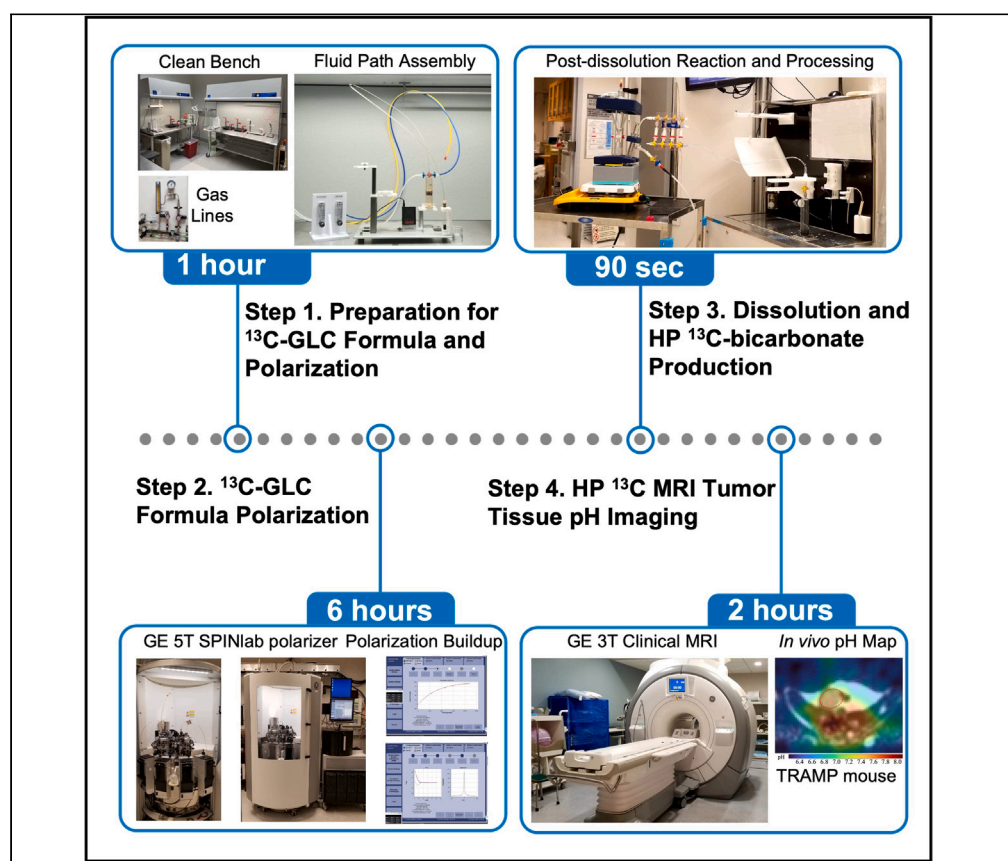


Protocol

Protocol for producing hyperpolarized ^{13}C -bicarbonate for clinical MRI of extracellular pH in aggressive tumors



Changhua Mu,
Xiaoxi Liu, Andrew
Riselli, ..., John
Kurhanewicz, David
M. Wilson, Robert R.
Flavell

changhua.mu@ucsf.edu

Highlights

Instructions for
preparation and
hyperpolarization of
 ^{13}C -GLC

Production of
hyperpolarized ^{13}C -
bicarbonate for
patient injection

Hyperpolarized ^{13}C
MRI data acquisition
and generation of pH
imaging maps

Tumor acidosis is one of the hallmarks indicating the initiation and progression of various cancers. Here, we present a protocol for preparing a hyperpolarized (HP) ^{13}C -bicarbonate tissue pH MRI imaging contrast agent to detect aggressive tumors. We describe the steps for the formulation and polarization of a precursor molecule ^{13}C -glycerol carbonate (^{13}C -GLC), the post-dissolution reaction, and converting HP ^{13}C -GLC to an injectable HP ^{13}C -bicarbonate solution. We then detail procedures for MRI data acquisition to generate tumor pH maps for assessing tumor aggressiveness.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Mu et al., STAR Protocols 5,
103091

September 20, 2024 © 2024

The Authors. Published by
Elsevier Inc.

<https://doi.org/10.1016/j.xpro.2024.103091>



Protocol

Protocol for producing hyperpolarized ^{13}C -bicarbonate for clinical MRI of extracellular pH in aggressive tumors

Changhua Mu,^{1,6,7,*} Xiaoxi Liu,¹ Andrew Riselli,^{1,5} James Slater,¹ Evelyn Escobar,¹ Duy Dang,¹ Scott Drapeau,² Romelyn Delos Santos,¹ Stacy Andosca,¹ Hao Nguyen,³ Peder E.Z. Larson,¹ Robert Bok,¹ Daniel B. Vigneron,¹ John Kurhanewicz,¹ David M. Wilson,¹ and Robert R. Flavell^{1,4}

¹Department of Radiology and Biomedical Imaging, University of California, San Francisco, San Francisco, CA 94158, USA

²Makers Lab, Library, University of California, San Francisco, San Francisco, CA 94143, USA

³Department of Urology, University of California, San Francisco, San Francisco, CA 94143, USA

⁴Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94158, USA

⁵Present address: Department of Bioengineering and Therapeutic Science, University of California, San Francisco, San Francisco, CA 94158, USA

⁶Technical contact

⁷Lead contact

*Correspondence: changhua.mu@ucsf.edu
<https://doi.org/10.1016/j.xpro.2024.103091>

SUMMARY

Tumor acidosis is one of the hallmarks indicating the initiation and progression of various cancers. Here, we present a protocol for preparing a hyperpolarized (HP) ^{13}C -bicarbonate tissue pH MRI imaging contrast agent to detect aggressive tumors. We describe the steps for the formulation and polarization of a precursor molecule ^{13}C -glycerol carbonate (^{13}C -GLC), the post-dissolution reaction, and converting HP ^{13}C -GLC to an injectable HP ^{13}C -bicarbonate solution. We then detail procedures for MRI data acquisition to generate tumor pH maps for assessing tumor aggressiveness. For complete details on the use and execution of this protocol, please refer to Mu et al.¹

BEFORE YOU BEGIN

Solid tumors typically develop an acidic interstitial microenvironment with pH 6.5–7.2, in contrast to pH 7.4 in normal tissues² due to heterogeneous perfusion, high metabolic activity, and rapid cell proliferation.^{3,4} Thus, the ability to non-invasively image tissue pH may facilitate detecting aggressive and potentially metastatic lesions, stratifying patients, and monitoring therapy responses. Despite a long-standing focus on measuring tissue pH, existing methods have not gained widespread clinical use due to inherent limitations.^{5–8}

We developed a tumor pH imaging method using dissolution dynamic nuclear polarization (DNP) to produce a highly polarized and human injectable ^{13}C -bicarbonate formula. The agent enables ^{13}C magnetic resonance imaging (MRI) to image the tumor's extracellular pH. The method involves polarizing a non-toxic precursor molecule, ^{13}C -glycerol carbonate (^{13}C -GLC), breaking it down to HP ^{13}C -carbonate via a base-catalyzed hydrolysis reaction, and converting HP ^{13}C -carbonate to HP ^{13}C -bicarbonate by neutralization.^{1,9} Additional solution processing steps include removing trityl radical AH111501 sodium (AH111501) and sterilization before injection. Following injection, HP ^{13}C -bicarbonate equilibrates with HP $^{13}\text{CO}_2$ in the tumor, enabling the ^{13}C MRI data acquisition of both HP ^{13}C -bicarbonate and HP $^{13}\text{CO}_2$ signals through our specifically designed ^{13}C multi-metabolite specific 2D gradient echo (GRE) sequence. Finally, we generate the tumor pH maps according to the modified Henderson–Hasselbalch equation.^{9–12}



We established this protocol following Good Manufacturing Practices (GMP) guidelines for producing positron emission tomography agents. Subsequently, we received approval for the Investigational New Drug application from the US Food and Drug Administration (FDA) to evaluate this method in clinical trials. It consolidates our designs, approaches, findings, and methodologies for achieving a high degree of polarization of the ^{13}C -bicarbonate formula suitable for human studies. The protocol includes three major procedures: formulation and polarization of ^{13}C -GLC sample, post-dissolution reaction and processing, and MRI image acquisition and reconstruction, with quality control mechanisms incorporated in each procedure.

Completing all the protocol steps on the same day of patient study is essential for preventing contamination. Therefore, the necessary preparatory steps, such as purchasing materials and supplies, inspecting workstations, setting up the instrument, customizing the apparatus, and sterilizing glassware and connectors, are imperative beforehand.

Institutional permissions

We produced polarized HP ^{13}C -bicarbonate MRI contrast following the GMP guidelines outlined in the U.S. Code of Federal Regulation Title 21, Part 212. We have received approval for the Investigational New Drug (IND) application from the U.S. Food and Drug Administration (FDA, IND# 165528) and subsequent Institutional Review Boards approval (IRB# 23–38344) to conduct our human clinical trials (NCT05851365).

We also conducted pre-clinical animal studies in compliance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

Configuration of the GE 5T SPINLab clinical polarizer for ^{13}C -GLC polarization

⌚ Timing: 7 h

Note: To mitigate inadvertent errors, such as those related to the pipetting technique, and to facilitate accurate recording and backtracking, we use an analytical balance to measure experiment quantities. We allow an uncertainty within $\pm 3\%$ from the specified weights.

