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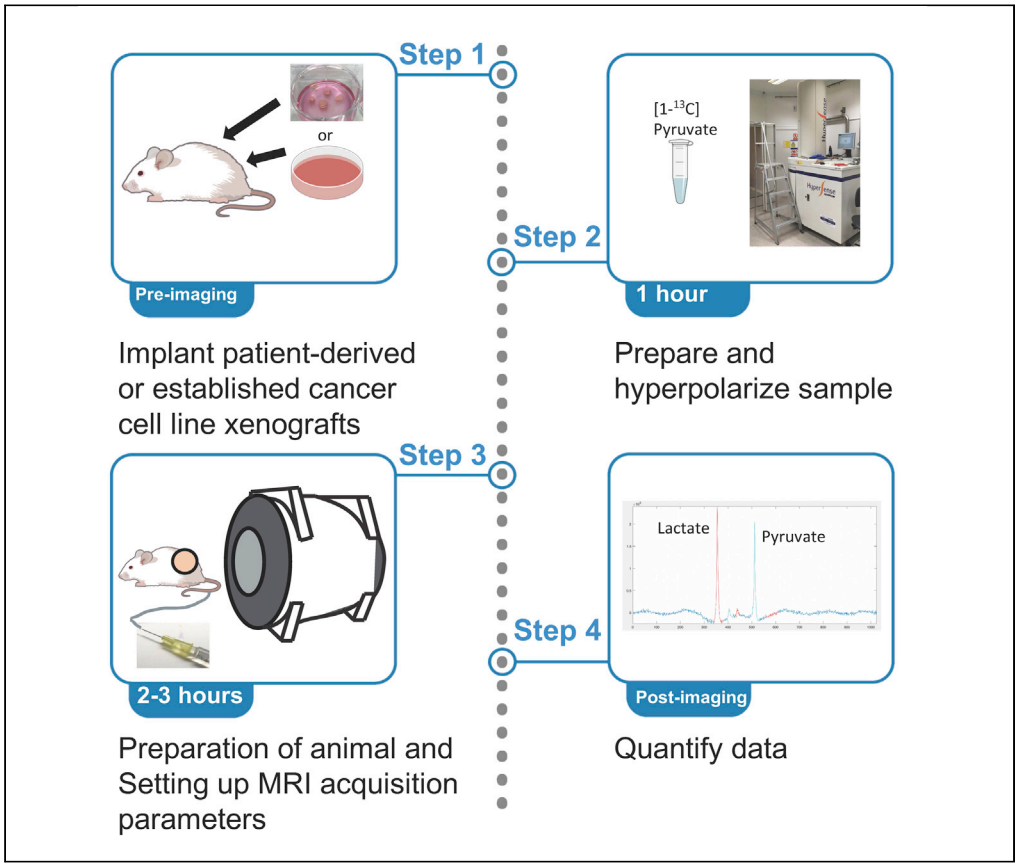
Metabolic imaging with hyperpolarized [1-^{13}C] pyruvate in patient-derived preclinical mouse models of breast cancer



^{13}C nuclear spin hyperpolarization can increase the sensitivity of detection in an MRI experiment by more than 10,000-fold. ^{13}C magnetic resonance spectroscopic imaging (MRSI) of hyperpolarized ^{13}C label exchange between injected [1-^{13}C]pyruvate and the endogenous tumor lactate pool can be used clinically to assess tumor grade and response to treatment. We describe here an experimental protocol for using this technique in patient-derived and established cell line xenograft models of breast cancer in the mouse.

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Highlights

Increased ^{13}C label exchange reflects enhanced tumor aerobic glycolysis

Higher levels of ^{13}C label exchange are observed in more aggressive tumors

It can be used to image treatment response to drugs perturbing lower glycolysis

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Metabolic imaging with hyperpolarized [1-^{13}C] pyruvate in patient-derived preclinical mouse models of breast cancer

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SUMMARY

^{13}C nuclear spin hyperpolarization can increase the sensitivity of detection in an MRI experiment by more than 10,000-fold. ^{13}C magnetic resonance spectroscopic imaging (MRSI) of hyperpolarized ^{13}C label exchange between injected [1-^{13}C]pyruvate and the endogenous tumor lactate pool can be used clinically to assess tumor grade and response to treatment. We describe here an experimental protocol for using this technique in patient-derived and established cell line xenograft models of breast cancer in the mouse.

For complete details on the use and execution of this protocol, please refer to Ros et al. (2020).

BEFORE YOU BEGIN

The technique has been used with patient-derived xenograft models of breast cancer, where we used it to detect response to PI3K alpha inhibitors (Ros et al. 2020), as had been done previously in established cell line xenograft models of glioblastoma (Venkatesh et al., 2012) and breast cancer (Ward, Venkatesh et al. 2010, Ros et al. 2020). The technique has been translated to the clinic (Nelson, Kurhanewicz et al. 2013, Gallagher, Woitek et al. 2020). We anticipate that in the longer term it could be used to detect early treatment response of ER+ breast cancer patients harboring PIK3CA mutations to drugs targeting the PI3K-Akt pathway in early-stage clinical trials of new drugs and subsequently in the clinic to guide treatment in individual patients.

Alternatives: We have used patient-derived breast cancer xenografts and xenografts produced from established human breast cancer cell lines in NSG mice. The technique can also be used with other mouse and rat models of cancer and indeed animal models of other diseases.

Preparation of solutions

Timing: [less than 1 h]

1. Preparation of [1-^{13}C]pyruvate for hyperpolarization
   a. To 5 g of [1-^{13}C]pyruvic acid (~4.1 mL, ~14 M) add 83.5 mg of tris (8-carboxy-2,2,6,6-tetra-(hydroxyethyl)-benzo-[1,2-4,50]-bis-(1,3)-dithiole-4-yl)-methyl sodium salt (OX063) (15 mM) and vortex for a minute. [1-^{13}C]pyruvic acid is the free acid, which is a liquid at room temperature. Note, the sodium salt cannot be used because of limited solubility.
   b. Aliquot 500 µL into Eppendorf tubes, using foil to prevent exposure to light. This will prevent photolysis of the radical. The solution can be stored for a month at 4°C. We recommend storing aliquots at −80°C.

