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OMICS International has organized 500 conferences, workshops and national symposiums across the major cities including San Francisco, Las Vegas, San Antonio, Omaha, Orlando, Raleigh, Santa Clara, Chicago, Philadelphia, Baltimore, United Kingdom, Valencia, Dubai, Beijing, Hyderabad, Bengaluru and Mumbai.

High Resolution NMR Metabolomics on Biological Tissues and Live Objects

Jian Zhi Hu

Pacific Northwest
NATIONAL LABORATORY

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NMR Metabolomics

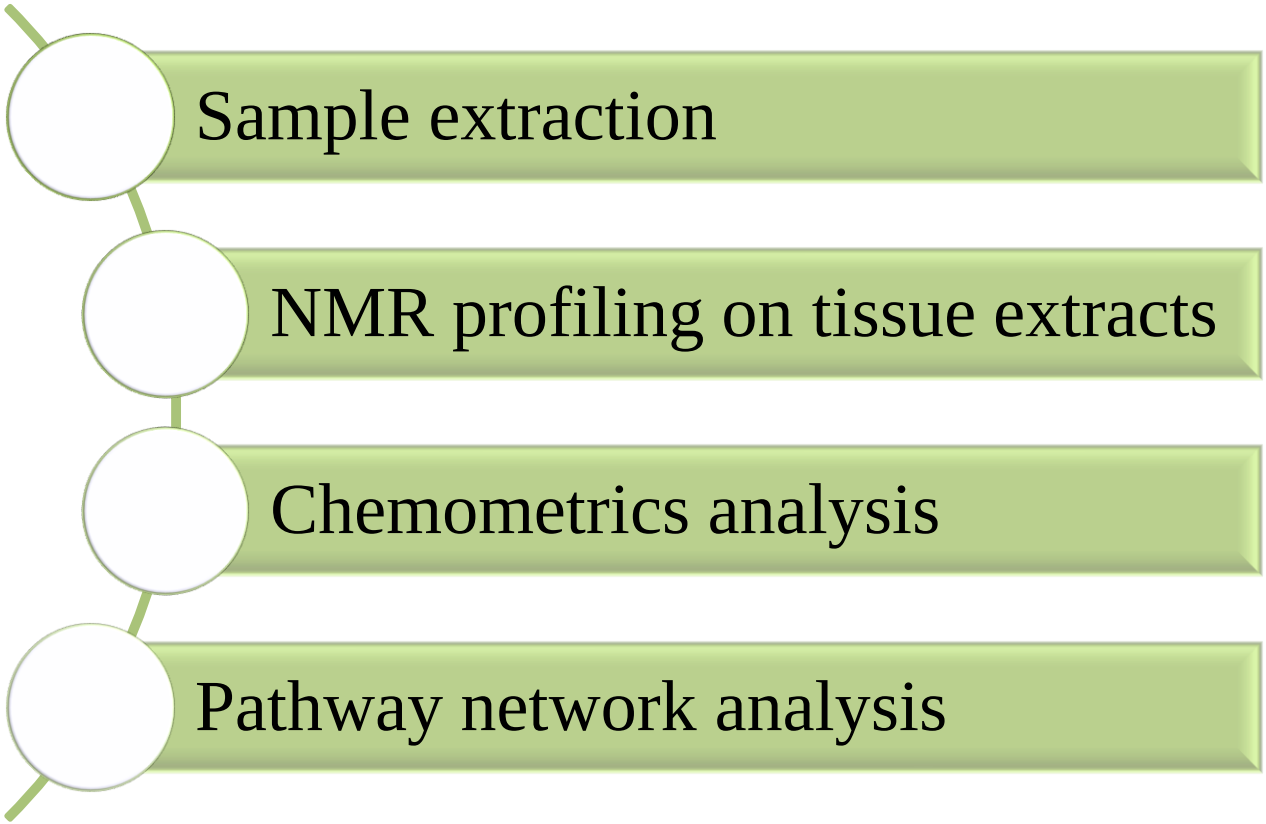
- Traditional liquid state NMR metabolomics on biofluids and tissue extracts
- High resolution magic angle spinning (fast and slow) NMR metabolomics on intact tissues
- Biostatistics to find out discriminary metabolites
- Bioinformatics (pathway network analysis)
- Metabolic flux measurement using ^{13}C isotope enriched nutrients such as glucose, etc, for tracing out the flow of biological pathways.

Liquid State ^{1}H NMR Metabolomics on tissue extracts

A case-guided introduction using ^{1}H NMR metabolomics study of metastatic melanoma in C57BL/6J mouse spleen.

X. Wang · M. Hu · J. Feng, M. Liu · J. Z. Hu. 1H NMR metabolomics study of metastatic melanoma in C57BL/ 6J mouse spleen. *Metabolomics* **10**:1129–1144 (2014)

Outline


Sample extraction
NMR profiling on tissue extracts
Chemometrics analysis
Pathway network analysis

Sample extraction protocol

- Grind the frozen tissue in the freeze mill;
- Homogenize the pre-weighted tissue powder after adding 4 ml MeOH and 0.85 ml H_2O per gram of tissue;
- Vortex the mixture and then adding 2ml chloroform per gram of tissue, vortex again;
- Add 2 ml g^{-1} chloroform and 2 ml g^{-1} water to the sample and then vortex again;
- Transfer different layers into glass vials separately with syringes after the mixture being left on ice for 15 min and centrifuged at 5000 rpm for 15 min at 4°C;
- Remove the solvents using lyophilizer ($MeOH/H_2O$ layer) or under dry nitrogen gas ($CHCl_3$ layer);
- Store the extracts at -80 ° C before performing NMR measurements.







Micro-Homogenizer

O. Beckonert, H.C. Keun, T.M.D. Ebbels, J. Bundy, E. Holmes, J. C. Lindon & J. K. Nicholson. ***Nature Protocols* 2, 2692-2703 (2007)**

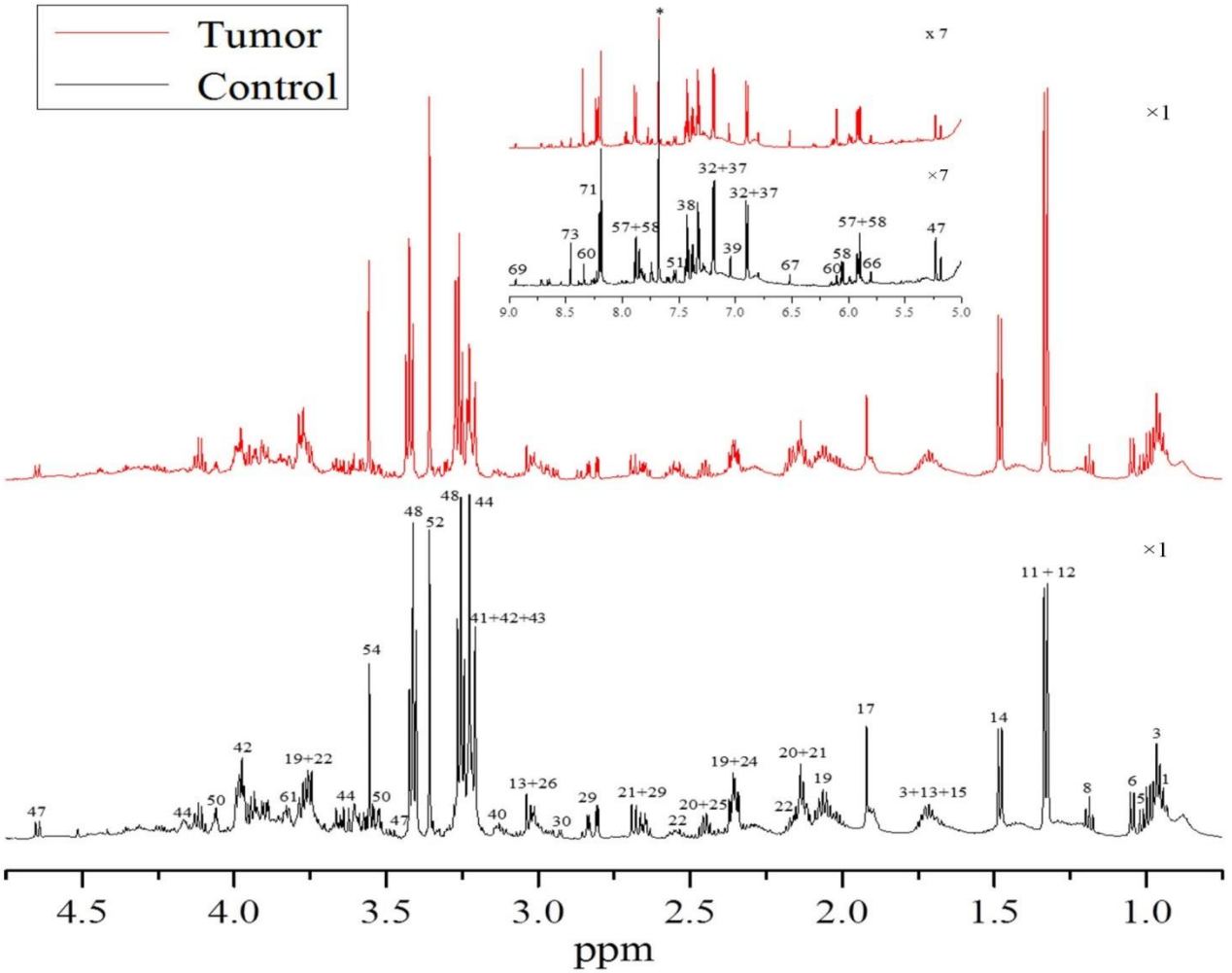
Solvents-Tissue ratio for extraction

Tissue	MeOH (ml)	H_2O (µl)	$CHCl_3$ (ml)	$CHCl_3$ (ml)	H_2O (ml)
1 g	4	850	2	2	2
0.5 g	2	425	1	1	1
0.25 g	1	213	0.5	0.5	0.5
125 mg	0.5	106	0.25	0.25	0.25
62.5 mg	0.25	53	0.125	0.125	0.125
31.3 mg	0.25	53	0.125	0.125	0.125
10.0 mg	0.25	53	0.125	0.125	0.125

