Membrane Protein

- **Expression**
- **Purification**
- **Reconstitution**
- **Characterization**
- **Sample Preparation**

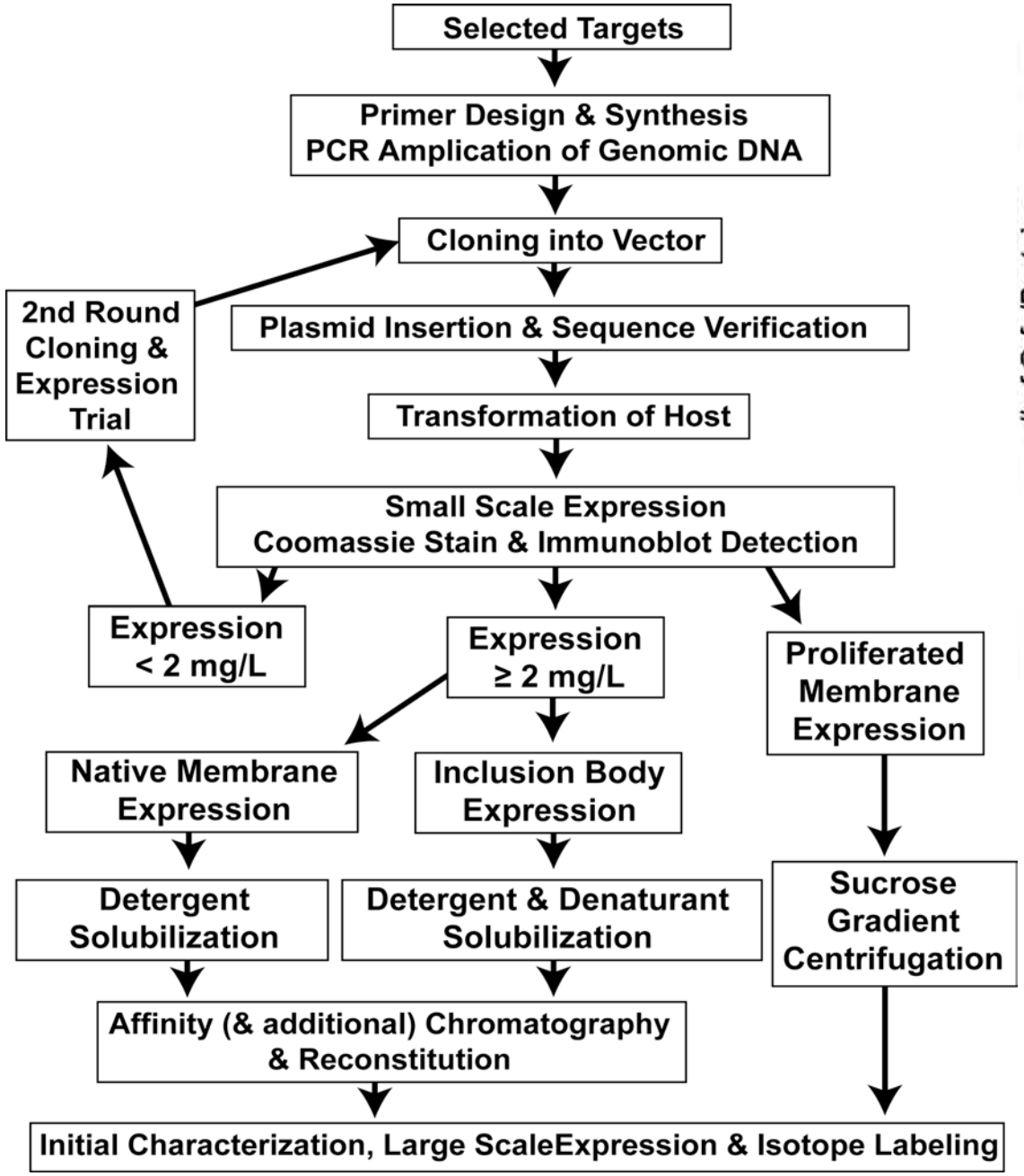
Tim Cross, Florida State University - if interested in help with any of the above contact us and arrange to spend a few days in my lab with people who have lots of first hand experience (i.e. not myself).

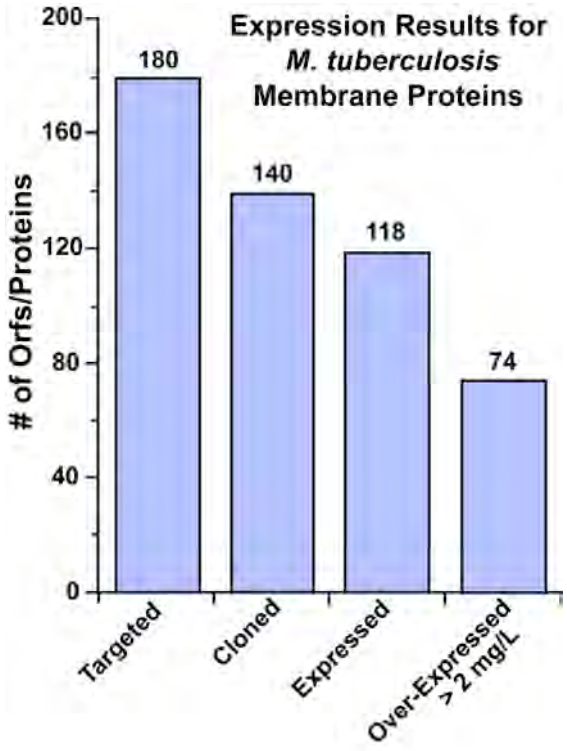
- **Preserving your sample (probe technology)**

Tim Cross, National High Magnetic Field Lab - if interested in this technology - please contact myself of Bill Brey at the NHMFL.

Sensitivity, High Field, Sensitivity, High Field, Sensitivity, H

Membrane Protein Expression and Purification Flow Chart

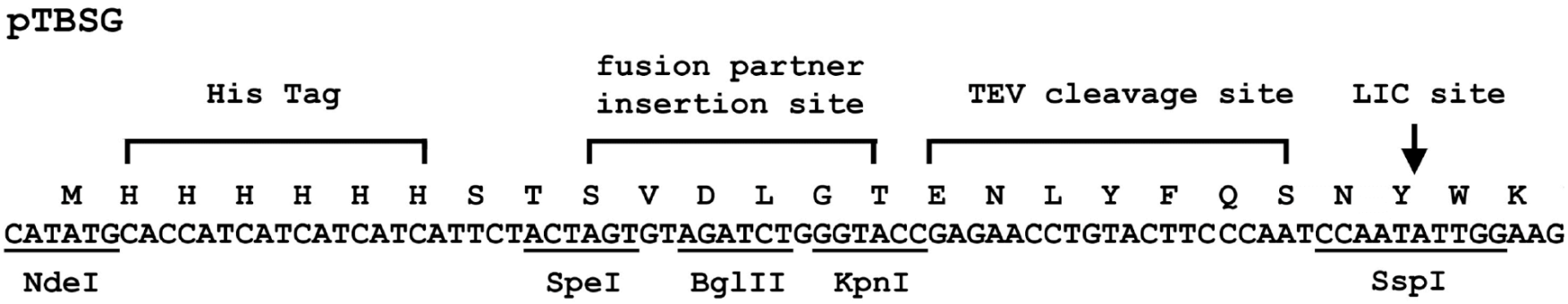


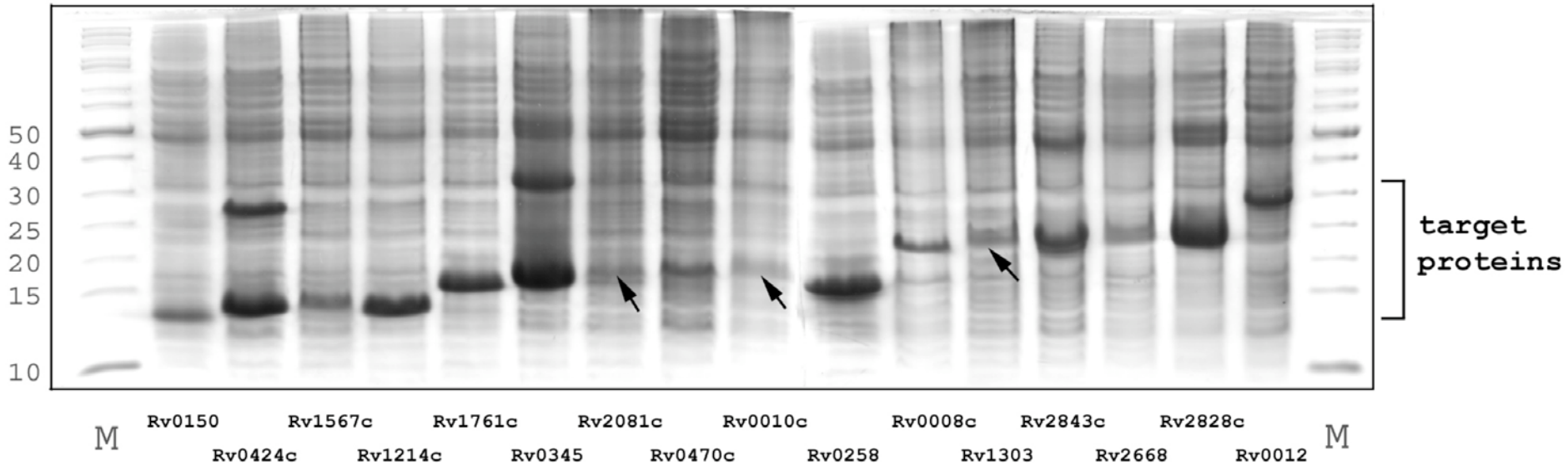


- Data above for Over-expression can be substantially improved

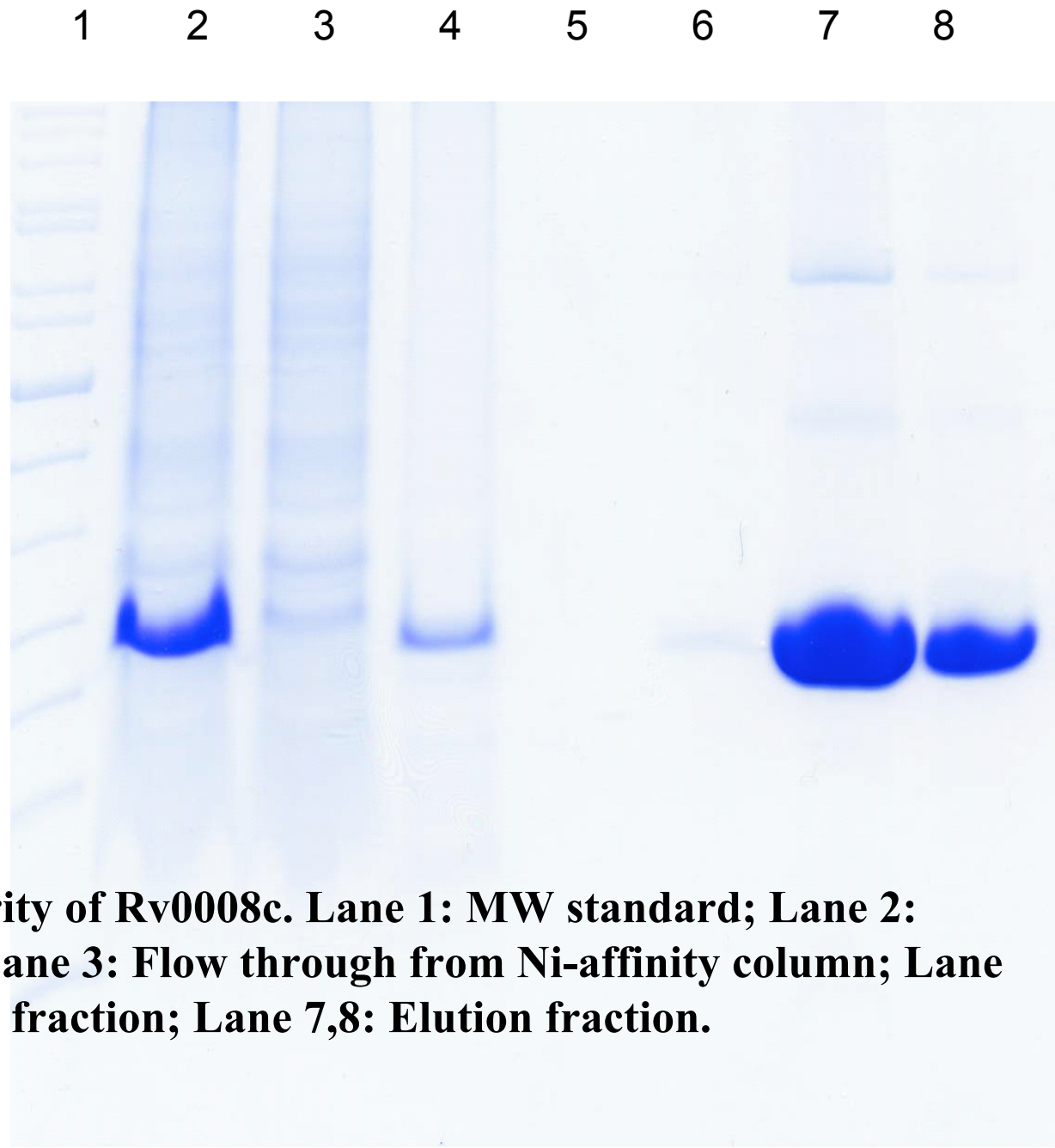
Engineered Vector

- **N-terminal His Tag, • Fusion partner insertion site, • TEV cleavage site,**
- **LIC site, • Target sequence**