2. Preparation of the buffer for dissolving the frozen hyperpolarized [1-^{13}C]pyruvate solution. Prepare a 40 mM HEPES, 94 mM NaOH, 30 mM NaCl and 50 mg/L EDTA solution, pH 12. This solution can be kept at room temperature for a month. The final pyruvate concentration in the injected solution is 83 mM.
   a. Weigh out 4.77 g of HEPES, 1.88 g of NaOH, 0.876 g of NaCl and 0.025 g of EDTA.
   b. Add to 500 mL MilliQ Water.
   c. Mix by stirring, until the solution is clear.
   d. Check pH and adjust to pH 12, with 1M HCl or 1M KOH.
   e. Filter the solution by vacuum filtration using a 0.2 µm pore size filter.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Patient-derived xenografts (PDX) for explants	(Bruna, Rueda et al. 2016) https://caldaslab.cruk.cam.ac.uk/bcape/	N/A
Human breast cancer cell lines	ATCC	MCF7: Cat# HTB-22; RRID: CVCL_0031 T47D: Cat# HTB-133; RRID: CVCL_0553 MDAMB231 Cat# HTB-22; RRID: CVCL_0062
Chemicals, peptides, and recombinant proteins		
Iodine	Ecolab	Cat#30377410
Tissue glue GLUture	Zoetis	Cat#ZE-10013
Vetivex	Dechra	Cat#1034/4079
Heparin	Wockhardt UK Ltd	Cat#PL29831/0105
[1-^{13}C]Pyruvic acid	Cambridge Isotope Laboratories	Cat#CLM-8077
OXO63	GE Healthcare Chicago, IL, USA	N/A
Dotarem	Guerbert	NDC 67684-2000
HEPES	Sigma	Cat#H3375
NaOH	Sigma	Cat#S8045
NaCl	AMRESCO	Cat#0241
EDTA	Fisher	Cat#BP120
Matrix, Basement membrane; Corning; Matrigel; Growth Factor Reduced; Phenol Red-Free	BD Biosciences	Cat#356231
Other		
Single use vacuum filter unit, e.g., Nalgene Rapid-Flow™ Media-Plus Sterile Filter Unit with 90 mm nylon membrane, 0.2 µm, 500 mL funnel with 500 mL receiver	Thermo Fisher Scientific	Cat#163-0020
Petri dishes	Corning	Cat#430167
6-Well dishes	Costar	Cat#3516
Cryovials	Thermo Fisher Scientific	Cat#377267
Eppendorfs	Thermo Fisher Scientific	Cat#11926955

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fine bore polyethene tubing 0.28 mm ID 0.61 mm	Smiths Medical	Cat#800/100/100
1 mL Syringe	BD Plastipak	Cat#00382903031726
30G needle	BD Microlance	Cat# 301700
DMEM	Gibco	Cat#41965-039
FBS heat inactivated	Gibco	Cat#A3840401
DMSO for tissue culture	Sigma	Cat#D8418
0.25% Trypsin-EDTA	Gibco	Cat#25200-056
3.35T Hypersense polarizer	Oxford Instruments	N/A
7.0T MRI instrument	Agilent	N/A
$^{13}C/^{1}H$ volume transmit coil, 42 mm diameter	RAPID Biomedical	N/A
^{13}C receiver coil, 20 mm diameter	RAPID Biomedical	N/A
Active decoupling unit	RAPID Biomedical	N/A
Experimental models: Organisms/strains		
Mouse: NSG: NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ	Charles River	Cat# 614; RRID: IMSR_JAX:005557
Software and algorithms		
MATLAB	MathWorks	http://www.mathworks.com/products/matlab/; RRID: SCR_001622
Script for analysis and examples		Apollo-University of Cambridge Repository: https://doi.org/10.17863/CAM.55683; https://doi.org/10.17863/CAM.62058

STEP-BY-STEP METHOD DETAILS

Generation of patient-derived xenografts

Timing: [less than 1 hr] for step 1

Timing: [~15 min] for step 2

Timing: [less than 30 min per mouse] for step 3

This step describes how to store patient-derived material that has been fragmented into pieces for future implantation and generation of subcutaneous xenografts in NSG mice. The experiments should be performed in accordance with relevant local guidelines and regulations.

1. Storing patient material for future implantation

⚠ CRITICAL: Being as fast as possible when taking tumor material for storage will increase the probability of successful subsequent tumor implantation.

a. Euthanize the tumor-bearing animal by exposure to gradually increasing concentrations of carbon dioxide and then surgically resect the xenograft. We implant xenografts subcutaneously, although they can also be implanted orthotopically in the mammary fat pad (Vareslija, Cocchiglia et al. 2017).
b. Clean the tumor by placing it in a petri dish containing DMEM (without any supplements).
c. Cut the tumor into ~1 cm^3 pieces, see Figure 1.
d. Store the 1 cm^3 fragments in 1 mL 1% DMSO - FBS solution (heat inactivated) in cryovials, usually 5 to 10 pieces in 1 mL of media.
e. Slowly freeze the samples in a container that allows a cooling rate of 1°C/min until the temperature reaches −80°C.

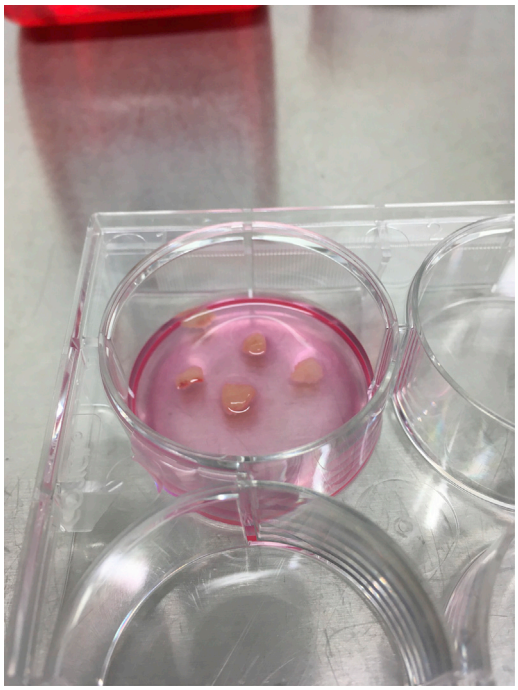

Figure 1. Tumor pieces collected from a xenograft before cryopreservation

f. After 24 hours, store the vials in a −80°C freezer, or in liquid nitrogen.

2. Preparing tumor material for implantation

⚠ CRITICAL: Implanting the material as fast as possible following thawing will increase the probability of successful subsequent tumor implantation. Avoid implanting fragments that contain traces of fat or are spongy. Defrost the Matrigel the day before at 4°C, and add Matrigel immediately prior to implantation.

a. Defrost the cryovials containing the tumor fragments (usually 5 to 10 pieces) by placing the vial in a tissue culture incubator at 37°C for 5 min, see Figure 1.
b. Open the vial in a cell culture hood and transfer the fragments into a petri dish containing 1 mL of DMEM medium (lacking supplements or FBS) to wash out the DMSO.
c. Transfer the fragments into an Eppendorf tube containing 500 µl of DMEM (lacking supplements or FBS).
d. Once the mouse is prepared as in 3 immerse the fragment in Matrigel and proceed to implantation, one 1 cm^3 piece per mouse.

3. Implantation of tumor material

⚠ CRITICAL: Implanting the material as fast as possible will increase the probability of successful tumor implantation. We recommend implanting up to 5 mice in one session.

a. Anesthetize 6–8-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Charles River) mice using 1%–2% isoflurane (Isoflo, Abbotts Laboratories Ltd, Maidenhead, UK) in air/O_2 (75/25% vol/vol, 2 L/min).
b. Administer analgesia, for example Vetergesic (Alstoe, York, UK), and Rimadyl LA (Pfizer, New York, USA).
c. Shave the mouse abdomen with clippers, see Figure 2.
d. Scrub the abdomen with iodine to sterilize.

