

NMR

- History
 - 1946 – 2 physicists (Purcell/Block) invent technique
 - 5 years later chemists took over after chemical shifts were discovered
 - 1960's biologists started to use in structural determinations
 - 1990's MRI
- Special Advantages
 - Theory allows, in principle, detailed atomic arrangement information from spectra
 - See H atoms – whereas x-ray diffraction cannot
 - H, N, C, P, etc. can be separately examined

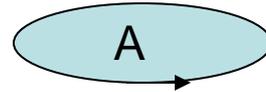
Introductory Theory

- Nuclei with odd numbers of either p and/or n possess nuclear spin (I), where I can be $1/2$, 1, $3/2$, etc
- Pauli exclusion principle applies to $1/2$ integral spins (e.g. protons ^1H , deuteron ^2H , ^{13}C (1% natural abundance), ^{15}N (~0.4%), ^{17}O , ^{19}F , ^{31}P (100%), ^{23}Na , etc.
- Nuclei with even no. of both p and n have $I = 0$; e.g. ^4He , ^{12}C , ^{16}O , ^{24}Mg , etc.

Magnetic moments

- As a consequence of having both spin and electric charge, these nuclei (with $I \neq 0$) will have magnetic moments, μ

where classically $\mu = i A$



- In quantum mechanics, we can relate μ to I

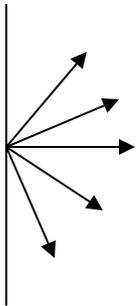
$$\mu = g_N \beta_N I, \text{ where}$$

$$\beta_N = \frac{eh}{4\pi M} = \textit{nuclear magneton}$$

and $g_N = \textit{nuclear g-factor} = 5.5855$ for free proton (value depends on environment)

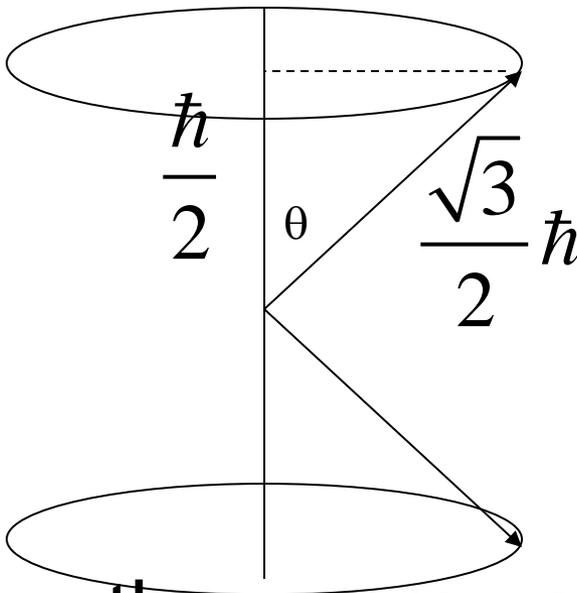
Magnetic moment in B field

- Suppose that a nucleus of spin I is placed in a uniform, static magnetic field B .
- Quantum mechanics tells us what happens: I must be in one of a set of quantized states – each having a different energy and spin orientation
 - For spin $\frac{1}{2}$ there are 2 possible states with spin components along the B field direction (z) given by $+\hbar/2$ and $-\hbar/2$.
 - In general there are $2I + 1$ possible orientations – e.g. if $I = 2$ then there are 5 states: $-2\hbar, -\hbar, 0, +\hbar, +2\hbar$
 - For a proton, there are 2 possible states with $I_z = \pm\hbar/2$
 - The magnitude of I is given by $|I| = \sqrt{I(I+1)}\hbar$



Spin orientation

- For a proton $|I| = \sqrt{(1/2)(1/2+1)}\hbar = \frac{\sqrt{3}}{2}\hbar$



θ is equal to $\pm 55^\circ$

- the energy of these two states differs by an interaction energy $E = -\vec{\mu} \cdot \vec{B} = \pm \mu_z B$
- We'll limit ourselves to proton NMR for now