NMR profiling on tissue extracts

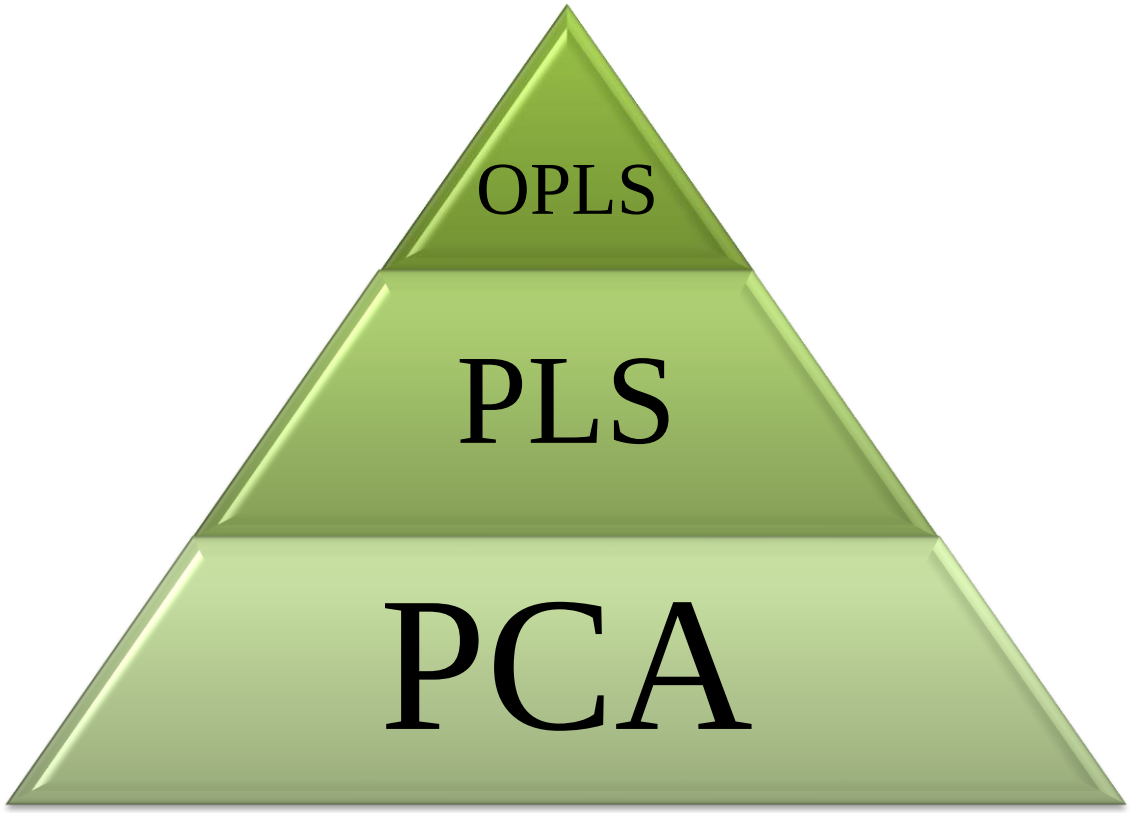
- Reconstitute the hydrophilic metabolites in 600 µl D_2O containing 0.5 mM TSP-d4 as chemical shift reference and internal concentration standard;
- Load the prepared sample into a standard 5mm NMR tube;
- Acquire one-dimension ^1H NMR spectra on a Varian 600 MHz NMR spectrometer at 20 °C using the standard Varian PRESAT pulse sequence;
- Preprocessing the NMR spectra in Chenomx (NMR suite 8.0, Professional).

O. Beckonert, H.C. Keun, T.M.D. Ebbels, J. Bundy, E. Holmes, J. C. Lindon & J. K. Nicholson. ***Nature Protocols*** **2, 2692-2703 (2007)**



600 MHz liquid state ^{1}H NMR metabolite spectra of the hydrophilic extracts of spleens excised from the control and the melanoma mice. 73 metabolites can be identified ranged from sugars, simple organic acids, amino acids, phospholipids and simple lipids, etc.

Chemometrics aspects



SIMCA: http://www.umetrics.com/downloads/simca

http://metabolomicsworkbench.org/data/DRCCDataDeposit.php

Principal Component Analysis (PCA)

- PCA can be considered as ‘‘the mother of all methods in multivariate data analysis.’’
- The aim of PCA is dimension reduction;
- PCA can be seen as a method to compute a new coordinate system formed by the latent variables, which is orthogonal, and where only the most informative dimensions are used;
- It is a method for exploratory data analysis and can be applied to practical any ***X***-matrix (metabolite concentration or binned peak data);
- No ***Y***-data (disease *vs* control) are considered and therefore it is unsupervised learning.

Partial least-squares (PLS)

- PLS stands for *partial least-squares or/and projection to latent structures by means of partial least squares*;
- PLS is a powerful linear regression method, insensitive to collinear variables, and accepting a large number of variables;
- Resulting model predicts a property y from the original dependent variables x_1 to x_m;
- Linear model contains regression coefficients b_1 to b_m and an intercept b_0;
- The number of PLS components determines the complexity of the model and can be optimized for high prediction performance.

Orthogonal projections to latent structures (OPLS)

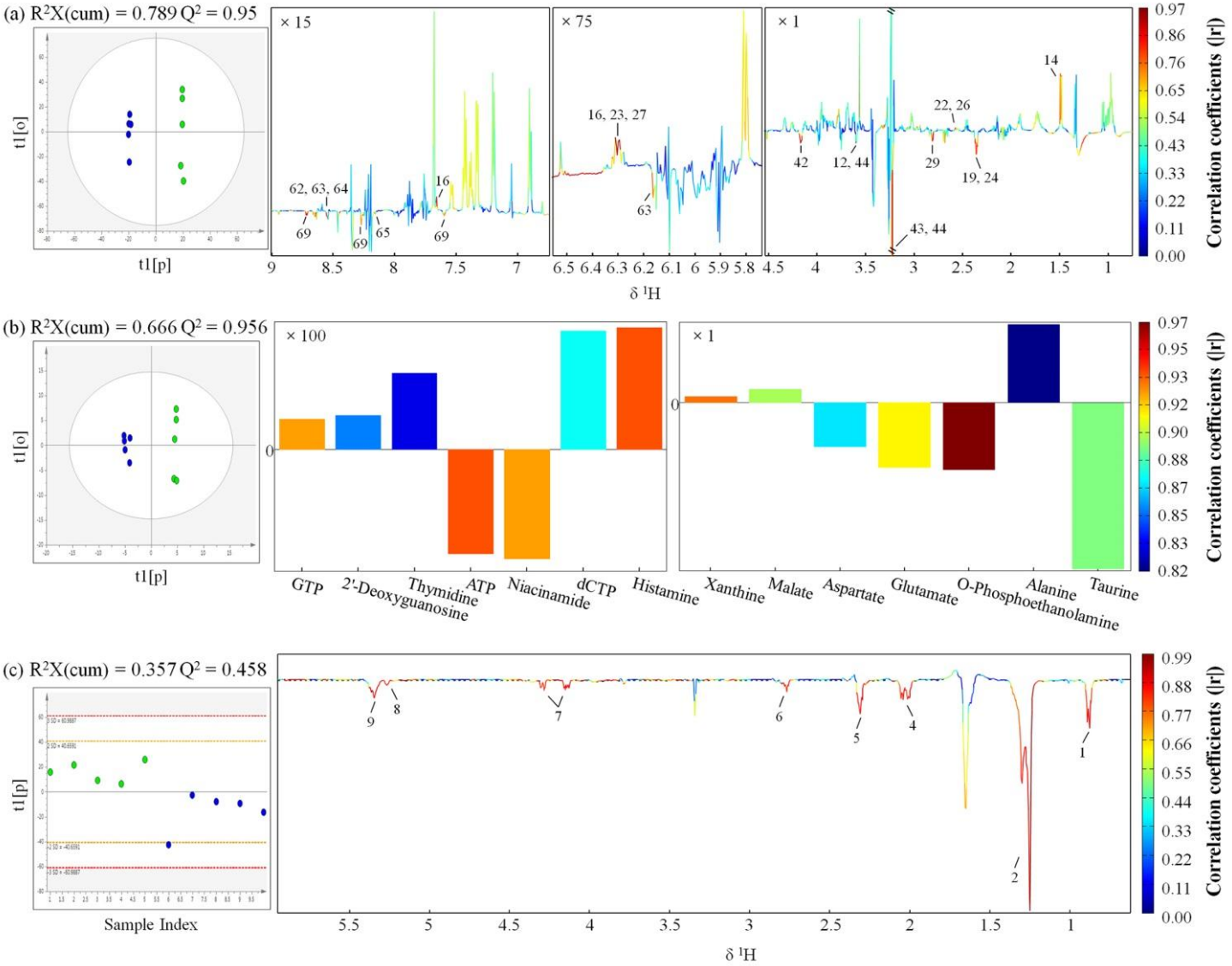
- OPLS provides a way to remove variability in ***X*** (metabolites) that is orthogonal to ***Y*** (control or treated/diseased);

- OPLS analyzes the disturbing variation in each regular PLS component. The non-correlated variation in ***X*** is separated from the correlated variation;

- Removing non-correlated variation in data prior to data modeling is not only interesting from a predictive point of view, but the interpretational ability of resulting models also improves.

Control (blue)

Tumor (green)



OPLS scores (left) and coefficients-coded loadings plot (right) of the model discriminating the control (blue dots) and the tumor (green dots) groups. (a) Binning and (b) metabolite concentration results of ^{1}H NMR spectra from hydrophilic extracts. (c) Using the data from binning results of ^{1}H NMR spectra from hydrophobic extracts. CV-ANOVA results gave *p* values of 0.046, 1.37 × 10-3 and 0.117 for models (a), (b) and (c), respectively.

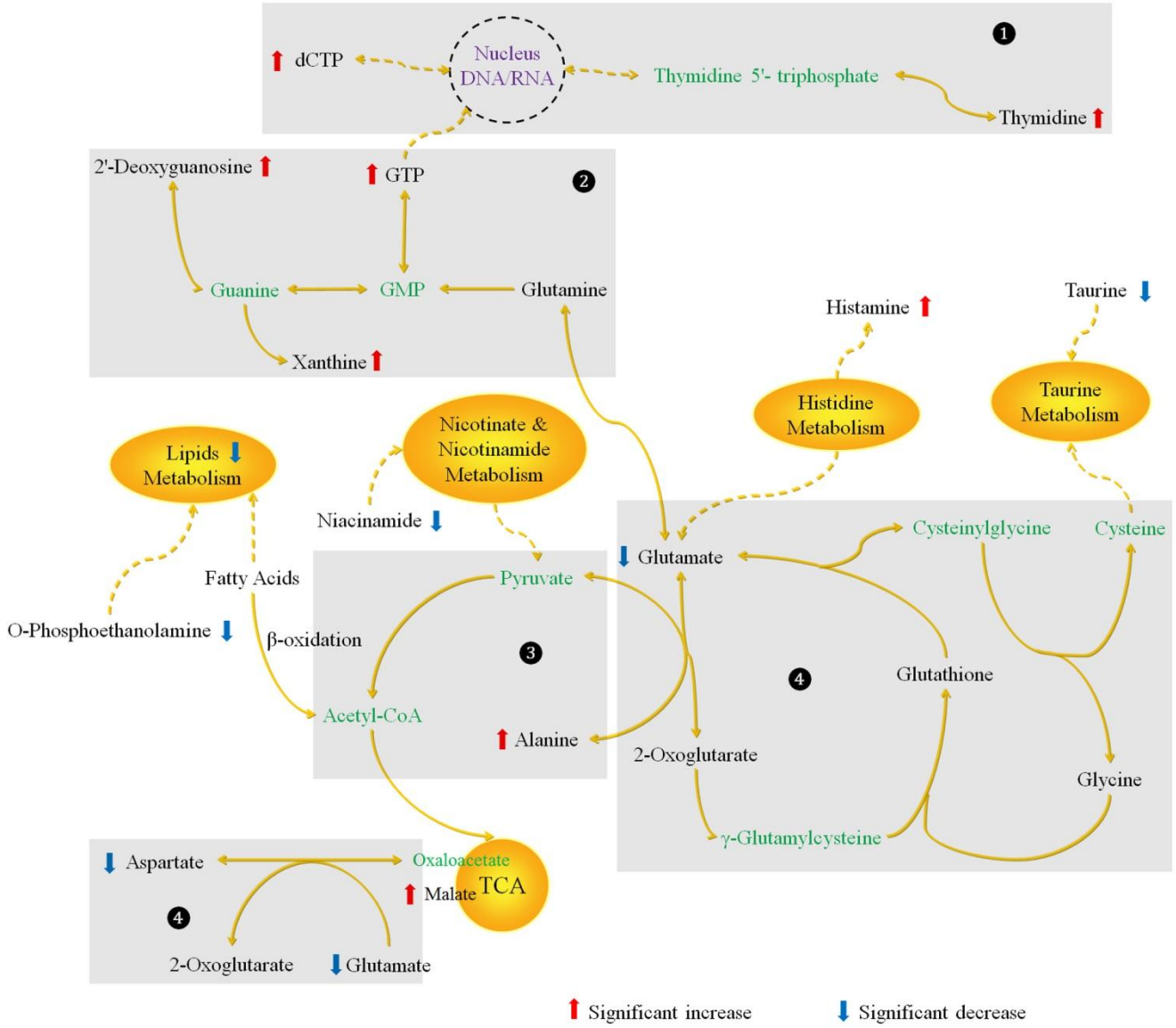
Melanoma induced metabolic changes in spleen tissue extracts

Key	Metabolites	Correlation coefficients
14	Alanine	0.817
16	Thymidine	0.832
19	Glutamate	-0.911
23	dCTP	0.874
24	Malate	0.897
27	2'-Deoxyguanosine	0.854
29	Aspartate	-0.870
33	Histamine	0.936
43	O-Phosphoethanolamine	-0.967
48	Taurine	-0.892
63	ATP	-0.936
65	GTP	0.924
69	Niacinamide	-0.925

Pathway network analysis



http://www.smpdb.ca/

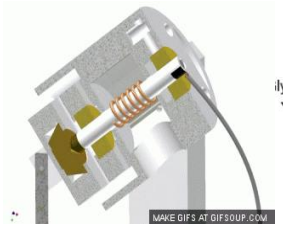


Schematic diagram of disturbed metabolic pathway networks associated with the significantly altered metabolites during melanoma development: ❶ pyrimidine metabolism, ❷ purine metabolism, ❸ pyruvate metabolism, ❹ glutamate metabolism.