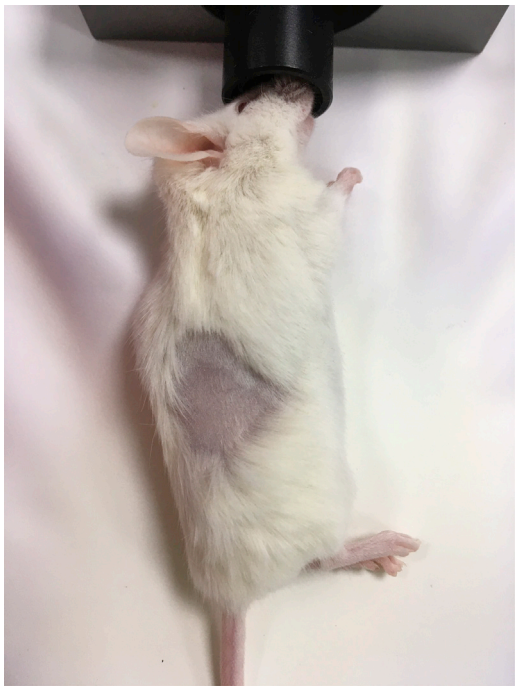

Figure 2. Shaved area in the mid-lateral abdomen
This is ready to be cleaned and an incision made for implantation of a patient-derived tumor fragment.

e. Make an incision with scissors parallel to the long axis of the mouse body. See Figures 2 and 4 for the site of tumor implantation. Implanting the tumor at this site reduces the effect of respiratory motion on acquisition of ^{13}C magnetic resonance images and spectra. Try to cut as small an incision as possible.
f. Clean and open the incision with a cotton wool bud soaked in PBS.
g. Loosen the skin with scissors and insert one fragment per mouse.
h. Take each side of the skin and cover the implanted tissue, closing the wound with tissue glue (GLUture).
i. Let the animal recover in a heated box at 37°C.

Generation of breast cancer cell line xenografts

Timing: [~15 min]

4. Preparing cell material for implantation (into 5 animals in this example)

⚠ CRITICAL: Defrost Matrigel the day before at 4°C. As it can solidify very quickly Matrigel is added to the cell suspension immediately prior to subcutaneous injection of the cell preparation.

a. Wash cells growing in 3 to 5 T-75 flasks, and which are 80% confluent, with 5 mL of PBS per flask.
b. Add 1 mL of 0.25% trypsin-EDTA solution per flask.
c. Incubate in a tissue culture incubator at 37°C for 5 to 10 min until the cells have detached.
d. Add 5 mL per flask of medium containing FBS, to inactivate the trypsin, and place the suspension in a 50 mL falcon tube.
e. Centrifuge at 1000 × *g* for 3 min.
f. Discard the supernatant and resuspend the cells in 20 mL of fresh medium.

g. Use a small volume to calculate cell viability and density. This could be done by using, for example, a Vi-CELL Blu analyzer (Beckman Coulter) that uses the Trypan Blue Dye Exclusion method for assessing cell viability.
h. Take the volume that corresponds to 5 × 10^6 cells and place in a 15 mL falcon tube.
i. Centrifuge at 1000 × *g* for 3 min.
j. Discard the supernatant and resuspend in 500 μl of PBS.
k. Transfer to an Eppendorf tube and place on ice.
l. Add Matrigel (1:1 vol:vol) and keep the tube on ice. The samples are now ready to be implanted; 150 μl to 200 μl per mouse via subcutaneous injection, in the same location as in 3 g – see Figures 2 and 4, using a 1 mL syringe and a 30G needle.

Note: Some breast cancer cell lines require estradiol for growth. This can be provided via the drinking water or via prior implantation of an estrogen pellet.

- If using estradiol pellets we recommend implanting one pellet per mouse (0.72 mg 17β-Estradiol 0.72 mg, 90 Day Release from Innovative Research of America (Cat# NE-121)). Subcutaneous implantation is performed as described in 3. We recommend implantation on the opposite flank from the tumor, four to six days prior to subcutaneous cell/tumor fragment implantation.
- If using estradiol in the drinking water (5 μg/mL). Make up a stock of ß-estradiol (Cat#E2758 from Sigma-Aldrich) in ethanol (Cat#51976 from Sigma-Aldrich) at 5 mg/mL and then add 300 μL of this stock to 300 mL of drinking water. We recommend starting treatment four to six days prior to subcutaneous cell/tumor fragment implantation.

Imaging

Timing: [2 to 3 hours per mouse]

Timing: [15 min] for step 5

Timing: [~10 min] for step 6

Timing: [~45 min] for step 7

Timing: [~45 min] for step 8

Timing: [~10 min] for step 9

Timing: [~10 min] for step 10

5. Preparing the mouse for the imaging session
We do not routinely fast animals before imaging. However, low intra-individual variability and a higher [1-^{13}C]lactate/[1-^{13}C]pyruvate signal ratio has been reported for a murine lymphoma model implanted subcutaneously if the mice were fasted for 18 hours prior to imaging (Serrao, Rodrigues et al. 2016).
a. When a tumor is approximately 0.6–1.2 cm^3 in volume, measured using calipers (1/2 × (length × $width^2$)) it is ready for imaging. For established breast cancer cell line tumor models this volume is reached typically after 2–4 months, for breast cancer PDX models this could be 2–8 months, depending on the model. Tumors smaller than 0.6 cm^3 can be imaged, although low signal-to-noise ratios might compromise the results obtained.
b. Prepare a tail vein cannula using fine bore polyethene tubing. At one end insert a 30G needle and attach a 1 mL syringe containing Vetivex and Heparin, prepared previously by adding

