

# Ex Vivo Spectroscopy - Linking the Benchtop to the Clinic

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## Introduction.

Magnetic Resonance Spectroscopy (MRS) has the unique advantage of detecting and quantifying metabolites *in situ* in living tissue, completely non-invasively. So why should one wish to sacrifice that advantage and use the same method on biopsies, *ex vivo*?

1. More powerful instruments can be used, and they have more sophisticated shim coils, as well as sample spinners. The overall result is better signal/noise, peak resolution and peak shape. The most powerful horizontal-bore magnets that are commonly used for laboratory MRS *in vivo* are currently 7-9.4T, whereas magnets of 14-21T can be used for high resolution studies on solutions.
2. Better spectra can be obtained from solid tissue biopsies than from the same tissue *in vivo* because of the higher field, sample spinning, longer scan times etc. Also, the intact biopsy can be recovered at the end of the experiment for histological examination.
3. Magic Angle Spinning of solid tissue biopsies gives still better spectral resolution than seen *in vivo*, with about 40 metabolites detectable in  $^1\text{H}$  and  $^{13}\text{C}$  2-D studies of brain (1). And the intact biopsy is still available at the end of the experiment for histological examination.
4. Still greater spectral resolution is available when extracted metabolites are dissolved a uniform aqueous solution. About 50 metabolites can be routinely detected in  $^1\text{H}$  spectra of tissue extracts compared with less than 10 *in vivo* (see Table 1).
5. The peaks can be quantified more precisely *ex vivo*, both because the magnetic field can be better shimmed and because the sample can be spun, thus improving the shape of the peaks; in addition, the baseline is flat.

All the methods developed for studies on pure organic molecules (e.g. various kinds of 2D NMR, shifting the pH of the sample etc) can be used to improve peak resolution and assignment, and a wealth of information is available. In this lecture I shall not deal with the basic methodology of *in vitro* NMR, as this is covered in numerous basic textbooks [e.g. (2)]. Instead I shall concentrate on the special techniques necessary to use the method for studying biological tissues, particularly in relation to Metabolomics, and some of the results that have been obtained.

## NMR for Metabolomics

In recent years the new science of metabolomics (which deals with the totality of metabolites in a living cell, tissue, disease state etc) has made much use of *ex vivo* NMR, since it gives data that usefully complement those obtained by transcriptomic and proteomic methods. Just as genes “use” proteins, so do proteins “use” small-molecule metabolites, and even the quite simple metabolomic datasets (“metabolic profiles”) obtainable by 1D  $^1\text{H}$  NMR can help in the interpretation of the data from the other -omic sciences (e.g. transcriptomics or proteomics). A variety of metabolites can be quantified simultaneously, in the same sample by NMR without any need for preliminary derivatisation, so the concentrations of the metabolites relative to one another are known with great precision. Chemical derivatisation is required before samples can be examined by Mass Spectrometry, the main competing technique. It is because of the uncertainty as to the completeness of derivatisation and the completeness of ionisation, particularly of an unknown compound, that MS is quantitatively less precise than NMR. This, and the much simpler sample

preparation, are the main reasons why NMR remains competitive with the MS methods in the field of metabolomics, even though they are cheaper and much more sensitive.

Metabolomic studies can take several forms.

1. Studies aimed at elucidating the entire metabolome of an organism. Metabolomics is still in its infancy, so little systematic work of this kind (which would have to be very large-scale) has yet been reported. A classic paper by Stephen Oliver et al. (3) introduced a method termed FANCY for studying the metabolomic impact of gene knockouts in yeast.
2. It is currently much easier to study the metabolomic effects of individual gene modifications. A metabolic profile of a cell or organism with a gene knockout, knockdown (by siRNA etc) or overexpression can be compared with that of the corresponding control. There are thousands of laboratory models of this kind, either in cultured cells or in mouse models and they are being widely studied by transcriptomic or proteomic methods, which usually find changes in numerous genes, many of which are poorly understood. Even the simple metabolomic data available from NMR spectra can add an extra dimension to the interpretation of such a study.
3. Most novel drugs are nowadays targeted on individual genes or gene products, so the same metabolomic strategy can be used to help elucidate whether the candidate drug is acting by the originally-intended mechanism (4). Toxicological studies on its effect on the metabolomes of normal tissues are possible, too.