High Resolution ^{1}H NMR Metabolomics on Intact Biological Objects Using Slow Magic Angle Spinning

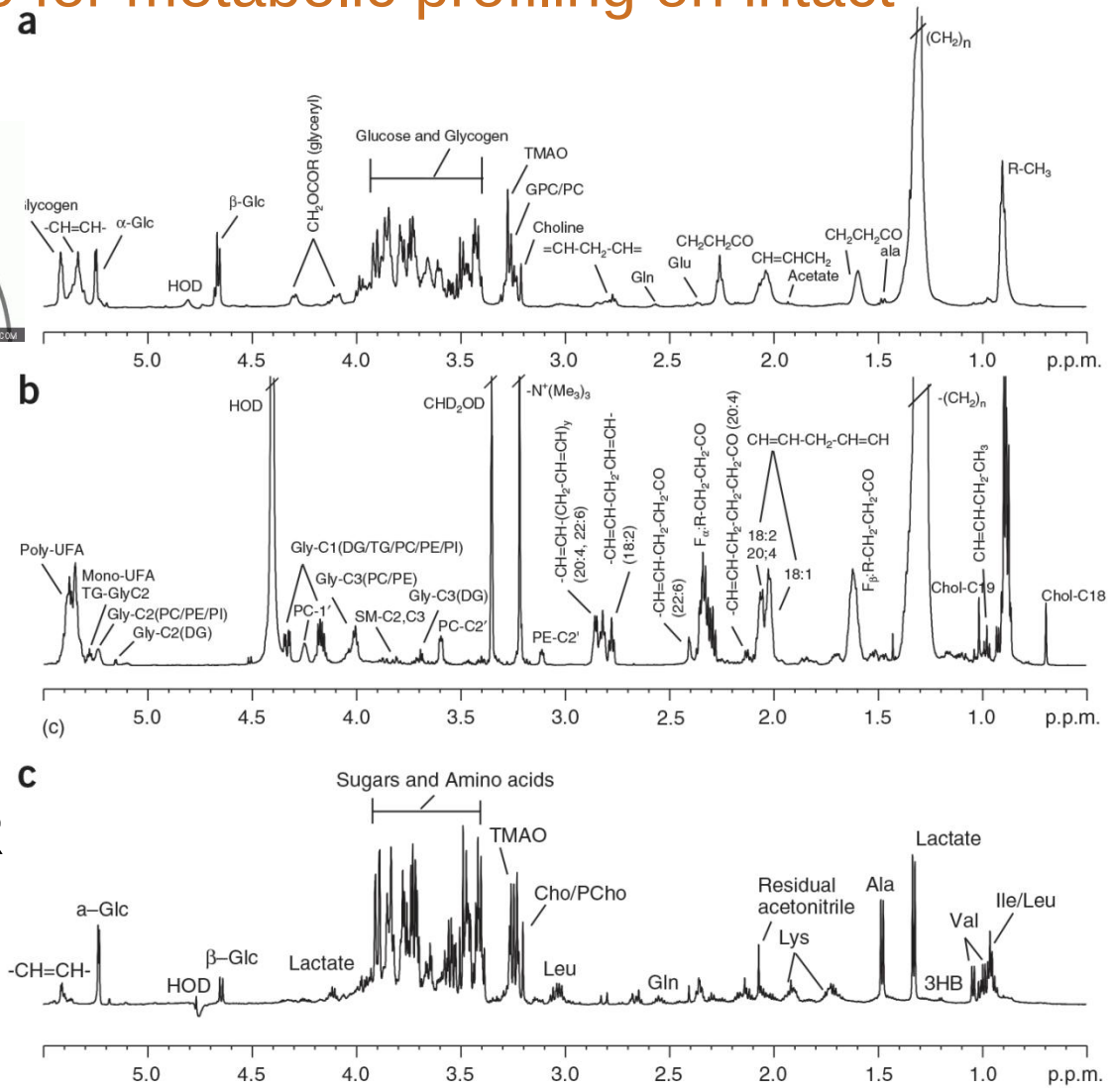
^{1}H HR-MAS NMR using a sample spinning rate of a few kHz is a powerful technique for metabolic profiling on intact biological tissues

(a) ^{1}H HR-MAS NMR of **intact** mouse liver tissue.

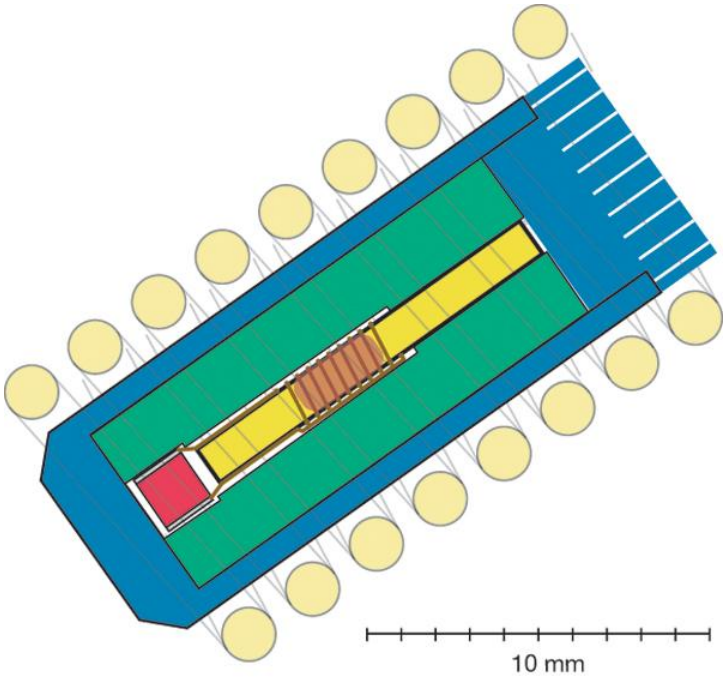


(b) Liquid state ^{1}H NMR spectrum of a **lipid soluble** mouse liver tissue **extracts**.

(c) Liquid state ^{1}H NMR of an **water soluble** liver mouse tissue **extracts**.



O. Beckonert, M. Coen, H. C. Keun, Y. Wang, T. M. D. Ebbels, E. Holmes, J. C. Lindon & J. K. Nicholson. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. ***Nature Protocols* 5(6), 1019-1032 (2010)**



< 1 μl sample volume can be analyzed

Sakellariou D., Goff GL and Jacquinot J-F. High-resolution, high-sensitivity NMR of nanolitre anisotropic samples by coil spinning. *Nature* **447**, 694-698 (2007).

HR-MAS is an excellent technique but the followings:

- Production of a 100% leakage free sample rotor for HR-MAS is challenging due to the use of a sample spinning rate of a few kHz or more.

- The large centrifugal force associated with fast sample spinning is destructive to the tissue structure and even some of the cells.

- Difficult to spin a large tissue sample (300 mg or more) to the desired spinning rate required for hr-MAS analysis.

- Work with small volume sample, e.g., ~1.0 μl or less is challenging.

Our Approach

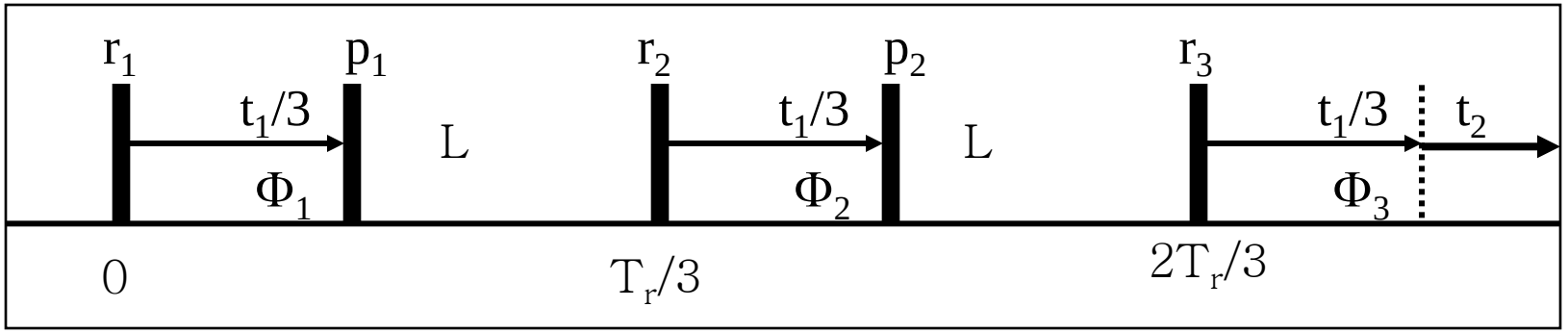
- **Aproach-1:** Slow-MAS using a sample spinning rate of 40-300 Hz. Non-destructive to intact biological tissues (This will be discussed in my next talk)

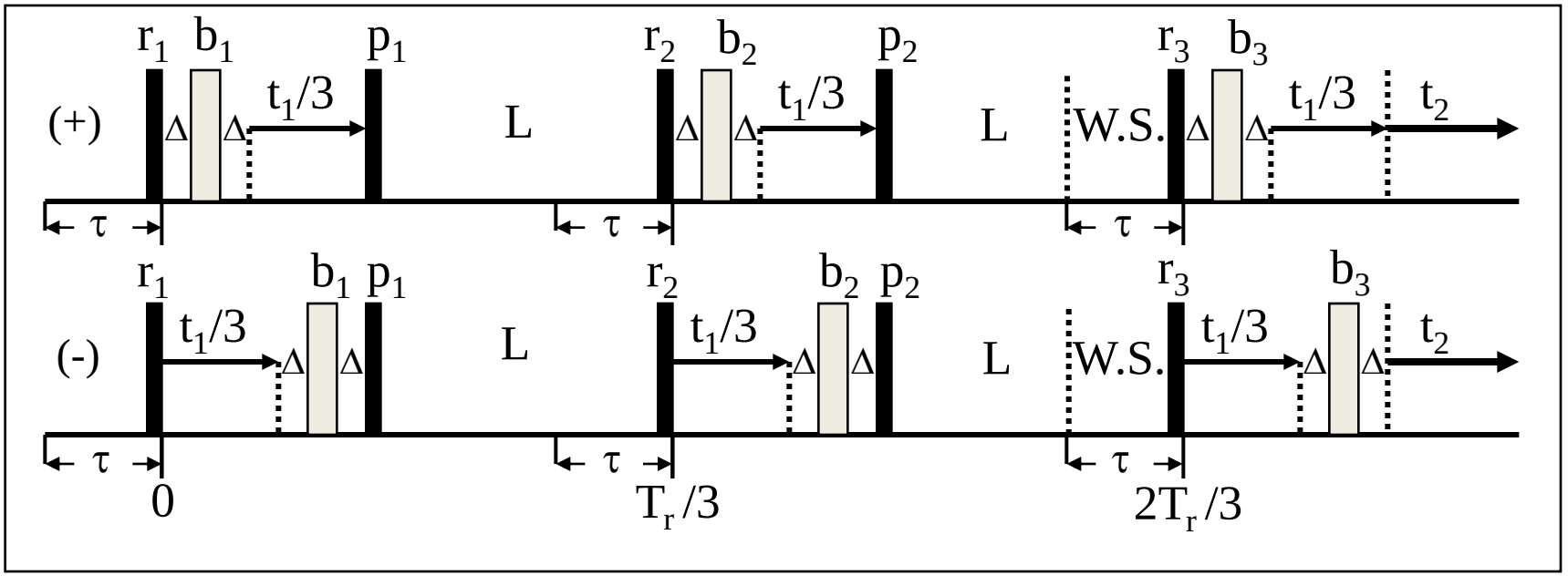
- **Aproach-2:** Ultra-slow-MAS using a sample spinning rate of 1 to < 6 Hz. Non-destructive to live small biological objects