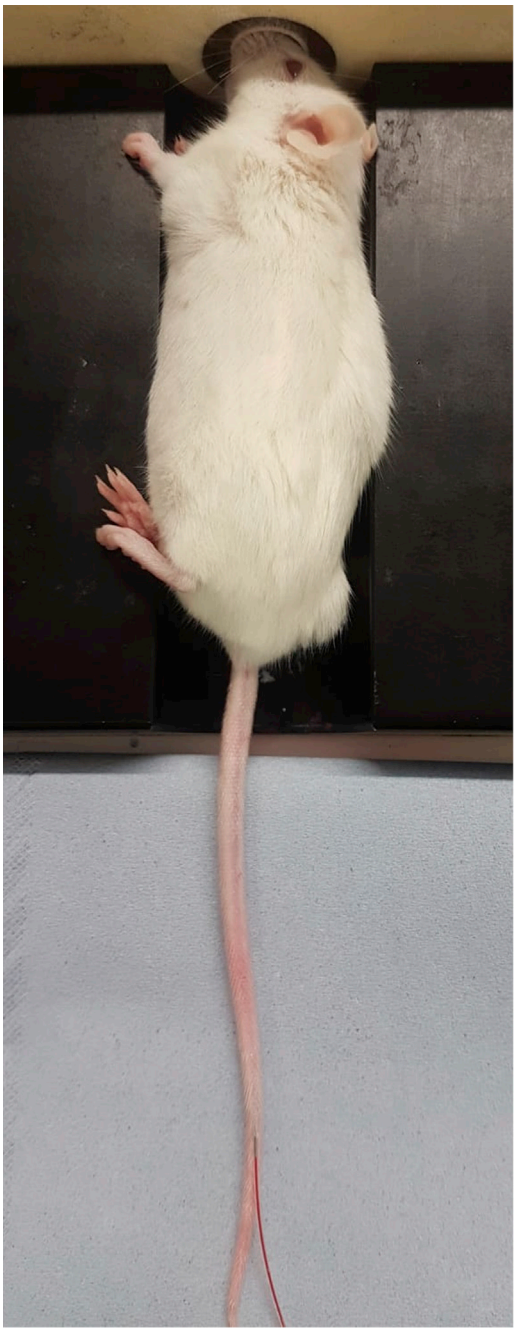

Figure 3. Tail vein cannulation of a mouse with a tumor

5000 units of Heparin to 100 mL of Vetivex. At the other end insert the blunt end of a 30G needle, after removing the needle from the plastic mount.

c. Anesthetize the mouse using 1%–2% isoflurane in air/O_2 (75/25% vol/vol, 2 L/min).

d. Insert the cannula prepared in b into the lateral tail vein.

e. If cannulation is successful, you will usually see blood refluxing (Figure 3). Fix the cannula using some autoclave tape (Figure 4).

f. Take the plunger of a 1 mL syringe and place it alongside the tail (Figure 4). Fix in place with autoclave tape (this will ensure that the tail does not move during injection).

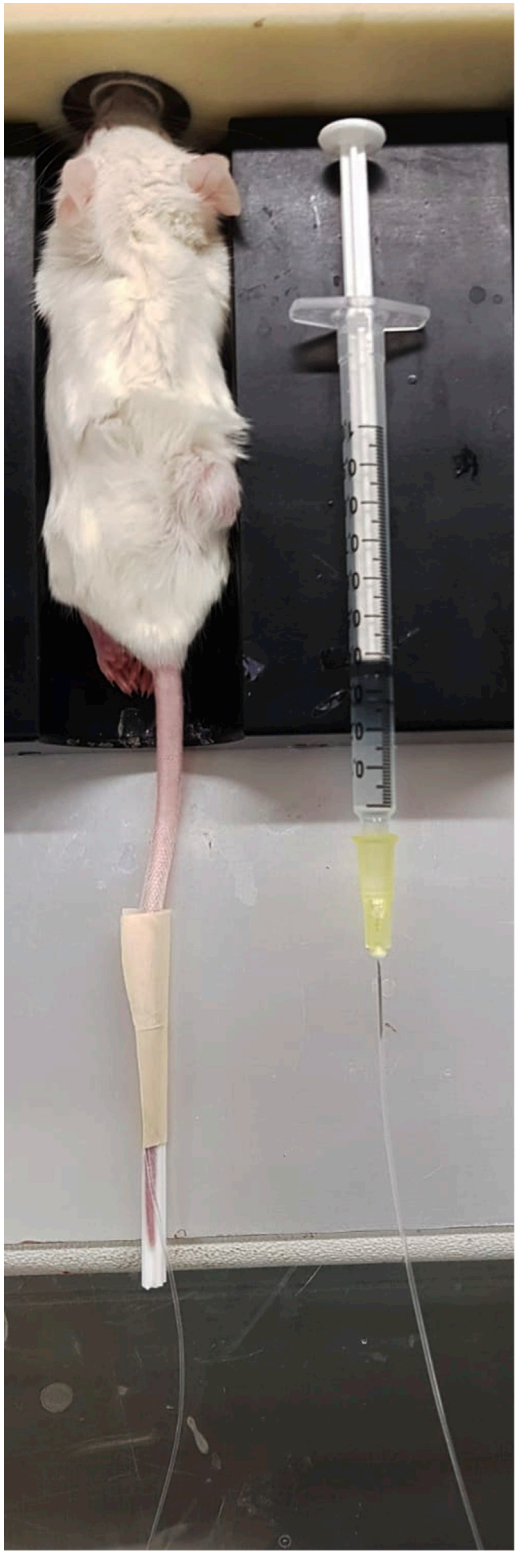

Figure 4. Fixation of the cannula using autoclave tape

6. Preparing the pyruvate sample for hyperpolarization
   a. Weigh out 44 mg (36 μL) of the mixture of pyruvic acid, containing 15 mmol/L of trityl radical (tris (8-carboxy-2,2,6,6-tetra-(hydroxyethyl)-benzo-[1,2-4,50]-bis-(1,3)-dithiole-4-yl)-methyl sodium salt (OX063); GE Healthcare, Amersham, UK), prepared as described above and transfer to an Eppendorf tube. This pyruvic acid and trityl radical solution can be stored at −80°C for several months, but if defrosted the aliquot can be stored at 4°C and should be used within a month.
   b. Add 1 μL of a 50 mM gadolinium chelate solution: (gadoterate meglumine (Dotarem), Guerbet, Roissy, France) prepared previously in water to the Eppendorf tube to give ~1.35 mM gadoterate meglumine and mix well.
   c. Pipette the solution into a hyperpolarizer sample cup, avoiding the formation of bubbles (Figure 5).
7. Polarizing the pyruvate sample
   a. Cool down the 3.35 T Oxford Instruments Hypersense polarizer (Figure 6).
   b. Insert the sample cup.
   c. The microwave frequency should be swept for a representative sample to determine the optimal polarization frequency. For these samples a 180 s irradiation at 4 MHz intervals over a range of frequencies. On our system 94.09 to 94.13 GHz is sufficient, although the user should make sure this range contains the frequency given in the installation information sheet for each hyperpolariser. Frequency sweeps should be started once the helium level in the variable temperature insert (VTI) has reached the set point (usually 55%).
   d. When an optimal frequency has been determined (i.e., the frequency that gives the maximum solid state ^{13}C polarization) this can be used for all subsequent measurements with the stock solution of [1-^{13}C]pyruvic acid and OX063.
   e. The sample is polarized at the optimal microwave frequency (approx. 94.1GHz) for at least half an hour with sampling of the solid state ^{13}C NMR signal every 300 s. A 45-min polarization at the optimal frequency and 1.25°K should achieve >90% of the possible polarization (Figure 7, for more detail see Figure 10). The solid-state polarization build-up can be viewed in the Hypersense software, although optimal polarization is reached after 30 to 45 min, and as shown in Figure 10 the polarized sample can be kept for longer until ready to be used.