1. Prepare ^{13}C -GLC polarization formula.
 - a. Add 936 mg of ^{13}C -GLC and 16 mg of AH111501 to a 3-mL brown glass vial.
 - b. Mix the resulting mixture with a vortex at 2000 RPM for 5 min to yield a homogeneous 15 mM AH111501 in ^{13}C -GLC. Wrap the vial with aluminum foil to protect it from light exposure.
2. Determine microwave frequency for ^{13}C -GLC polarization.
 - a. Load 793.3 mg of the ^{13}C -GLC/AH111501 polarization formula to the cryovial and 41.8 g of deuterium oxide (D_2O) to the syringe of a GE research fluid path.
 - b. Assemble, prepare, and load the research fluid path to the SPINLab polarizer per GE manual.
 - c. Perform microwave frequency sweep using the recommended parameters in [Figure 1A](#).
 - d. Expect to observe a microwave sweep diagram in [Figure 1B](#). The first peak signifies the microwave frequency required for polarizing ^{13}C -GLC (e.g., 139.87 GHz, adjusting parameters if needed for optimization).
3. Build up polarization and execute dissolution.
 - a. Build up the polarization on ^{13}C -GLC using the specified polarization frequency and other parameters in [Figure 1C](#) for approximately 5 h until the polarization time is about 3 times the time constant as shown in [Figure 1D](#).
 - b. While the polarization is building up, pre-tune, match, and shim a 1.4 T NMR spectrometer on ^{13}C NMR channel per the manufacturer manual and apply the following recommended acquisition parameters: individual scan number = 128, repetition time = 5 s, flip angle = 10° , bandwidth = 129 ppm, center frequency = 160 ppm, and ^1H decouple for the hyperpolarized ^{13}C NMR data acquisitions.

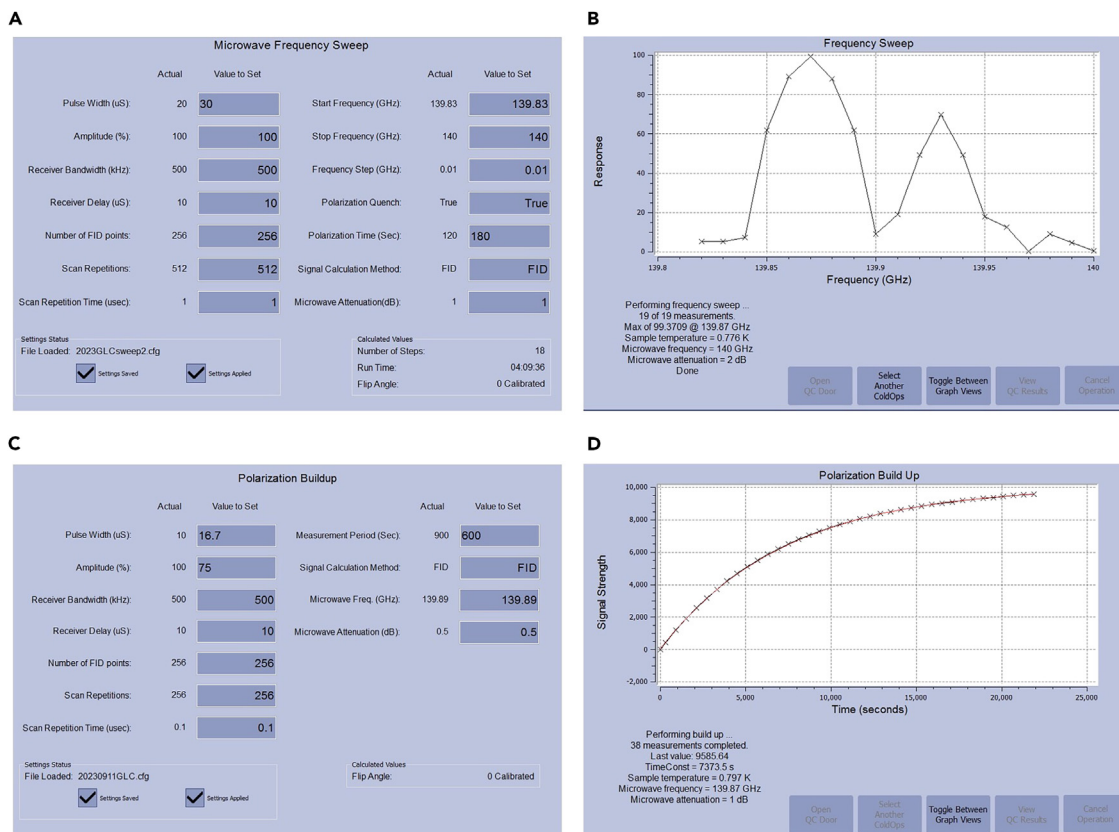


Figure 1. Configuration of the GE 5T SPINLab polarizer for ^{13}C -GLC polarization

(A) Parameters for the microwave frequency sweep.

(B) Diagram illustrating the microwave frequency sweep and the determined frequency on the first peak.

(C) Parameters for building up the solid-state polarization of ^{13}C -GLC.

(D) Diagram illustrating the buildup of solid-state polarization of ^{13}C -GLC.

- c. Upon the buildup of solid-state polarization of ^{13}C -GLC, swiftly execute the following actions within 30–40 s.
 - i. Dissolve HP ^{13}C -GLC into a 250-mL flask and start a stopwatch simultaneously.
 - ii. Transfer the solution into an NMR tube.
 - iii. Load the tube into the pre-set 1.4 T NMR spectrometer.
 - iv. Initiate ^{13}C NMR data acquisition and stop the stopwatch.
 - v. Record the transfer time (t) as the duration from the initiation of dissolution to the start of ^{13}C NMR data acquisition.
- d. Following the completion of HP ^{13}C NMR data acquisition, add 0.1% (volume) of Gd^{3+} (Magnevist-brand of gadopentetate dimeglumine) to the ^{13}C -GLC D_2O solution in the NMR tube and ensure thorough mixing.
- e. Perform the thermal equilibrium ^{13}C NMR data acquisition with the recommended acquisition parameters: averaged number of scans = 1024, repetition time = 10 s, flip angle = 90° , while keeping other parameters consistent with Step b.
4. Analyze ^{13}C -GLC polarization properties.
 - a. Determine the relaxation constant (T_1): Measure the hyperpolarized ^{13}C -GLC signal intensity (S_{measured}) as absolute integral (same as below) in the ^{13}C NMR spectra at each time point. Correct for magnetization loss depleted by radiofrequency excitation.

$$S_{corrected}(t) = \frac{S_{measured}(t)}{(\cos(\text{flip angle}))^n}$$

where n is the number of times the data acquisition repeats ($n = \text{Number of Time Points} - 1$). Plot the corrected signal intensity ($S_{corrected}$) against time and fit the data with the T_1 relaxation equation.

$$S(t) = S_0 e^{-\left(\frac{1}{T_1}\right)t}$$

where $S(t)$ is the signal intensity at time t , and S_0 is the initial signal intensity (at $t = 0$).

- b. Determine the present ^{13}C -GLC polarization level.
 - i. Measure the hyperpolarized ^{13}C -GLC signal intensities in both the first observed hyperpolarized and the averaged thermal equilibrium (ThE) ^{13}C NMR spectra.
 - ii. Incorporate the following equation to calculate the present polarization level of ^{13}C -GLC. Percentage Polarization Level (%Pol) = Enhancement Factor \times Polarization at ThE. where Enhance Factor = HP Signal Intensity/(ThE Signal Intensity/ $\sin(\alpha)$ /1024), where α is excitation flip angle, $\sin(\alpha)$ corrects for 90° full excitation, 1024 is the number of scans.

$$\text{Polarization at ThE} = \frac{n_+ - n_-}{n_+ + n_-} = \tanh\left(\frac{h\gamma B_0}{2K_B T}\right)$$

where h is the Planck constant (6.626×10^{-34} J/Hz), γ is the gyromagnetic ratio of carbon-13 (10.705×10^6 Hz/T), B_0 is the magnetic field in tesla (T), K_B is the Boltzmann constant (1.38×10^{-23} Hz/T), and T is the temperature in Kelvin (K).

- c. Determine the initial ^{13}C -GLC polarization level (%Pol₀) by incorporating the T_1 value, the present ^{13}C -GLC polarization level (%Pol_t), and the transfer time (t) to back-calculate the initial ^{13}C -GLC polarization at the time of dissolution. [Troubleshooting 1](#).

$$\%Pol_0 = \%Pol_t e^{\frac{t}{T_1}}$$

△ CRITICAL: The GE 5T clinical SPINLab polarizer requires regular calibration (such as monthly) or immediately after any service, maintenance, or adjustment to ensure a high level of polarization.

Note: The initial and present ^{13}C -GLC polarization levels obtained here establish baselines for the achievable polarization of ^{13}C -GLC and facilitate assessing the level of polarization loss in the final HP ^{13}C -bicarbonate solution after the subsequent post-dissolution reaction and processing steps.

Customization of the post-dissolution reaction and processing apparatus

⌚ **Timing:** 3 days

Note: To transform HP ^{13}C -GLC into a human-injectable HP ^{13}C -bicarbonate formula while maintaining polarization and ensuring safety, we must perform four crucial processing steps, including the breakdown of HP ^{13}C -GLC to HP ^{13}C -carbonate, conversion to HP ^{13}C -bicarbonate by neutralization, removal of AH111501, and sterilization. However, the essential accessories and quality control systems, such as those integrated with the SPINLab polarizer for the clinical HP ^{13}C -pyruvate studies, are not commercially available to manage these procedures. Consequently, we need to customize an apparatus before conducting studies, including acquiring glassware, preparing fluid flow adapters and transfer tubing, and fabricating a rack via 3D printing to hold these components.

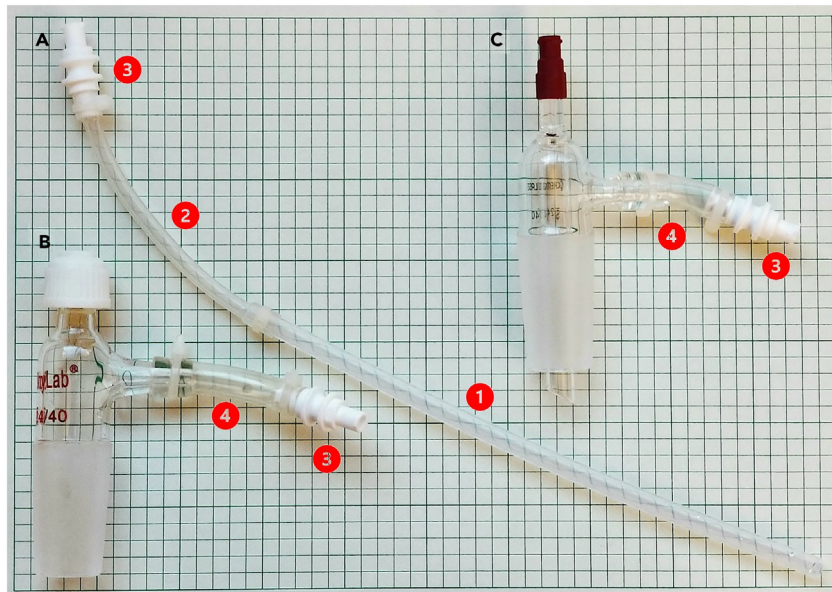


Figure 2. Preparation of fluid flow adapters and transfer tubing

(A) Fluid transfer tubing, (B) Fluid transfer adapter, and (C) Dissolution inlet adapter. The size of the background grid is 5 × 5 mm.