Spin flips

- If EM radiation with photon energy

$$E_{\text{photon}} = hf = \Delta E_{\text{spin}} = 2\mu_z B$$

is absorbed by a proton in a nucleus in the ground state it will “flip” its spin to the higher energy (anti-parallel) state = **resonance**

- Similarly, a transition from the high to low energy state results in the emission of a photon with the same energy
- For $B = 10 \text{ kG} = 1 \text{ Tesla}$, $f = 10 - 100 \text{ MHz}$ depending on μ_z (corresponds to RF)

Spin populations

- It's a general result that the probability of an upward or downward transition is the same (Einstein); therefore, in a collection of atoms/molecules if spin up and down states have equal populations, there will be no net absorption or emission of radiation
- Only get net absorption if there are more atoms/molecules in the lower state
- But, in thermal equil, Boltzmann says:

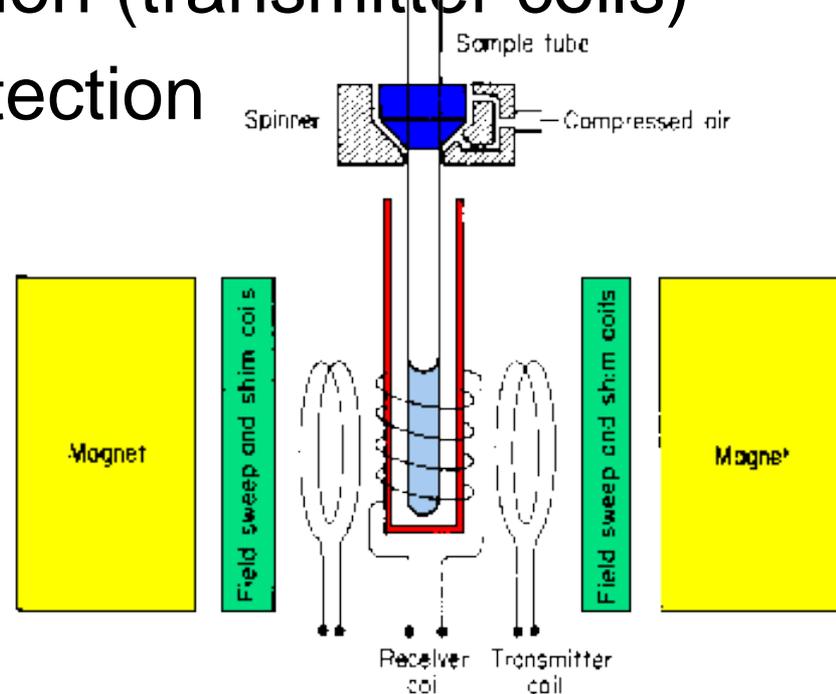
$$\frac{n_2}{n_1} = e^{-\Delta E/kT}$$

where $\Delta E = hf = 2\mu_z B$

- At $f = 220$ MHz, for example, $n_1 = n_2(1 + 35/10^6) = 35$ ppm difference - only 35 ppm of the population of atoms/molecules will yield net absorption - clearly do better if cooled, so n_1 increases with respect to n_2 .

Basic Experiment

- Requirements:
 - Strong DC magnet (yellow) to orient magnetic dipoles
 - Source of RF radiation (transmitter coils)
 - Receiver coil for detection
 - Peripherals



NMR scans

- In a typical set-up, instead of varying RF field frequency to tune : $hf = 2\mu_z B$, the RF frequency is fixed and B is varied by small amounts using an auxiliary magnet.
- To scan through the resonance condition, need a stable homogeneous field – often samples are spun at ~ 1000 rpm between magnet poles to reduce inhomogeneities
- Commonly used RF frequencies are
 - 60 MHz --- 14 kG = 1.4 T
 - 100 MHz --- 24 kG = 2.4 T = largest EM
 - 270 MHz --- 63 kG = 6.3 T need superconducting magnets
 - 600 MHz --- 150 kG = 15 T
 - 800 MHz --- 200 kG = 20 T
 - 900 MHz --- 225 kG = 22.5 T