Figure 5. Sample cup for an Oxford Instruments Hypersense polarizer

Figure 6. The Hypersense Hyperpolariser

8. Placing the animal in the magnet and setting up the transmit and receiver coils.
   This step can be performed while the sample is polarizing. Spectra in this publication were acquired at 7.0 T using an Agilent scanner (Palo Alto, CA) running Vnmrj 3.1 software. However, similar experiments have been performed on other vendor instruments at fields of between 1 T and in excess of 9.4 T ((Day, Kettunen et al. 2007, Hill, Orton et al. 2013, Tee, DiGialleonardo et al. 2016). We acquire the MR data using a 42 mm diameter $^{13}C/^{1}H$ volume transmit coil (Rapid Biomedical, Rimpar, Germany) with a single loop 20 mm diameter ^{13}C receiver coil (Rapid

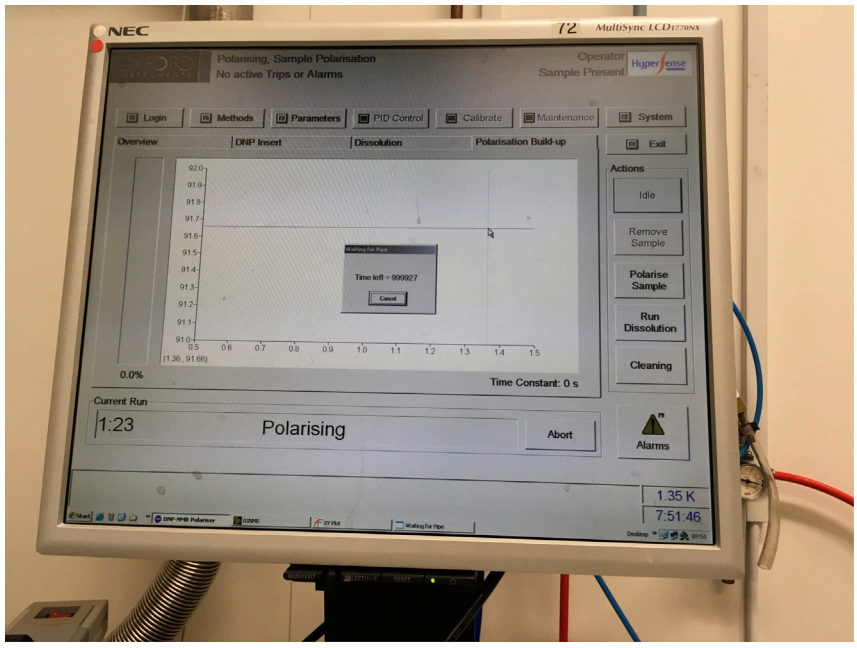

Figure 7. The Hypersense software interface at the start of polarization

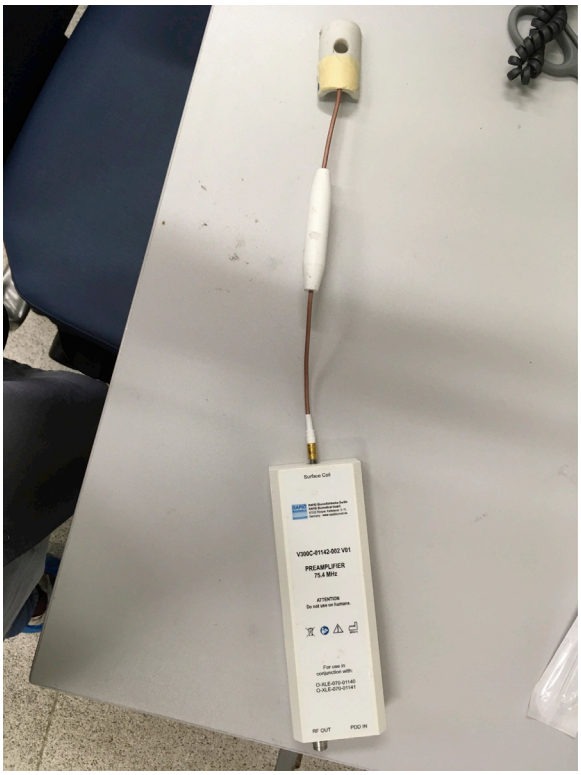

Figure 8. ^{13}C receive coil with preamplifier interface

Biomedical) (Figure 8). Anatomical proton images are acquired using the ^{1}H volume coil acting in transmit and receive mode and the ^{13}C data are acquired using the volume coil in transmit mode and the surface coil in receive mode. Both frequency channels are connected to an active decoupling unit (Rapid Biomedical) for detuning/retuning the coils during the ^{13}C pulse sequence.

a. Place the animal on the bed, with a pad underneath the chest for respiratory monitoring and a rectal thermometer to monitor body temperature (Figure 9).
b. Inhalational anesthesia is provided via a nose cone. A 1:2 ratio of air:oxygen at 2 L per minute and containing 1%–2% isoflurane is sufficient to maintain anesthesia.
c. Vital signs are monitored using a small animal monitoring system (Small Animal Instruments Inc., NY, USA) that records respiration rate and body temperature. Anesthesia can be adjusted to maintain respiratory rate at 40 to 80 breaths/min and body temperature at approximately 37°C. We maintain body temperature by passing warm air over the animal but a water-heated bed can also be used.
d. With the mouse at the magnet isocentre, the volume coil is tuned for both proton and carbon frequencies, the carbon coils are then disconnected from the pre-amplifier to reduce noise for proton imaging.
e. The proton carrier frequency and coil transmit power are determined and manual shimming is used to optimize the water linewidth using a non-localized pulse and acquire sequence.
f. A set of anatomical MR Images are then acquired with the following parameters: A localizer image is acquired in three planes (axial, coronal and sagittal) to confirm positioning of the tumor close to the magnet isocenter. Three sets of three spoiled gradient echo images, with an echo time of 3 ms and a repetition time of 30 ms, are acquired with a pulse flip angle of 20°. Each data set consists of 3 slices, 2 mm thick with 2 mm spacing between slices, a field of view of 100 mm × 100 mm acquired into 128 × 128 data points.
g. A set of axial T_2-weighted images are acquired from the abdomen. Fast spin echo images, 15 contiguous slices, each 2 mm thick, are acquired with a 40 mm × 40 mm field-of-view into

Figure 9. Positioning the ^{13}C receiver coil over the tumor

256 × 256 data points. An effective echo time of 48 ms is used with a train of 8 echoes and the center of k-space collected at the 4th echo. Fat saturation is also included and the repetition time is 2 s.

h. A set of sagittal T_2-weighted images are acquired from the full length of the animal. The acquisition parameters are the same as for the axial T_2-weighted images but the field-of-view is 80 mm × 40 mm acquired into 512 × 256 data points with 18 slices (to achieve full mouse coverage), a 49 ms effective echo time and a repetition time of 2.05 s.

i. These anatomical images (axial and sagittal) can now be used for planning the slice-selective pulse and acquire acquisition sequence used to shim on this volume.

j. Set up a pulse and acquire sequence using a Shinnar-Le Roux excitation pulse and a gradient combination to give excitation of a specified slice, with a thickness between 6 and 10 mm depending on tumor size. The slice can be rotated from an initially coronal plane so that it maximizes coverage of the tumor while minimizing overlap with the surrounding body. Acquiring ^{1}H spectra with this sequence in manual shimming mode allows optimization of the shim to give water linewidths of between 40 and 100 Hz by adjusting the first and second order shims.

k. Serial hyperpolarized ^{13}C spectra are then acquired from this same volume with a similar pulse sequence. In this publication we describe acquisition of ^{13}C spectra, but ^{13}C spectroscopic images can also be acquired (see (Day, Kettunen et al. 2007)). The ^{13}C channel of the volume coil is reconnected and the active decoupler unit set for ^{13}C acquisition. The carrier frequency is set to a predetermined value close to approximately 179 ppm and the flip angle is preset to a nominal 5°, as previously calibrated on a phantom. This value is not varied between mice, with no adjustment for different coil loading by different animals. The phantom consists of a 5 mm diameter glass tube containing 1.2 mL of a 4 M sodium [1-^{13}C]lactate solution.