5. Acquire the necessary glassware, materials, and parts. Refer to items listed in [key resources table](#) and the following figures.
6. Assemble fluid transfer tubing ([Figure 2A](#)).
 - a. Cut a 200 mm length of polypropylene tubing ([Figure 2, A-①](#)).
 - b. Cut a 100 mm length of silicone tubing ([Figure 2, A-②](#)).
 - c. Connect one end of the silicone tubing to the polypropylene tubing and the other to a female-female connector ([Figure 2, A-③](#)).
 - d. Secure the connection with zip ties.
7. Assemble adapters ([Figures 2B and 2C](#)).
 - a. Cut two 50 mm-length Tubclair tubing ([Figure 2, B and C-④](#)).
 - b. Connect one end of the tubing to the side hose of the fluid transfer adapter ([Figure 2B](#)) and the Dissolution Inlet Adapter ([Figure 2C](#)).
 - c. Attach the other end of the tubing with a female-female connector ([Figure 2, B and C-③](#)).
 - d. Secure the connection with zip ties.
8. Autoclave all the components using a gravity cycle (121°C) for at least 40 min.

Note: Components include the above-prepared fluid transfer tubing and adapters, a two-neck round-bottom flask, a separatory funnel, a stir bar, and an autoclave indicator strip in an autoclave bag.

- a. Confirm the success of autoclaving by verifying the color change of the autoclave indicator strip.
 - b. Securely store the autoclaved items sealed inside the autoclave bag in a sterile environment for immediate use (within 7 days).
9. Fabricate a rack to hold the glassware securely ([Figure 3](#)).
 - a. Download [Data S1](#): the STL model files for the 3D design of the holding rack of the post-dissolution apparatus, related to [Figure 3](#).

Note: There are four files named according to the function of parts of the rack indicated in [Figure 3](#): fluid transfer adapter holder ([Figure 3, A-①](#)), two-neck round-bottom flask holder

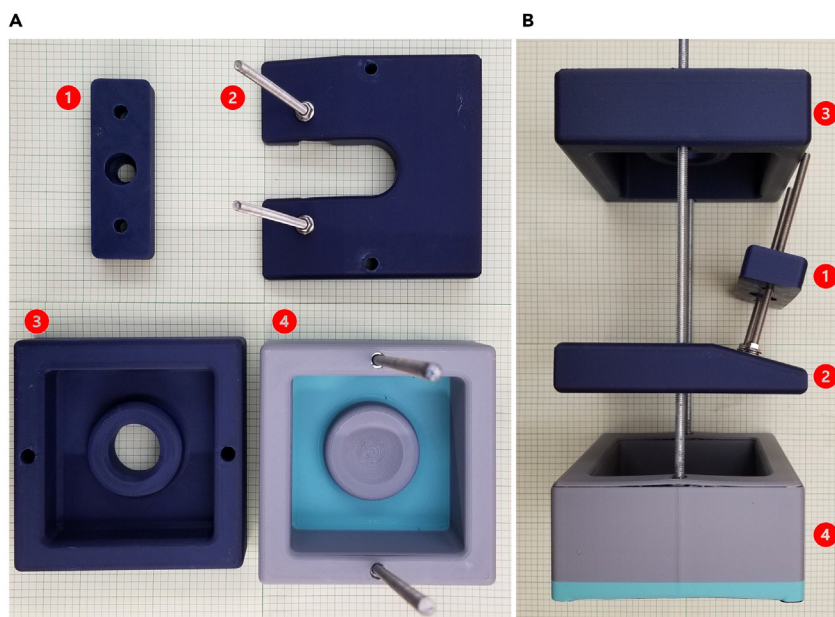


Figure 3. Fabrication of the holding rack of the post-dissolution reaction and processing apparatus

(A) ① fluid transfer adapter holder, ② two-neck round-bottom flask holder, ③ separatory funnel and dissolution inlet adapter holder, and ice-bath base ④.

(B) The assembly of the pieces of the holding rack. The size of the background grid is 5 × 5 mm.

(Figure 3, A-②), separatory funnel and dissolution inlet adapter holder (Figure 3, A-③), and ice-bath base (Figure 3, A-④).

- b. Import them into a slicing program for the 3D printer.
- c. Print the parts using a standard setting with 15% or more infill, and at least 2 wall perimeters.
- d. Assemble all the parts as shown in Figure 3B.

Configuration of the GE 3T clinical MRI scanner for hyperpolarized ^{13}C data acquisition

Note: The clinical MRI scanner primarily images proton signals, such as H_2O , in the region of interest (ROI) within humans. Acquiring HP ^{13}C signals, the scanner requires a specially designed system comprising ^{13}C receiver coils, a radiofrequency (RF) pulse sequence, an updated operating system, and data reconstruction and analysis. Given the diversity and complexity of MRI facilities and operating systems in the different clinical hyperpolarization MRI research centers, direct implementation of our methods may not be applicable. Providing an example from our study, we use the GE MRI system to localize the tumor, identify the position of the ROI, and determine the ^{13}C central frequency. Subsequently, we use an RTHawk system from HeartVista, Inc., which interfaces with the GE MRI system, to execute a ^{13}C multi-metabolite specific 2D gradient echo (GRE) sequence with spiral readout to acquire HP ^{13}C bicarbonate and HP $^{13}\text{CO}_2$ signals. We need four essential installation and setup steps to prepare the MRI scanner for HP ^{13}C data acquisition.

10. Choose and install proper ^{13}C receiver coils for specific cancer study.

Note: For example, we use a 4-Channel Torso Coil by GE Healthcare and a 1-Channel ^{13}C -Tuned Endorectal coil for prostate cancer imaging.

11. Set up the GE 3T clinical scanner console for anatomy references per manufacturer instruction.

12. Set up the RTHawk system for HP ^{13}C imaging per manufacturer instruction.
13. Install a metabolite-specific 2D GRE sequence with spiral readout to the RTHawk system for executing HP ^{13}C imaging data acquisition.

⚠ **CRITICAL:** Check the functionality of the MRI scanner and coils at least a day before the clinical trial experiment.

Inspection of materials, supplies, and workstation

⌚ **Timing:** 30 min

14. Inspect all the reagents, supplies, and kits listed in the [key resources table](#), verifying their availability, adequacy, and expiry status.
15. Perform a visual inspection of the GE clinical pharmaceutical fluid path, ensuring its integrity by checking for missing parts, kinks, ruptures, and disconnection. Apply additional UV curving glue if necessary to enhance the connections between joints.
16. Inspect the workstation, ensuring cleanliness and disinfection of the working area, functionality of all equipment, and accuracy of measurement tools (check calibration expiry status or perform calibration) per manufacturer instructions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1,2-Glycerol carbonate-(carbonyl- ^{13}C)	MilliporeSigma	cas# 1379618-83-3 (GMP Grade)
Deuterium oxide	MilliporeSigma	cas# 7789-20-0 (Bioburden tested)
Deuterium chloride solution – 35 wt % in D_2O	MilliporeSigma	cas# 7698-05-7
Tromethamine (Tris)	Letco Medical by Fagron	cas# 77-86-1 (USP grade)
Sodium hydroxide	Letco Medical by Fagron	cas# 1310-73-2 (NF grade)
Ethylenediaminetetraacetic acid disodium salt dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$)	Letco Medical by Fagron	cas# 6381-92-6 (USP grade)
Methyl, tris[8-carboxy-2,2,6,6-tetrakis (2-methoxyethyl)benzo[1,2- <i>d'</i> :4,5- <i>d'</i>]bis [1,3]dithiol-4-yl]-, trisodium salt (AH111501)	Symeres	identifier# AH111501; cas# 874536-54-6
Ethyl alcohol, pure	MilliporeSigma	cas# 64-17-5, 200 proof, HPLC/ spectrophotometric grade
Sodium chloride	MilliporeSigma	cas# 7647-14-6, ACS reagent, $\geq 99.0\%$
Gadopentetate dimeglumine (Magnevist)	Bayer HealthCare Pharmaceuticals, Inc.	cas# 86050-77-3
Software and algorithms		
MATLAB	MathWorks	https://www.mathworks.com/?s_tid=gn_logo
3D design software: Solidworks		https://www.solidworks.com/
3D printer software: Bambu Lab and PrusaSlicer		https://bambulab.com/en-us and https://www.prusa3d.com/
Other		
5T GE SPINlab multi-sample polarizer		
3T GE 750 whole body MR scanner	GE	
GE multiprobe QC & fluid handler	GE	
SpinSolve 60 carbon spectrometer	Magritek	