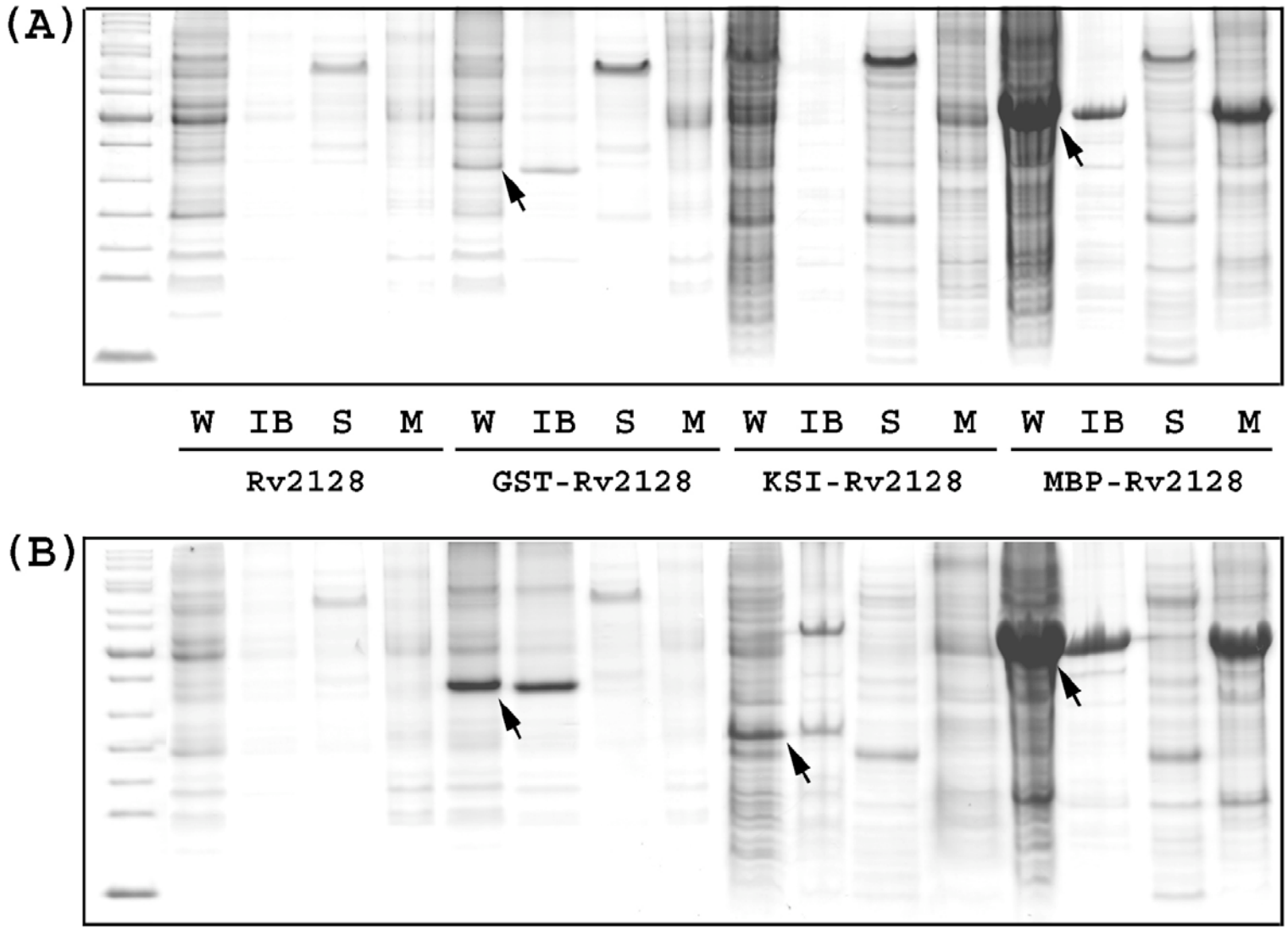




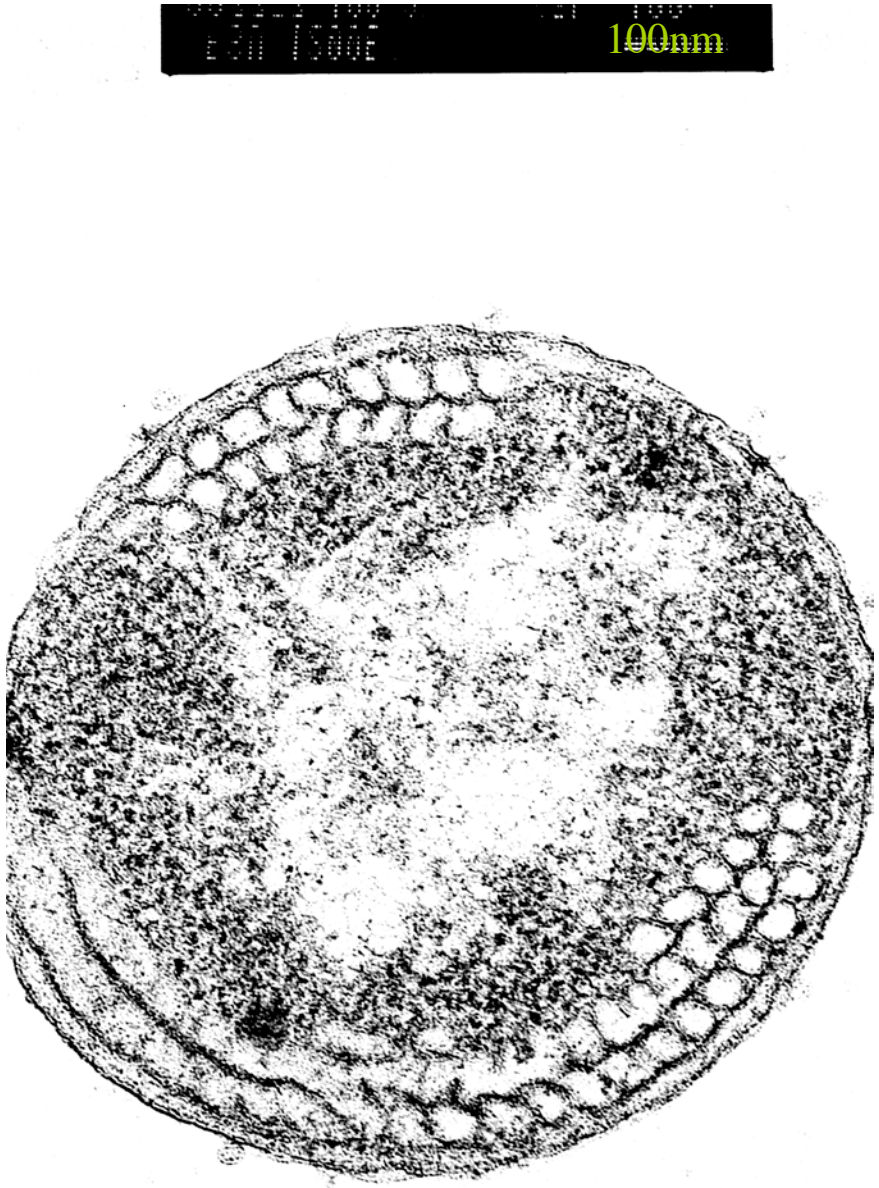
Expression and Purification Using a Ni-affinity Column



Gel showing high purity of Rv0008c. Lane 1: MW standard; Lane 2: solubilized protein; Lane 3: Flow through from Ni-affinity column; Lane 4,5,6: imidazole wash fraction; Lane 7,8: Elution fraction.


(A)
W IB S M W IB S M W IB S M W IB S M
Rv2128
GST-Rv2128
KSI-Rv2128
MBP-Rv2128
(B)
W IB S M W IB S M W IB S M W IB S M
Rv0011c
GST-Rv0011c
KSI-Rv0011c
MBP-Rv0011c

Expression In Proliferated Membranes



- Rv 2433c
- 11.3 kDa
- pET-16b/BL21-CodonPlus-(DE3)-RP
- Ampicillin
- Electron Microscopy
- JEM-1200EX
- 65k Mag
- 100kV

Membrane Protein Environment

	Membrane Environment	Aqueous Environment
Dielectric Constant:	**2 to 220**	**80**
[H_2O]:	**10^{-6} to 55 M**	**55 M**
Fluidity S_{cd}:	**0 - 0.4**	**0**
Lateral Pressure:	**1 to 300 atm**	**1 atm**

Heterogeneous Environment

•

Altered Amino Acid Composition

•

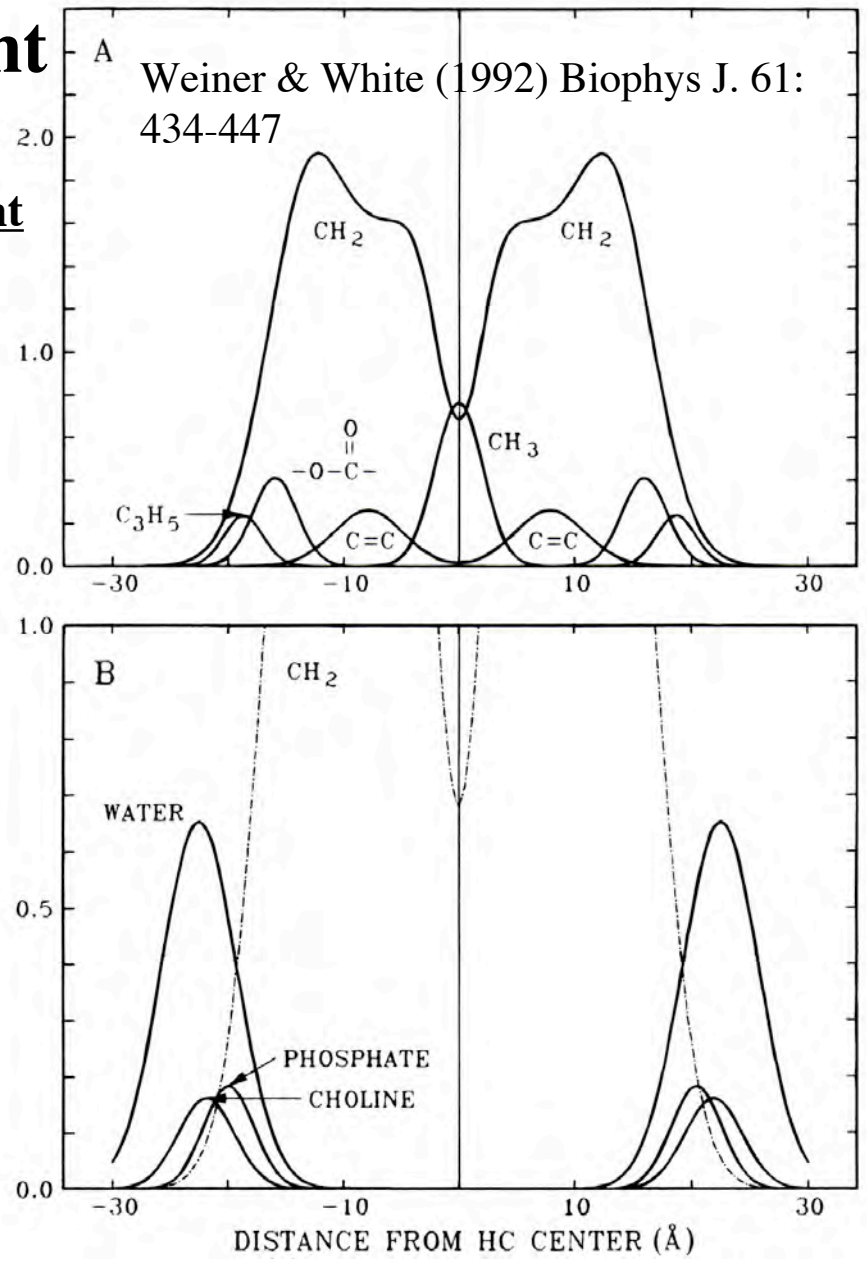
Unique Physical Properties: Curvature Frustration; Hydrophobic Mismatch; Electrical, Chemical, & Mechanical Potentials