9. Running the dissolution process, injecting the hyperpolarized sample and acquiring ^{13}C spectra

a. Once optimal polarization is reached, usually after 30 to 45 min (Figure 10), place a "sample catcher" next to the MRI magnet to receive the hyperpolarized [1-^{13}C]pyruvate sample. The catcher has a 3 way tap that can be used to fill a 1 mL syringe (Figure 11).

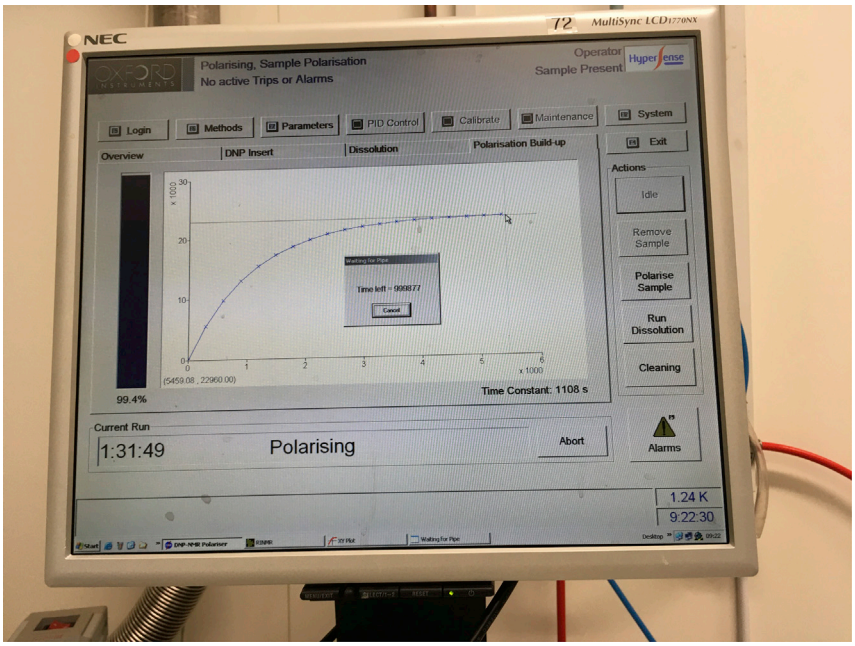

Figure 10. The solid-state polarization build-up can be viewed in the Hypersense software

Optimal polarization is reached after 30 to 45 min, and as shown here the polarized sample can be kept for longer until ready to be used.

b. Place 6 mL of dissolution buffer into the Hypersense pressure vessel. Close the tap at the top of the pressure vessel and move the dissolution stick into position.
c. The sample will be dissolved at 180 °C but will cool rapidly to around 22°C–25°C as it arrives in the sample catcher. Draw the hyperpolarized [1-^{13}C]pyruvate solution into the syringe and push back and withdraw 5 times rapidly to mix the solution (see Methods video S1, step 9). Remove the syringe and inject 0.3 mL (final pyruvate concentration in the injected solution is 83 mM) into the mouse by changing the syringe at the end of the cannula with the new syringe containing the hyperpolarized [1-^{13}C]pyruvate sample.

d. Start the MR acquisition at the start of injection (this usually requires a second person). The time between dissolution and injection is usually less than 30 seconds. This is a critical step which needs to be performed rapidly as the polarization of the sample decreases with time after dissolution. An extended delay between dissolution and injection into the mouse will result in poor signal-to-noise in the acquired ^{13}C spectra or images.
e. A series of spectra are then acquired from a slice within the tumor every second for three min.
f. Data are acquired with a nominal flip angle of 5° from a 6–10 mm thick slice, excited with a 600 ms sinc pulse. The spectral width is 6010 Hz with the Free Induction Decays (FID) acquired into 1024 data points, with a delay of 0.93 ms between the end of the pulse and the start of signal acquisition.
g. If post-treatment data acquisition is required the position of the slice selected after treatment needs to be as close as possible to that used for pre-treatment data acquisition (Figure 12, from (Ros et al. 2020)).
h. It is possible to check that the experiment has worked during data acquisition. Go to vnmrJ and select the Current Scan box (right top). Type the command wft (Weighted Fourier transform (FT). Weighting applies a window function to FID to enhance resolution or signal-to-noise and reduce truncation artifacts from finite data collection). The spectra acquired will appear and by using the forward and reverse arrows (right top) you will be able to see these over

Figure 11. Sample catcher used to receive the hyperpolarized [1-^{13}C]pyruvate solution

time. If the experiment has worked a pyruvate peak will appear and the lactate peak will build up later. If no signal is detected see the Troubleshooting section.

10. Removing the animal from the magnet and cleaning the system
    a. When data acquisition is complete, remove the mouse from the bed and place it on a heated blanket or plate.
    b. Carefully remove the cannula from the tail vein.
    c. Press the hole until it stops bleeding and let the mouse recover from the anesthesia in a heated box.
    d. Measure the pH of the injected solution with a pH indicator strip. This should usually be 7.5. If the pH is lower than 4 or higher than 8 the imaging results should be discarded since pH can affect the injection. The pH can be measured prior to injection but the resulting delay inevitably results in further loss of polarization. Since an out-of-range pH is an infrequent occurrence, we usually make retrospective pH measurements.
    e. To clean the Hypersense system use a 200 mg/L EDTA solution, as described in the manual.

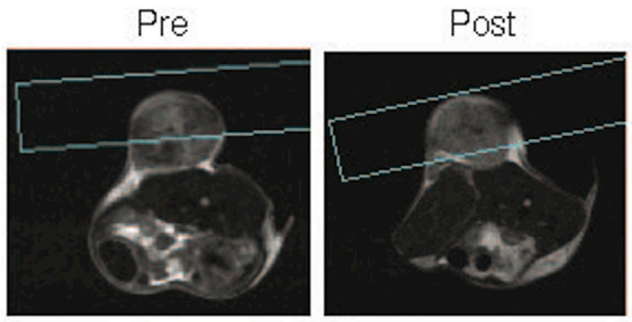

Figure 12. Example of slice selection pre and post-treatment] from (Ros et al. 2020)

Analysis of the acquired data

Timing: [~15 min]

Timing: [~10 min] for step 11

Note: The script 'Matlab_pyrlac_script' is imported into Matlab where it is used to Fourier transform, with line broadening, the acquired spectra and the spectrum with the maximum pyruvate peak intensity is identified. Each spectrum is then referenced to this spectrum. The spectra are phased automatically by maximising the peak height using a zero order phase correction. The first order phase correction is set to 0.125 radians/ppm in order to compensate for the delay between the pulse and the beginning of signal acquisition.