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Labconco Purifier 4 ft. and 6 ft. horizontal clean benches	Labconco	
Analytical balance XSR105	METTLER TOLEDO	Manufacturer Part# 30355059
Oakton pH 700 benchtop meter	Oakton	Item# UX-35419-12
Eco-Heat magnetic stirrer	United Scientific	SKU# UNMGSTR-2L
Magnetic stirrer bar (Polygon, length: 30 mm, diameter: 8 mm, FDA grade, USP Class VI Teflon PTFE coated)	Fisher Scientific	Cat# 16-800-510
GE Pharmaceutical Kits		
Synthware separatory funnel with standard taper joint (capacity: 125 mL, joints: 24/40)	Kemtech America	Manufacturer Part# F454125
Synthware round bottom two neck flask (capacity: 100 mL, joints: side 24/40, center 24/40)	Kemtech America	Manufacturer Part# F414400
Inlet thermometer adapter with hose connection and compression cap	StonyLab	SKU #B07YBWPXXB
Chemglass Life Sciences adapter, female luer lock, vacuum, 1/4"-28 thread, 24/40 inner	Chemglass Life Sciences	Manufacturer Part# CG-1049-F-24
4-Way stopcock manifold with swivel male luer lock, high-flow	Smiths Medical ASD, Inc.	Manufacturer Part# MX934Z4LM
Namic vascular access adapters and caps – rotating male adaptor/male luer lock, 2" L	Medline	Manufacturer Part# H749700271801
Sep-Pak tC18 Plus Long double luer-lock cartridge	Waters	SKU# WAT036800DL
Tight-Ties Self-locking nylon ties, overall length: 101.6 mm	Spectrum Chemicals & Laboratory Products	Cat# 630-12882
Hi-Flo 3-way stopcock	Smiths Medical	Manufacturer Part# MX4311L
Female-female luer-lock connector	International Medical Industries, Inc.	REF# 57-13
Multi-functional male/female luer lock sterile cap	ISO-MED	Item# ISOCAP-R
Tubing	Tubclair AL	OD 10 mm, ID 6 mm
FEP tubing	PharmaFluor	OD 6 mm, ID 4 mm
0.22 µm Hydrophobic vent filter (white)	Minisart	
0.20 µm Hydrophobic PTFE filter (blue)	Minisart	Cat: SLFGL25BS
ZenPure PureFlo 0.2 µm PES 65 mm disc capsule	Saint-Gobain	Manufacturer Part# D65RS020LMLF-R-PH-ETO-1
MedRad syringe (disposable MRI kit for 65/115 MR injector system)	Bayer Medical Care, Inc.	REF# SSQK 65/115 VS
3D printer: Bambu X1 carbon and Prusa i3 MK3		
3D print material: polylactide acid	Bambu Filament	https://us.store.bambulab.com/products/pla-basic-filament
Zinc-coated steel threaded rods (suggested diameter of 8–9 mm)	Hardware store	
Stainless steel washers	Hardware store	
Nuts (outside measurement of 11.2 and 10.5 mm to fit threaded rods)	Hardware store	
Nylon-insert lock nuts	Hardware store	
Corn nuts	Hardware store	

STEP-BY-STEP METHOD DETAILS

The protocol comprises two primary components for imaging tumor interstitial pH–production of the HP ^{13}C bicarbonate contrast agent and ^{13}C MRI data acquisition and reconstruction. We produce HP ^{13}C bicarbonate formula through two major steps: Step One, which includes the procedure for preparing a highly polarized ^{13}C -GLC solution, and Step Two, which performs the post-dissolution reaction and solution processing procedure to produce a safe (sterilized and AH111501 free), highly polarized, and high-concentration ^{13}C -bicarbonate contrast formula. Immediately after injection, we initiate the data acquisitions with a pre-calibrated and pre-set GE 3T clinical scanner equipped with our multi-metabolite GRE with spiral readout RF pulse sequence.¹³ Finally, we perform the reconstruction and processing of data using MATLAB software with our specific scripts.

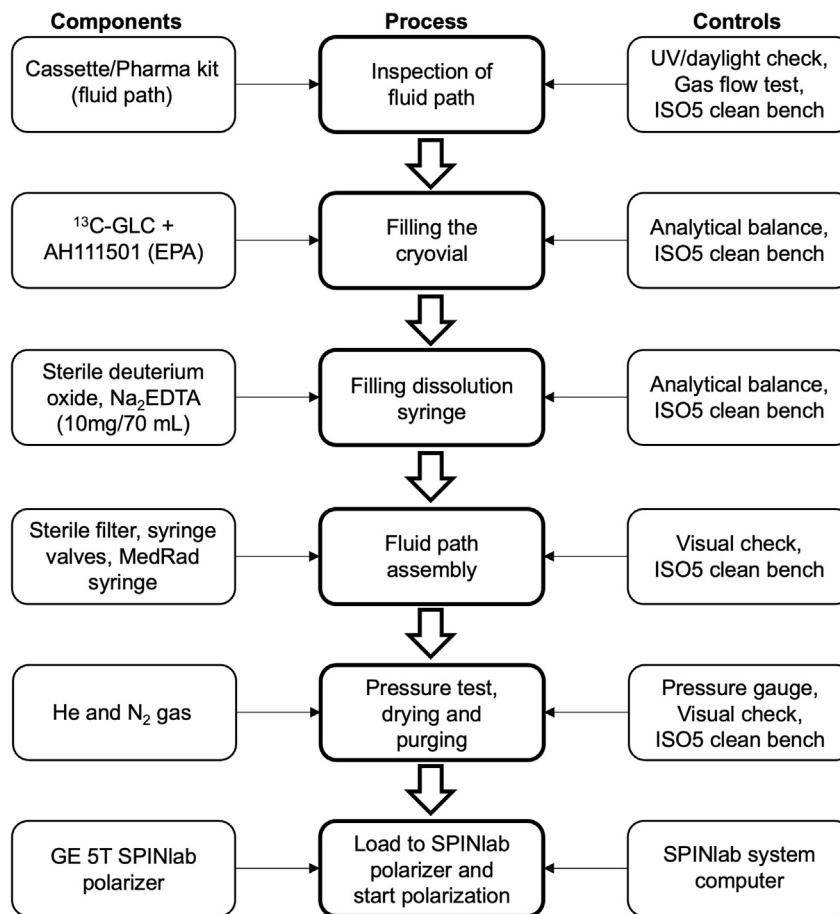


Figure 4. Flowchart of major steps of preparing GE clinical fluid path for polarizing ^{13}C -GLC, incorporating essential components and quality controls for each step

Preparation and hyperpolarization of ^{13}C -GLC

⌚ Timing: 1 h (preparation time) and 6 h (sample polarization time)

This step describes the workflow for preparing the ^{13}C -GLC polarization formula, assembling the GE clinical fluid path, and polarizing the formula in the GE 5T SPINlab polarizer to produce an HP ^{13}C -GLC solution in D_2O .

⚠ CRITICAL: These steps require strict adherence to the GMP guidelines and FDA-approved procedures.

Note: Figure 4 illustrates the workflow of essential steps, incorporating necessary components and quality controls for each step.

Note: We allow an uncertainty within $\pm 3\%$ from the specified weights

1. Laminar flow clean bench setup.
 - a. Fill liquid nitrogen Dewar.
 - b. Clean and disinfect the clean bench, fluid path, parts, and apparatuses.

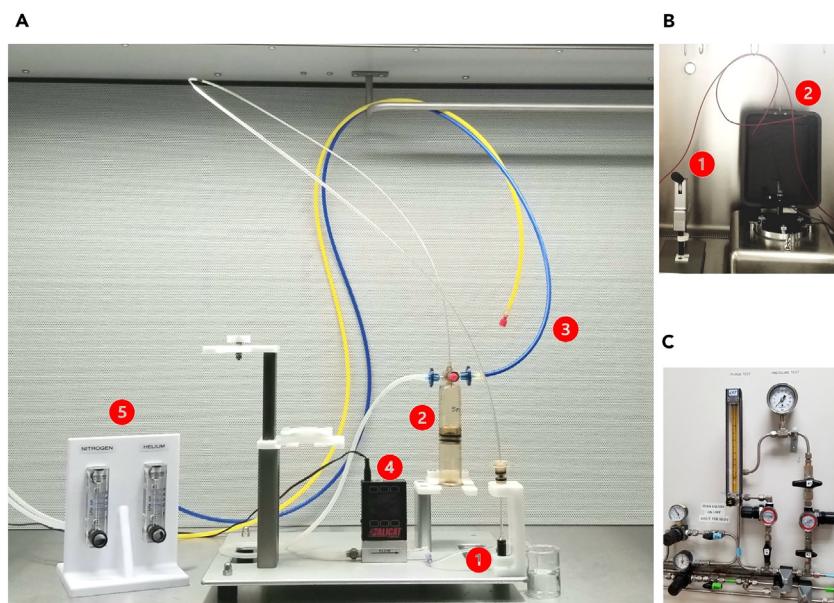


Figure 5. The setup for preparing the GE clinical fluid path within ISO 5 clean bench for ^{13}C -GLC polarization

(A) The setting of preparing GE clinical fluid path for polarization: ① Sealed cryovial loaded with ^{13}C -GLC/AH111501 polarization formula, ② MedRad syringe loaded with EDTA/D₂O solution, ③ Gas lines, nitrogen gas (blue) and helium gas (yellow), ④ Digital gas flow meter, and ⑤ Gas flow regulator.

(B) Cryovial sealing tools: ① vial capper and ② Laser sewing system.

(C) The settings of gas lines.

2. Preparation of the ^{13}C -GLC/AH111501 polarization formula.
 - a. Add 936 mg of ^{13}C -GLC and 16 mg of AH111501 to a 3-mL brown glass vial.
 - b. Mix the resulting mixture with a vortex at 2000 RPM for 5 min to yield a homogeneous 15 mM AH111501 in ^{13}C -GLC. Wrap the vial with aluminum foil to protect it from light exposure.
3. Load the ^{13}C -GLC/AH111501 polarization formula into the cryovial and securely seal it.
 - a. Pipette 793.3 mg of ^{13}C -GLC/AH111501 mixture into the cryovial (Figure 5, A-①).
 - b. Attach the fluid path tubing cap onto the cryovial, secure it using a vial capper with pressure (Figure 5, B-①), and then laser weld it (Figure 5, B-②). Ensure sealed.
4. Preparation of EDTA/D₂O solution.
 - a. Dissolve 10 mg of Na₂EDTA•2H₂O in 70 g of D₂O within a glass bottle.

Note: We specified the vendor to aliquot 70 g of bioburden-tested D₂O into each glass bottle and seal them, with one bottle designated for single use.

 - b. Mix the mixture using a vortex at 2000 RPM for 3 min to make Na₂EDTA solution in D₂O (EDTA/D₂O solution, 1.58 mg/mL, 67.3 μM).
5. Load EDTA/D₂O into the dissolution syringe (Figure 5, A-②).
 - a. Insert the dispensing pin into the dissolution syringe of the fluid path.
 - b. Inject 42.2 g of EDTA/D₂O into the dissolution syringe through the inlet port of the dispensing pin.
6. Adjust and verify the pressure reading of compressed air (80 PSI), nitrogen (50 PSI), and helium (50 PSI) tanks, and attach a hydrophobic PTFE filter to the incoming main gas line.
7. Fluid path flow test.
 - a. Submerge the cryovial in liquid nitrogen in a dewar.