100 ton
magnet



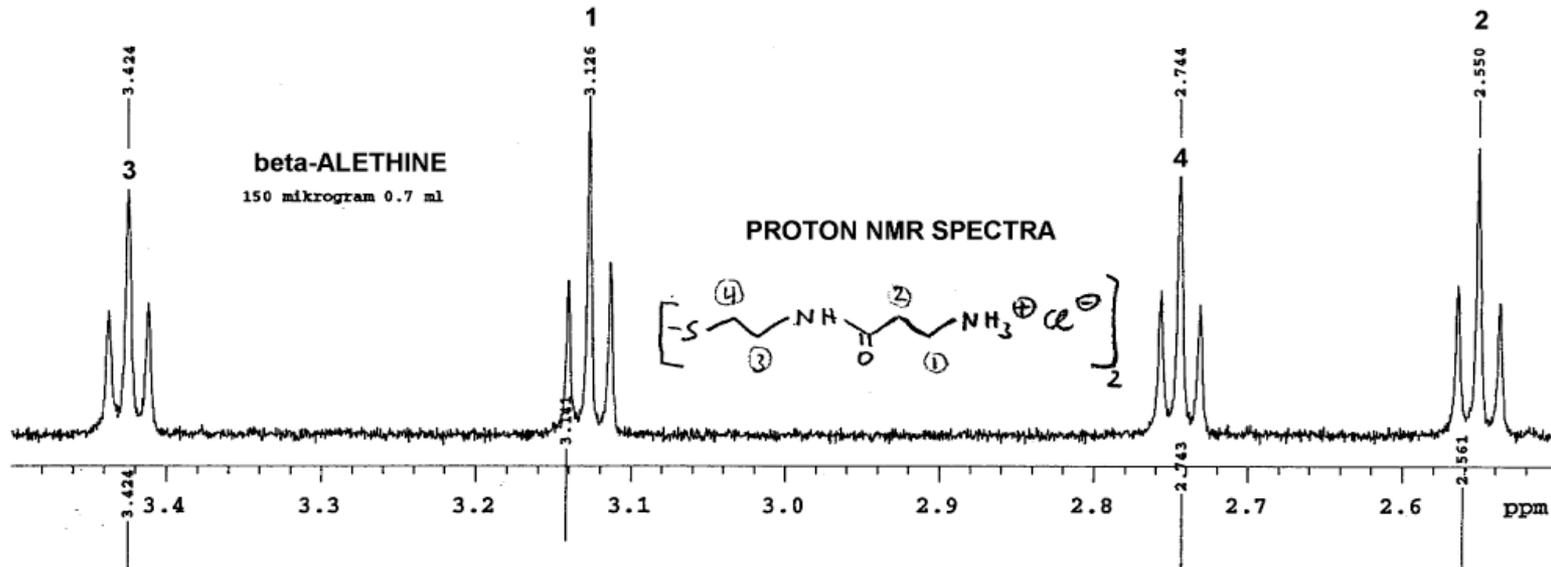
900 MHz Varian NMR machine (ready for mars launch!!)

NMR signal

- NMR transition induces an emf in the receiver coil (search coil) from Faraday's

Law:
$$\mathcal{E} = -\frac{d\Phi_B}{dt}$$

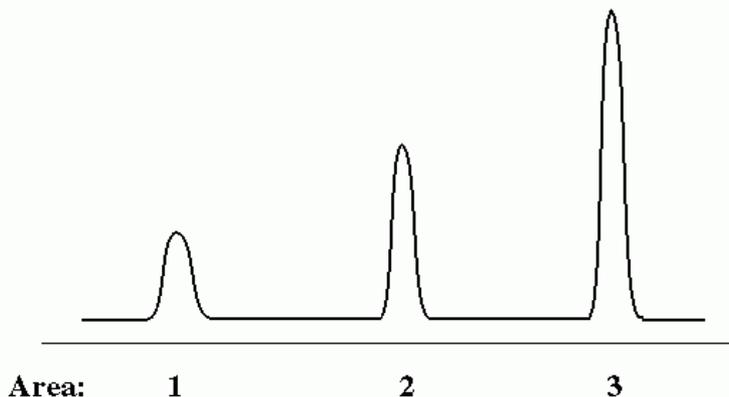
- The signal is amplified and might look like this:



