NMR-based prostate cancer metabolomics

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Abstract

Prostate cancer is the second most common malignancy, and the fifth leading cause of cancer related death among men, worldwide. A major unsolved clinical challenge in prostate cancer is the ability to accurately distinguish indolent cancer types from the aggressive ones. Reprogramming of metabolism is now a widely accepted hallmark of cancer development, where cancer cells must be able to convert nutrients to biomass while maintaining energy production. Metabolomics is the large-scale study of small molecules, commonly known as metabolites, within cells, biofluids, tissues or organisms. Nuclear magnetic resonance (NMR) spectroscopy is commonly applied in metabolomics studies of cancer. This chapter provides protocols for NMR-based metabolomics of cell cultures, biofluids (serum and urine) and intact tissue, with concurrent advice for optimal biobanking and sample preparation procedures.

Keywords: biobanking, biofluids analysis, cell extracts analysis, metabolite quantification, metabolomics, NMR pulse sequences, NMR spectroscopy, prostate cancer, sample preparation, targeted metabolic pathway analysis, tissue analysis

1. Introduction

Prostate cancer is the second most common malignancy, and the fifth leading cause of cancer related death among men, worldwide [1]. Prostate cancer is a manifested heterogeneous disease, ranging from slow growing and indolent to aggressive with high risk of fatal outcome. A major unsolved clinical challenge in prostate cancer is the ability to accurately distinguish indolent cancer types from the aggressive ones. This leads to substantial overtreatment of patients with harmless cancers, as well as undertreatment of patients with aggressive disease.

Reprogramming of metabolism is now a widely accepted hallmark of cancer development [2]. Cancer cells must be able to convert nutrients to biomass while maintaining energy production, which requires reprogramming of central metabolic processes in the cells. This phenomenon is increasingly recognized as a potential target for treatment, but also as a source for biomarkers that can be used for prognosis, risk stratification and therapy monitoring. Metabolomics is the large-scale study of small molecules, commonly known as metabolites, within cells, biofluids, tissues or organisms. Collectively, these small molecules and their interactions within a biological system are known as the metabolome. Since metabolites not only represent the downstream output of the genome, but also the upstream input from the tumor microenvironment, they contain a rich source of information that is currently underexploited. Metabolomics have shown that prostate cancer cells display significantly altered metabolism compared to normal cells [3, 4]. It has repeatedly been shown that distinctive metabolites are associated with prostate cancer aggressiveness and recurrence, and further that these metabolites are associated with gene regulation of cancer-associated pathways [3, 5-9].

Nuclear magnetic resonance (NMR) spectroscopy is one of the most widely applied methodologies in metabolomics studies of cancer, using sample material such as cell culture extracts, biofluids or even intact tissue [10]. NMR spectroscopy is a robust and reproducible technique with safe metabolite identification, requiring limited sample preparation leaving the sample intact after analysis. Additionally, in conjunction with the use of stable isotope tracers, NMR spectroscopy is an excellent method for exploring the dynamics and compartmentation of metabolic pathways and networks. The major limitation compared to mass spectrometry (MS) is the lower sensitivity (micromolar range compared to picomolar range for some MS-based methods), however, metabolites from important cancer pathways (glycolysis, choline metabolism, amino acids) are detected by MR analysis. The aim of the current chapter is to provide protocols for NMR-based metabolomics of cell cultures, biofluids (serum and urine) and intact tissue, with concurrent advice for optimal biobanking and sample preparation procedures.

2. Materials

All work in the laboratory should be carried out wearing a lab coat and disposable gloves when handling chemical reagents and biological material. When handling liquid nitrogen, safety glasses and cryo-gloves, lab coat with long sleeves, and pants worn outside the shoes to prevent shoes from filling should be worn. Chloroform and methanol must be handled in a ventilated fume hood.

2.1. Biobanking

2.1.1. Biofluids collection

1. For serum: Hypodermic needles, serum-separating tubes, pipettes, and cryo-compatible tubes.
2. For urine: Sterile urine containers, pipettes and cryo-compatible tubes.

3. Centrifuge and -80 °C freezer.

2.1.2. Tissue

1. Biopsy needle and scalpel or double bladed knife.
2. Cryo-compatible container, tube or bag.
3. Two solid metal plates (thickness ≥ 5mm), metal plate with a pit suitable for drilling, drill fixed to a stand, bits for drill to cut out cylinders, tweezers and styrofoam surface.
4. Liquid nitrogen and nitrogen-compatible thermos.

2.2. Biofluids analysis

1. Eppendorf tubes, 3mm NMR tubes with caps, syringes with long needles (>10 cm), micropipette(s) (60-550µL) and tips, and ruler for serum analysis.
2. Vortex or shaker and centrifuge.
3. Buffer solutions for serum (*See* **Note 1**) and/or urine analysis (*See* **Note 2**).