⚠ **CRITICAL:** Ensure the complete freezing of the ^{13}C -GLC/AH111501 polarization formula inside the cryovial before proceeding to the next step.

- b. Connect the helium line to the gas inlet port on the dispensing pin.
- c. Run helium at 0.2 L/min for 2 min, ensuring the gas flow is smooth and not blocked.
8. Fluid path holding pressure test.
 - a. Keep the cryovial submerged in liquid nitrogen in a dewar.
 - b. Connect the helium line to the gas inlet port and cap the outlet port with a sterile cap on the dissolution syringe.
 - c. Open the helium line valve, adjust the pressure to 40 PSI, and close the valve.
 - d. Monitor the pressure gauge that the pressure holds at 40 PSI for at least 2 min, ensuring no leaks.

Note: Typically, we apply additional UV curving glue if necessary to reinforce the connections between joints. If the leak still happens, it may indicate that the laser weld of the cap to the cryovial is not secure or that it is a defective fluid path. In such cases, restart from step 2.

- e. Slowly disconnect the helium line to release the pressure, and carefully remove the cryovial from liquid nitrogen.
- f. Cap inlet and outlet ports with sterile caps on the dispensing pin and the dissolution syringe, respectively.
9. Dry fluid path (Figures 5A and 5C).
 - a. Allow the cryovial to stand at 25°C for 15 min to complete thawing ^{13}C -GLC/AH111501 inside.
 - b. Connect the nitrogen gas line to the inlet port of the dispensing pin through a hydrophobic PTFE filter.
 - c. Attach a hydrophobic PTFE filter, followed by a flow meter, to the outlet port of the dissolution syringe.
 - d. Flush the fluid path with nitrogen gas (0.1–0.15 L/min) for 15 min.
 - e. Switch to the helium gas line and flush with helium gas (0.1–0.15 L/min) for 5 min.
 - f. Replace the dispensing pin with the dissolution valve and attach a dissolution transfer tubing to the outlet port on the dissolution syringe. The fluid path is ready to load to the polarizer.
10. Polarize ^{13}C -GLC with the GE 5T SPINLab polarizer.
 - a. Load the fluid path to the polarizer per GE manual.
 - b. Start ^{13}C -GLC polarization using the microwave frequency and other parameters determined in the above “before you begin” section shown in Figures 1C and 1D.
 - c. After 5–6 h of polarization, when polarization time is about three times the polarization constant, it is ready for dissolution to produce the HP ^{13}C -GLC solution in D_2O .

Preparation for post-dissolution reaction and processing of HP ^{13}C -bicarbonate

⌚ **Timing:** 2 h

This step describes the workflow for preparing the post-dissolution reaction reagents and assembling the apparatus for handling the production and processing of the HP ^{13}C bicarbonate contrast agent.

⚠ **CRITICAL:** This major step requires strict adherence to the GMP guidelines and FDA-approved procedures.

Note: Figure 6 illustrates the workflow of essential steps, incorporating necessary components and quality controls for each step.

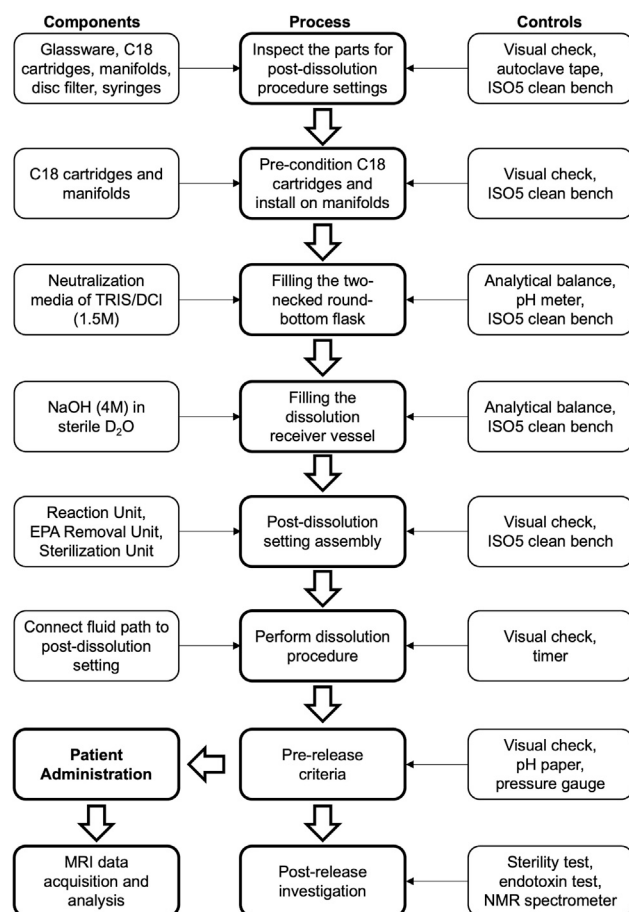


Figure 6. Flowchart of major steps of preparing post-dissolution reaction and processing apparatus and performing dissolution procedure to produce hyperpolarized HP ^{13}C -bicarbonate formula human administration, incorporating essential components and quality controls for each step

11. Preparation of reagents and solutions.

△ CRITICAL: For sterilization and safety, prepare the reagents on the same day of the clinical study in the ISO-5 cleanroom.

- Add 2.73 g of Tris in 10.00 g of EDTA/D₂O solution to make Tris/EDTA/D₂O solution.
- Dilute 3.00 g of 35% concentrated DCl in 4.40 g of EDTA/D₂O solution to make DCl/EDTA/D₂O solution (7 M).

Note: The percentage of DCl in D₂O may vary between batches. Check the Certificate of Analysis for the tested percentage. If it differs from 35%, calculate the accordingly.

- Add 5.99 g of 7 M DCl/EDTA/D₂O solution (step b) to all Tris/EDTA/D₂O solution (step a) to make Tris-DCl/EDTA/D₂O buffer solution.
- Vortex the mixture at 2000 RPM for 1 min until Tris dissolves.
- Measure the pH of the resulting Tris-DCl/EDTA/D₂O buffer solution within pH 4.0–7.5.

△ CRITICAL: Ensure the Tris-DCl/EDTA/D₂O buffer solution pH falls within 4.0–7.5 to preserve polarization and maintain the correct pH for human injection.

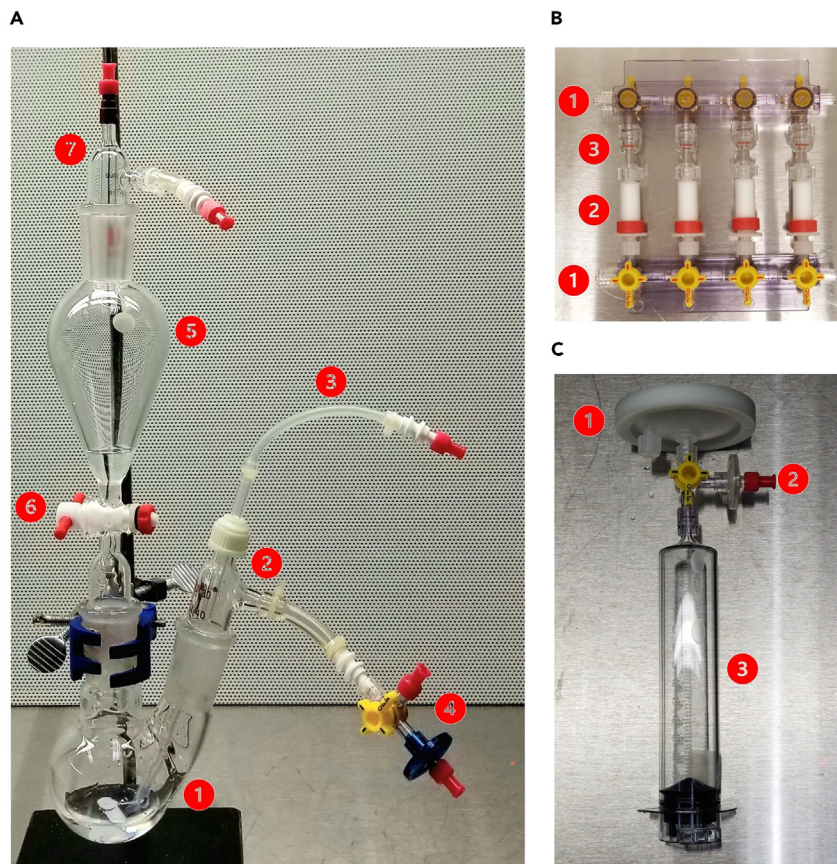


Figure 7. Assembly of each unit of post-dissolution reaction and solution processing system

(A) The assembly of Reaction Unit. ① 2-neck round bottom flask, ② Fluid transfer adapter, ③ Fluid transfer tubing, ④ 3-way stopcock and a hydrophobic air filter, ⑤ Separatory funnel, ⑥ Valve, and ⑦ Dissolution inlet adapter.
(B) The assembly of AH111501 Removal Unit. ① Four-way stopcock manifold, ② Pre-conditioned C18 cartridges, and ③ Rotatory male-male Luer-Lock connectors.
(C) The assembly of Sterilization and Collection Unit. ① PES sterility filter, ② 3-way stopcock and vent filter, and ③ 60-mL MedRad syringe.

f. Perform endotoxin test per manufacturer instruction.

⚠ **CRITICAL:** Ensure the endotoxin test passes. Otherwise, check for possible contamination and repeat the above steps.

Troubleshooting 2.

12. Assemble the Reaction Unit (Figure 7A).
 - a. Weigh 15.8 g of Tris-DCI/EDTA/D₂O buffer (1.5 M) with a 20-mL syringe and add it to a 2-neck round bottom flask (Figure 7, A-①), followed by adding a stir bar in the flask.
 - b. Attach the fluid transfer adapter (Figure 7, A-②) to the side neck of the 2-neck round bottom flask.
 - c. Insert a fluid transfer tubing (Figure 7, A-③) through the compression cap of the fluid transfer adapter to the bottom of the 2-neck round bottom flask and screw it tightly.
 - d. Connect to the side hose connection of the fluid transfer adapter with a 3-way stopcock and then a hydrophobic air filter (Figure 7, A-④).
 - e. Attach a separatory funnel (Figure 7, A-⑤) to the straight neck of the 2-neck round bottom flask.
 - f. Ensure the separatory funnel valve (Figure 7, A-⑥) is closed.