Approach-2: Ultra-Slow MAS (1-6 Hz)



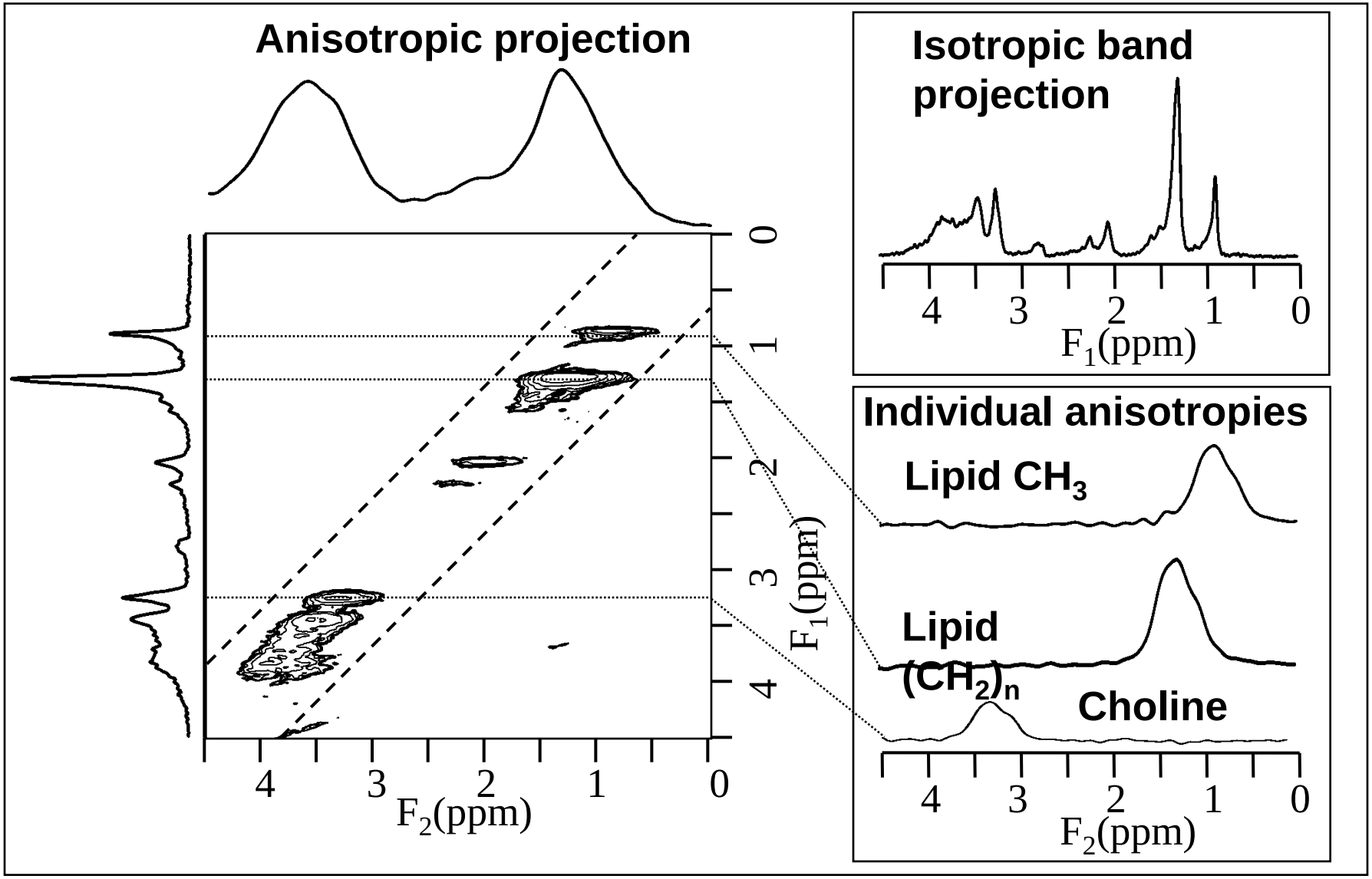
The Pulse Sequence (PHORMAT)





J. Z. Hu and R. A. Wind. "Sensitivity-enhanced phase corrected ultra-slow magic angle turning using multiple-echo data acquisition". *J. Magn. Reson*.163, 149-162 (2003).

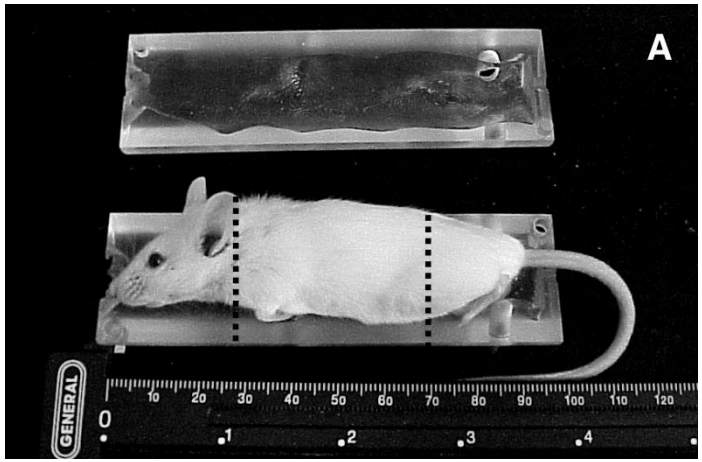
Ex vivo 300 MHz, 1 Hz MAS ^{1}H PHORMAT of excised rat liver*)

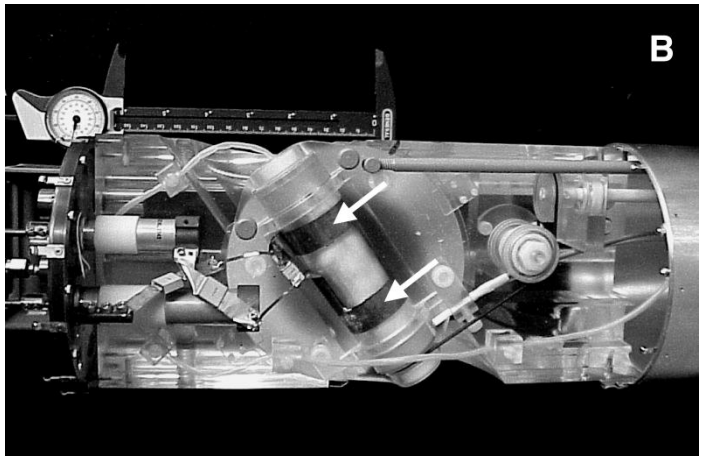


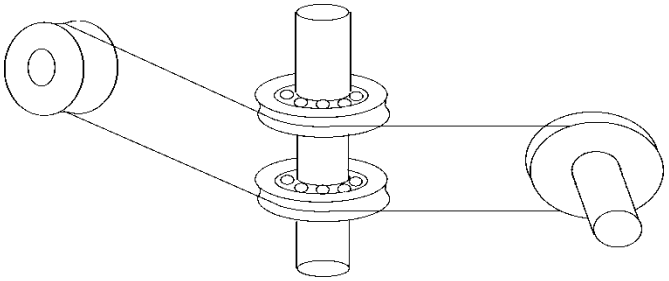
Hu and Wind et al, MRM 47, 829 (2002)

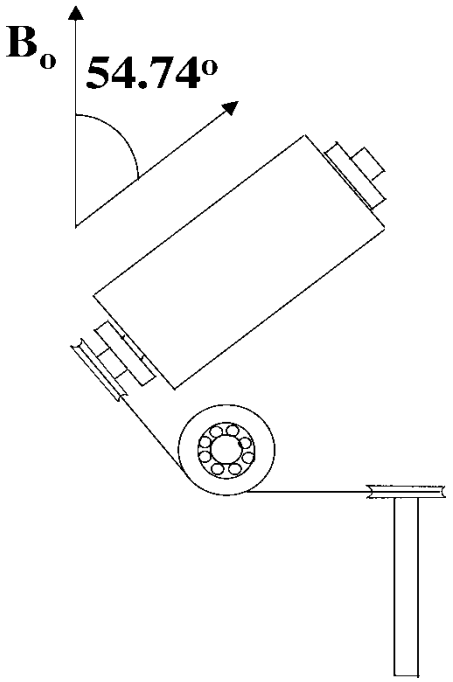
In vivo 85 MHz, 1.5 Hz MAS, ^{1}H PHORMAT on a live mouse*)