2.3. Polar cell extract analysis

1. Pipette(s), T225 flasks, tubes and cell scrapers. 5 mm NMR tubes with caps.
2. Sterile cell culture medium (*See* **Note 3**), phosphate buffered saline (PBS), D2O (99.8%)-based PBS solution, 70% methanol and ice.
3. Centrifuge, vortex and -80°C freezer.
4. Parafilm, 27G-30G needles and a lyophilizer for freeze-drying of cell extracts.

2.4. Dual phase tissue extract analysis

1. Cryo-comaptible tubes (2 mL and 15 mL), pipettes, ice cooled porcelain mortar and pestile and spatula. 5 mm NMR tubes with caps.
2. Methanol (-20°C), chloroform (-20°C), liquid nitrogen and distilled water (ice cold).
3. Analytical balance, vortex, centrifuge and lyophilizer.

2.5. Intact tissue analysis

1. Rotors with caps, disposable inserts, plugs and screws, filling funnel and screw driver.
2. Micropipette and tips, tweezers, weighing boats and permanent marker.
3. 25 mM sodium formate (HCOONa) in D2O (99.8%) solution and liquid nitrogen.
4. Analytical balance and a cooled work station (described in [11]) or ice block.

3. Methods

3.1. Biobanking: Collection and storage of biological material

Human biological material is regularly collected and stored in biobanks across the world. The stored material may include tissue, full blood, serum, plasma and urine. This collection plays a crucial role in modern biomedical research. For any biobank it is of utmost importance to have standardized protocols for handling biological material, to ensure reproducibility in research projects. Biobanks also have a responsibility to strictly adhere to national and international ethical guidelines regarding patient consent, privacy and safety of donors and proper archiving of samples.

3.1.1. Biofluids

Biofluids include full blood, serum, plasma and urine. Blood and urine should be collected in the morning after a fasting night, unless the research question requires other collection conditions.

Serum

For NMR experiments, serum is preferred over whole blood and plasma, as the latter two contain cells, clotting factors and additives which can interact with metabolites of the sample [12]. Serum can be separated from whole blood through centrifugation in serum-separating tubes or sedimentation with EDTA. Serum separating tubes includes a gel which

fractionalizes and accelerates blood clotting. This method is preferred over sedimentation, as EDTA is a contaminant for NMR analysis (*See* **Note 4**).

Protocol for serum

1. Collect venous blood from the donor in a serum separating tube with a hypodermic needle (This step has to be performed by an official health care professional).
2. After collection, invert the tube five times to mix the sample with the gel included in the serum separating tube.
3. Leave the sample at room temperature for 30 minutes for coagulation to occur.
4. Centrifuge the blood sample at 1800 x g for 10 minutes.
5. Carefully transfer the top layer of supernatant to one or more cryo-compatible tubes using a pipette (*See* **Note 5**).
6. The serum samples can be used immediately (continue at **subheading 3.2.1**, **Protocol for serum**, step 2), or placed in a -80 °C freezer.

Urine

Urine is a true non-invasive way of biological sampling and does not require the competence of a health care professional for collection.

Protocol for urine

1. The urine is collected by the donors themselves in a sterile urine container and closed with the screw lid
2. Pipette appropriate volumes of urine from the urine container into cryo-compatible tubes.
3. The urine samples can be used immediately (continue at **subheading 3.2.1**, **Protocol for urine**, step 2) or placed in a -80 °C freezer (*See* **Note 6**).

4. Before analysis, large containers may be thawed overnight in the fridge and centrifugation may be needed to remove precipitation.

3.1.2. Tissue

Prostate cancer tissue is more difficult to collect compared to biofluids due to the limited amount of material and the unavoidable invasiveness of the procedure. However, tissue offers a more direct and valuable view into the metabolism of the tumor. Tissue from the prostate can either be collected as transrectal needle biopsies directly from the patient, as surgical biopsies after radical prostatectomy or as thin tissue slices after radical prostatectomy [13]. To stop tissue degradation, the material has to be snap frozen as fast as possible but within 30 minutes after blood supply is cut to [14, 15].

Protocol for needle biopsies

1. Collect the tissue with a sterile biopsy needle (This step has to be performed by a health care professional).
2. Place the biopsy in a cryo-compatible tube with a clean tweezer. Drop the closed tube in liquid nitrogen and store the sample in either -80 °C or keep in liquid nitrogen.
3. Before NMR analysis, place the biopsy on a plastic weighing boat on top of a metal plate, which has been pre-cooled in liquid nitrogen, and use a scalpel to cut off approximately 1 mm at each end (*See* **Note 7**).

Tissue slice collection

This protocol is adapted from a highly standardized biobank collection of 2 mm research slices from the middle of the resected prostate. A homemade drilling system is developed for extraction of small tissue cylinders. Details on the equipment can be found in [13].