- g. Weigh 4.2 g of NaOH/EDTA/D₂O solution (4 M) with a 5-mL syringe and add it to the separatory funnel.
- h. Cap the separatory funnel with the dissolution inlet adapter (Figure 7, A-⑦).
- i. Cap all the ports with sterile caps to prevent contamination.
- j. Set aside this Reaction Unit for further assembly.
13. Assemble the AH111501 Removal Unit (Figure 7, B).
 - a. Pre-condition four Sep-Pak tC18 plus cartridges.
 - i. Slowly pass through 5 mL of anhydrous ethanol.
 - ii. Retain the remaining ethanol in the cartridge for 3 min.
 - iii. Slowly pass through 25 mL of sterile water for injection.
 - iv. Retain the remaining water inside of the cartridge.
 - b. Attach the pre-conditioned cartridges (Figure 7, B-②) to a 4-way stopcock manifold (Figure 7, B-①) via their outlet luer-locks.
 - c. Connect the cartridges' inlet Luer-Locks to four rotatory male-male Luer-Lock connectors (Figure 7, B-③), which connect to another 4-way stopcock manifold on the flipped side.
 - d. Verify the flow direction (the red band on the cartridge indicates the outlet), identify the inlet and outlet ports on the manifolds, and adjust the stopcocks accordingly.
 - e. Set aside this AH111501 Removal Unit for further assembly.
14. Assemble the Sterilization Unit (Figure 7, C).
 - a. Cap the side port of a PES sterility filter with a clean cap included in the package (Figure 7, C-①).
 - b. Attach the straight male port of a 3-way stopcock to the female outlet port of the sterility filter.
 - c. Attach a vent filter to the side port of the 3-way stopcock (Figure 7, C-②).
 - d. Attach a 60-mL MedRad syringe (Figure 7, C-③) to the female port of 3-way stopcock.
 - e. Attach sterile caps to the inlet port of the sterility filter and the vent filter.
 - f. Set aside this Sterilization Unit for further assembly.
15. Assemble the whole post-dissolution reaction and processing apparatus (Figure 8).
 - a. Mount the Reaction Unit (Figure 7A) onto the 3D print rack and tighten it.
 - b. Connect the fluid transfer tubing (Figure 7, A-③) to the inlet port on the AH111501 Removal Unit manifold.
 - c. Connect an IV extension tubing from the outlet port on the AH111501 Removal Unit manifold to the inlet port of the PES sterility filter (Figure 7, C-①). Keep the other sterile caps in place to prevent contamination.
 - d. Keep the whole apparatus on the Laminar flow clean bench until 30 min before the dissolution.

Preparation for GE 3T clinical MRI to execute HP ¹³C-bicarbonate tissue pH imaging

⌚ Timing: 30 min (phantom calibration scan) and 30 min (patient scan)

This step describes the workflow for setting GE 3T clinical MRI to execute HP ¹³C-bicarbonate tissue pH imaging and provides corresponding reference parameters.

Note: Given the diversity and complexity of MRI facilities and operating systems across the clinical hyperpolarization MRI research centers, direct implementation of our methods may not be applicable. Therefore, we provide our settings and experience as references.

16. Calibrate Power and Frequency of the 3T GE MRI scanner with a phantom.
 - a. Connect the specified coils, such as 4-Channel Torso Coil and 1-Channel ¹³C-Tuned Endorectal coil for prostate cancer study, to the GE 3T scanner.
 - b. Place a polyethylene glycol phantom and load the pre-set protocol.
 - c. Run Localizer to find the phantom and confirm the connection is good.

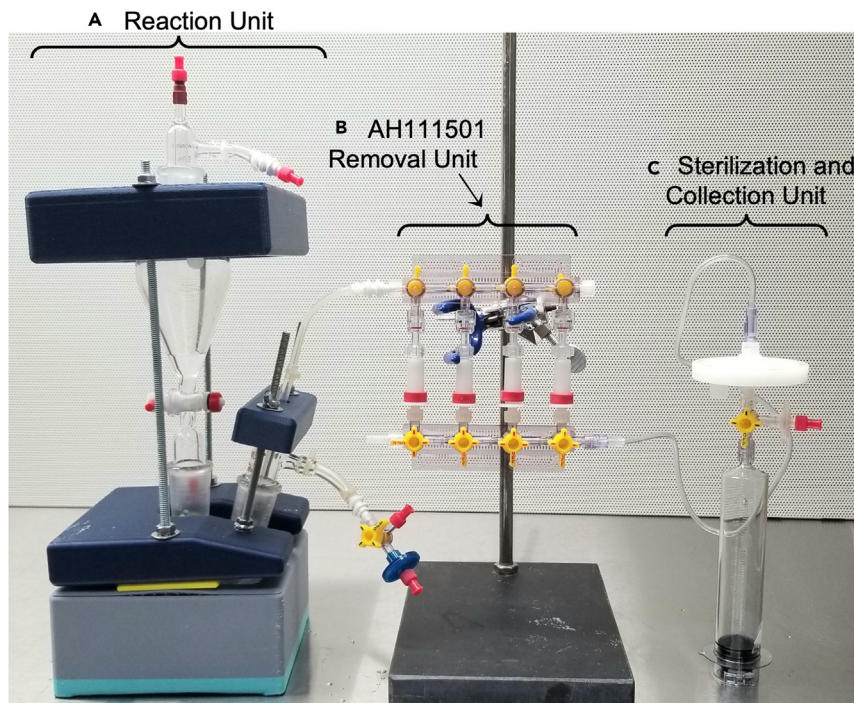


Figure 8. Assembly of the post-dissolution reaction and solution processing apparatus

(A) Reaction Unit mounted in 3D holding rack.

(B) AH111501 Removal Unit.

(C) Sterilization and Collection Unit.

- d. Run Spectroscopy Prescan and set the center frequency of phantom to zero.
- e. Determine the Transmit Gain (TG) value:
 - i. Set the excitation RF pulse to 180 degrees.
 - ii. Set both Analog Gain and Digital Gain to the maximum.
 - iii. Adjust Transmit Gain (TG) until the phantom signal reaches the null point and record this TG value.
- f. Verify the TG value:
 - i. Under this TG value, set the RF pulse to 30 degrees and measure the signal intensity amplitude (A1).
 - ii. Then set the RF pulse to 90 degrees and measure the signal intensity amplitude (A2).
 - iii. The signal intensity ratio of A1/A2 should be close to $\frac{1}{2}$ since $\sin(30^\circ)/\sin(90^\circ) = \frac{1}{2}$.
- g. Remove the phantom.
17. Determine tumor position, powder, and ^{13}C -bicarbonate central frequency with a patient.
 - a. Load the patient onto the pre-calibrated GE 3T MRI scanner and place the coils.
 - b. Conduct sequentially functional runs of "Localizer", "Calibration Scan Breath Holding Pelvis", and "Sagittal Scout" to identify the coil position and the tumor region.

△ **CRITICAL:** If the A1/A2 ratio deviates from $\frac{1}{2}$, recalibrate the TG value.

△ **CRITICAL:** It would be necessary to adjust the patient's position to ensure the tumor or region of interest is at the isocenter.

Table 1. Parameters for real time board band calibration

Dropdowns menu	Frequency cal	Plot zoom	2
Center Frequency	0	B1 Scaling	65.22
Flip Angle	90	TR	150
Slice Thickness	1000	CF Cal Offset	0
NumAverages	1		

Note: The scout image is a survey of the region of interest to select the area of dedicated image acquisition.

- c. Acquire Axial T₁- and T₂-weighted images and determine the parameters of the tumor's position.
- d. Run the "IDEAL IQ" function to check the B₀ map, covering the tumor region in Rx.
- e. Setup targeted shim on volume covering whole body region.
- f. Conduct "Manual Prescan" to determine the ¹H center frequency (cf) and the shimming values in x, y, and z dimensions.
- g. Calculate the center frequency for ¹³C-bicarbonate by multiplying the ¹H center frequency by the constant 0.251489272704124.
(cf (¹³C-bicarbonate) = cf(H₂O) × 0.251489272704124).
- h. Conduct "Power calibration" to identify the Transmit Gain (TG) value.

Note: This step is the same as the procedures described in the above section (steps 15-e and f) for phantom calibration.

18. Set up the RTHawk system for data acquisition.
 - a. Launch the RTHawk system, choose the coils, and load the pre-set protocol.
 - b. Meanwhile, on the GE console, validate or adjust the TG value, the ¹³C-bicarbonate center frequency, and the shimming values as determined above, and set both Analog Gain (R1) and Digital Gain (R2) to 10.
 - c. Set "Real Time Board Band Calibrations"
 - i. Set "Geometry" by inputting the parameters of the tumor's position determined from the above T₁- or T₂-weighted image (Step 17-c).
 - ii. Set the parameters listed in [Table 1](#) to run a high Flip Angle (90 degrees) and a large slab (Slice Thickness: 1000 mm) to check the ¹³C NMR spectrum.
 - iii. Change Flip Angle to 3 degrees and Slice Thickness to 30 mm for the HP ¹³C-bicarbonate scan.
 - d. Set "Bolus Tracking ¹³C"
 - i. Select "No Tracking" and run the sequence on the default geometry.
 - ii. Set "Geometry" as above and the parameters listed in [Table 2](#).
 - iii. Set "Noise Calibration": Set ROI with the parameters of the tumor's position (Step 17-c), and set the typical ROI size (25 mm, 25 mm, 30 mm).
 - iv. Run the sequence to calibrate noise and select "Tracking Arrival" for the HP ¹³C scan.
 - e. Set "B1 Mapping ¹³C"
 - i. Similar to the above, set "Geometry" and ROI with the parameters of the tumor's position (Step 17-c) and set the ROI size (40 mm, 40 mm, 30 mm).
 - ii. Set the parameters listed in [Table 3](#).