•

Altered Balance of Molecular Forces to Stabilize Structure

•

A Challenge for all Structural Technologies



»» *very difficult to mimic*

Oriented Sample Preparation on Glass Substrate: *General Strategy*

(Disclaimer - the protocol has been successful with half a dozen different proteins, but we are continuously refining this protocol and learning why some things work and others don't….)

- **The basic idea for oriented sample preparation is to induce alignment of bilayers with respect to the glass surface.**
  - **assembly of lipid molecules into a planar bilayer on the surface**
  - **flattening a liposome preparation onto the surface.**
- **Bilayer assembly is typically not viable for proteins as opposed to peptides, so reconstitution of full length protein into liposomes is necessary step.**
- **Hydrophobic peptides are usually soluble and stable in organic solvents and relatively easily aligned in lipid bilayers by rehydrating a peptide/lipid film to induce bilayer assembly - I will not discuss this further.**

Membrane Protein Aligned Sample Preparation: I

- **Exposure to extreme pH, organic solvents and elevated temperature must be avoided. Length of time from expression to stable environment must be minimized.**
- **The general protocol involves detergent/lipid exchange by dialysis to prepare liposomes from lipid-protein-detergent mixed micelle.**
- **Best results are usually achieved in mild "non denaturing" detergents with high CMC like octylglucoside (OG). Other high CMC detergents can be used such as SDS if the protein is stable in them.**
- **e.g. mixed micelles prepared by addition of a 30% OG solution dropwise to a white chalky suspension of lipids in very little buffer until the solution clears. usually up to 10% OG (20*CMC of OG, not a lot considering high amount of lipids). This solution can be sonicated to homogenize the solubilization. Point of onset of liposome solubilization by OG is about 1 OG/lipid (molar ratio)**
- **Concentration of protein should be high so as not to be too dilute in the mixed micelles, usually more than 1-2 mg/ml.**
- **Lipid to protein molar ratio should be kept at no less than 150. Larger extra-membranous domains may induce curvature in the bilayer at higher concentrations. Recommended place to start for lipid/protein molar ratio is 200-250 and working downward until protein can be aligned in maximum quantity without loss of alignment.**

MP Aligned Sample Preparation: II

- **Choice of detergent for protein:**
- **Protein preparations should be highly homogenous in the detergent chosen for reconstitution. Many proteins may not be soluble in octylglucoside or even in any of the high CMC detergents.**
- **Case I:**

  ChiZ was not soluble in OG but highly soluble in DPC. Solution NMR results confirmed homogeneity of the preparation in DPC. Oriented sample were prepared by dissolving protein in minimum amount of DPC required to solubilize the protein, followed by mixing with octylglucoside-lipid mixed micelles.
- **Case II:**

  Rv0008c formed highly homogeneous preparations in SDS micelles as judged from HSQC spectra in SDS. The oriented samples were prepared by combining protein in 5% SDS with OG-lipid-mixed micelles. Final concentration of SDS before dialysis was 0.5% (2-3 CMC).

MP Aligned Sample Preparation: III

- **Factors affecting choice of lipids:**

  - Lipid chain length : Hydrophobic thickness, tilt angle.

  - Phase transition temperature: Protein exposure to high temperature may not be desirable, experiments need to be performed at temperature higher than T_m, adds to heat generated by high RF field.

  - Ester linkages in lipids are prone to hydrolysis, for longer stability of oriented samples ether linked lipids should be used. Hydrolysis is most effective at temperatures close to phase transition temperature. This temperature range should be avoided if using pH other than 6.5-7.5 range.

  - Inter bilayer separation is dependent on bilayer charge and can be modulated by using ionic lipids. This inter bilayer separation plays an important role if the protein has a large domains outside the membrane. PG appears to be helpful in a number of membrane protein preparations.

MP Aligned Sample Preparation: IV

- **Dialysis:**

  CMC of the detergent is dependent on ionic strength, pH and temperature. CMC of ionic detergents is highly dependent on ionic strength, care should be taken while dialyzing SDS. Lower temperatures make detergent removal slower, sometimes crystalline aggregates with octylglucoside form.

- **Buffer:**

  Protein stability should be considered when choosing buffer. Initial dialysis steps are usually performed in high concentration of buffer strength, final dialysis should be performed in low buffer concentration (5-10mM) so as to avoid additional. Preferred buffers are

  - Bis-Tris-propane > HEPES > Sodium phosphate >Tris –HCl

  based on electric conductivity.

- **Extensive dialysis is necessary, two buffer changes daily in the reservoir over 4-5 days for OG and 8-10 days for SDS. Biobeads should be used if low CMC detergents are used, such as DPC, DDM, DM.**

- **Turbidity of the liposomes can be measured at 500nm to monitor vesicle formation.**

  - Sodium azide should be added to buffer for long-term storage.

MP Aligned Sample Preparation: V

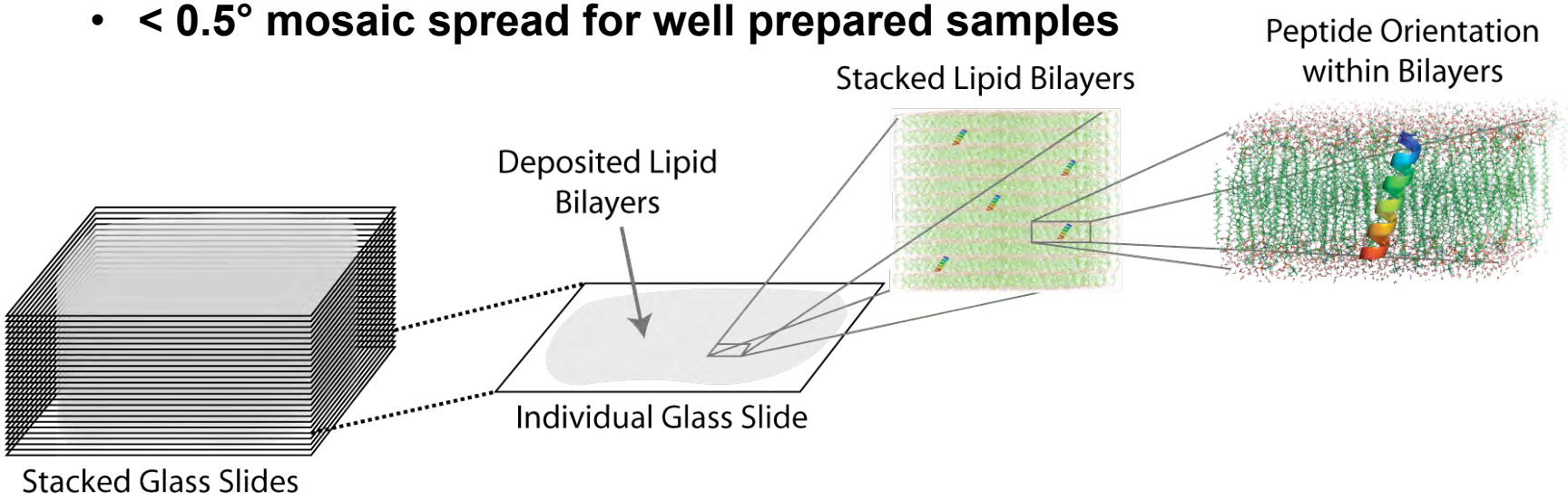
- **Ultracentrifugation should be performed for 2-3 hours at 90,000g. Incomplete reconstitution can be judged at this point by measuring the UV absorbance of the supernatant at 280 nm for the presence of protein in micelles.**
- **Pellet should be resuspended in buffer or ddH_2O (1-2 ml) and can be homogenized by freeze thaw cycles, sonication and vortexing. Extensive sonication or freeze thaw cycles should be avoided since extramembranous portion of the protein might not be stable.**
- **Glass slides (ours are 5.7 x 11.5 mm) are washed with chlorofrom and dried in the oven.**
- **Liposome solution is deposited on 35-40 glass slides spreading 1.5 - 2 mgs lipids per slide. Slides with more sample are prone to inhomogeneous drying, are difficult to rehydrate and high mosaicity, due to bilayer defects.**
- **Slides are air dried at room temperature overnight, stacked and placed into sample cell. Care should be taken not to shear the slides on each other.**

MP Aligned Sample Preparation: VI

- **Measure the weight of dry stacked slides, place the sample cell in 98% humidity chamber (saturated solution of K_2SO_4) at 37°C.**
- **Monitor the weight gain until it stabilizes, seal the sample cell with a glass slide and wax. Usually we hydrate our samples for 5-7 days for DMPC:DMPG mixtures.**
- **Well hydrated samples usually are somewhat transparent.**
- **Water droplets forming on the sample cell wall is an indication that there is severe sample heating taking place: The recycle delay and cooling air are not particularly effective in controlling this problem.**
- **^{31}P is only partially effective for quality control - signals from the protein are needed for assessing the quality of alignment.**