Protocol for tissue slice collection

1. After surgical removal of the prostate, extract a complete prostate tissue slice by placing the prostate in a holder with a double bladed knife [13].
2. Place the tissue slice in a pre-cooled cryo-clamp to prevent tissue bulging.
3. Plunge the cryo-clamp with the tissue into liquid nitrogen.
4. Transfer the frozen tissue into a cryo-compatible bag or other container and place it in a -80 °C freezer.

Protocol for tissue cylinder drilling from 2 mm whole-mount tissue slices (*See* **Note 8**)

1. Pre-cool metal plates (designed for drilling) by placing them in liquid nitrogen using cryo-glowes. The metal is sufficiently cooled down when the nitrogen has stopped boiling.
2. Take out the metal plates from the liquid nitrogen using cryo-gloves and place them on the styrofoam surface.
3. Before proceeding to **subheading 3.2.4**, **Protocol for intact tissue**, place the tissue slice on the metal plate. Hold the tissue stable and drill out cylinders (1-3 mm diameters) (*See* **Note 9**).
4. Optional: The drilled sample may be cryo-sectioned and stained to obtain a histopathological evaluation of the sample before using it for analyses. Use isotonic saltwater for fixating the sample during sectioning to avoid contamination of your sample (*See* **Note 10**).
5. In order to fit the sample into the insert, cut the cylinder into smaller pieces with a biopsy core device or scalpel as described in **subheading 3.1.2**, **Protocol for needle biopsies**, step 3.

3.2. Sample preparation

3.2.1. Biofluids

Liquid biopsies are easy to access and less invasive than tissue biopsies. The metabolic profiles of biofluids such as serum and urine can be investigated by NMR spectroscopy in a quantitative, automated and high-throughput fashion.

Protocol for serum

1. Thaw samples at room temperature (*See* **Note 11**).
2. Slowly invert the tubes containing the thawed serum sample a couple of times to mix.
3. Pipette an equal volume (≥120 µL) of serum (*See* **Note 12**) and serum buffer (*See* **Note 1**) into an Eppendorf tube. Slowly invert the tube three times to create a homogenous 50:50 serum:buffer mix.
4. Mark a 3mm NMR tube at a height of 4 cm, excluding the curved bottom from the measurement.
5. Use a long needle and syringe to fill the NMR tube with the serum:buffer mix to the 4 cm mark without air bubbles.
6. Cap the NMR tube by pressing the cap against the tube with your index finger until a click is heard.

Protocol for urine

1. Thaw samples at room temperature (*See* **Note 11**).
2. Centrifuge the samples at 3000 x g for five minutes to remove debris.
3. Pipette 540 µL of urine and 60 µL of urine buffer (*See* **Note 2**) into an Eppendorf tube. Mix using vortex or shake.
4. Use a long needle and syringe to fill the NMR tube with the urine:buffer mix without air bubbles. This should fill the NMR tubes at exactly 4 cm.

5. Continue at **subheading 3.2.1**, **Protocol for serum**, step 6.

3.2.2. Cell extracts

For *in vitro* metabolomics studies, extracts of cultured cancer cells can be prepared for analysis using high-resolution NMR spectroscopy (*See* **Note 13**). The choice of extraction protocol depends on the polarity and cellular concentration of the metabolites of interest. Below, we describe a protocol that allows analysis of culture medium and polar cell extracts from experiments where cells have been cultured with a ^{13}C-enriched substrate (*See* **Note 14**) for targeted studies of specific metabolic reactions (*See* **Note 15**). It should be noted that the choice of ^{13}C tracer, cell line, culture medium composition and experimental conditions depend on the objective of the experiment. It is, however, important to select the total number of cells, substrate concentration and incubation time so that the amount of ^{13}C substrate consumed by the cells will give sufficiently high signal-to-noise ratio in the NMR spectra in an acceptable experiment time.

Protocol for polar cell extraction

1. Culture approximately 1.5 x 10^7 cells to roughly 70% confluency in T225 flasks.
2. At T0, replace medium with 40 mL fresh culture medium-containing tracer of choice.
3. Transfer 2 mL of the culture medium to a centrifuge tube and store at -80°C.
4. At time of harvest (*See* **Note 16**), collect 2 mL spent culture medium in cryo-compatible tubes and immediately store in -80°C.
5. Wash cells twice with 25 mL PBS.
6. Place 25 mL of -20°C 70% methanol on cells.
7. Using a cell scraper, remove cells off flask bottom, transfer them to a centrifuge tube and store on ice.
8. Vortex for 5 minutes.
9. Repeat steps 6-7, transferring to a separate cryo-compatible tube.