Table 2. Parameters for ¹³C bolus tracking setting

FOV	33.6	Slick thickness	30	# Of noise cal	50
Flip Angle	5	B1 Scale	65.22	Noise Std	-
TR	1000	Signal Threshold	3	# of Cumulative Signals	-
Noise Cal TR	200	Send Trigger After	0	Signal Decay Tolerance	-

Table 3. Parameters for ^{13}C B₁ mapping setting

Items	Values	Items	Values	Items	Values
FOV	33.6	Slick Thickness	30	B1 scale	65.22
Flip Angle	10	NumAverages	1	Signal Threshold	2
TR1	200	TR2	200	Slices	1
Slice separation	30	Calibration Slice	1		

- iii. Run the sequence. Check the geometry, FOV, and displayed image within the range.
- f. Set "Multi-Slice Spiral GRE C1BicCO₂"

Note: This is the main sequence for the data acquisition.

- i. Similar to the above, set "Geometry" and ROI with the parameters of the tumor's position, and set the ROI size (40 mm, 40 mm, 30 mm).
- ii. Set the following parameters listed in Table 4.

Note: The acquisition parameters provided here are for reference purposes only. They may vary depending on the exact characteristics of the tumor under investigation.

- iii. Run the sequence to ensure it is running correctly.
- iv. Change NumTimePoints to 90 and select "Triggered by BSB1 Cal".
- v. The RTHawk system is now ready to execute the data acquisition for the HP ^{13}C -bicarbonate and HP $^{13}\text{CO}_2$ signals.

Production of hyperpolarized ^{13}C -bicarbonate for patient injection

⌚ Timing: 30 min

This step describes the workflow for the setting of the apparatus, followed by executing the dissolution and post-dissolution reaction and solution processing procedures to produce HP ^{13}C -bicarbonate solution with quality control for human injection.

19. Set up the dissolution procedure (20 min before dissolution).
 - a. Connect the dissolution transfer tubing (Figure 9, ①) from the fluid path loaded in the polarizer to the dissolution inlet adapter (Figure 7, A-⑦) in the Reaction Unit.
 - b. Mount the MedRad syringe on the GE MPQC system and create a vacuum inside per GE manual (Figure 9, ②).
 - c. Adjust the 3-way stopcock attached to the fluid transfer adapter with "OFF" facing the hydrophobic air filter (Figure 9, ③).
 - d. Connect the nitrogen gas line to the hydrophobic air filter (Figure 9, ④) and adjust the pressure to 40 PSI.
 - e. Turn on the stir plate and ensure the stir bar is spinning smoothly.

Table 4. Parameters for multi-slice spiral GRE sequence setting for data acquisition

Items	Values	Items	Values	Items	Values
FOV (cm) / res (mm)	25.6 (res 0.8)	Slice Thickness	8	B1 scale	65.22
Flip Angle 1	45	Flip Angle 2	10	NumTimePoints	5
TR1	80	TR2	320	nMets	2
FOV scale 1	1	FOV scale 2	1		
Freq 1	−1140	Freq 2	0		
Slices	1	Slice separation	20		

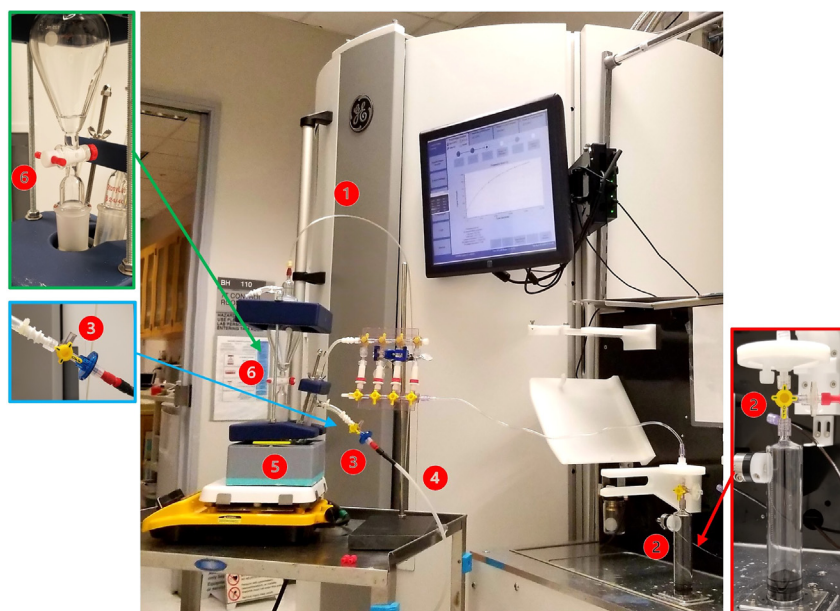


Figure 9. The assembly and procedures of producing human-injectable hyperpolarized ^{13}C -bicarbonate contrast formula

- f. Place 520 g of NaCl/crushed ice mixture (3:10 by weight) into the cooling bath base of the apparatus (Figure 9, ⑤). Secure all the screws.
- g. When the temperature decreases to -18°C , remove all sterile caps.
- h. It is ready to perform dissolution.
20. Perform dissolution and post-dissolution procedures to produce HP ^{13}C -bicarbonate.
 - a. Perform dissolution procedures on the polarizer per GE manual.
 - b. The HP ^{13}C -GLC dissolution comes into the separatory funnel receiver (in 10 s).
 - c. Turn on the valve of the separatory funnel receiver (Figure 9, ⑥) to allow the solution to flow into the 2-neck round bottom flask in the ice-bath base (Figure 9, ⑤) (in 10 s).
 - d. Close the valve (Figure 9, ⑥) and turn the two 3-way stopcocks on the fluid transfer adapter (Figure 9, ③) and MedRad syringes (Figure 9, ②) immediately with the “Off” handler facing side port. The solution flows through all the units into the MedRad syringe (in 15 s).
 - e. Simultaneously withdraw the sample with a 3-mL sterile syringe via the side port of 3-way stopcock for pH testing, perform a bubble test to check the integrity of the disc filter, and mount the MedRad syringe onto the auto-injector (15 s). [Troubleshooting 3](#).
 - f. Verify all injection criteria in 10 s, including AH111501 residual color (no green), solution pH (4–9), and the bubble test (pass) for the integrity of the sterile filter disc. [Troubleshooting 4](#).

Note: Compare with a standard solution of $5\ \mu\text{M}$ of AH111501 solution in the same type of MedRad syringe to ensure the accuracy of visual inspection. The produced solution should be colorless or less green than the reference solution for injection.

△ CRITICAL: Should not inject the solution into the patient if any criterion fails or the entire procedure time is over 90 s.

- g. Administer the HP ^{13}C -bicarbonate formula to the patient and initiate HP ^{13}C MRI data acquisition.
- h. After injection, perform endotoxin and sterility tests per manufacturer instruction with the left-over solution.

HP ¹³C tissue pH MRI data acquisition and reconstruction

⌚ Timing: Data acquisition (2 min) and data reconstruction (1 h)

This major step describes the MRI data acquisition of HP ¹³C-bicarbonate and HP ¹³CO₂ signals and data reconstruction to obtain the tissue pH map.

21. Once the bolus reaches the ROI in 5–10 s after administration, the MRI scanner automatically initiates the data acquisition with the specified parameters mentioned above.
22. Imaging reconstruction and data analysis.
 - a. Perform k-space gridding using the Kaiser-Bessel gridding method¹⁴ (<http://web.stanford.edu/class/ee369c/mfiles/gridkb.m>).
 - b. Apply the inverse Fourier transform to convert the gridded k-space into the reconstructed image.
 - c. Correct B₀ inhomogeneity-induced phase errors among different echoes by combing the phasing maps measured from ¹³C-bicarbonate at the time point with the highest signal.
 - d. Estimate the multi-echo metabolite area-under-curve (AUC) images by summing all time points signals.
 - e. Calculate the signal-to-noise ratio by dividing the signal magnitude by the standard deviation of the real part of the noise.
 - f. Generate the pH imaging maps using normalized AUC signals of HP ¹³C-bicarbonate and HP ¹³CO₂ with the modified Henderson–Hasselbalch equation.

$$pH = pK_a + \log \left(\frac{S_{HCO_3^-}}{S_{CO_2}} \times \frac{\sin(\alpha_{CO_2})}{\sin(\alpha_{HCO_3^-})} \right)$$

Note: Please refer to our research center’s published manuscripts for more details about imaging data acquisition, reconstruction, and analysis methods.^{13,15,16}

EXPECTED OUTCOMES

This protocol delineates the strategy and methodology that we have created to address the significant challenges associated with producing clinically applicable hyperpolarized ¹³C-bicarbonate for MRI imaging of tissue pH. The primary four challenges include a profound loss of polarization found using non-optimal conditions during the procedure, unavailability of post-dissolution settings for the reaction and processing, and the imperative to meet stringent criteria adhering to the GMP and FDA standards for clinical application, as well as a lack of magnetization-efficient pulse sequence for MRI data acquisition. Therefore, we expect this protocol to overcome these challenges, making it suitable for clinical translation to routinely produce a highly polarized, high-concentration, sufficient volume of ¹³C-bicarbonate contrast agent that is crucially safe for human injections. Subsequently, our dynamic metabolite-specific GRE pulse sequence with spiral readout ensures efficient data acquisition, enabling accurate measurement of tissue pH and its inhomogeneity.^{1,16}

In summary, this protocol consistently yields about 35 mL of a polarized ¹³C-bicarbonate contrast agent with a polarization level of 20%–25% within 60 s from the start of dissolution. The concentration of ¹³C-bicarbonate is approximately 120 mM, supplemented with glycerol (130 mM), Tris buffer (380 mM), and NaCl (255 mM) in 35 mL of D₂O. Furthermore, the resulting injection solution is AH111501-free, maintains a pH of 7.8–8.2 within the safe range of pH 4–9, and passes both sterility and endotoxin tests. Please refer to our primary research paper for comprehensive details and *in vivo* images.¹

LIMITATIONS

The primary limitations of this protocol arise from the lack of an automated control system for precise execution of post-dissolution steps, such as optimal timing for valve and stopcock adjustments. For example, if the operation is too fast, it may result in an incomplete breakdown of ^{13}C -GLC, inadequate neutralization, and insufficient temperature reduction. Conversely, an unnecessarily extended operation will lead to a low signal intensity in imaging studies due to the rapid decay nature of hyperpolarization to thermal equilibrium. Moreover, the success of the procedures depends on the seamless incorporation of a sophisticated team, with each member responsible for specific roles in the procedures. However, with the efforts in configuring the essential equipment settings and establishing the research team, the protocol will become valuable for conducting tissue pH studies in various phenotypes and conditions. Meanwhile, it is also worth exploring the feasibility of creating a more automated control system to enhance the precision of operation and eliminate potential errors.