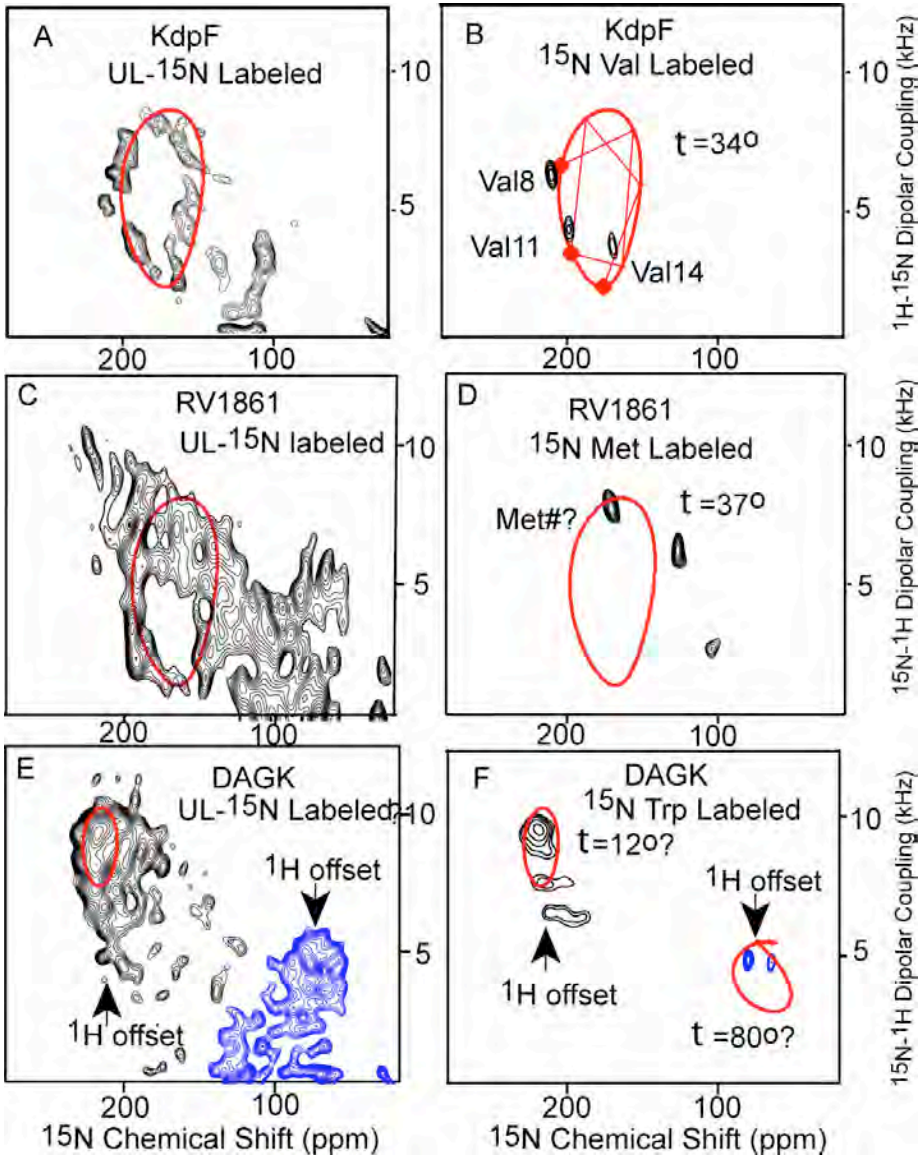
Aligned Sample on Glass Slides

- **Detergent Mediated reconstitution**
- **Bilayer Environment**
  - **Native like environment**
  - **Position and orientation within bilayer attainable**
  - **$^{1}H/^{15}N$-PISEMA**
  - **Oriented Bilayer samples**
    - **Proteoliposomes deposited onto thin glass slides**
    - **Dry, stack, rehydrate**
    - **30-40 stacked slides per sample**
    - **≈5,000 bilayers per slide**
  - **Mosaicity**
    - **< 0.5° mosaic spread for well prepared samples**



Membrane Proteins: Solid State NMR

- **Sample Preparation is everything**
- **Homogeneous and Uniformly Aligned Preparation**
- **Spectral Resolution**
- **Long Term Stability**
- **Utilized Liquid-crystalline Lipid Bilayers**
- **Requires isotopic labeling**
- **Can obtain structural information from 1st spectrum**
- **1st Spectrum includes Structural Restraints, but need more**
- **Finally build a Structural Model**

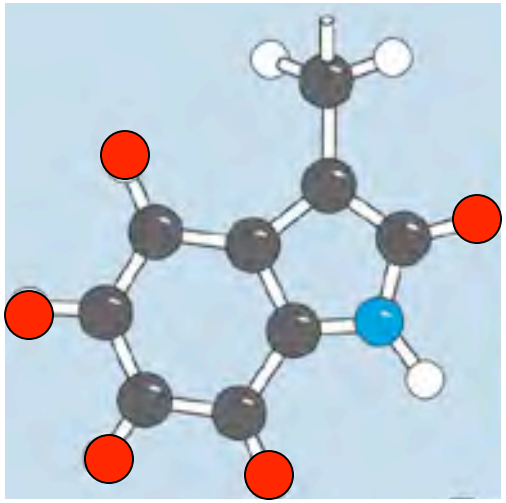


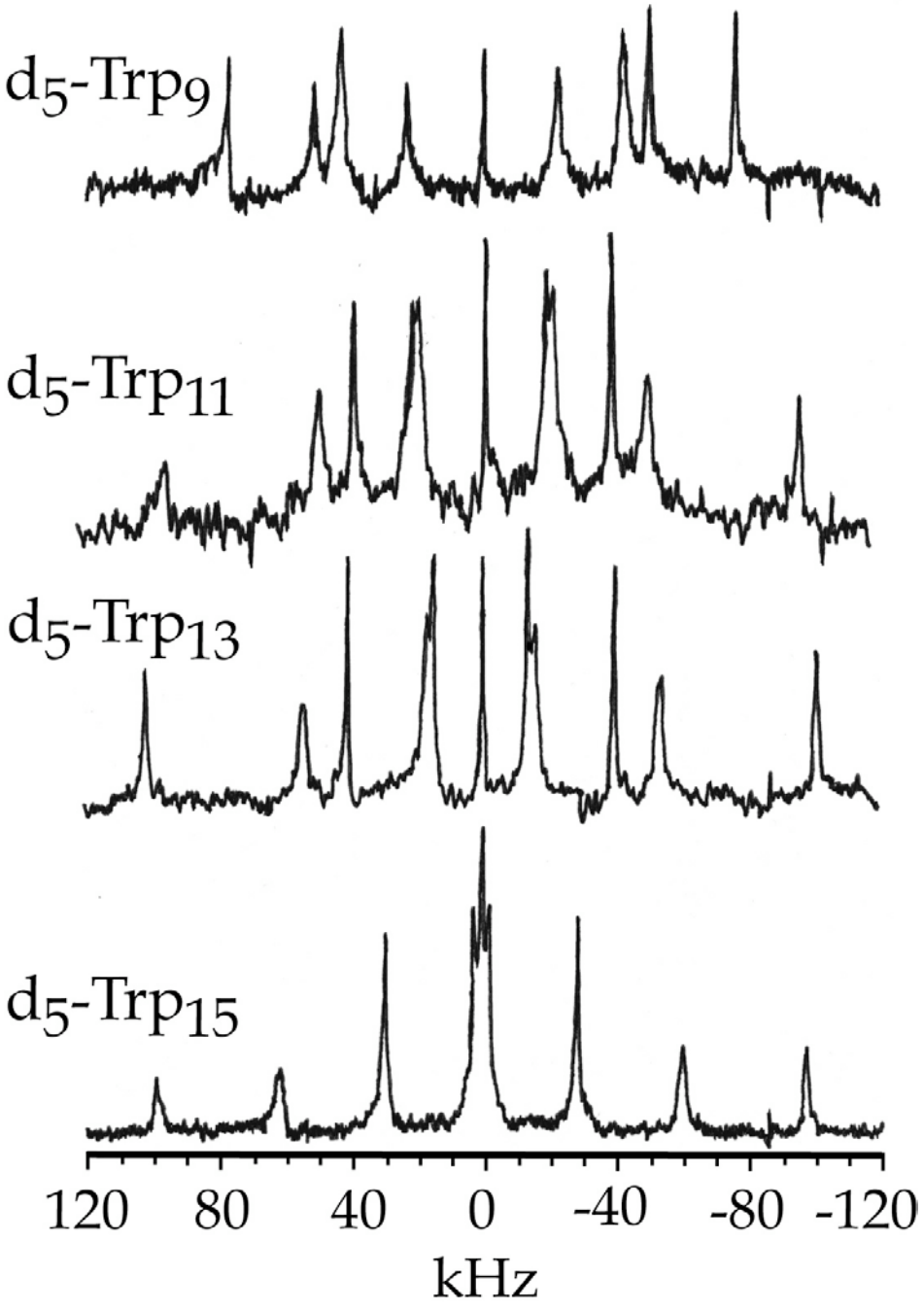
Spectra of three full length MPs in Uniformly Aligned Liquid-Crystalline Lipid Bilayers - Li et al., JACS 2007

The Precision of Time Averaged Orientational Restraints

»» ^{2}H spectra of d5 Trp labeled gramicidin A in uniformly aligned lipid bilayer preparations.

»» The linewidths are consistent with an orientational dispersion of just ±0.3° - consequently very precise indole orientations.