10. Repeat step 8.
11. Centrifuge extract tubes at 18000 x g for 15 minutes at 4°C.
12. Pool the metabolite extracts from the two tubes (for each sample) to one 50 mL tube and store at -80°C. Store cell debris pellets at -80°C for determination of total protein content (*See* **Note 17**).
13. Additional control samples can be prepared according to steps 1-10 to compensate for naturally abundant ^{13}C:

    -Extracts from cells cultured in the absence of ^{13}C labeled tracer

    -Fresh and used culture medium (without ^{13}C substrate)

Culture medium and cell extracts should be stored at -80°C until further analysis. Cell extracts must be freeze-dried and reconstituted in a small volume of NMR-compatible buffer before analysis, whereas culture media can be analyzed directly after addition of a small amount of NMR-compatible buffer.

Protocol for freeze-drying of cell extracts

1. Distribute cold (-80°C) cell extracts into 50 mL tubes in small volumes (5-10 mL) in each tube.
2. Put parafilm on the top of each open tube and pierce/stick the parafilm 20-25 times with a needle.
3. Freeze dry the sample until all liquid has evaporated (typically takes several hours). Store at -80°C.

Protocol for NMR sample preparation of polar cell extracts

1. Dissolve the metabolite pellet thoroughly in 700 µL PBS dissolved in D_2O.

2. Centrifuge at 2300 x g (4°C) for 10 minutes. On visual inspection, the reconstituted sample should be clear and free from particles.
3. Using a pipette, transfer 600 µL of the supernatant into 5 mm NMR tubes for NMR acquisition.

Protocol for preparation of culture medium prior to NMR acquisition

1. Centrifuge the culture medium at 2300 x g for 10 minutes. On visual inspection, the sample should be clear and free from particles.
2. Mix 500 µL of medium with 100 µL PBS in D_2O
3. Using a pipette, transfer the solution (600uL) into 5 mm NMR tubes for NMR acquisition.

3.2.3. Tissue extracts

Chemical extractions can be performed on prostate tissue to yield solutions enriched with tissue metabolites that can be analyzed with high resolution NMR spectroscopy in the same way as biofluids and cell extracts. The extraction method should be chosen according to the polarity of the metabolites of interest. For example, for extraction of polar metabolites only, extraction protocols based on perchloric acid can be used. Below we describe a dual-phase extraction procedure using methanol and chloroform. This procedure yields two fractions that can be analyzed with NMR separately: an aqueous (polar) phase, containing water-soluble low-molecular weight metabolites, and an organic phase containing non-polar lipophilic molecules.

Protocol for dual-phase tissue extraction

1. Weigh the tissue on a weighing boat with an analytical balance and register the weight (*See* **Note 18**).

2. Crush and grind the tissue into a fine powder in liquid nitrogen using a pre-cooled mortar and pestile.
3. Using a pre-cooled spatula, transfer the powder into a 2mL cryo-compatible tube sitting on ice, which will be referred to as **tube 1**.
4. Add three times the tissue weight in cold methanol into **tube 1.**
5. Add ca 100 µL of water into **tube 1**.
6. Add six times the tissue weight in cold chloroform into **tube 1** (*See* **Note 19**).
7. Vortex until a milky appearance is achieved.
8. Centrifuge **tube 1** at 4°C at 1800 x g for 15 minutes.
9. Using a pipette, transfer the resulting top layer, i.e. the polar methanol phase, to a new cryo-compatible tube, which will be referred to as **tube 2** (*See* **Note 20**). Keep **tube 2** on ice.
10. Add six times the tissue weight in methanol, four times the tissue weight in water and four times the tissue weight in chloroform into **tube 1** and vortex.
11. Repeat steps 8 and 9.
12. Add three times the tissue weight in methanol, two times the tissue weight in water into **tube 1** and vortex.
13. Repeat steps 8 and 9.
14. Leave **tube 1** in the fume hood so that the chloroform evaporates for subsequent total protein content determination (*See* **Note 21**).
15. Add three times the tissue weight in chloroform and 20 times the tissue weight in water into **tube 2** and vortex.
16. Centrifuge **tube 2** at 4°C at 1800 x g for 15 minutes.

17. Using a pipette, transfer the resulting top layer, i.e. the polar methanol phase, to a new cryo-compatible tube, which will be referred to as **tube 3** (*See* **Note 20**). Keep **tube 3** on ice.
18. Add six times the tissue weight in water and 1.5 times the tissue weight in methanol into **tube 2**.
19. Repeat steps 16 and 17.
20. Freeze **tube 2** and **tube 3** at -80°C.
21. Evaporate any chloroform from **tube 2** and the methanol and water from **tube 3** using the lyophilizer as described for cell extracts in **subheading 3.2.2**, **Protocol for freeze-drying of cell extracts**.
22. Store **tube 2**, containing the organic phase, and **tube 3,** containing the polar phase, at -80°C, or continue with the next steps
23. For polar cell extracts, prepare the samples for NMR acquisition as described in **subheading 3.2.2**, **Protocol for NMR sample preparation of polar cell extracts**.