Below some pictures of the mouse-MAS probe are shown





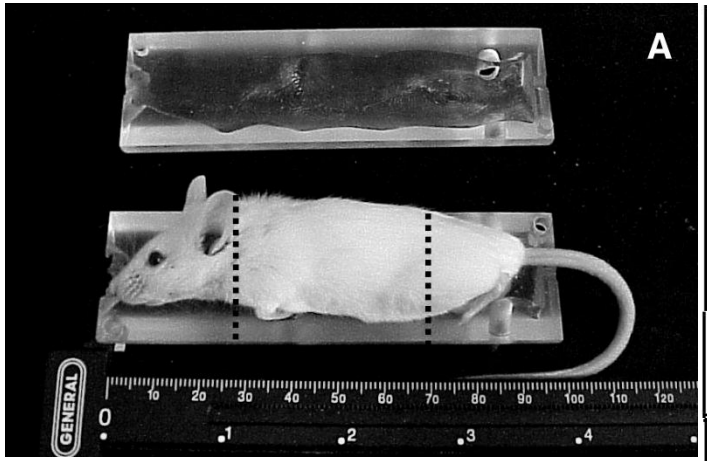


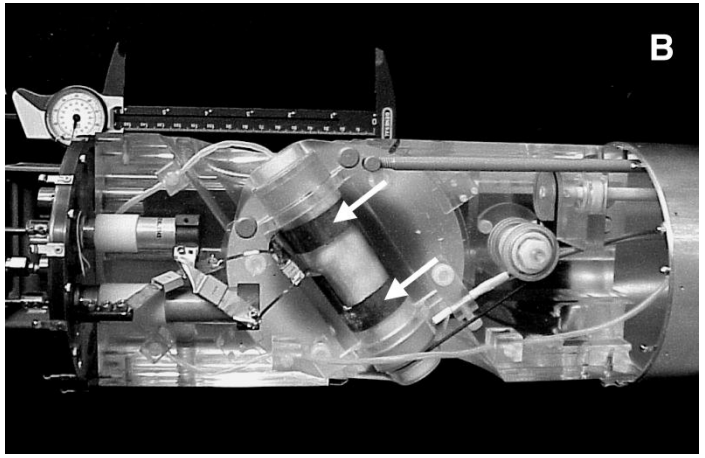


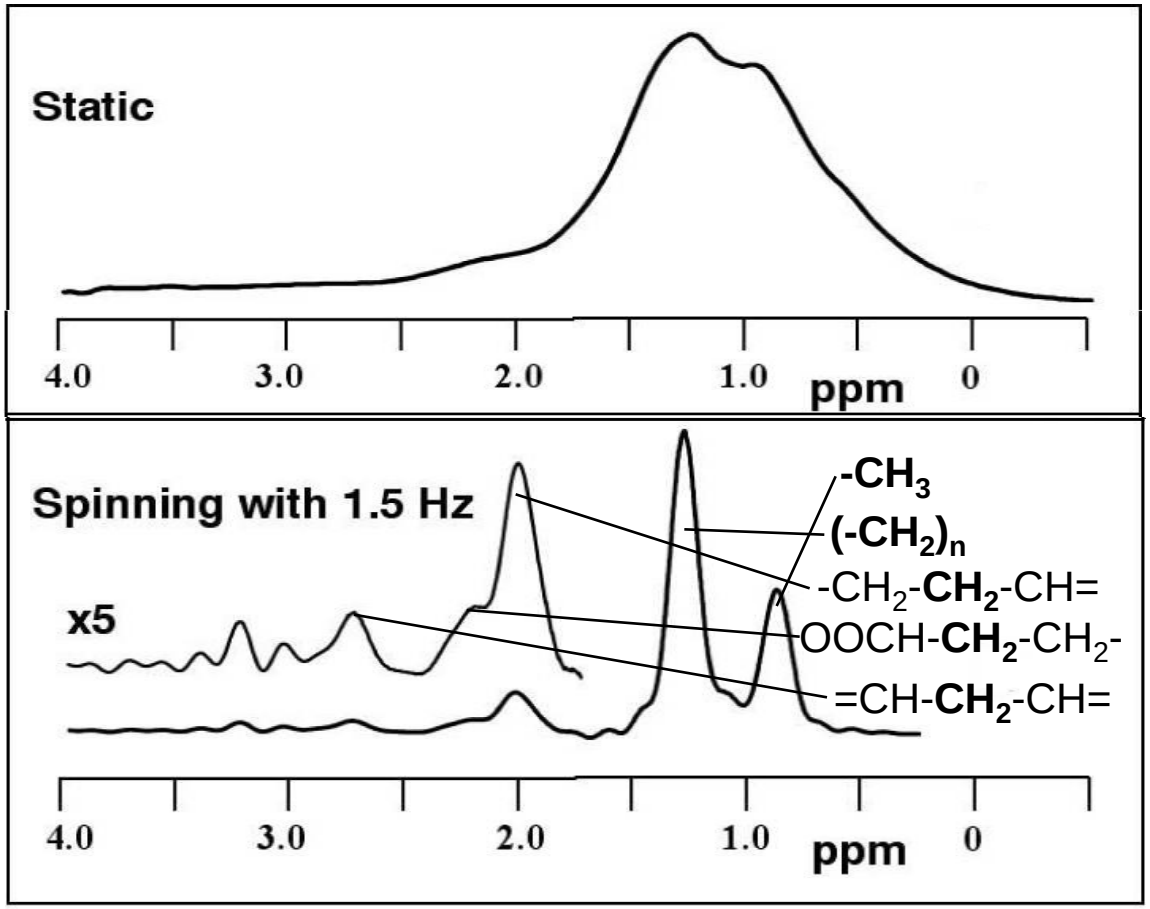
Wind, and Hu et al, MRM 150,1113 (2003)

In vivo 85 MHz, 1.5 Hz MAS, ^{1}H PHORMAT on a live mouse*)

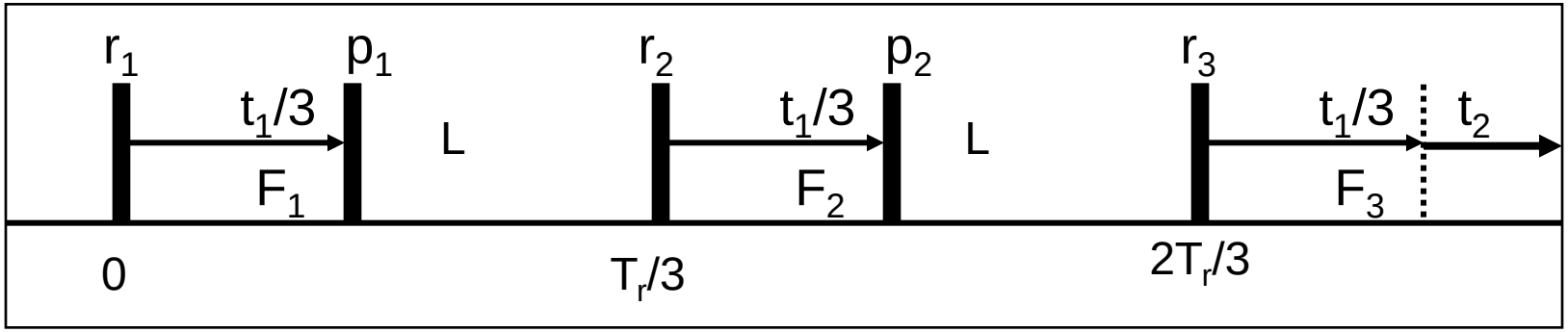
Below some pictures of the mouse-MAS probe are shown, and proton NMR spectra are given obtained on a stationary mouse and a spinning live (anesthetized) female BALBc mouse in a 2 T magnet. Even in this relatively low field a significant spectral resolution enhancement is obtained. Application: body fat content+composition.



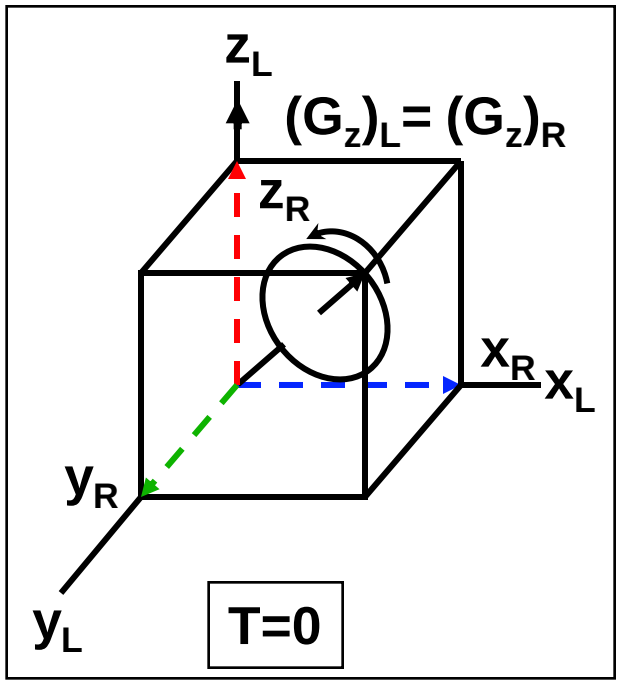


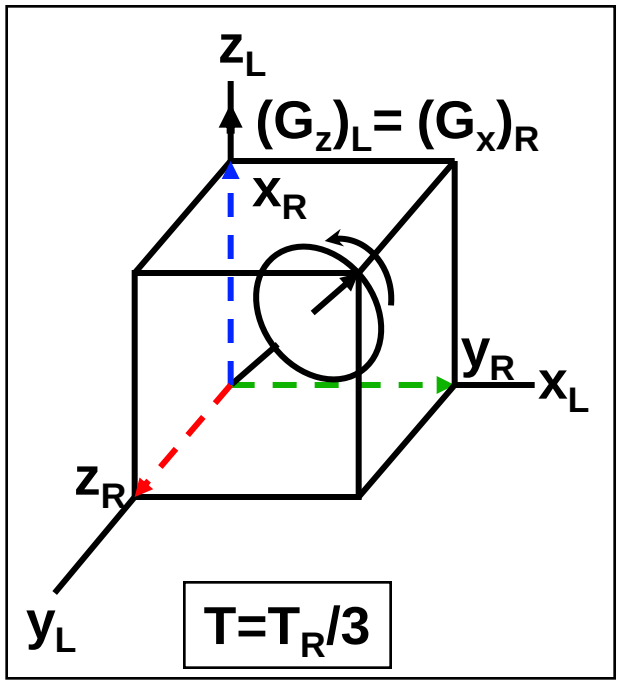


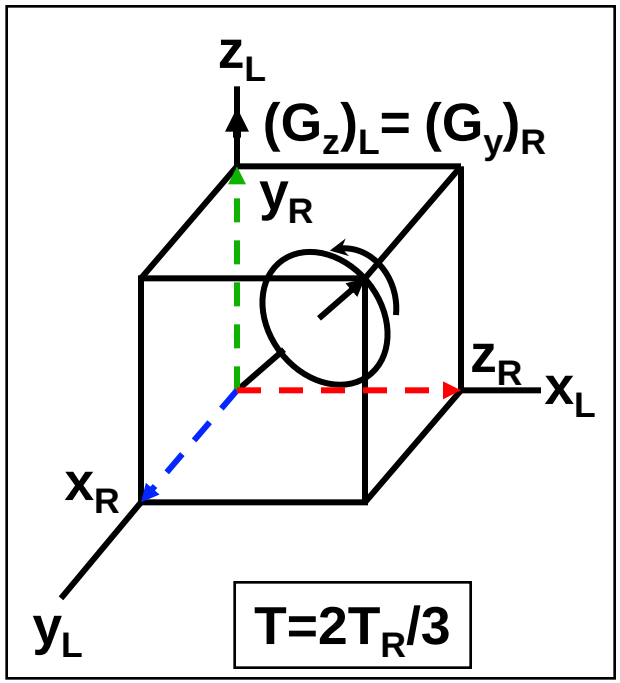
Localized PHORMAT (LOCMAT)



Magic angle spinning occurs along the [1, 1, 1] direction. Hence a single, e.g., z-gradient in the laboratory frame becomes a z-, x-, and y-gradient at 120^0 intervals in a frame rotating synchronously with the rotor*).