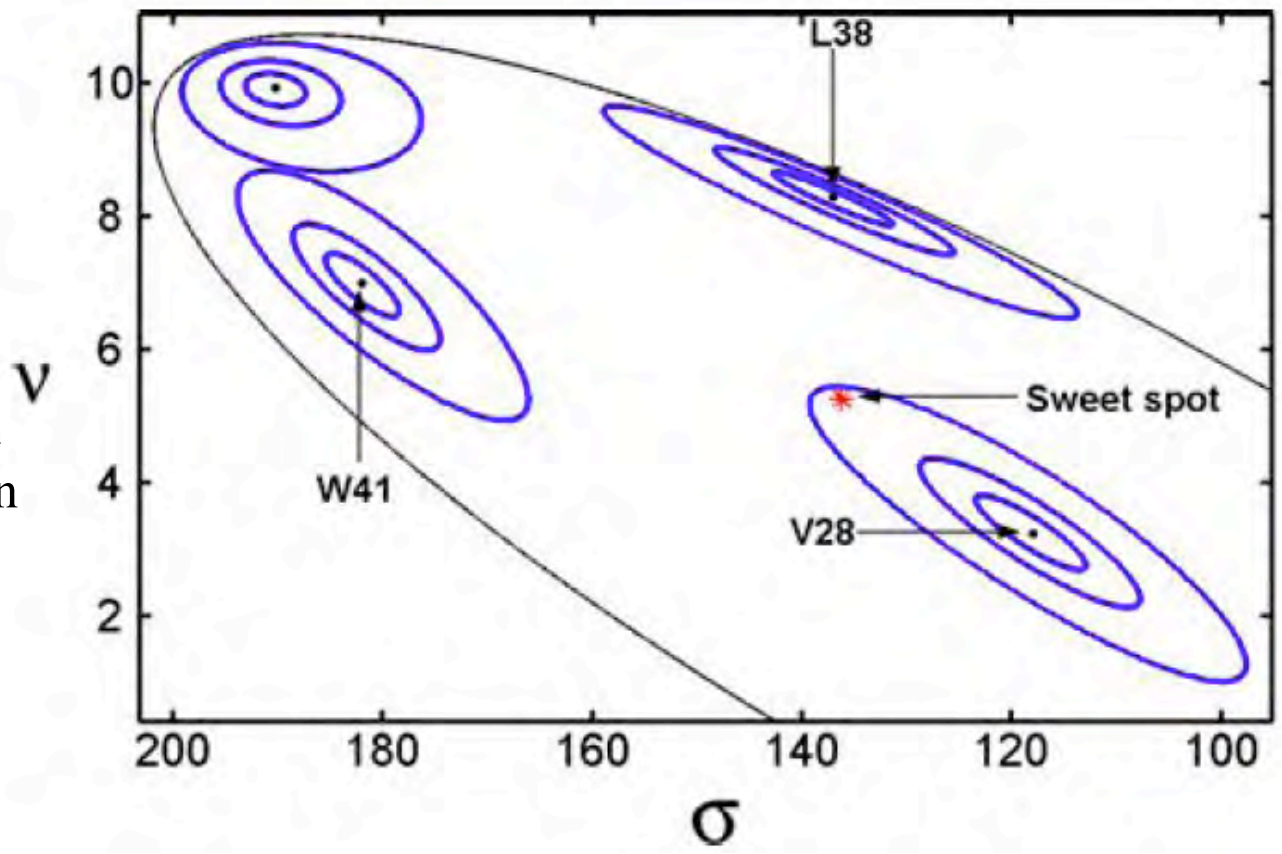




The Precision of Time Averaged Orientational Restraints

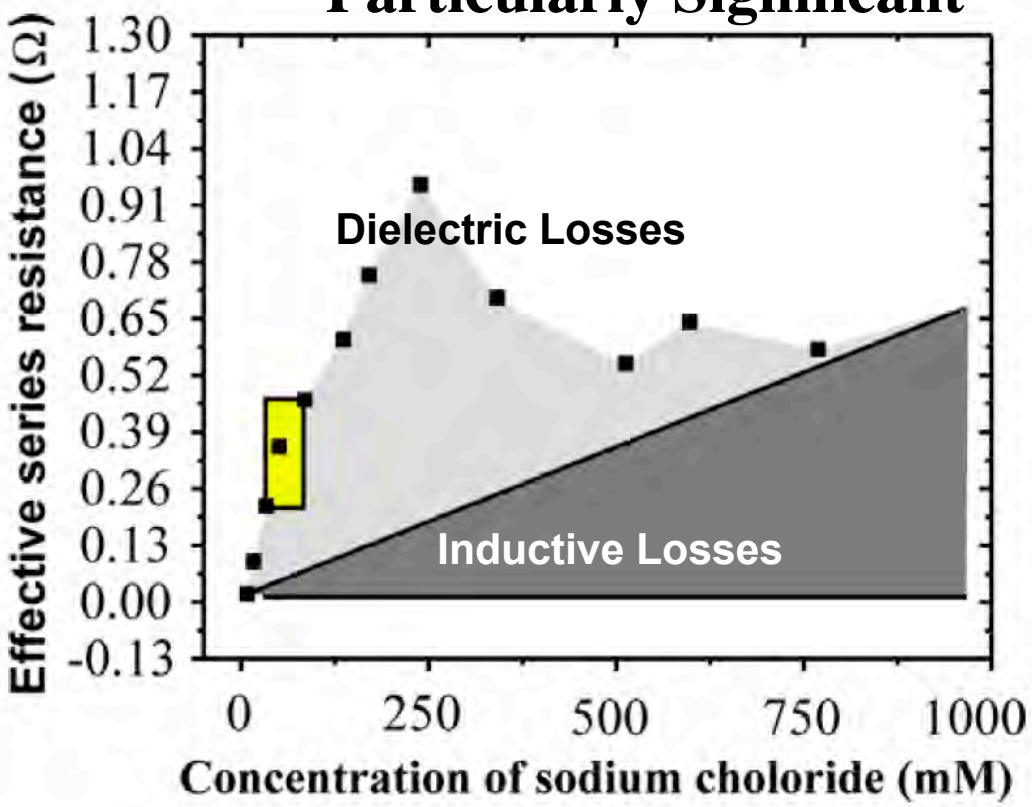
»» ^{15}N lineshapes predicted from mosaicity radii of 2, 4, and 8° .

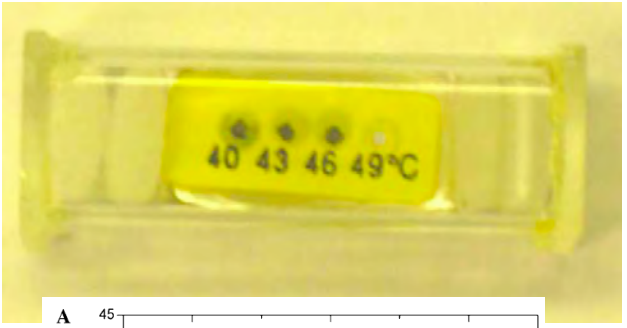
»» 2° radii generate linewidths of 6 to 12 ppm - substantially greater than the linewidths "typically" observed. Note that linewidths in the middle of the accessible spectral space are significantly greater than those on the periphery.

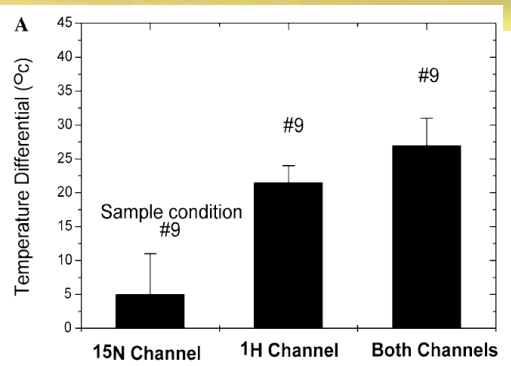


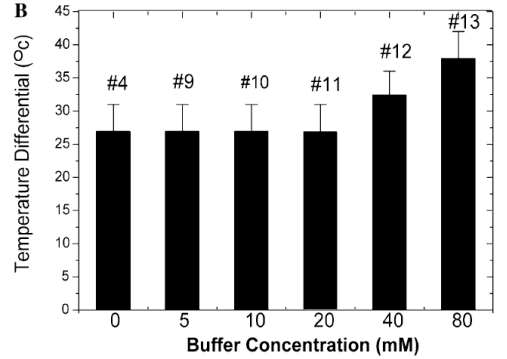
Quine et al. (2006) JMR 179:190

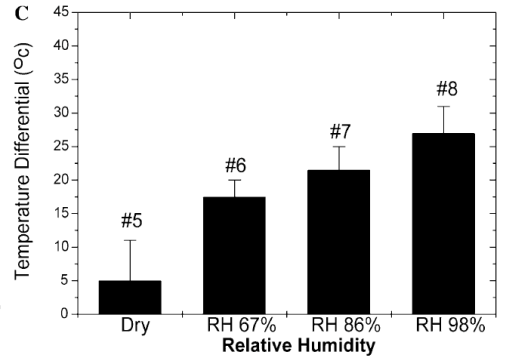
RF Heating of Lipid Bilayer Preparations is Particularly Significant









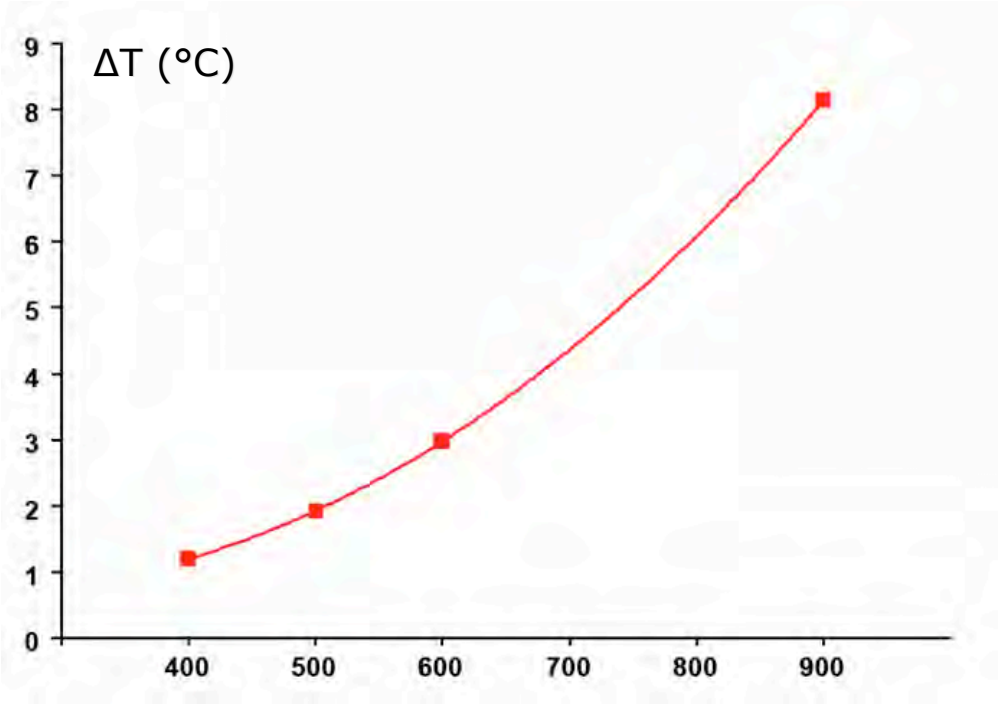


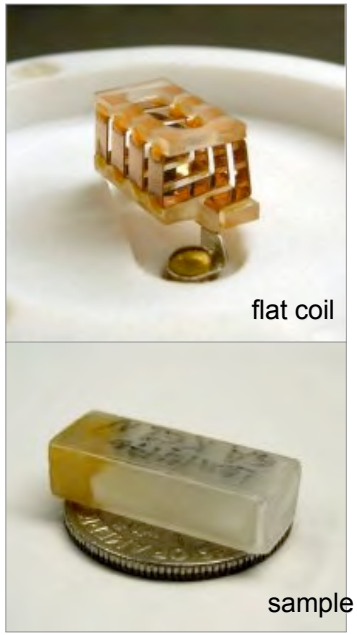
- **Heating is primarily from the ^{1}H Channel**
- **At modest ion concentrations the heating is independent of ion concentration**
- **The heating is highly dependent on hydration**
- **Dielectric Losses dominate Inductive Losses**

Li et al., 2006 JMR 180:51; Gor'kov et al., 2007 JMR 185:77

Sample heating in solenoids

Temperature rise in mechanically aligned preparation
after single ^{1}H decoupling pulse: **50** kHz, **10** ms long





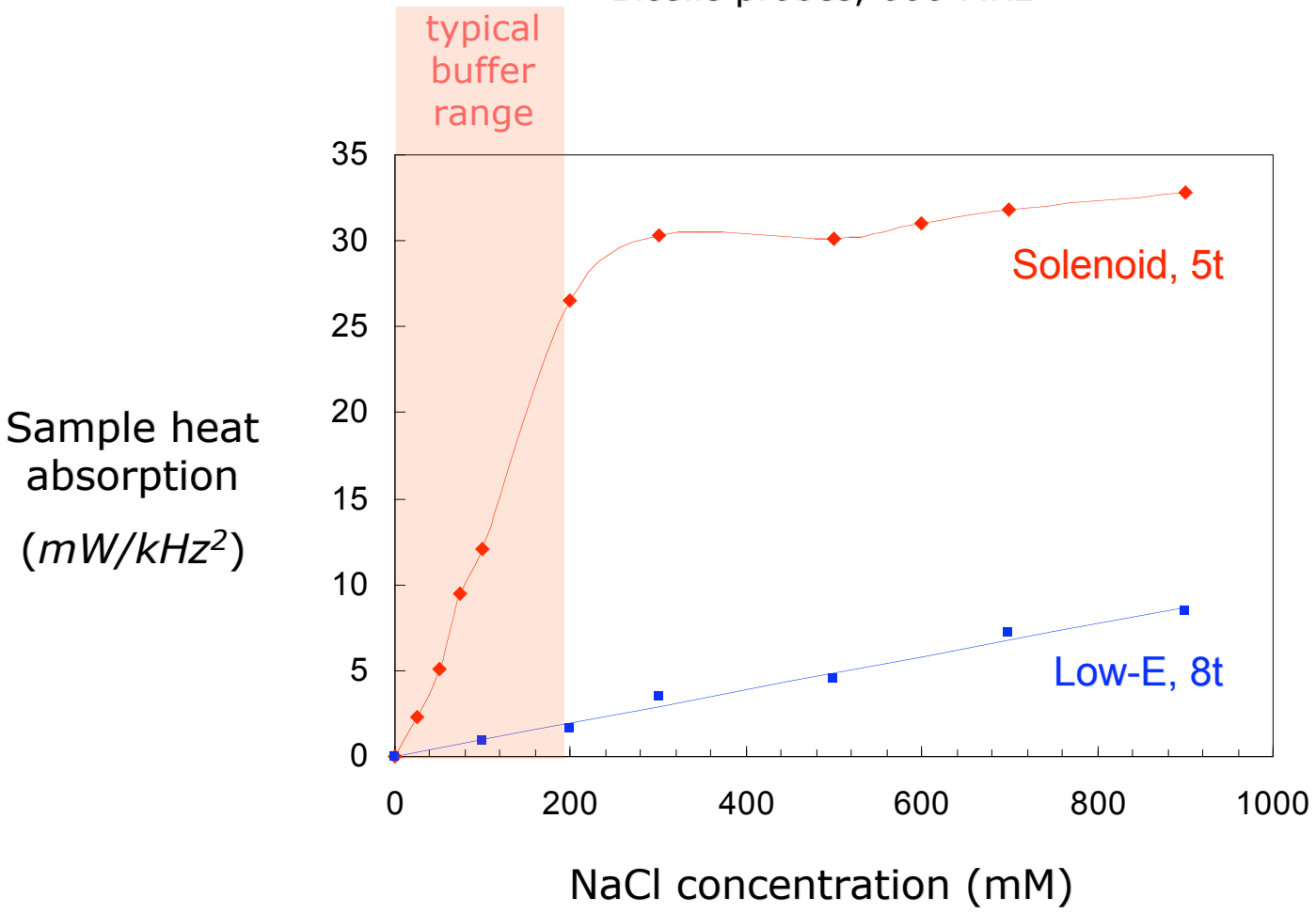
S. McNeil et al., Magn. Reson. Chem. (2007), in press

