate was observed, the liver and gut contained considerable amounts of alanine.

Figure 2: In vivo $^{13}$C chemical shift imaging of SABRE-hyperpolarized [1-$^{13}$C]pyruvate-$d_3$ acquired starting 15 s after beginning the HP bolus injection. a, regions of interests with anatomical $^1$H MRI for reference selecting aorta and vena cava (top), the gut and liver (middle), and the kidneys (bottom) and, b, corresponding summated $^{13}$C-NMR spectra from these regions with metabolite $^{13}$C signals from pyruvate (blue), alanine (green), and lactate (red). c, 2D metabolite maps of lactate, alanine, and pyruvate (same colors as in b) superimposed with an anatomical $^1$H MRI of the same axial slice ($^{13}$C CSI recorded: 3 mm slice thickness, 20 x 14 matrix, 1.5 x 1.5 mm$^2$ in-plane resolution). The unprocessed $^{13}$C MRI data are presented in the SI.

In a second experiment, we tested metabolic imaging with higher spatial and temporal resolution using a fast, metabolite-selective 3D $^{13}$C bSSFP sequence. Here, we focused on the pyruvate-to-lactate conversion and started the $^{13}$C MRI acquisition approximately 5 s prior to the start of the injection of HP [1-$^{13}$C]pyruvate-$d_3$ (Fig. 3). This setting enabled us to monitor the conversion kinetics.
clearly showing the arrival of the HP pyruvate bolus and the subsequent formation of lactate. From the 3D time-resolved data we reconstructed images depicting pyruvate that was observed mostly in the heart, aorta and vena cava, and lactate, that was formed predominantly in the kidneys.

![Figure 3: 3D dynamic in vivo $^{13}$C metabolic imaging of pyruvate-to-lactate conversion.](image)

Figure 3: 3D dynamic in vivo $^{13}$C metabolic imaging of pyruvate-to-lactate conversion. a, coronal (top) and axial view (bottom) of anatomical $^{1}$H MRI (left), superimposed with time-summated $^{13}$C-pyruvate (center) and $^{13}$C-lactate signal (right). b, metabolite conversion kinetics of pyruvate and lactate located to aorta and vena cava (left) and the kidneys (right). $^{13}$C MRI acquired: frequency-selective bSSFP sequence$^{[50]}$, 21 x 11 x 8 matrix, 2.5 x 2.5 x 2.5 mm$^3$ resolution, temporal resolution of $\approx 0.85$ Hz). The measurement was started before beginning the injection. The unprocessed data is presented in the SI.

In total, we performed metabolic MRI in 4 mice and observed no adverse effects to the injection for the remainder of the MRI investigation ($\approx$20-30min). During the imaging experiment, we observed only a temporary increase in breathing rate following the injection (see SI Fig. S18). After approximately 1-2 min, the breathing rate returned to pre-injection values and remained stable throughout the
experiment. This bolus-induced temporary increase in breathing rate is a commonly observed side effect to the increased volume entering the right ventricle and lung circulation. The mice were sacrificed at the end of the imaging experiment.

Overall, the in vivo experiment confirmed that SABRE-polarized pyruvate is well suited for metabolic imaging and demonstrated its power for monitoring metabolic processes in vivo.

Discussion

It is noteworthy that the metabolic conversion kinetics of pyruvate is not affected by the presence of deuterium isotopes, as evidenced by previous studies.\(^\text{51}\) Therefore, for metabolic HP MRI, pyruvate-\(d_3\) can be utilized successfully in place of non-deuterated pyruvate. The isotope-enriched \([1-^{13}\text{C}]\text{pyruvate-}\,d_3\) is available commercially and requires no further chemical modification for SABRE hyperpolarization. A commercially available agent is a significant advantage compared to "hydrogenative" \(\text{pH}_2\)-based hyperpolarization techniques that necessitate suitable unsaturated precursors for the permanent addition of \(\text{pH}_2\).\(^\text{13,17–20}\) Furthermore, SABRE offers some notable advantages over current dDNP methods, such as faster production and lower costs. Currently, the methods allow us to polarize a dose in less than 10 min, or several doses during one imaging session (i.e. one anesthesia). Undoubtedly, this rate can be increased significantly by further optimizing the process – not a simple task, but worthwhile now that the proof-of-principle has been established in this study.