Protocol for NMR sample preparation of non-polar tissue extracts

1. Dissolve the samples in 600 µL $CDCl_3$/CD_3OD (2:1).
2. Using a pipette, transfer the solution (600 µL) into 5 mm NMR tubes for NMR acquisition.

3.2.4. Intact tissue

Although more metabolites can be detected using NMR spectroscopy when analyzing tissue extracts, the metabolites detected in intact tissue are less likely to be a product of changes other than the innate biological processes of the tumor. The global metabolic profile has been found to sustain significant alterations when exposed to elevated temperatures commonly used in gas chromatography-mass spectrometry for processes such as derivatization, vaporization, and ionization [16]. The degree of chemical degradation of the tissue that the

extraction procedure entails, which gives rise to issues similar to the effect of thermal degradation, is unknown.

NMR of solids and semisolids, such as biological tissue, exhibit nuclear interactions that are anisotropic, i.e. dependent on the orientation or direction of the molecules which the involved nuclei are part of with respect to the external magnetic field. These arise due to the lack of molecular mobility [17] and give broad signals that can cause overlap between metabolite peaks. The signals may even be so broad that the signal is completely obscured. This does not occur when analyzing liquids, since the rapid movement of the molecules averages out the anisotropic interactions. Rapid spinning of a solid sample on its axis at an angle of 54.7 degrees with respect to the external magnetic field, referred to as the magic angle, mimics a liquid solution state, where the anisotropy of the interactions is averaged to zero. This method is called high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS) [18, 19] and produces spectra from tissue samples with resolution that is comparable to conventional liquid solution NMR. HR MAS MRS is a non-destructive method, leaving the tissue intact for subsequent RNA/DNA/protein extraction or histology [13].

When preparing samples for HR MAS MRS, these should be kept frozen to minimize tissue degradation. For this, an ice block or a nitrogen-filled cooling workstation [11] can be used. The following procedure should not exceed five minutes.

Protocol for intact tissue

1. Pipette 3.0 µL of sodium formate in D_2O solution (*See* **Note 22**) into a 30 µL disposable insert. Be careful not to make bubbles.
2. Place the insert on the analytical balance and tare the weight
3. Place a weighing boat on the ice block or cooling station.

4. Cut a frozen sample to fit the insert using a scalpel, biopsy puncher or drill [11] (*See* **Section 3.1.2, Protocol for tissue cylinder drilling from 2 mm whole-mount tissue slices**) (*See* **Note 23**) on a weighing boat.
5. Transfer the sample carefully into the bottom of the insert using tweezers or the biopsy punch. Make sure the content is evenly distributed in the insert (*See* **Note 24**).
6. Weigh the sample in the insert and record the weight.
7. Introduce a plug into the insert using tweezers. The plug should stay fit at the neck of the insert without requiring any further adjustment.
8. Place a screw cap on the insert and screw tight using the screwdriver (*See* **Note 25**).
9. Set the insert all the way inside a rotor, so that it is fixed and immovable. The tightness inside the rotor can be adjusted by tightening or loosening the screw cap with the screwdriver.
10. Introduce a spinning cap upside down into the filling funnel, followed by the upside down rotor with the insert. Push the bottom of the rotor gently against the filling funnel until a click is heard.
11. Trace half the diameter of the bottom of the rotor with a black permanent marker to enable spin counting.
12. Store the rotor at -16 °C or lower until transferred into the magnet.
13. Once NMR acquisition has been completed, the sample can be recovered by using liquid nitrogen to loosen the rotor cap and then remove the insert from the rotor. For a detailed procedure, we refer to [11].

3.3. NMR acquisition

Prior to NMR spectral acquisition, temperature calibration should be performed to correct for any difference between the displayed probe temperature and the true sample temperature. For HR MAS MRS acquisition, an additional prior step is the adjustment of the magic angle to

optimize the spectral resolution. Although it is not necessary to perform these steps before every sample, we suggest doing it at least once a week and when starting the analysis of a new batch of samples and/or following insertion of the NMR probe into the magnet. For a detailed procedure on magic angle adjustment and temperature calibration for HR MAS MRS, we refer to [11].

3.3.1. Sample acquisition

The following is the general procedure for acquiring NMR metabolomics data from *in vitro* and *ex vivo* samples. For more detailed procedures specific for the analysis of biofluids and extracts and for HR MAS MRS of intact tissue, we refer to [20] and [11], respectively:

Protocol for NMR acquisition

1. For HR MAS MRS: Load the sample into the magnet and stabilize the spin-rate (*see* **Note 26**).
2. Set the temperature to an appropriate value (*See* **Note 27**) based on the last temperature calibration (*See* **subheading 3.3.**) and let stabilize.
3. For steps 4-10 (*See* **Note 28***)*
4. Perform tuning and matching to the nucleus of interest.
5. Lock on to the solvent signal.
6. Perform shimming.
7. Optimize the 90° pulse (P1).
8. Optimize the value for water suppression (O1).
9. Acquire the spectrum using the optimal parameters (i.e. shim, P1 and O1) and pulse sequences (*See* **subheading 3.3.2.**).
10. For acquisition of biofluids and tissue extracts, *see* **Note 28**.