»» measured using the indicator TmDOTP

Reduction of ^{1}H decoupler heating

Different buffer concentration

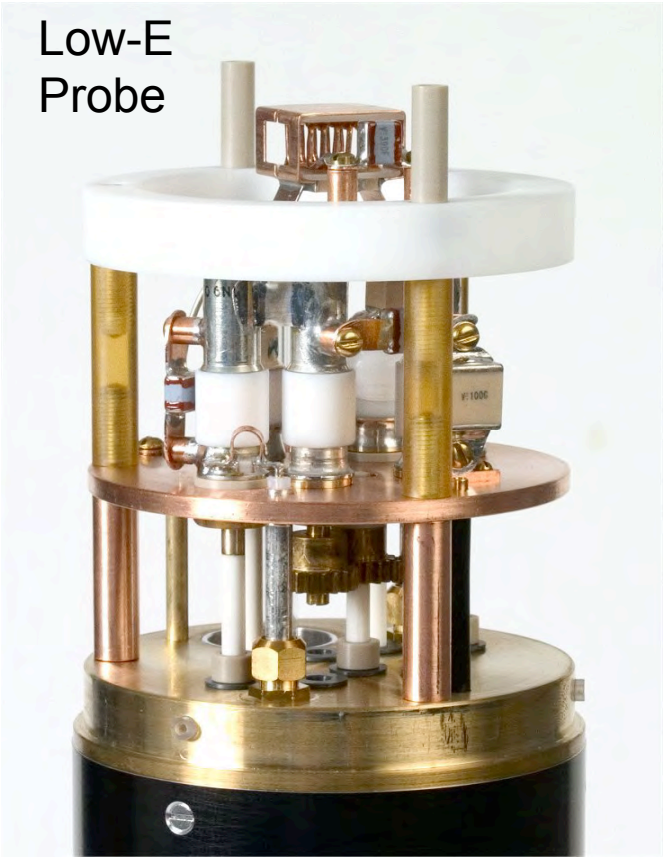
Bicelle probes, 600 MHz



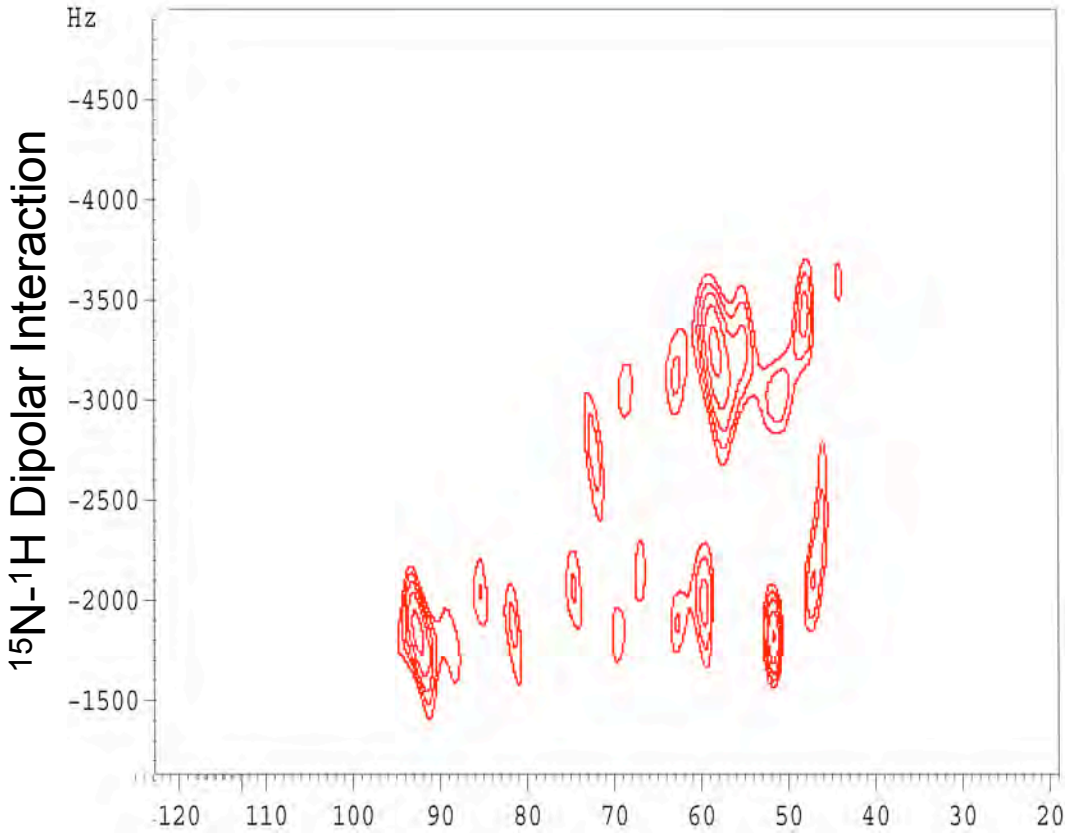
Data by C. Qian

Uniform labeled M2 in DMPC/DHPC Bicelle

- Membrane fraction purified M2 protein reconstituted by detergent(OG) dialysis in DMPC/DHPC Bicelles.
- Recorded with SAMMY sequence 2500 scans and 32 increments with 0.8 ms contact time at 600 MHz.

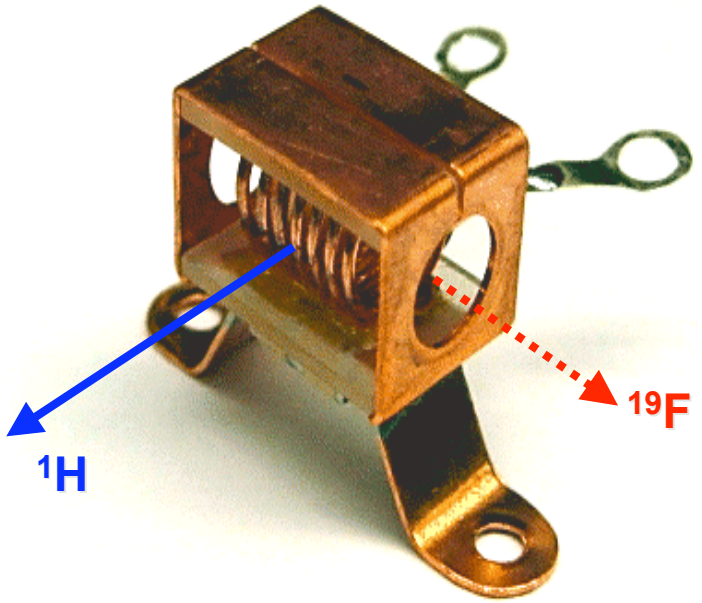


M. Sharma,



^{19}F Detection in Membrane Proteins

- ✓ No natural biological background
- ✓ Exceptional sensitivity
- ✓ Strong dipolar coupling
- ✓ Large chemical shift > 100 ppm
- ✓ Easy to substitute for H or OH group in amino acids

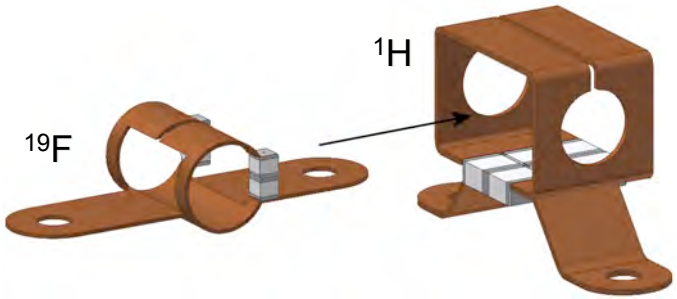


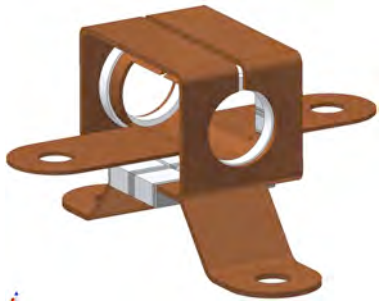
- × ^{19}F frequency is only 6% apart from ^{1}H
- × Detection with solenoid is prohibited
- × Sequences like CPMG would heat and destroy sample