The purification scheme proposed here is fast and easy to install, reducing both methanol and catalyst below acute toxic levels. Hence, we foresee this technique being available to many researchers. While a human trial would require more sophisticated cleaning and quality assurance methods, these results clearly show that obtaining clean solutions is feasible and can be optimized further by engineering. In future, a faster and more complete evaporation of methanol may be accomplished through optimizing vacuum and temperature, using larger evaporation chambers, and increasing the surface area between the liquid and gas phases.
It is important to consider the current levels of residual methanol and catalyst in the purified samples. Our current purification scheme results in a methanol concentration of approximately 12.5 mM or 400 mg/L in the bloodstream of injected mice, equivalent to 40 mg/kg of methanol per mouse weight. Already now, this concentration is well below the reported oral LD50 (5.628 g/kg) and LD00 (143 mg/kg) values for rodents,\cite{52} and close to the threshold beyond which humans get treated after methanol poisoning (300 mg/L blood).\cite{53} With regard to the SABRE catalyst, we were unable to identify toxicity studies in the literature. However, our tests have shown toxicity to cells only at concentrations higher than the final concentration found in the bloodstream. We therefore conclude that the concentrations of methanol and catalyst are low enough for first preclinical applications, although we will continue to work towards reducing them in future studies.

In our pursuit to enhance the signal-to-noise ratio for metabolic in vivo studies further, we aim to achieve higher polarization levels and pyruvate concentrations through our SABRE approach in future. Our current work is dedicated to reducing the polarization losses during purification by reducing the sample-handling time. However, the signal-to-noise ratio achieved in this study was already high enough to monitor pyruvate metabolism in vivo in mice using $^{13}$C MRI.

In conclusion, our study demonstrates that non-invasive in vivo metabolic imaging is feasible with SABRE hyperpolarized pyruvate, produced in aqueous solution. The entailed bioburden appears to be low, whereas the production process is much faster (<10 min) and more cost efficient than a dDNP experiment. Further optimization of the method is expected to lead to cleaner, more-concentrated, and higher-polarized samples.

The efficient hyperpolarization of pyruvate and its translation to preclinical imaging have been a major focus of SABRE research since its discovery in 2009. In this study, we successfully hyperpolarized $[1^{-13}\text{C}]$pyruvate-$d_3$ using SLIC-SABRE at microtesla field,\cite{48} and the long-lived polarization allowed us to extract highly-hyperpolarized $[1^{-13}\text{C}]$pyruvate-$d_3$ in a purified aqueous solutions. This approach
enabled us to conduct the first \textit{in vivo} metabolic MRI with SABRE hyperpolarized pyruvate. This is a promising accomplishment, given that pyruvate is the most widely applied HP MRI agent.

Our results demonstrate that SABRE is now a viable option for preclinical metabolic pyruvate MRI, with a sufficiently high signal-to-noise ratio to monitor pyruvate metabolism in mice accurately. We envision this technique to be widely adopted by researchers, paving the way for improved metabolic imaging and a better understanding of metabolic changes in cancer and other pathologies.

\textbf{Methods}

\textbf{Setup:} To conduct the SABRE reaction (Fig. 4a) at the ideal physical conditions for SLIC\cite{48}, we developed a dedicated hyperpolarizer. The device consisted of a resistive solenoid coil to generate a static magnetic field $B_0$\cite{54} a saddle-shaped coil to play out the radiofrequency SLIC pulse on the $^{13}$C Larmor frequency, a three-layered mu-metal to shield external fields, and an NMR-tube based bubbling system to supply pressurized pH$_2$ to the SABRE samples (Fig. 4b). The homogeneity of $B_0$ and $B_1$ of our setup was ±1 % over the NMR tube sample region as shown in SI Fig. S2. The SLIC pulse was generated in a custom-written Python software controlling a digital-analog converter driving a 12-Watt audio amplifier module. Both $B_0$ and $B_1$ amplitudes were adjusted using a field probe. A more detailed description of the setup and the used materials is presented in the SI.