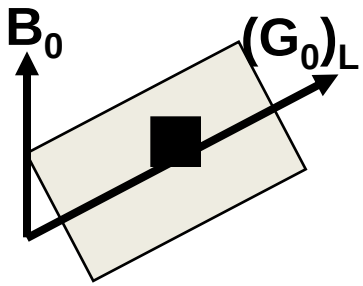


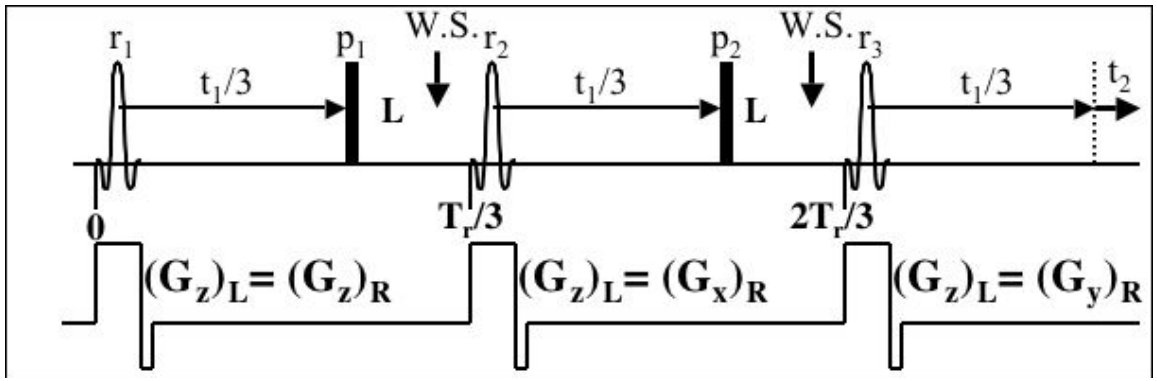




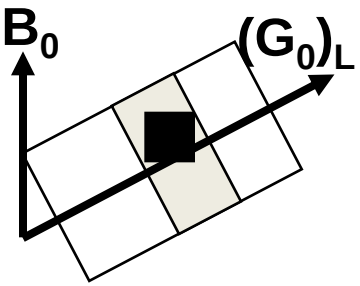
Localized PHORMAT (LOCMAT)

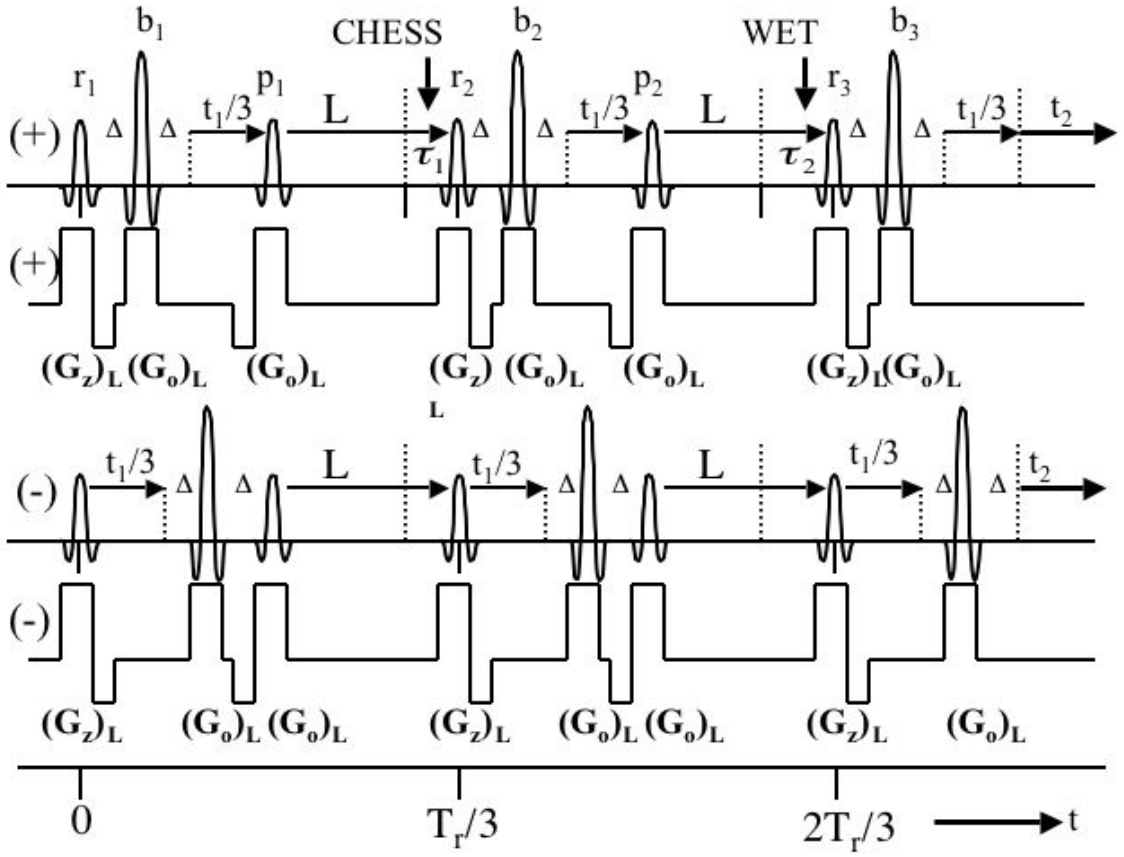
simple version



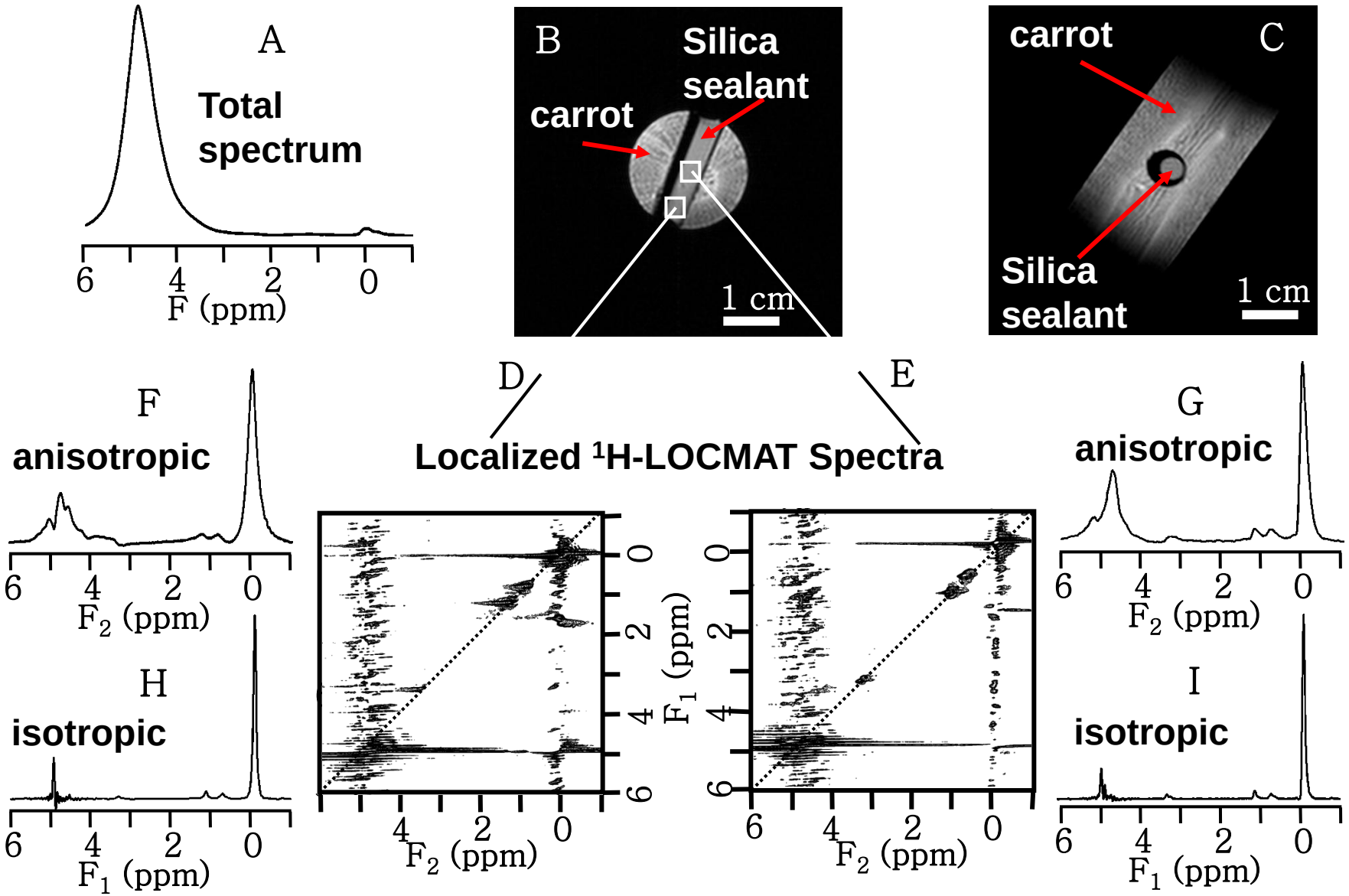


used version

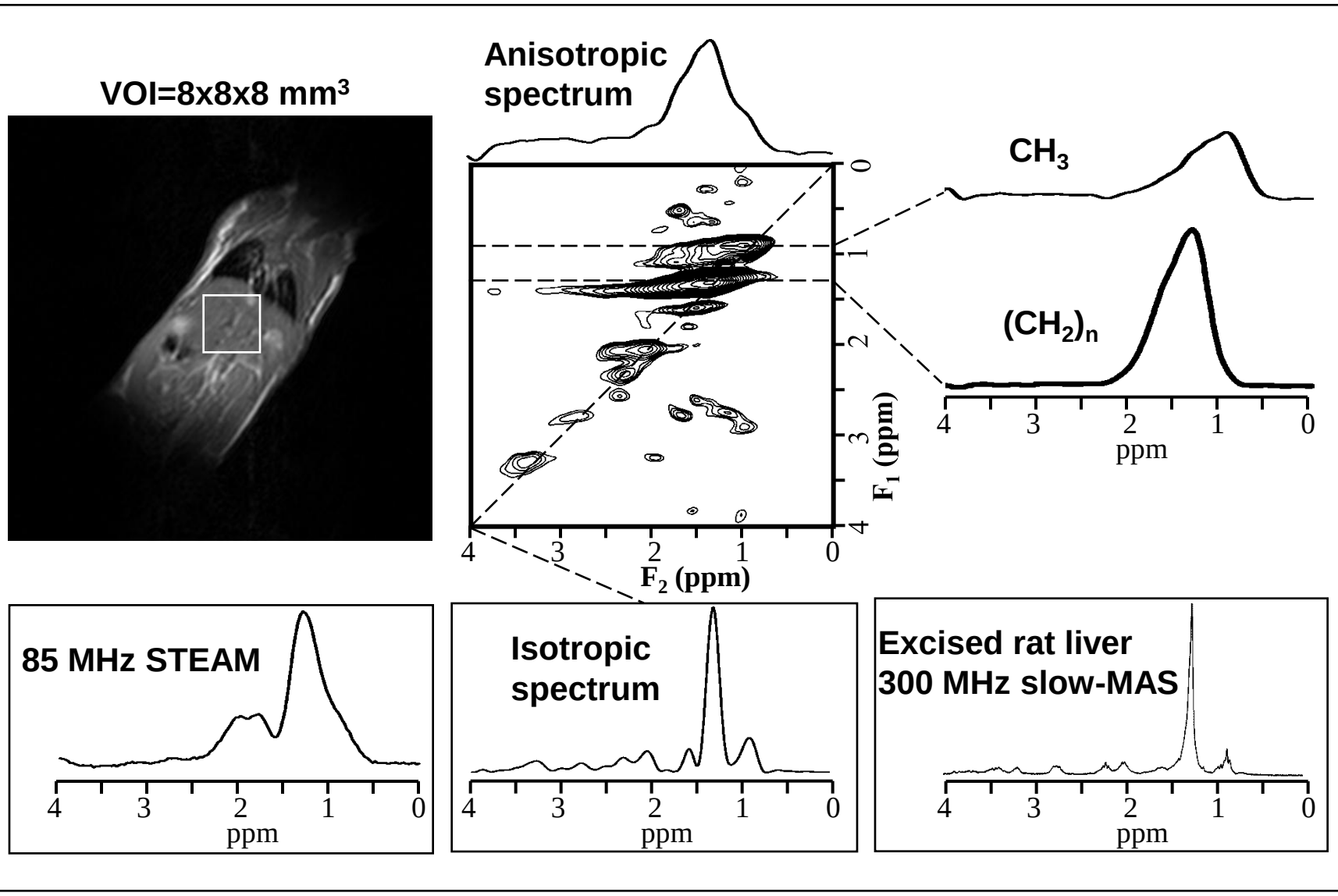




2.5 Hz Localized ^{1}H-PHORMAT (LOCMAT) Performance Test



In vivo 85 MHz, 4 Hz MAS ^{1}H LOCMAT in the liver of a female BALBc mouse



Approach-2: Summary

•LOCMAT separates the isotropic line width from the line broadening induced by both microscopic and macroscopic susceptibility gradients.