3.3.2. Pulse sequences

The most commonly measured nucleus in NMR spectroscopy is ^{1}H, but other nuclei like ^{13}C, ^{31}P, ^{17}O, ^{19}F and ^{23}Na can also be utilized in NMR studies. Various NMR pulse sequences are available to optimize the acquired spectrum according to the biological questions of interest. Arguably the most widely used pulse sequence in NMR metabolomics is the one-dimensional Nuclear Overhauser Effect Spectroscopy (NOESY) [21], with which spectra are acquired while suppressing the water signal through presaturation. The sequence begins with a long or continuous-wave, low-power pulse for presaturation of the water signal. This is followed by three high-power 90° pulses where the first and second pulses are separated by a delay, and the second and third pulses are separated by a mixing time. The free induction decay (FID), i.e. the signal produced by relaxing nuclei [22], is acquired after the third 90° pulse.

NMR signals from macromolecules, such as lipids and proteins, are broad and can mask signals from low-molecular weight metabolites. Because the mobility of small molecules is much higher than that of macromolecules, the latter exhibit shorter T2, i.e. faster transverse relaxation [22-24]. The difference in T2 can thus be exploited to suppress lipid signals by employing T2-weighted pulse sequences which generate NMR spectra where the signal intensity of nuclei is directly proportional to its T2. One such sequence is the Carr-Purcell Meiboom-Gill (CPMG) pulse sequence, which is particularly useful for lipid suppression in serum and tissue spectra. This sequence acts as a T2 filter, suppressing the faster-relaxing macromolecules while enhancing the smaller metabolites. It begins with a presaturation and 90° pulse, similarly to the NOESY sequence. This is followed by a delay and a series of 180° pulses, each with a short delay before and after. Each 180° pulse inverts or refocuses the signals dephased by the transverse relaxation [22]. The rephasing and subsequent dephasing of the signals produces an echo of the original 90° pulse.

If instead contrast enhancement of the macromolecules is desired, a diffusion-editing pulse sequence can be used. Diffusion-editing pulse gradients provide a diffusion filter which excludes signals from the fast-diffusing smaller molecules prior to spectral acquisition. The simplest form of a diffusion-edited pulse sequence begins with a 90° pulse followed by a gradient to disperse the magnetization, a 180° pulse to invert the dispersed magnetization and a second gradient to refocus the signal, prior to the acquisition of the FID [24]. The refocusing of signals is inversely proportional to the diffusion coefficient of the molecules they arise from, resulting in an attenuation of the faster-diffusing smaller molecules.

Carbon NMR only detects the ^{13}C isotope whose natural abundance is 1.1%, and since ^{13}C has a low gyromagnetic ratio, ^{13}C NMR spectroscopy is less sensitive than ^{1}H NMR spectroscopy. Nevertheless, ^{13}C NMR spectroscopy is a valuable tool for investigating fluxes of carbon through various metabolic pathways after administration or incubation of ^{13}C labelled substrates. The most frequently used one-dimensional ^{13}C NMR pulse sequences are ^{1}H decoupled, and these pulse sequences use a repeated set of pulses for continuous irradiation of ^{1}H. ^{13}C-^{1}H coupled multiplets are consequently collapsed into singlets and enhanced signal-to-noise ratio is achieved.

Two-dimensional NMR pulse sequences are particularly useful for the unambiguous identification of metabolites. The pulse sequence for J-resolved (JRES) experiments, for example, produces a plot of the chemical shift versus the spin-spin coupling constants [25]. Comparing this plot to a one-dimensional spectrum allows for the elucidation of the shape and number, i.e. multiplicity, of peaks arising at a particular chemical shift. This facilitates metabolite assignment and detection of overlapping peaks. The JRES pulse sequence begins with a relaxation delay, followed by a 90° pulse with a consecutive incremented mixing time (t1) and a 180° pulse also with consecutive t1, prior to the FID acquisition. For a description of other two-dimensional NMR experiments for metabolite identification, including chemical

shift correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and heteronuclear single quantum correlation (HSQC) spectroscopy, we refer to [26].

3.4. Quantification

The proportionality of the NMR signal intensity to the number of nuclei producing that signal makes NMR spectroscopy highly quantitative. There are several methods available to perform quantification of metabolites from NMR spectra. Curve fitting routines, such as PeakFit (Systat Software Inc., USA), that determine the area of metabolite peaks, can handle overlapping signals. However, this approach can be time consuming as each metabolite peak of interest must be fitted manually, and is therefore unfeasible for high numbers of samples and untargeted metabolomics approaches. Another peak deconvolution software that is simple to use is Chenomx NMR Suite (Chenomx, Edmonton, AB, Canada). Chenomx allows both identification and quantification of peaks simultaneously, and can be performed doing both manually or semi-automatic adjustment of peaks.