\textbf{Hyperpolarization:} The NMR tubes were filled with 600 \(\mu\)L of the SABRE solution containing 6 mM catalyst, 40 mM DMSO, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM [1-$^{13}$C]pyruvate-d$_3$ in methanol-d$_4$ and centered in the hyperpolarizer and in a \(\approx\)7 °C water bath (SI Fig. S4) using a custom-build holder. The preparation and chemicals used for the SABRE samples are described in detail in the SI. $B_0$ was set to 50 \(\mu\)T and the SLIC pulse amplitude $B_1$ was set to 2 \(\mu\)T amplitude, adjusted to the $^1$H-$^1$H indirect dipolar coupling of catalyst-bound pH$_2$ (10.4 Hz \cite{55}). The $^{13}$C Larmor frequency at 50 \(\mu\)T was calculated to 535.25 Hz. At the end of the SLIC pulse we added an adiabatic pulse to rotate the hyperpolarization along $B_0$ (Fig. 4c). Samples were hyperpolarized for 5 min by playing out the SLIC pulse while simultaneously guiding pH$_2$ with 90% enrichment through the sample at \(\approx\)8 bar (SI Fig. S1).
**Purification:** After the polarization, we added 600 µL phosphate-buffered D$_2$O (see SI) to the HP sample then placed it in a hot water bath and 450 mT magnetic field while applying vacuum from the top of the NMR tube. Subsequently, the solution was extracted through a 1.2 µm syringe filter (Puradisc 13, Whatman, UK) using a 1 mL syringe combined with a catheter (Fig. 4d).

**In vivo experiments:** The MRI setup, protocols and raw data are reported in the SI. The experimental procedures followed internationally accepted recommendations and guidelines for the handling of laboratory animals. The local animal ethics committee approved the study (Regierungspraesidium Freiburg, Talstr. 4-8, 79095 Freiburg; AZ: 35-9185.81/G-19/162). C57BL/6N mice with no genetic modifications weighing approximately 24 g were used for the study. Anesthesia was induced using sevoflurane (2-4% in >99.5% O$_2$, ~1.0 L·min$^{-1}$, during spontaneous breathing) and the animal's vital signs were continuously monitored (SA Instruments 1030, Stony Brook, NY 11790). To maintain a respiration rate of about 70 min$^{-1}$, the anesthesia depth was adjusted as needed. Respiration was monitored using a pressure-sensitive cushion. The animal's body temperature was monitored using a rectal thermometer and stabilized using a custom-made water circulation system driven by a water pump. All necessary measures were taken to minimize the animal's suffering, and it was humanely euthanized at the end of the experiment.
Figure 4: Process of SLIC-SABRE hyperpolarization and purification of [1-13C]pyruvate-d3 samples. a, schematic representation of the SABRE hyperpolarization reaction involving the binding of pH2 and [1-13C]pyruvate-d3 to the SABRE catalyst for polarization transfer to pyruvate. b, experimental setup for the SLIC-SABRE method, which includes the static magnetic field B0, radiofrequency field B1, NMR tube bubbling system to introduce pH2 to pyruvate in methanol-d4, and a mu-metal to shield external fields. c, during SLIC-SABRE, B0 was set constant at 50 µT, and the B1 amplitude was set to 2 µT. After the SLIC pulse, an adiabatic pulse was applied, rapidly increasing and subsequently ramping down the B1 amplitude while detuning the resonance frequency by +50 Hz to rotate the hyperpolarization along B0. d, stepwise purification and application protocol consisting of buffered-D2O addition, solvent evaporation under vacuum in a hot water bath, solution filtering, and in vivo administration.

Data availability

Data generated during this study are available from the corresponding authors upon reasonable request.
References:


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Competing interests

SK is an employee of NVision Imaging Technologies GmbH. EYC holds a stake of ownership in XeUS Technologies Ltd. and serves on the scientific advisory board of Visma Life Sciences LLC. FS serves on the scientific advisory board of NVision Imaging Technologies GmbH. The other authors declare no competing interests.