11. Uploading the script and calculating the ratio
    a. Upload the script 'Matlab_pyrlac_script' and fid files. The Matlab script integrates the [1-^{13}C] pyruvate and [1-^{13}C]lactate peaks and sums the integrals over the three min of signal acquisition. The ratio of these summed integrals (lactate/pyruvate) is proportional to the rate of lactate labeling (Hill, Orton et al. 2013). Provided that the pyruvate inflow is captured differences in the time of pyruvate injection and subsequent initiation of signal acquisition have no significant effects on this calculation.
    b. The summed spectrum is baseline corrected by fitting a 15th order polynomial to points selected manually as not containing any metabolite signal and subtracting this baseline from the real part of the spectrum. The regions containing the pyruvate and lactate peaks, which are selected manually, are then summed to give the integrated amplitudes of these two resonances. These provide the numerator and denominator in the following equation

$$\frac{Area\ Under\ the\ Curve\ [1-^{13}C]Lactate}{Area\ Under\ the\ Curve\ [1-^{13}C]Pyruvate}$$

    c. This ratio is then used as a metric for the rate of lactate labeling.

EXPECTED OUTCOMES

We include an example of a dataset, containing serial ^{13}C spectra, which was used in the publication by Ros et al (Cancer Cell. 2020 Oct 12; 38(4): 516–533.e9; https://doi.org/10.1016/j.ccell.2020.08.016). The Matlab script used to analyze these data can be found at Apollo-University of Cambridge Repository: [https://doi.org/10.17863/CAM.55683] and the example dataset at Apollo-University of Cambridge Repository: [https://doi.org/10.17863/CAM.62058].

The folder name for the script is Matlab_pyrlac_script, and the example dataset can be found at MRdata/Figure1F/HCI001_VEH/pre1.

To run the example, copy the raw MR_data folder onto the Desktop, where you will find the example used here. Write the path at the Matlab interface as follows base_path='/Desktop/MRdata/Figure1F/HCI001_VEH/pre1'; experiment_path='/20170223_17600R1pre_pyruvate_tc_TC_20170223_01.fid'

Run the script and a window will appear as in Figure 13. This is the Matlab interface used to define the Pyruvate peak (between point numbers 498 and 533 in this example) and Lactate peak (between point numbers 340 and 380 in this example).

Once you have defined the Pyruvate peak (larger cyan peak in Figure 13) and Lactate peak (red peak in Figure 13) click 'Save and Quit'' (Figure 14).

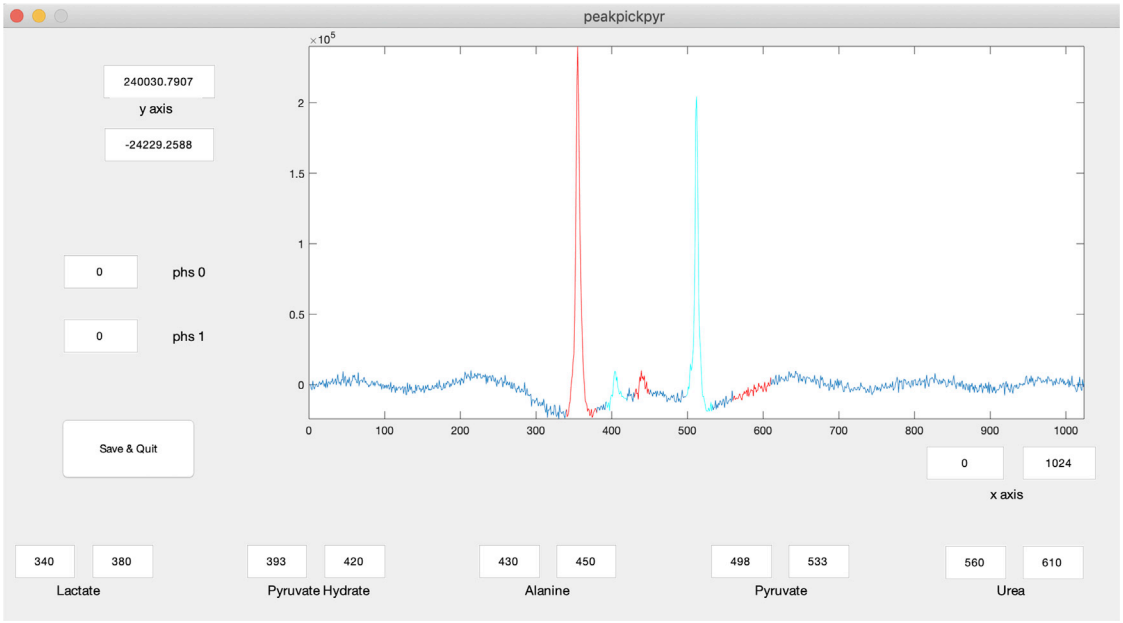

Figure 13. The Matlab interface used to define the Pyruvate and Lactate peaks

The spectrum shown in Figure 15 (Window 1) will appear and you will be able to modify it using the 'View' > 'Figure Palette' in Matlab. In the Workspace the fitted area-under-the-curve (AUC) ratio will be plotted, in this example it is 1.3116 (Figure 16). No estimates of the fitting quality are calculated in the script thus visual inspection of the data is necessary to determine whether there is sufficient signal-to-noise to reliably calculate the ratio.

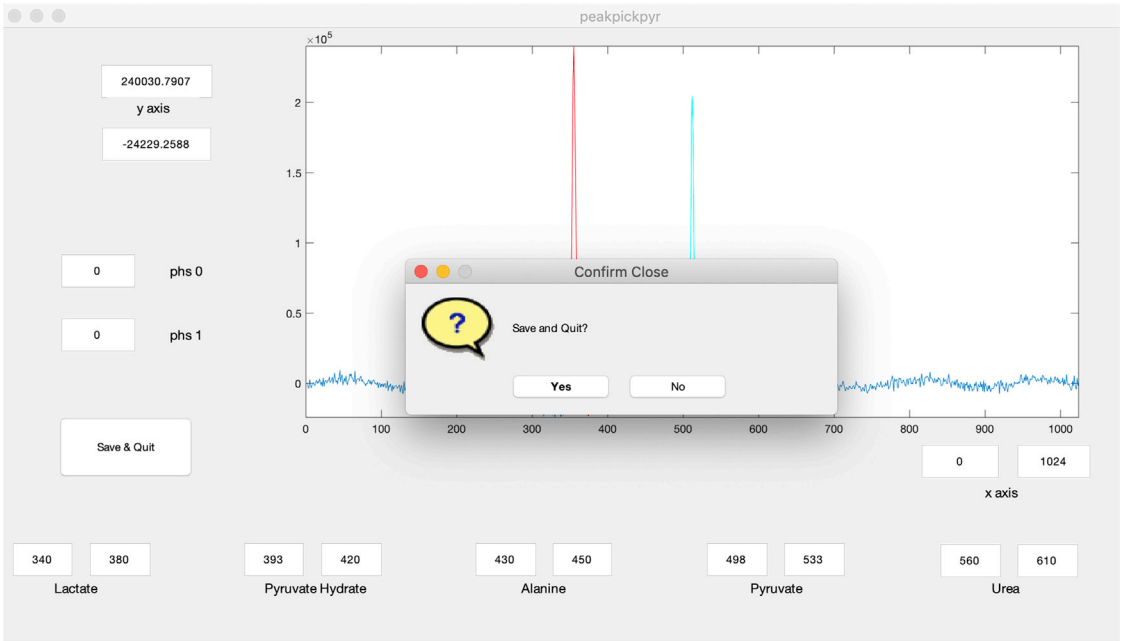

Figure 14. Once defined click 'Save and Quit"