Another quantification method that has been successfully employed for the quantification of *ex vivo* spectra of prostate tumor tissue [3, 5] is LCModel [27]. This method uses linear combinations of spectra of individual metabolites, collectively referred to as a basis set, to fit the spectra to be quantified.

Protocol for peak fitting using LCModel

1. If a suitable basis set (*See* **Note 29**) is not already available, generate a basis set through computer simulation of spectra of individual metabolites based on chemical shifts and coupling constants [28] using e.g. NMR-SIM (Bruker BioSpin, Germany).
2. Run LCModel using the graphical user interface (GUI) or in-house scripts and with the time domain data of the spectrum to be quantified (*See* **Note 30**) and the basis set (*See* **Note 31**).

3. Check the quality of the fit based on the residuals and uncertainties of the quantification i.e. Cramer-Rao lower bounds (*See* **Note 32**) produced as part of the output.

Relative quantification can be performed automatically by integrating fixed spectral regions of metabolites of interest once the spectra are preprocessed, particularly properly aligned. Although relative concentrations are not comparable with other studies, they allow comparisons between two groups within the same study, similarly as for gene expression microarray measurements. An important drawback of peak integration is that areas of overlapping peaks cannot be distinguished. This can lead to peak areas being influenced by signals other than those belonging to the metabolite of interest. To avoid this, only metabolite regions known to be isolated, i.e. not having overlap, should be selected for integration. The procedure can be evaluated by visually inspecting the regions selected for integration for each spectrum.

Once the area of the metabolite peaks, i.e. relative concentration, is determined, calculating absolute concentration requires a reference signal with known concentration. Deuterated trimethylsilyl propionic acid (TSP) [29] formate, or the electronic reference to access *in vivo* concentration (ERETIC) [30] can be used as internal references for the absolute quantification of NMR spectra. TSP is more suitable for quantification of biofluids and extracts, as it may bind to proteins and other membrane components in tissue, which may lead to overestimation of metabolite concentrations [17]. ERETIC is an electronically-simulated reference which can be positioned at any region of the spectrum, therefore avoiding reference-analyte interactions and peak overlap [31]. Pulse length based concentration (PULCON) [32] is another alternative for new spectrometers where the enhanced stability and reproducibility allows direct comparison to a standard acquired under identical conditions. Standard curves for quantification with PULCON and ERETIC should be established.

Protocol for standard curve preparation

1. Prepare standard solutions for a metabolite, e.g. creatine or formate, in a relevant concentration range (*See* **Note 33**).
2. For each concentration value, acquire spectral data for a known amount of standard solution (*See* **Note 34**) including three technical replicates using the same experimental set up, i.e. similar probe, acquisition parameters, etc., as the spectra to be quantified.
3. Calculate the peak area of the standards using the same method used for the spectra to be quantified (e.g. PeakFit, Chenomx or LCModel).
4. Make a standard curve by plotting the concentration versus the peak area for each standard solution.
5. Use the standard curve to relate the peak area to the added number of moles for each metabolite to calculate the absolute concentration of the unknown metabolites.

When absolute quantification is to be performed, it is important to use a sufficiently long repetition time (TR) during NMR acquisition, which ideally should be at least 5 times the longest T1 to achieve more than 99% of the equilibrium magnetization. If a short TR is used, a compensation for this must be implemented for absolute quantification, as described in [33].

Absolute quantification from spectra acquired using T2-weighted sequences like CPMG can also be misleading, as metabolite peak intensities can differ slightly due to variations in T2 relaxation times rather than just reflecting differences in concentrations [28]. In order to ensure accurate absolute quantification based on CPMG spectra, the T2 relaxation effect should be calculated and corrected for [34].

Protocol for T2 relaxation correction

1. For each metabolite to be quantified, repeat NMR spectroscopy experiments with different echo times (TE).
2. Relate each resulting metabolite peak area with the corresponding TE to fit a monoexponential decay function representing T2 [34].
3. Correct for the T2 effect by multiplying the peak areas by a T2 correction factor (f): $f = \exp[-TE_{eff}/T2]$, where TE_{eff} is the effective echo time.