#### Methods for Tissue Extraction

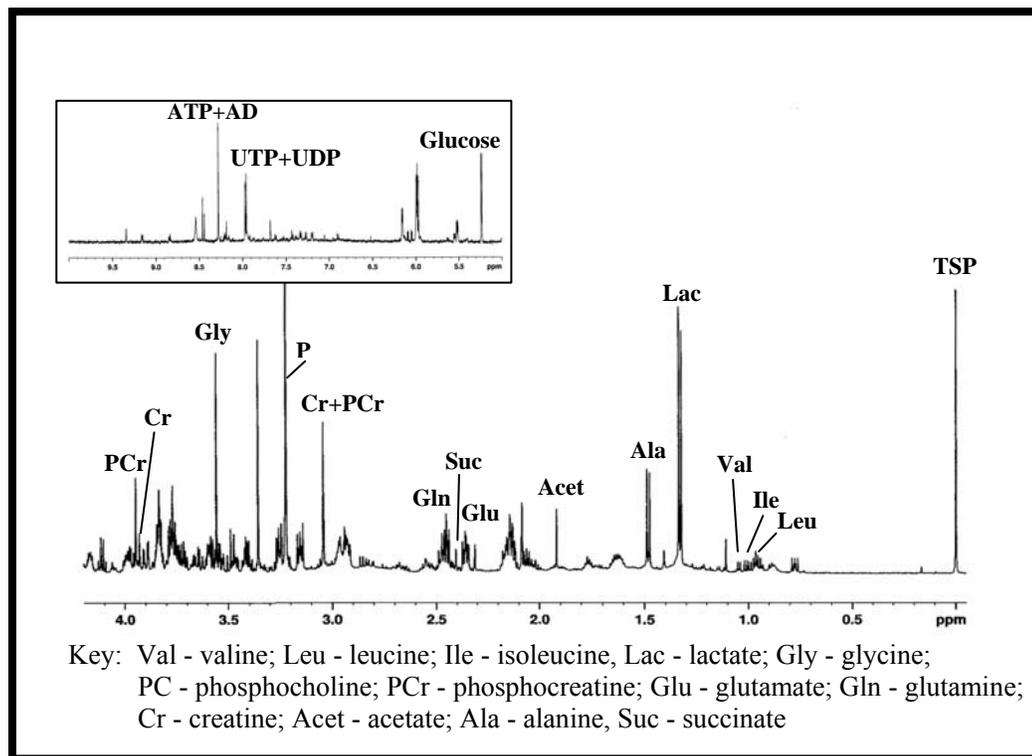
A chemical extract of a biopsy gives a snapshot of metabolism at the moment the biopsy was taken. Once the biopsied tissue loses its blood supply its metabolic profile will begin to change. Enzyme-catalysed reactions will begin to destroy many substances (e.g. phosphocreatine and glucose) and create others (e.g. inorganic phosphate and lactate) within minutes or even seconds. In conventional metabolic biochemistry, therefore, the sample is frozen instantly while still in the anaesthetised animal, by clamping it between metal blocks cooled in liquid nitrogen (“freeze-clamping”). Dropping the sample into liquid nitrogen is not so effective - a layer of bubbles forms which insulates it for several seconds. In practice, particularly when

$\alpha$ -Glucose	Glutamine	Phosphatidylethanolamine
$\alpha$ -Ketoisovalerate	Glutathione	Phosphatidylglycerol
$\beta$ -Glucose	Glycerol	Phosphocholine
$\beta$ -Hydroxybutyrate	Glycerophosphocholine	Phosphocreatine
Acetate	Glycerophosphoethanolamine	Phosphoethanolamine
ADP and ATP	Glycine	Plasmalogen
Alanine	Histidine	Scyllo-inositol
Asparagine	Inorganic phosphate	Succinate
Aspartate	Inosine	Sugar Phosphates
Betaine	Isoleucine	Taurine
Cholesterol and esters	Lactate	Threonine
Creatine	Leucine	Triacylglycerol
Dimethylamine	Lysine	Tyrosine
Formate	Myo-inositol	UTP and UDP
Free choline	NAD and NADH	Valine
Fumarate	Phenylalanine	
Glutamate	Phosphatidylcholine	

**Table 1** Metabolites routinely detected by *ex vivo*  $^1\text{H}$  and  $^{31}\text{P}$  NMR of tissue extracts.

collecting biopsies from patients during surgical operations, one has to compromise – freeze-clamping a tissue in a patient is impossible and the time taken to obtain and freeze the samples often depends on the progress of the operation.