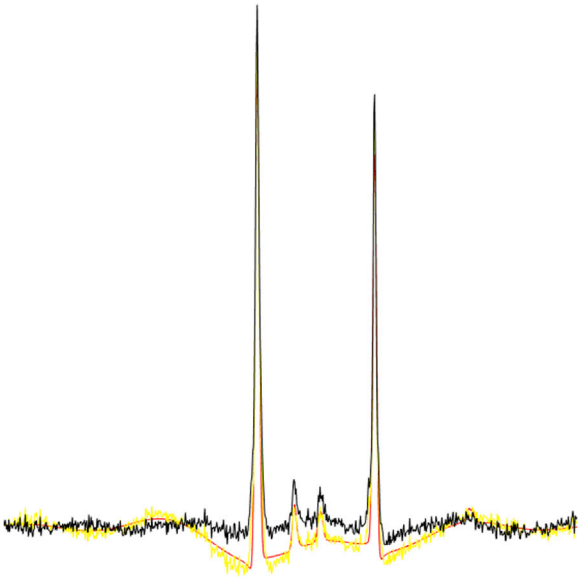

Figure 15. Figure generated using Matlab, with Pyruvate (right) and Lactate (left) peaks

LIMITATIONS

The short half-life of the polarization limits imaging with hyperpolarized [1-^{13}C]pyruvate to relatively rapid reactions, such as those involved in lactate labeling and precludes further tracking of the isotope label into other molecules. In tissues with high mitochondrial activity, such as the brain and heart, labeling of bicarbonate has been observed due to oxidative decarboxylation of the hyperpolarized [1-^{13}C]pyruvate by mitochondrial pyruvate dehydrogenase. The only tumor models in which we have observed bicarbonate labeling are glioblastoma PDXs. Tumors also frequently show alanine labeling from hyperpolarized [1-^{13}C]pyruvate, for example in a genetically engineered mouse model of pancreatic cancer (Serrao, Rodrigues et al. 2016).

TROUBLESHOOTING

Problem 1

Very low pyruvate or lactate signals are detected in the spectra (step 9 (h))

Potential solution

Check the pH of the injected hyperpolarized [1-^{13}C]pyruvate solution, see step 10 (d). If it is less than 4 or greater than 8 this may have affected the injection.

Problem 2

No pyruvate peak is present in the spectra (step 9 (h))

Potential solution

Check if canula is still in place and patent, see step 5 (e). If not then consider re-canulating the animal.

Problem 3

No signal can be detected in the spectra (step 9 (h))

Potential solution

Check for signal using the phantom, see step 8(k).

Problem 4

No signal can be detected (step 9 (h) and there is not a problem with the MRI scanner, see problem 3)

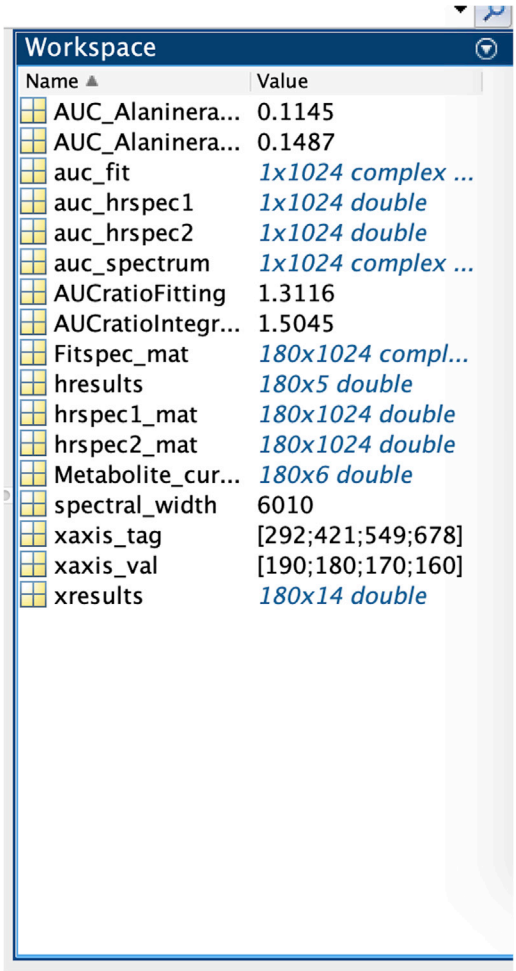

Figure 16. Calculated AUC values for different ratios

Lactate / Pyruvate AUC ratio is 1.3116 and Alanine / Pyruvate AUC ratio is 0.1145 in this example.

Potential solution

Make a new [1-^{13}C]pyruvate mixture and dissolution buffer, see [Preparation of solutions].

Problem 5

Extended delay between dissolution and injection (step 9 (c-d)).

Potential solution

The delay should be kept to a minimum in order to obtain a high signal-to-noise ratio (SNR), typically less than 30 s. A low SNR could compromise estimates of the ratio of the areas under the curves for labeled lactate and pyruvate. We do not recommend same day re-scanning since prior injection of hyperpolarized [1-^{13}C]pyruvate could affect the results obtained with a second injection (Saito, Matsumoto et al. 2012).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Kevin M. Brindle (email: kmb1001@cam.ac.uk).

Materials availability

Materials generated in this study are available upon request.

Data and code availability

Raw data for an example and script for analysis can be found in here:

Raw data

https://doi.org/10.17863/CAM.55683 and https://data.mendeley.com/datasets/vjj4sdwhjf/draft?a=43464dd5-acb8-4e51-8d28-d3c540a6421a

Script

https://doi.org/10.17863/CAM.62058

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100608.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.R. and K.M.B.; writing, S.R., A.J.W., A.B., C.C., and K.M.B.

DECLARATION OF INTERESTS

The hyperpolarizer is on loan from GE Healthcare (GEH) and is the subject of a research agreement between the University of Cambridge, Cancer Research UK, and GEH. K.M.B. holds patents with GEH on some aspects of dissolution dynamic nuclear polarization (DNP) technology. C.C. reports receiving speakers' bureau honoraria from Illumina, is a member of the AstraZeneca (AZ) External Science Panel, and reports receiving research grants from Roche, Genentech, AZ, and Servier that are administered by the University of Cambridge.

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