General Features of NMR Spectrum

- Each absorption line is characterized by 3 parameters:
 - Position of peak
 - Area under peak
 - Line shape and multiplicity (fine structure)
- Area: proportional to # of equivalent nuclei (protons) in the sample – comparison of areas under peaks gives relative abundance of different types of protons (in different environments)
- Position: Chemical shift (from some reference pt) Different protons (nuclei in general) are in different chemical environments (different electron distribution and neighboring nuclei) leading to different local B fields

For example: Ethanol $\text{CH}_3\text{CH}_2\text{OH}$ – 3 peaks with areas in ratio of 3:2:1 at low resolution (more later about fine structure)



Local B and Chemical Shift

- Local $B = B_{\text{ext}}(1-\sigma)$ where σ is a dimensionless “screening constant” $\sim 10^{-5}$
- - σ depends on local chemical environment
- Two different protons in different environments will have different resonant fields and the difference in these B's is called the chemical shift

$$B_1 - B_2 = B_{\text{ext}}(1-\sigma_1) - B_{\text{ext}}(1-\sigma_2) = B_{\text{ext}}(\sigma_2 - \sigma_1) = B_{\text{ext}}\delta_{12}$$

or $\Delta f = f_1 - f_2 = f_{\text{ext}}\delta_{12}$

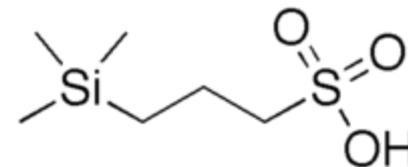
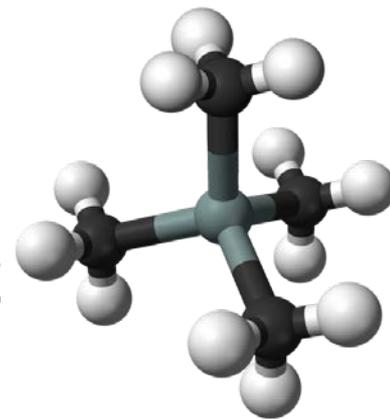
(since $\Delta B/B_{\text{ext}} = \Delta f/f_{\text{ext}} = \delta_{12}$)