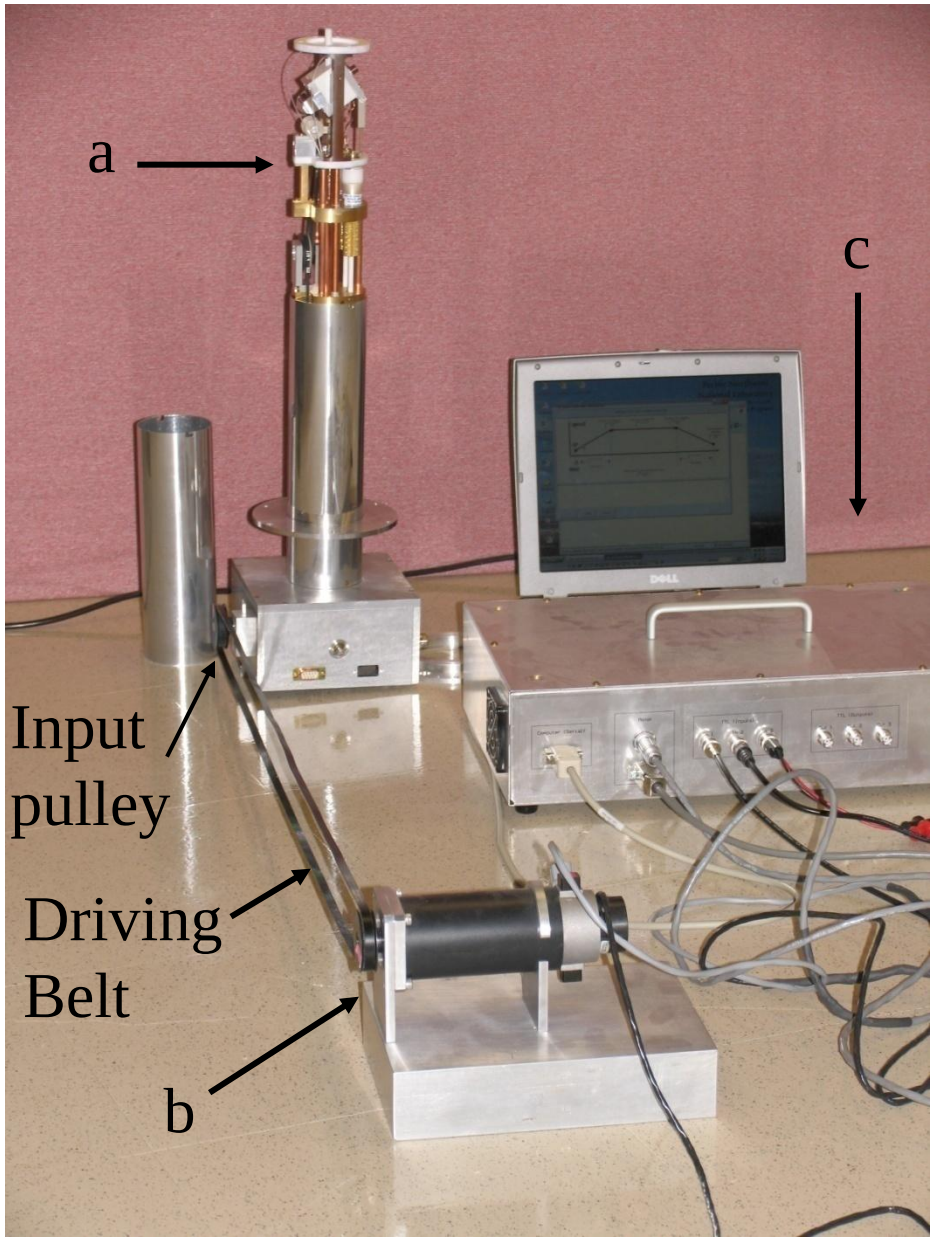
• The isotropic line width is mainly determined by the intrinsic T_2 and the residual B_0 inhomogeneity.

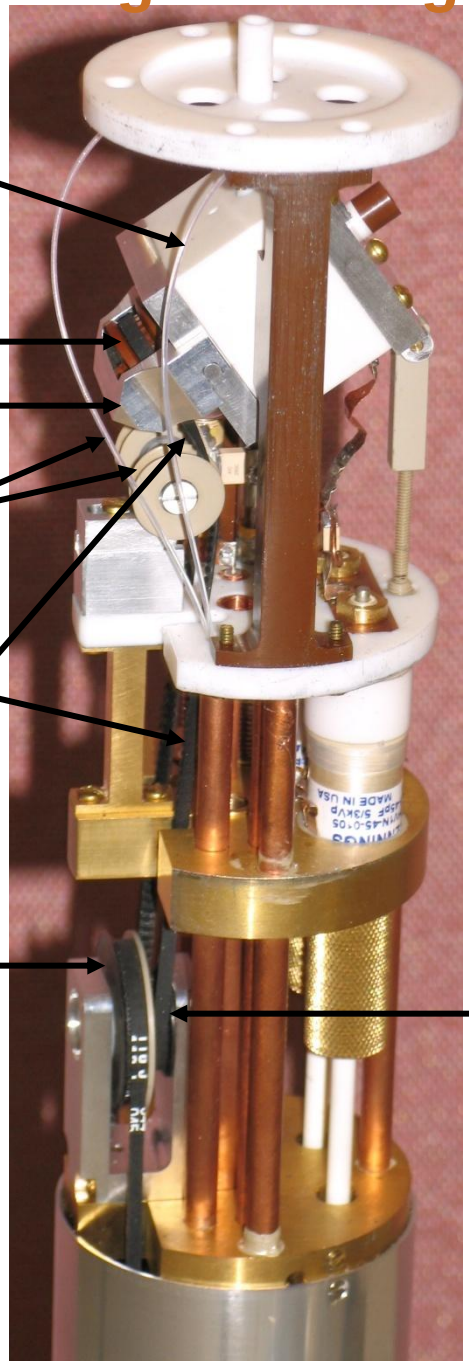
• Even without respiratory and/or cardiac triggering *in vivo* LOCMAT significantly increases the spectral resolution in areas where major motions occur.

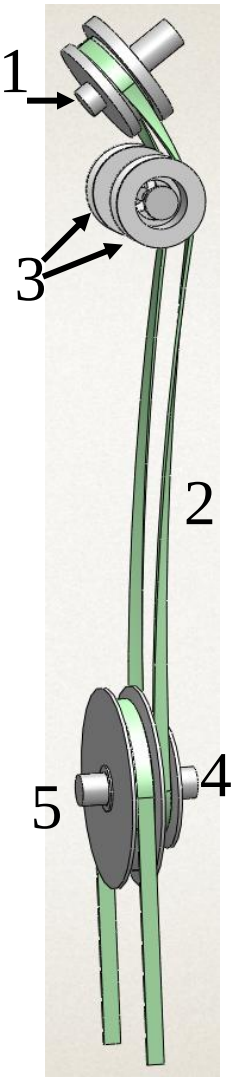
• LOCMAT retains the anisotropic information of the individual metabolite signals, which could become useful for, e.g., diagnosis.

• The main limitation in sensitivity is the t_1-noise, introduced by unwanted signal coherences, which in first order appear to be similar in a live and dead animal. If the sensitivity would have determined by the thermal noise, with the present setup the VOI could be reduced to 5x5x5 mm^3 or less.

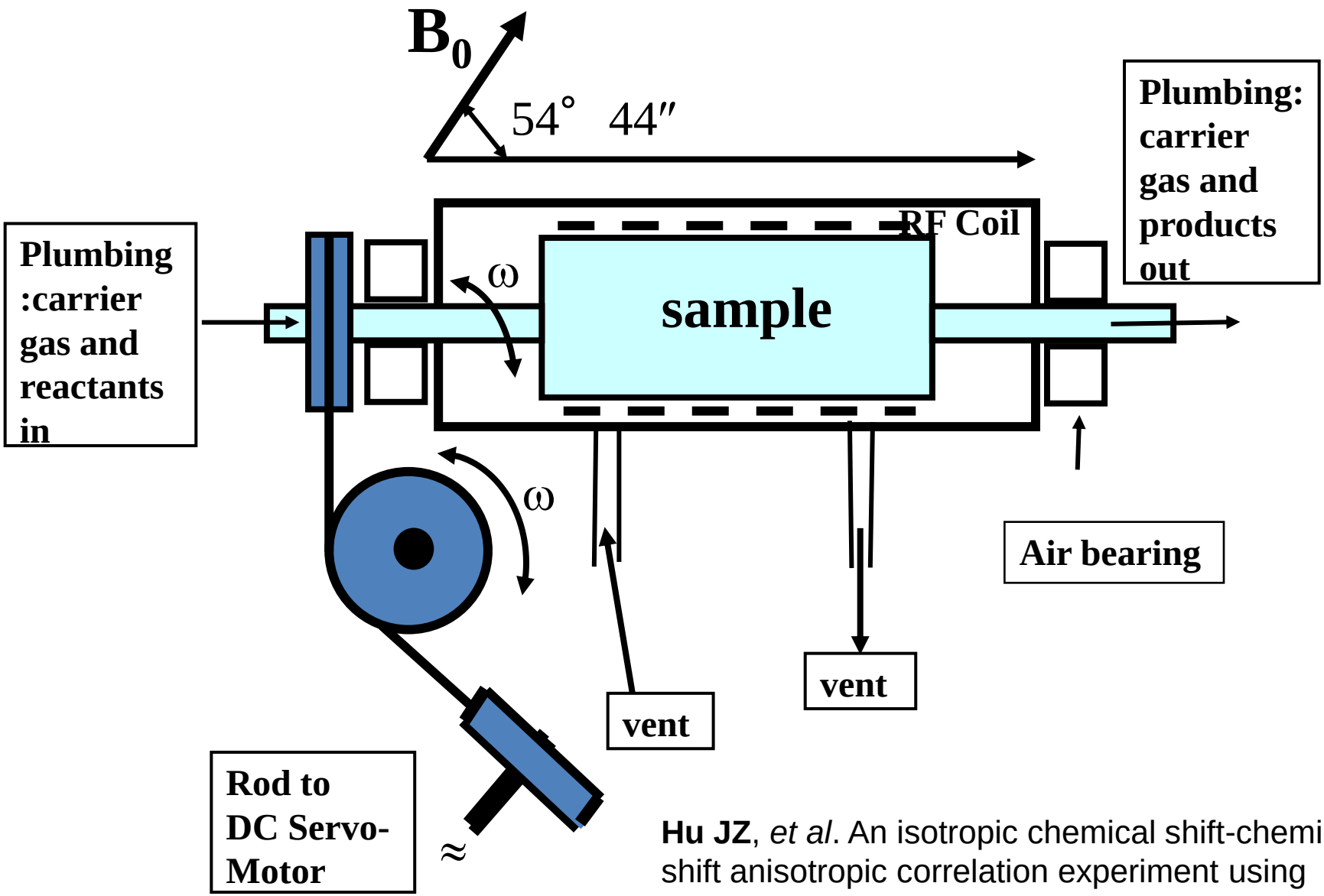
Alternative-1: The Discrete Magic Angle Turning (DMAT)