The next step is to extract the water-soluble metabolites, denaturing enzymes and other macromolecules and liberating bound metabolites that would not otherwise give sharp peaks. The standard procedure in metabolic biochemistry is to powder the tissue in liquid N<sub>2</sub> and then grind it in ice-cold perchloric acid, a sufficiently strong acid to denature enzymes and other proteins but which can be removed (after the denatured material has been removed by centrifugation) by slowly adding potassium hydroxide solution and spinning down the potassium perchlorate crystals. The final extract, now much diluted, is then titrated to neutrality and lyophilised by freeze-drying. The dried pellet can be kept frozen and redissolved in <sup>2</sup>H<sub>2</sub>O (to minimise the <sup>1</sup>H signal from water) just before NMR spectroscopy. A chemical shift and quantification standard (e.g. sodium 3-trimethylsilyl -2,2,3,3-tetradeuteriopropionate) can be added during the extraction procedure. This will ensure that any losses in the metabolites during the extraction are matched by losses of the quantification standard. Because the baseline is almost flat and the peaks are well separated and uniformly narrow, it is usually possible to perform quantification simply by measuring peak heights. Perchloric acid can also be used to extract metabolites from cultured cells.



**Fig 1.** <sup>1</sup>H NMR spectrum of a perchloric acid extract of prostate tumour cells. Bruker 600MHz spectrometer (pulse angle 45°; repetition time 6.5 seconds; 256 scans). The water resonance was suppressed by gated irradiation centered on the water frequency.

Perchloric acid extracts the soluble metabolites; it is also possible to use organic solvents (classically chloroform/methanol) to extract the lipids – the more polar ones dissolve in the methanol and the rest in the chloroform. It is possible to combine all three extractants in a single method (5).

## Magic Angle Spinning NMR

Much of the line broadening in solid tissues is due to dipolar coupling, which occurs because there is restricted movement of neighbouring nuclei. Many of the local interactions have an orientation dependence determined by  $(3\cos^2\theta - 1)$ , where  $\theta$  is the angle between the interaction vector and the applied magnetic field. If we spin the sample at the 'magic angle' ( $54^\circ 44'$ ) relative to the  $B_0$  field these dipolar coupling effects are removed. Thus one can obtain well-resolved spectra from solid tissue samples of around 20mg without having to extract them (Fig. 2) (1). The spinning speed used depends on the nucleus being studied and the field of the magnet, and is usually chosen to be fast enough to push the spinning sidebands outside the spectral region of interest. Speeds of 4-6 KHz are typical on a 600MHz instrument.