Dual-LGR low-E H/F probe

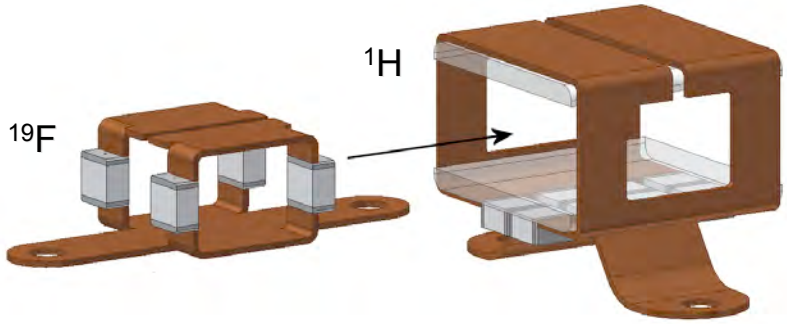
with two orthogonal loop-gap resonators

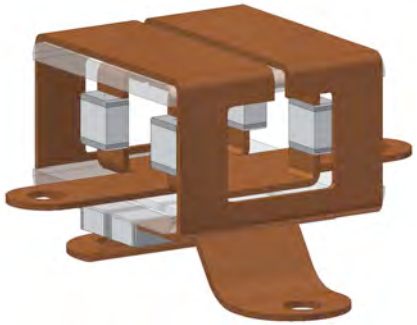
Bicelles





Mechanically aligned

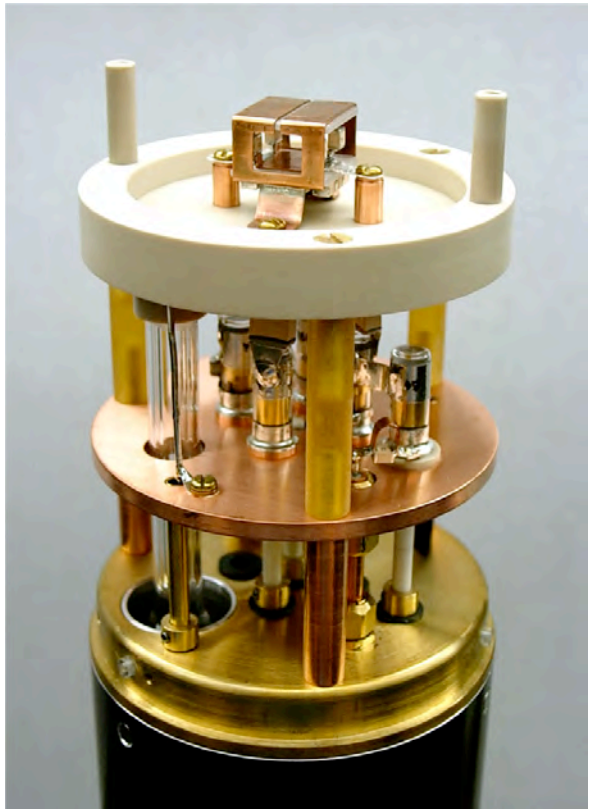


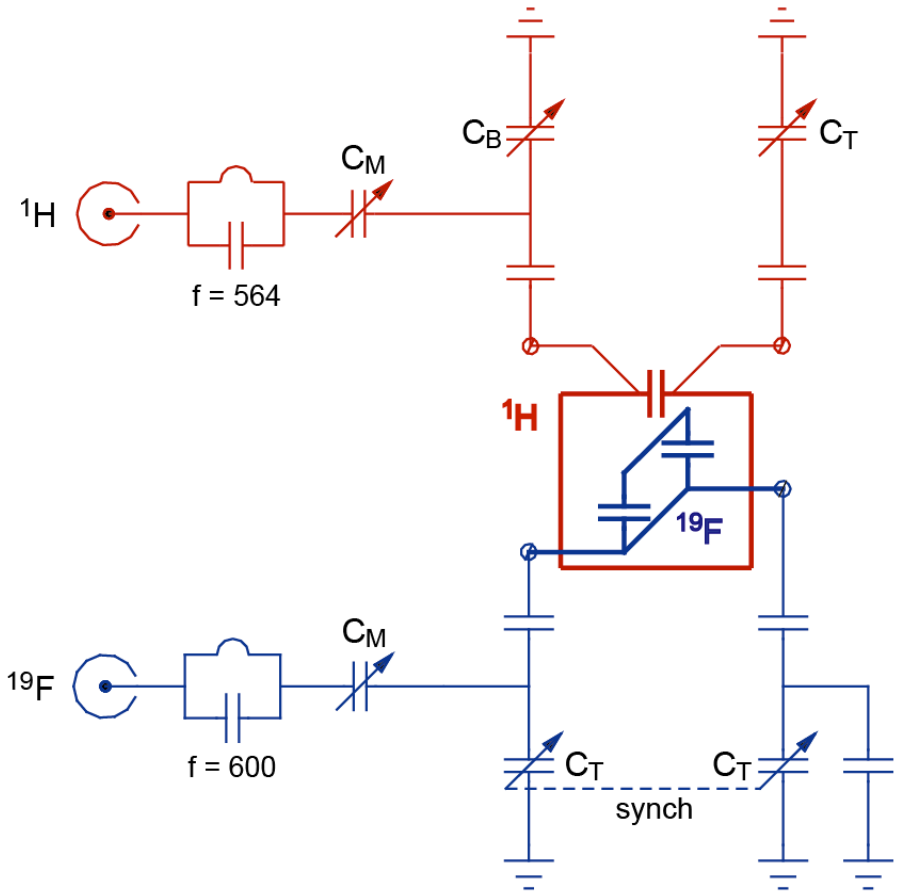


P. Gor'kov et al., J. Magn. Reson. 189 (2007) 17-24

RF circuit of H/F low-E probe

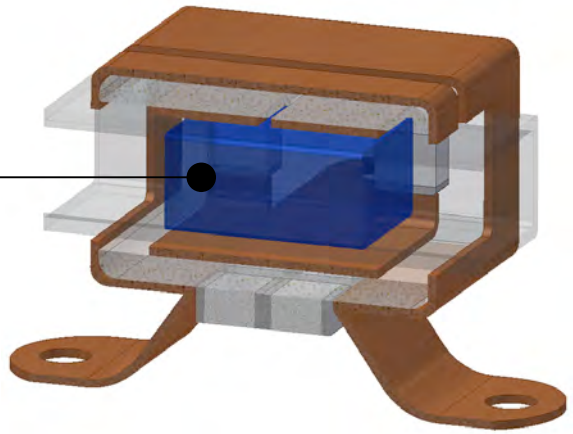
- ✓ Independent tuning despite very close frequencies
- ✓ Isolation within probehead ≥ 23 dB





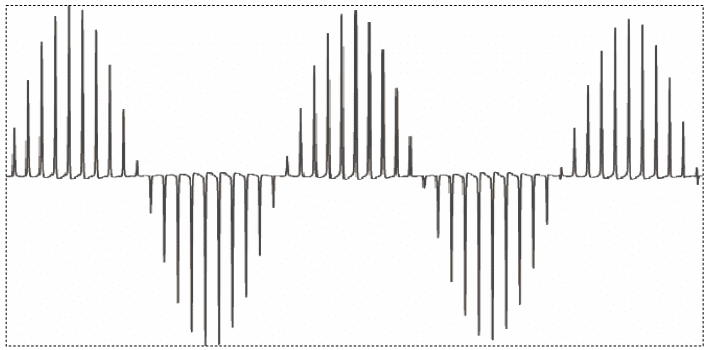
RF field homogeneity

6x4x9 mm
sample volume

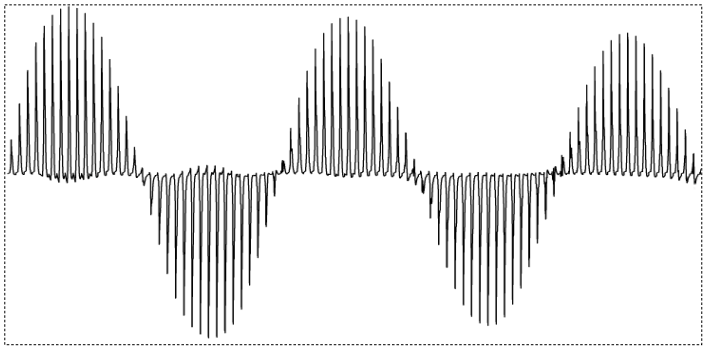


B_1 = 100 kHz:
@ 330 W (^{19}F)
@ 695 W (^{1}H)

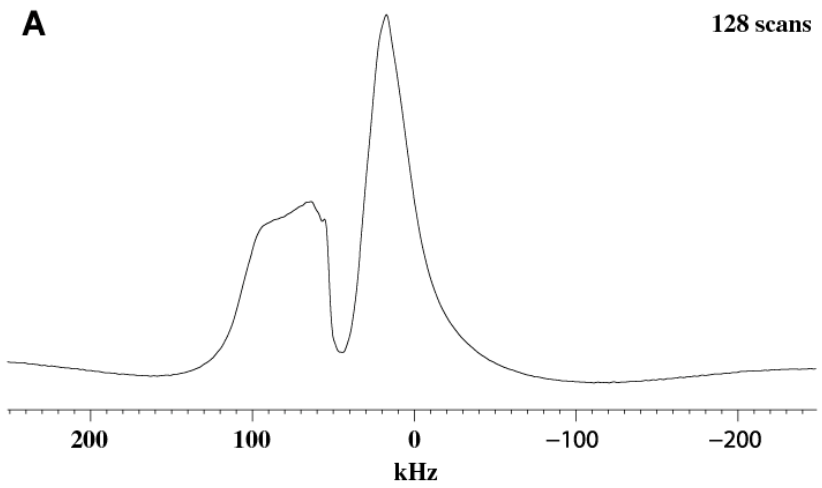
^{1}H 93%

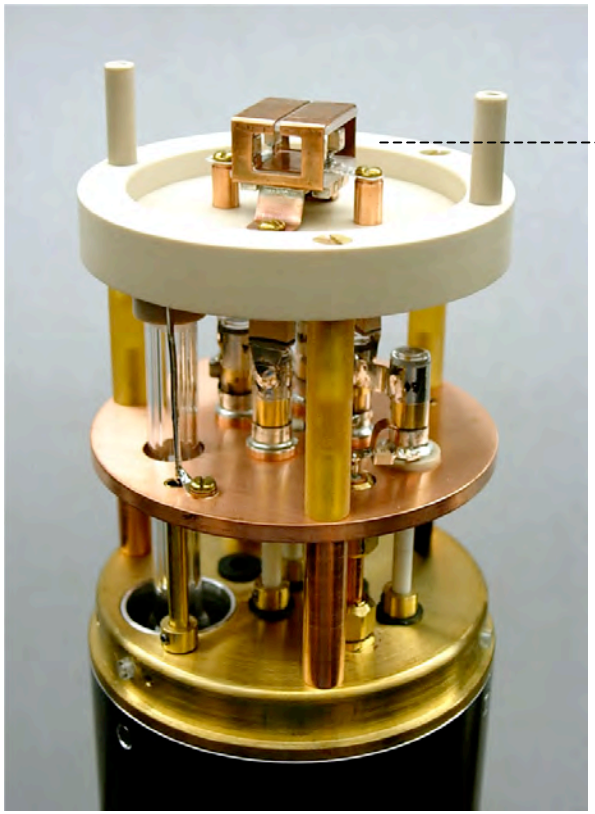


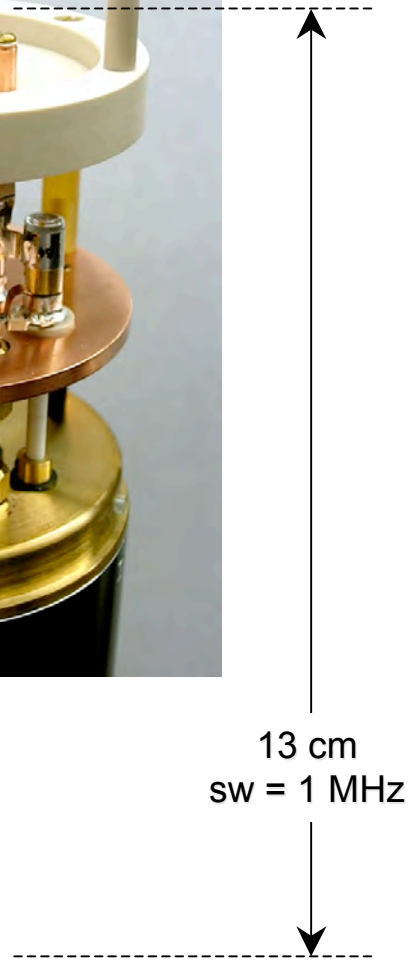
^{19}F 83%



Sources of ^{19}F background signal





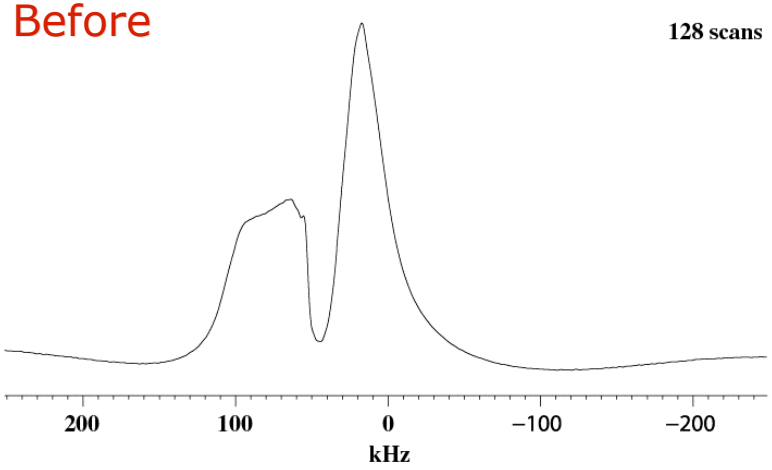


1. Fluorinated materials in sample coil vicinity
2. Fluorinated materials near current-currying conductors, within spectral window "distance" from the coil:
   - PTFE in RF coaxial cables
   - Space-age lubricant in glass tuning capacitors!

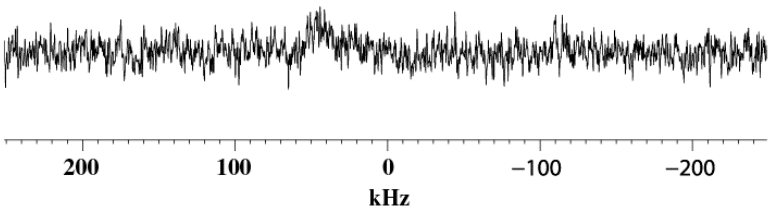
Removal of ^{19}F background signal

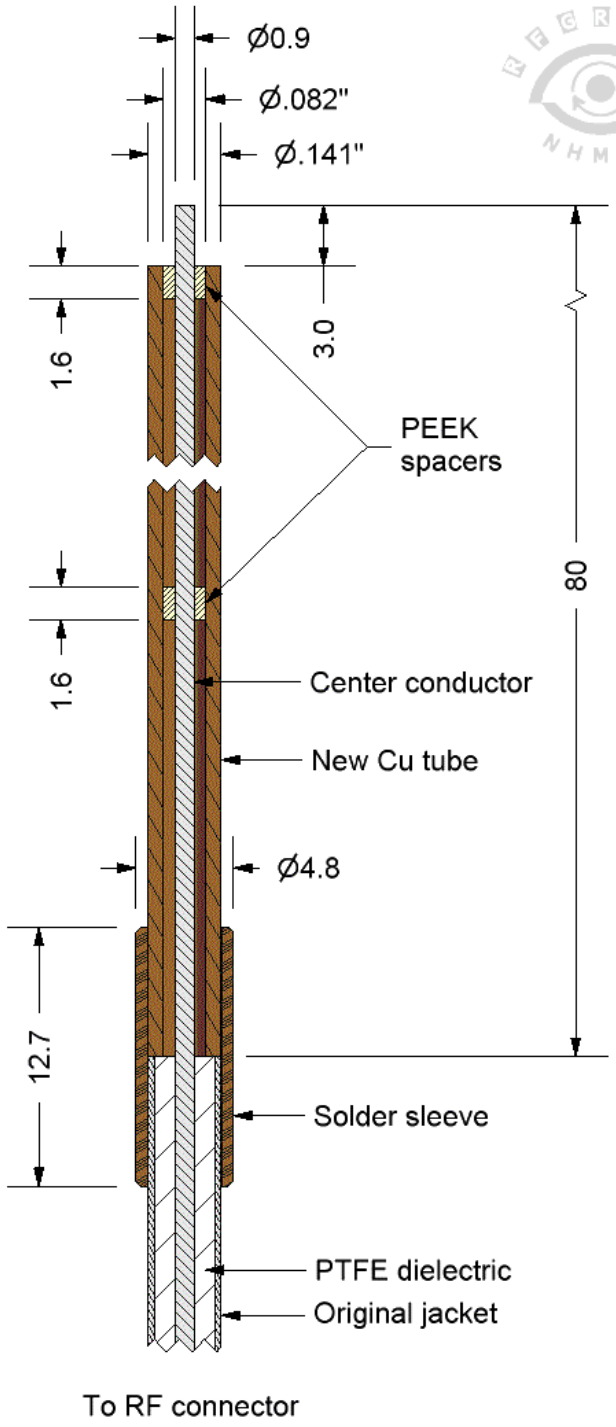
1. Made custom RF cables with air dielectric
2. Replaced lubricant in tuning capacitors