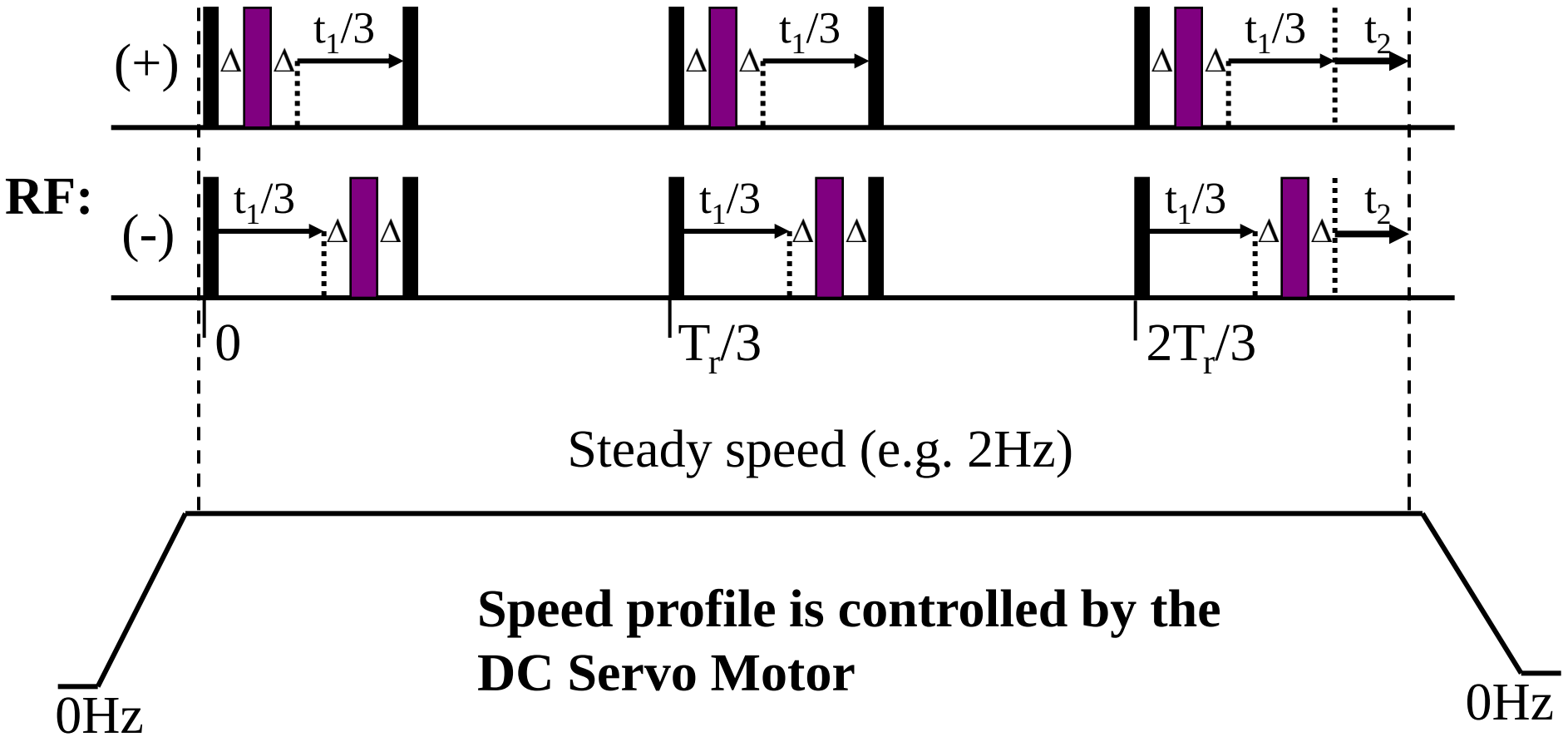


The rotation mechanism inside the DMAT probe

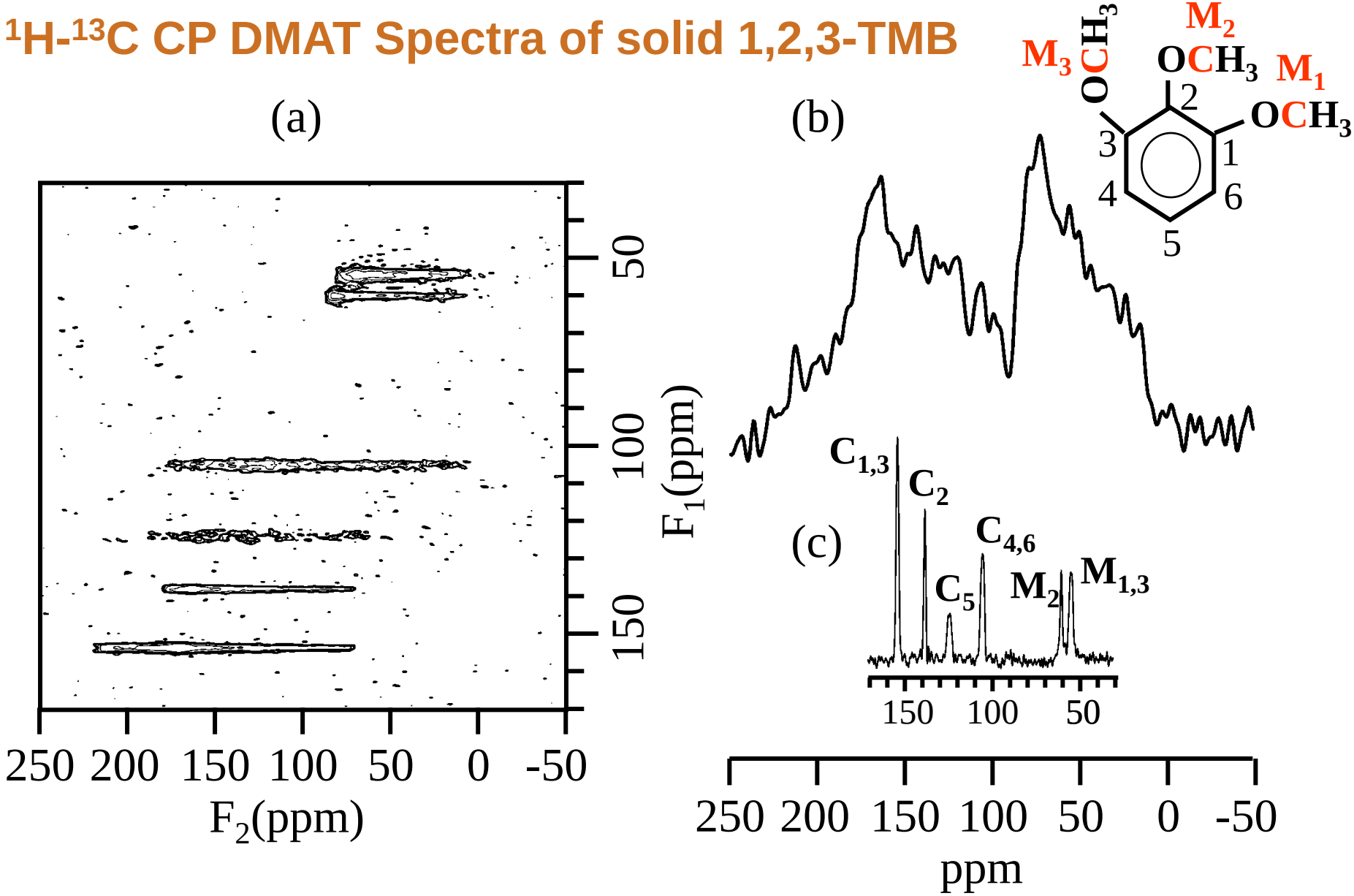


Hu JZ, *et al*. An isotropic chemical shift-chemical shift anisotropic correlation experiment using discrete magic angle turning. *J. Magn. Reson*. 198, 105-110, **2009**.

The Special RF Pulse Sequence for the DMAT Experiment



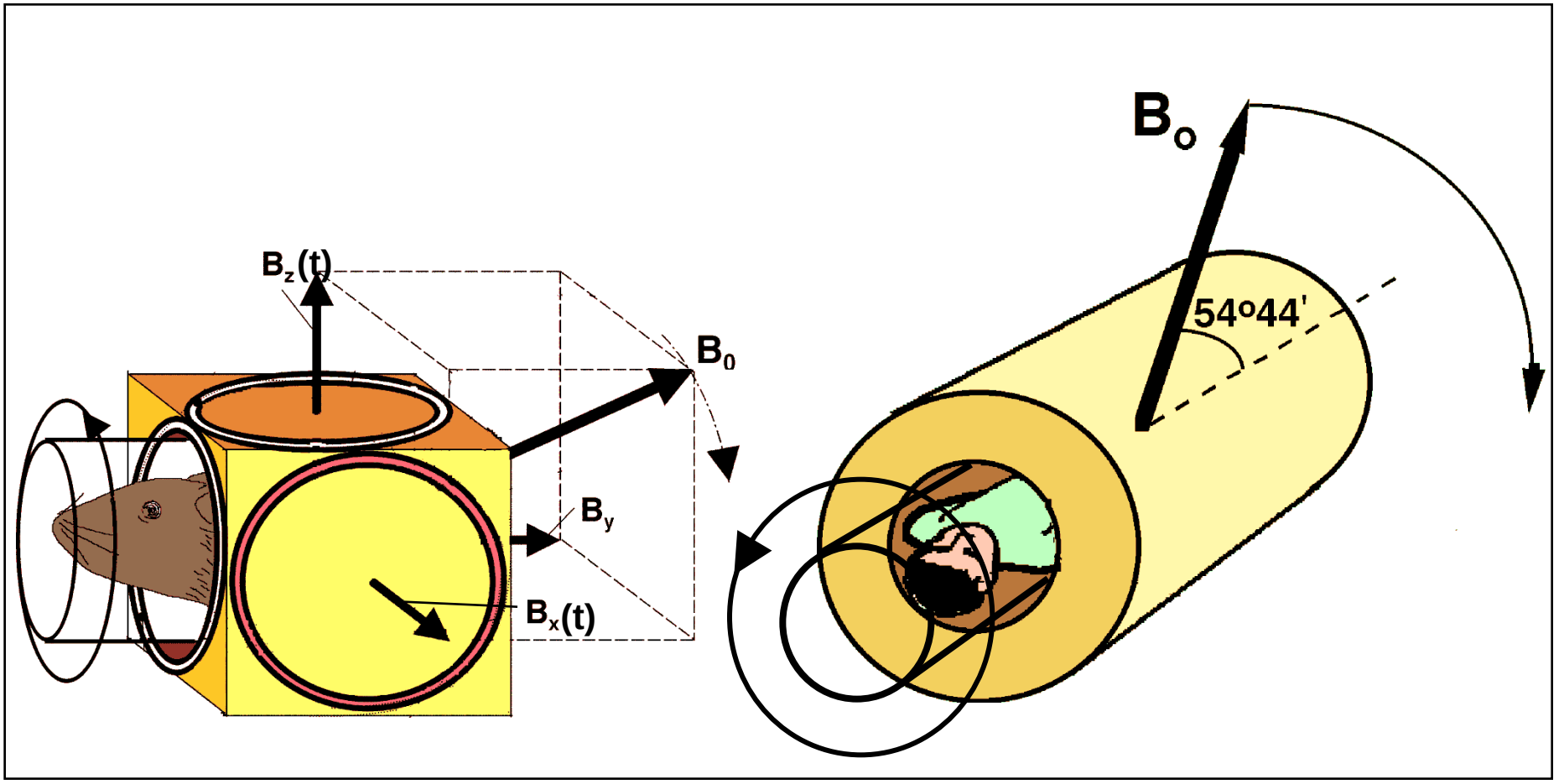
^{1}H-^{13}C CP DMAT Spectra of solid 1,2,3-TMB



(a) 2D contour plot of DMAT spectrum; (b) Anisotropic power pattern as obtained from a stationary sample; (c) High resolution isotropic projection as obtained from fast MAS at a sample spinning rate of several kHz.

Alternative Aproach-2: Rotate the B_o field or both the subject and the field

The same spectral resolution enhancement can also be obtained by rotating the magnetic field instead of the subject. This might make the slow-MAS approach even amenable for humans.



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Kevin Minard
Robert A. Wind

Biologists

Richard A. Corley
Karla D. Thrall

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b. NIH/NCRR 1R21RR025785 and in part by NIH/R01EB013231

c. NASA Space Based Radiation Biology Grant

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