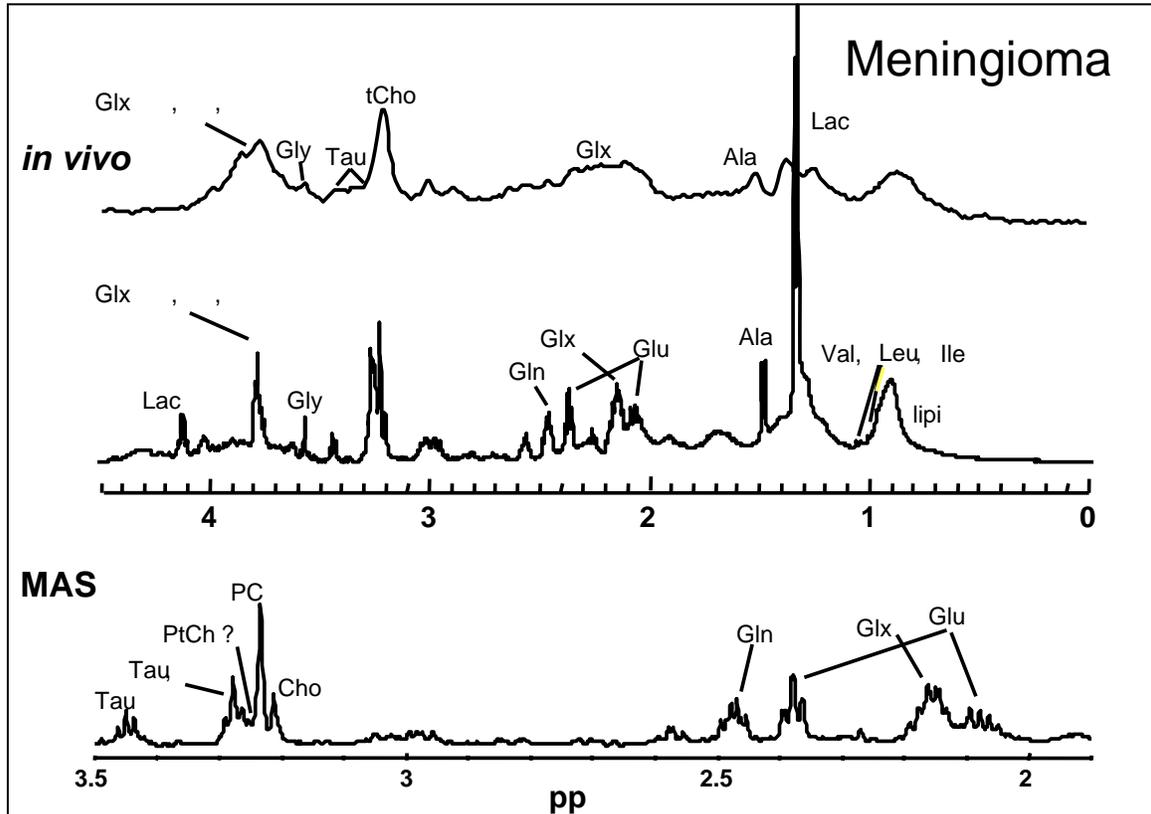


Fig 2

**The same meningioma studied *in vivo*, in the patient and by MAS of a biopsy.** Top, *in vivo* on a GE 1.5T Signa (STEAM TE 30ms); middle, MAS of a biopsy on a Bruker 500MHz with a 4kHz spinning rate; bottom, zoomed in region of a CPMG spectrum of the same biopsy (TE 60ms) Note the flat baseline because the macromolecule signal has died away. Ala, alanine; Cho, choline; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Ile, isoleucine; Lac, lactate; Leu, Leucine; Lipi, lipids; PC, phosphocholine; PtCh? Possibly phosphatidylcholine; Tau, taurine; tCho, total cholines; Val, valine.

MAS spectra contain all the metabolites, lipids and macromolecules that would be present in an *in vivo* spectrum, but gives less well-resolved peaks than would be obtained from a solution (since not all the broadening processes are eliminated). Whereas PCA extraction will only reveal the water soluble metabolites, and other extraction process using chloroform/methanol are needed to obtain the lipid spectra. However, MAS also risks "scrambling" or degradation of some metabolites while the MAS rotor is being packed with tissue and the spectrum is being taken, because the enzymes in the tissue will still be active even if all procedures are performed at  $4^\circ$ . However, many of the metabolites are reasonably stable at  $4^\circ$  over the time necessary to obtain an MAS spectrum (typically 15 minutes to 1 hour for tissue specimens).

The MAS method is less satisfactory for cultured cell studies. If, for instance, one were to centrifuge down a suspension culture in order to perform MAS on the pellet of cells, the cells would rapidly consume all available oxygen and become hypoxic once they became compressed together in the pellet during the centrifugation procedure. Thus the energy-pathway changes that ensued would be likely to dominate the metabolic profile.

## **NMR Studies on Biofluids**

Body fluids are particularly easy to study by NMR, of course, since in most cases little or no preparation is required. In recent years body fluid studies have been particularly identified with a method called Metabonomics (6), defined as “The quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification.” Metabonomics clearly covers some of the same territory as Metabolomics, and some people use the terms synonymously, but much of the literature on Metabonomics is in fact concerned with NMR measurements of body fluids, particularly in the context of toxicology, and with the use of pattern recognition methods to interpret the data.

### **1. Blood and Blood Plasma.**

Blood is the basic biofluid in animals, and the others are initially formed by ultrafiltration of blood plasma. Whole blood was the first biological tissue to be subjected to NMR spectroscopy (7), but it is now usual to remove the cellular components by centrifugation and to perform NMR studies on the blood plasma. Alternatively one can allow the blood to clot and study the serum - this is effectively plasma without the fibrinogen that forms the clot). Two main classes of compound can be detected: plasma proteins (8) and small metabolites such as glucose (8).

### **2. Urine**

Urine is initially formed by ultrafiltration of blood in the glomeruli of the kidney. Some metabolites (e.g. glucose) are then reabsorbed, while others, including many oxidised foreign compounds, are actively secreted against the concentration gradient. Finally, urine is concentrated by removal of water. In consequence, urine often contains much higher concentrations of interesting compounds than blood plasma. Another advantage is that normal urine contains negligible quantities of protein, which simplifies the spectra. NMR of urine is widely used for studying inborn errors of metabolism [9] - rare, genetically determined diseases, usually due to the absence of an enzyme, that cause substrate to accumulate, and often to undergo abnormal metabolism.

## **Acknowledgements**

I thank Franklyn Howe and Yuen-Li Chung for their help and advice, and Cancer Research UK for financial support.

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