4. Notes

1. The serum buffer is prepared as follows: i) Dissolve 10.05 g $Na_2HPO_4 \bullet 7H_2O$ in 380 mL H_2O; ii) add 0.4 g TSP; iii) mix well by ultrasonic mixing; iv) add 5 mL of a 4% NaN_3/H_20 solution; v) adjust pH to 7.4 with 1M HCl (1M NaOH); vi) add H_2O until total volume is 400 mL; vii) add 100 mL D_2O and mix well.
2. The urine buffer is prepared as follows: i) Dissolve 10.2 g KH_2PO_4 in 40 mL D_2O; ii) dissolve 50 mg TSP in 3-5 mL D_2O; iii) mix both solutions together by ultrasonic mixing; iv) adjust pH to 7.4 with KOH tablets or strong KOD solution (KOH tablets in D_2O); v) add D_2O until total volume is 50 mL and mix well.
3. The choice of medium depends on the nutrient requirements of the cell line and the objective of the experiment.
4. There are many different ways of isolating serum that are compatible with running an NMR experiment. The most important point is that the protocol should be standardized for all samples.
5. Separating the serum sample into several tubes of appropriate volume for analysis is recommended to avoid unnecessary freezing-thawing cycles.
6. Previous studies have reported that the samples can be stored at 4 °C for a maximum of 30 days [35] before further processing, but in general, this should be avoided. Remix the sample by pipetting before transferring into smaller containers.

7. One side of the biopsy may contain blood and rectal material; cutting off part of the ends will reduce the risk of contamination.
8. Selection of the tissue area to drill out can be based on histopathological evaluation of the two adjacent tissue slices collected for clinical pathology.
9. A 3 mm diameter is recommended for a suitable tissue weight for NMR spectroscopy analysis (*See* **Note 23**).
10. Other commonly used fixatives will contaminate the final NMR spectra.
11. Thawing takes 20-60 minutes, depending on the samples size.
12. Serum should be pipetted slowly because it is viscous.
13. It is also possible to analyze intact cells directly using HR MAS MRS (*See* **subheading 3.2.4.),** but this technique is less developed.
14. Cells can be incubated with various ^{13}C-enriched substrates. Normally, the substrate present in the culture medium is substituted with >99% pure ^{13}C-enriched substrate, to maximize the amount of substrate consumed during the experiment. The choice of substrate, substrate concentration and incubation time depend on the objective of the experiment.
15. The same extraction protocol can be used for studies of cells cultured under normal conditions (i.e. no ^{13}C-enriched tracer). The number of cells needed for high resolution ^{1}H NMR will be considerably lower.
16. Incubation duration and condition depend on the choice of ^{13}C substrate.
17. Total protein content can be determined to help normalize the NMR data to the number of cells in the extraction experiment. Alternatively, the cell number can be estimated from growth curves or cell counting under identical conditions.
18. Ideal weight for extraction is 20-50 mg.

19. All work with chloroform should be performed in a fume hood and with glass material.
20. Depending on the volume, use a 2 mL or 15 mL cryotube.
21. Total protein content can be determined for the sample from the pellet in **tube 1** after dissolving it in NaOH.
22. Formate is added for shimming purposes and D_2O is added to provide a frequency lock for the NMR spectrometer. Adding a larger volume than 3 µL of the D_2O solution can enhance the shimming. Formate can also be used as an internal quantification reference.
23. Ideal weight for intact tissue NMR analysis is 10-15 mg.
24. The tissue and the formate in D_2O solution should be evenly distributed in the insert, otherwise it may be difficult to reach the recommended spinning speed of 5000 Hz within the magnet.
25. Do not screw the screw cap too tight, as this will cause the insert neck to expand and not fit in the rotor.
26. 5000 Hz is appropriate when using 600 mHz NMR spectrometers.
27. The recommended temperature for intact tissue samples is ≤ 5°C to minimize tissue degradation. The recommended temperature for serum is 37°C and for urine, cell extracts and medium and tissue extracts, it is 27°C.
28. For biofluids and extracts, the acquisition for samples within the same batch can be automated using IconNMR (Bruker BioSpin, Germany). If performed this way, tuning and matching, shimming and P1 (90° pulse) optimization is carried out automatically for each sample. The O1 (water suppression) value is optimized manually for the first sample and set for subsequent samples.

29. LCModel requires all peaks observed in the target spectra, including those considered irrelevant, e.g. lipids or contaminants, to be comprised in the basis set; a signal that is unaccounted for will negatively affect the area calculation for the rest of the peaks in the spectrum. For spectra with high degree of lipid peaks, e.g. serum, simulating lipid peaks can prove to be a challenge when using this method.

30. When using in-house scripts, LCmodel prefers the time domain data to be organized into a text file of 8 columns containing 4 complex pairs (real, imaginary, real, imaginary…) in each row.

31. Some parameters, including chemical shift range of quantification, number of points and references for phasing and frequency alignment, need to be set before running LCModel. For more information we refer to the LCModel manual [36].

32. Typically a fit with Cramer-Rao lower bounds <15, shown as percentage standard deviationin the LCModel output, is considered unsuitable.

33. Typically solutions with at least five different concentrations are prepared. For creatine, these can be 0.25 mM, 1.0 mM, 2.5 mM, 5 mM and 10 mM.

34. Weigh the amount of sample analyzed using an analytical balance to know the exact amount of moles analyzed.

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