TROUBLESHOOTING

Problem 1

The solid-state polarization buildup of ^{13}C -GLC is slow, yielding a low polarization level of ^{13}C -GLC.

Potential solution

- Optimize the parameters for microwave frequency and power sweeps to ensure the accurate use of frequency and power for sample polarization.

Problem 2

The pH of Tris/DCl/EDTA/D₂O buffer (1.5 M) significantly deviates from the specified range of pH 4.0–7.5.

Potential solution

- If the pH exceeds 7.5, incrementally add the diluted DCl solution to bring it back within the specified range.
- Reanalyze the concentration of the concentrated DCl precisely through dilution and then titration with standard NaOH solution. Use the corrected concentration in the calculation for the preparation.

Problem 3

The final pH of ^{13}C -bicarbonate formula exceeds pH 9, and the volume of the final injection solution is less than 35 mL.

Potential solution

This issue indicates insufficient neutralization, possibly due to the incomplete melting of the frozen Tris-DCl buffer in the 2-neck round bottom flask in the cooling bath. Consider this a failed procedure and should not inject the solution into the patient. In the next run, it is essential to exercise caution to ensure the complete thawing of the buffer before proceeding to the next step.

Problem 4

The removal of AH111501 is incomplete, as evidenced by the green color in the solution.

Potential solution

If this is not a manufacturing defect of the C18 cartridge, the issue arises from inadequate temperature reduction of the solution in the cooling bath. A high temperature (> 50°C) can comprise the C18 cartridges and is unsafe for injection. Consider this a failed procedure and should not inject

the solution into the patient. In the next run, it is critical to allocate 15–20 min for the cooling bath to decrease to -18°C .

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Changhua Mu (changhua.mu@ucsf.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Dr. Changhua Mu (changhua.mu@ucsf.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2024.103091>.

ACKNOWLEDGMENTS

R.R.F. acknowledges research funding support from the U.S. Department of Defense W81XWH-19-1-0866 (PC180733) and Bristol Myers Squibb. C.M. acknowledges the Cancer Imaging Research Feasibility Fund sponsored by the Helen Diller Family Comprehensive Cancer Center at the University of California, San Francisco (UCSF), as part of P30 CA082103. All authors acknowledge funding from National Institutes of Health P41EB013598 and S10 (S10OD025077) and access to the core facilities in the UCSF Hyperpolarized MRI Technology Resource Center, notably GE 5T SPINLab polarizers and GE 3T clinical MRI scanner. The authors thank Maya Aslam and Alya Briceno for assistance with the IND and IRB documentation and submission. The authors also thank the Markers Lab at UCSF for fabricating the post-dissolution apparatus using the 3D printer and facility.

AUTHOR CONTRIBUTIONS

C.M. conceptualized, performed, and participated in all the experiments; developed methodology; conducted data analysis; created and validated the protocol; prepared the SOPs and the IND application documents; wrote the original manuscript; and secured feasibility funds. X.L. designed the HP ^{13}C MRI RF pulse sequence, acquired and reconstructed imaging data, and wrote, reviewed, and edited the corresponding MRI contents. A.R. and J.S. reviewed and edited the SOPs and IND documents and participated in PQ runs. E.E., D.D., R.D.S., and S.A. constituted the pharmacy team for executing the preparation procedures for the clinical studies, participated in PQ runs, and fulfilled project administration work. S.D. computer-aided and designed the structure of the post-dissolution rack and 3D printed and assembled it. H.N., P.E.Z.L., R.B., D.B.V., J.K., and D.M.W. advised the projects. R.R.F. conceptualized and supervised the project, secured research funding, and wrote, edited, and revised the manuscript. All authors read, discussed, edited, and revised the manuscript.

DECLARATION OF INTERESTS

Some elements of the original work are published in Mu et al. ACS Sens. 8, 4042–4054. 10.1021/acssensors.3c00851.

P.E.Z.L. receives research support from GE Healthcare. R.R.F. receives research funding from Bristol Myers Squibb.

REFERENCES

- Mu, C., Liu, X., Kim, Y., Riselli, A., Korenchan, D.E., Bok, R.A., Delos Santos, R., Sriram, R., Qin, H., Nguyen, H., et al. (2023). Clinically translatable hyperpolarized ^{13}C bicarbonate pH imaging method for use in prostate cancer. *ACS Sens.* 8, 4042–4054. <https://doi.org/10.1021/acssensors.3c00851>.
- Gatenby, R.A., Gawlinski, E.T., Gmitro, A.F., Kaylor, B., and Gillies, R.J. (2006). Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res.* 66, 5216–5223. <https://doi.org/10.1158/0008-5472.CAN-05-4193>.
- Webb, B.A., Chimenti, M., Jacobson, M.P., and Barber, D.L. (2011). Dysregulated pH: a perfect storm for cancer progression. *Nat. Rev. Cancer* 11, 671–677. <https://doi.org/10.1038/nrc3110>.
- Mu, C., Korenchan, D.E., Wang, S., Wilson, D.M., and Flavell, R.R. (2021). Tumor Microenvironment Biosensors for Hyperpolarized Carbon-13 Magnetic Resonance Spectroscopy. *Mol. Imaging Biol.* 23, 323–334. <https://doi.org/10.1007/s11307-020-01570-0>.
- Gillies, R.J., Liu, Z., and Bhujwalla, Z. (1994). ^{31}P -MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate. *Am. J. Physiol.* 267, C195–C203. <https://doi.org/10.1152/ajpcell.1994.267.1.C195>.
- Gillies, R.J., Raghunand, N., Garcia-Martin, M.L., and Gatenby, R.A. (2004). pH imaging. A review of pH measurement methods and applications in cancers. *IEEE Eng. Med. Biol. Mag.* 23, 57–64.
- Stubbs, M., Bhujwalla, Z.M., Tozer, G.M., Rodrigues, L.M., Maxwell, R.J., Morgan, R., Howe, F.A., and Griffiths, J.R. (1992). An assessment of ^{31}P MRS as a method of measuring pH in rat tumours. *NMR Biomed.* 5, 351–359. <https://doi.org/10.1002/nbm.1940050606>.
- Anemone, A., Consolino, L., Arena, F., Capozza, M., and Longo, D.L. (2019). Imaging tumor acidosis: a survey of the available techniques for mapping in vivo tumor pH. *Cancer Metastasis Rev.* 38, 25–49. <https://doi.org/10.1007/s10555-019-09782-9>.
- Korenchan, D.E., Flavell, R.R., Baligand, C., Sriram, R., Neumann, K., Sukumar, S., VanBrocklin, H., Vigneron, D.B., Wilson, D.M., and Kurhanewicz, J. (2016). Dynamic nuclear polarization of biocompatible (^{13}C -enriched carbonates for in vivo pH imaging. *Chem. Commun.* 52, 3030–3033. <https://doi.org/10.1039/c5cc09724j>.
- Gallagher, F.A., Kettunen, M.I., Day, S.E., Hu, D.-E., Ardenkjaer-Larsen, J.H., Zandt, R.I., Brindle, K.M., Jensen, P.R., Karlsson, M., Golman, K., and Lerche, M.H. (2008). Magnetic resonance imaging of pH in vivo using hyperpolarized ^{13}C -labelled bicarbonate. *Nature* 453, 940–943. <https://doi.org/10.1038/nature07017>.
- Korenchan, D.E., Bok, R., Sriram, R., Liu, K., Santos, R.D., Qin, H., Lobach, I., Korn, N., Wilson, D.M., Kurhanewicz, J., and Flavell, R.R. (2019). Hyperpolarized in vivo pH imaging reveals grade-dependent acidification in prostate cancer. *Oncotarget* 10, 6096–6110. <https://doi.org/10.18632/oncotarget.27225>.
- Korenchan, D.E., Gordon, J.W., Subramaniam, S., Sriram, R., Baligand, C., VanCriekeing, M., Bok, R., Vigneron, D.B., Wilson, D.M., Larson, P.E.Z., et al. (2019). Using bidirectional chemical exchange for improved hyperpolarized [^{13}C]bicarbonate pH imaging. *Magn. Reson. Med.* 82, 959–972. <https://doi.org/10.1002/mrm.27780>.
- Liu, X., Cui, D., Xu, D., Bok, R., Wang, Z.J., Vigneron, D.B., Larson, P.E.Z., and Gordon, J.W. (2024). Dynamic T2* relaxometry of hyperpolarized [^{13}C]pyruvate MRI in the human brain and kidneys. *Magn. Reson. Med.* 91, 1030–1042. <https://doi.org/10.1002/mrm.29942>.
- Jackson, J.I., Meyer, C.H., Nishimura, D.G., and Macovski, A. (1991). Selection of a convolution function for Fourier inversion using gridding [computerised tomography application]. *IEEE Trans. Med. Imaging* 10, 473–478. <https://doi.org/10.1109/42.97598>.
- Tang, S., Meng, M.V., Slater, J.B., Gordon, J.W., Vigneron, D.B., Stohr, B.A., Larson, P.E.Z., and Wang, Z.J. (2021). Metabolic imaging with hyperpolarized ^{13}C pyruvate magnetic resonance imaging in patients with renal tumors-Initial experience. *Cancer* 127, 2693–2704. <https://doi.org/10.1002/cncr.33554>.
- Liu, X., Tang, S., Mu, C., Qin, H., Cui, D., Lai, Y.-C., Riselli, A.M., Delos Santos, R., Carvajal, L., Gebrezgabier, D., et al. (2022). Development of specialized magnetic resonance acquisition techniques for human hyperpolarized [^{13}C , $^{15}\text{N}_2$] urea + [^{13}C] pyruvate simultaneous perfusion and metabolic imaging. *Magn. Reson. Med.* 88, 1039–1054. <https://doi.org/10.1002/mrm.29266>.