Before

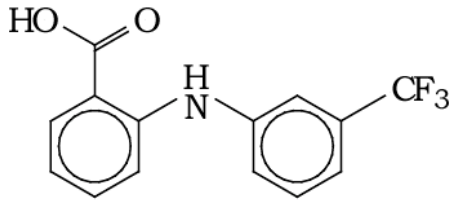


After



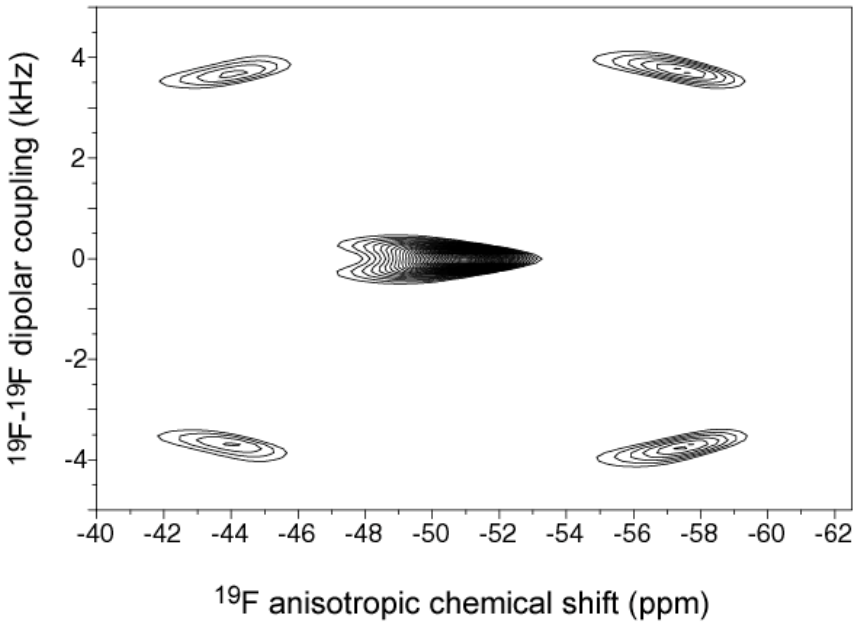


Two-dimensional ^{19}F CPMG

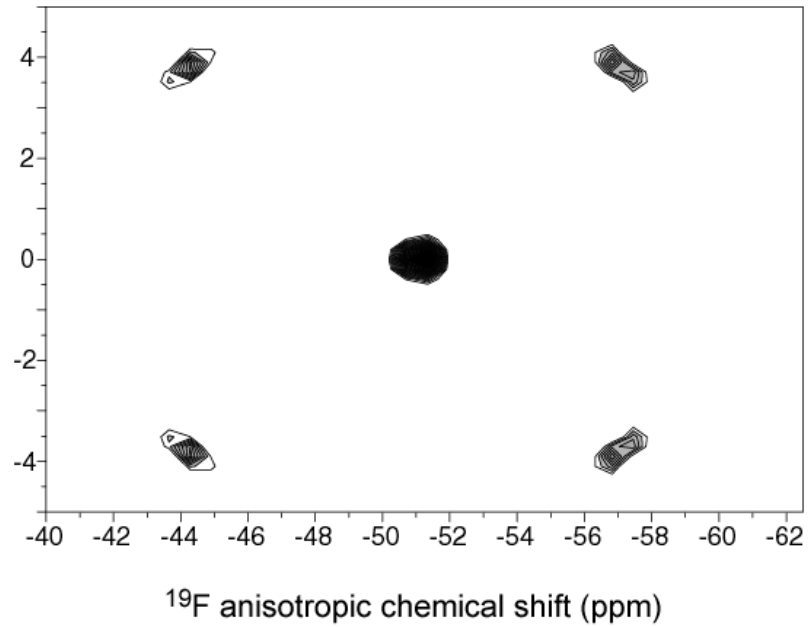


Flufenamic acid (FFA), an anti-inflammatory drug

B_1= 100 kHz (^{19}F), 70 kHz (^{1}H dec.), 256 t_1 incr. x 64 transients, 1 s delay, 34 min



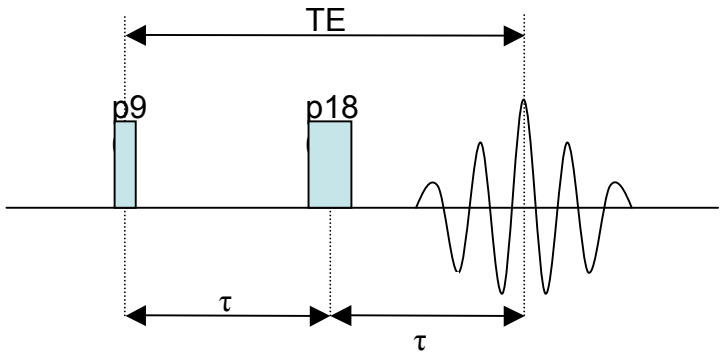
Experimental

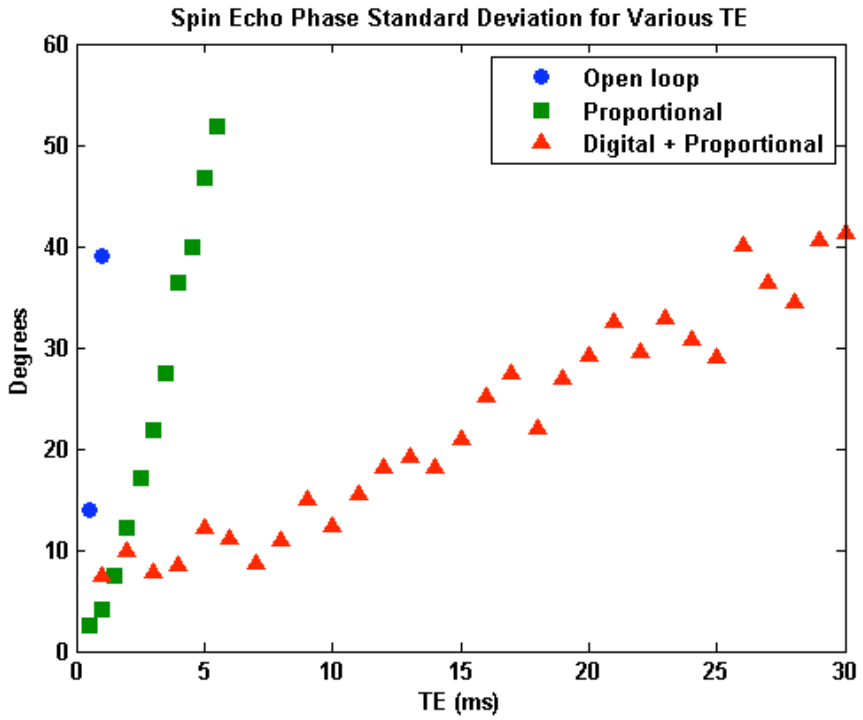


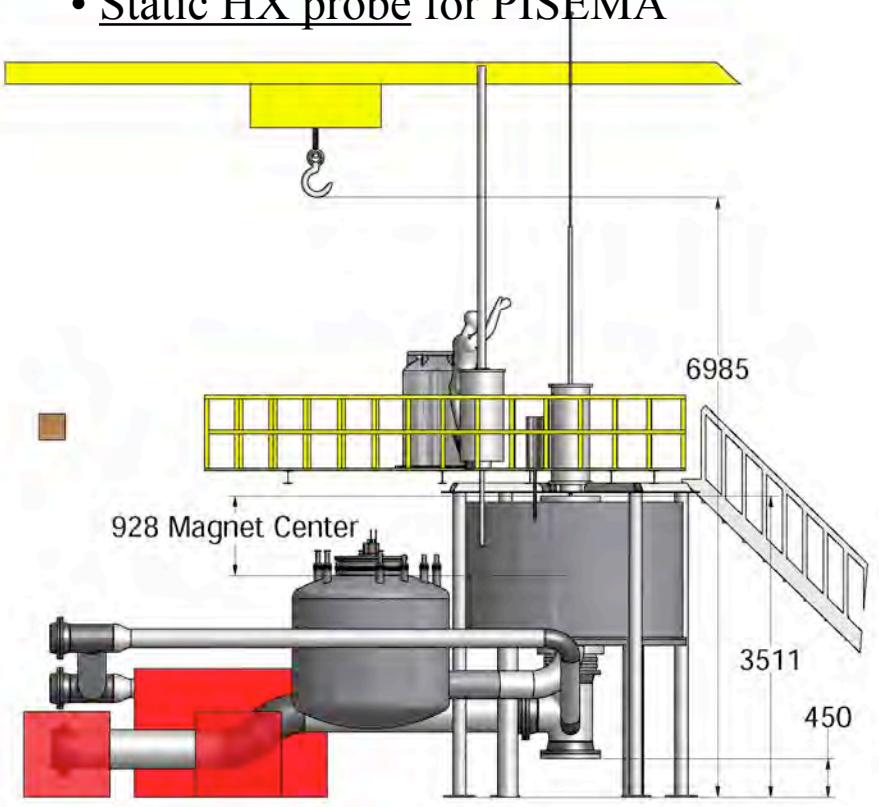
Simulated w/ SIMPSON

Sensitivity, Sensitivity, Sensitivity
High Field, High Field, High Field……
36T ~1.5 GHz 2011

- $^{1}H\{13C\}$ HR-MAS with ^{2}H Lock
- $^{1}H\{13C\}$ Solution probe with ^{2}H lock
- CP MAS probe for broadband observe and ^{1}H decouple
- Single-channel MAS probe for quadrupolar nuclei
- Static HX probe for PISEMA







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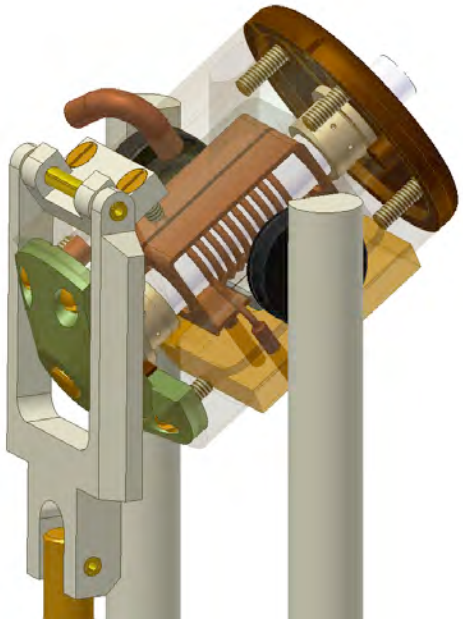

900 MHz Low-E H/C/N probe in the works
4 mm stator by Revolution NMR