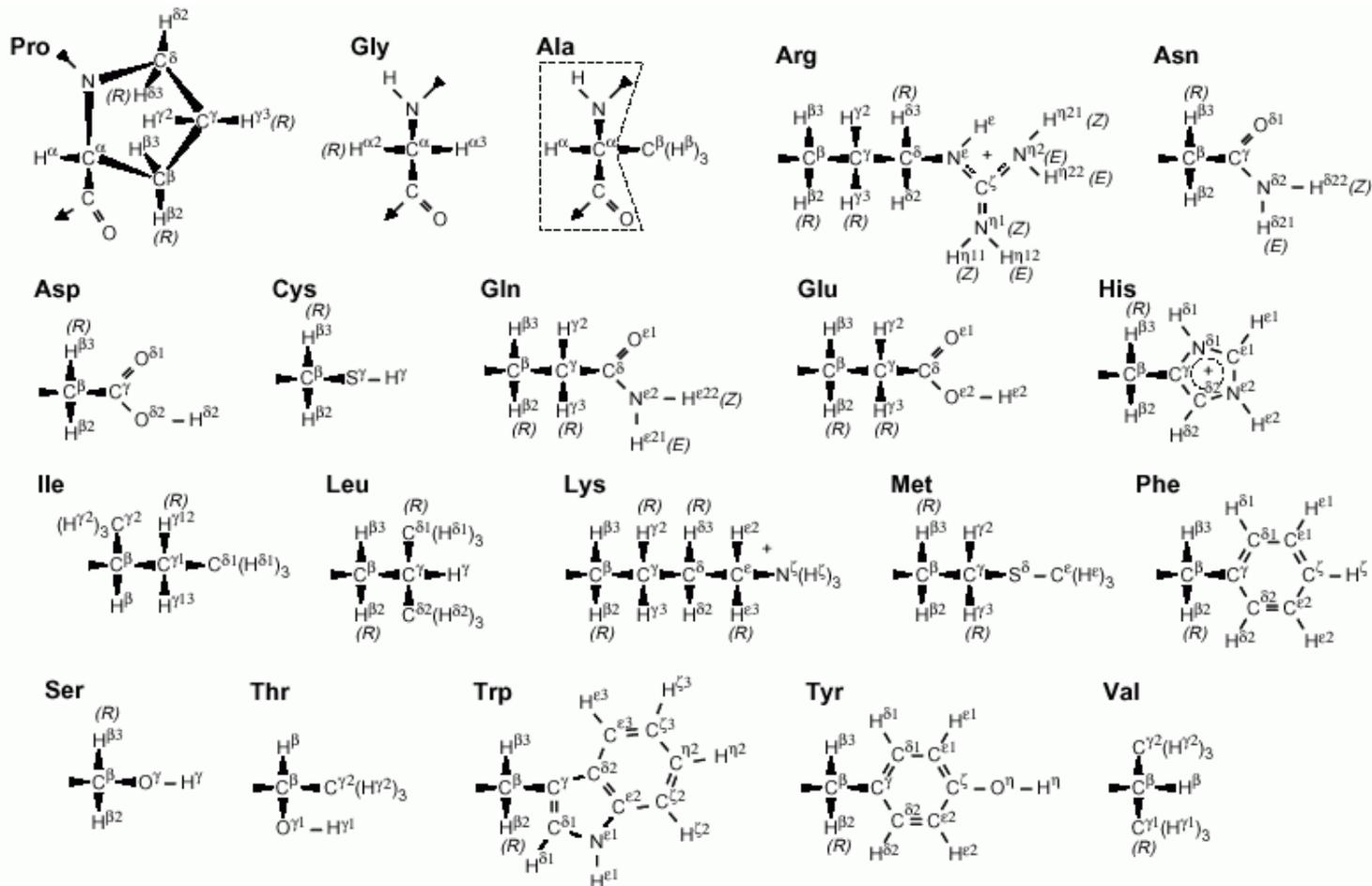
Usually δ_{12} is expressed in ppm or $\delta_{12} = \{\Delta f/f_{\text{ext}}\} \times 10^6$

δ is dimensionless and independent of f_{ext} - ppm is used since $\delta \sim 1 - 10$ ppm or so

NMR standard

- In practice δ is referred to a standard that is often added to the sample. Usually it is TMS (tetra-methyl-silane = $(\text{CH}_3)_4\text{Si}$ with 12 identical C's)
- TMS is chemically inert, has a strong 12 C signal, has a resonance at higher B than most protons – so shifts of other H's are “downfield”
- Then, $\delta_{\text{sample, TMS}} = (f_{\text{sample}} - f_{\text{TMS}})/f_{\text{ext}} \times 10^6$
- Note that TMS is immiscible in water – not generally a problem since most aqueous NMR is done in D_2O to avoid ^1H water peak – DSS is used as stnd in water





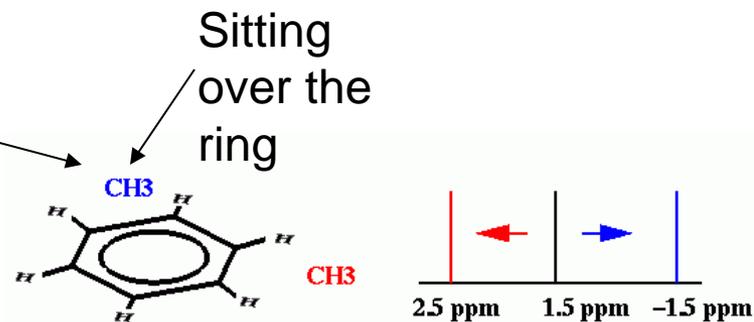
The main constituents of the natural amino acids are not too different from simple, small organic molecules. We find various arrangements of CH_2 and CH_3 groups (Val, Ala, Leu, Ile, Met) and OH groups (Ser, Thr). Additional groups are the aromatic side chain of Trp, Tyr, Phe and His, the backbone carbonyls, the positive and negative charges on Asp, Glu, Arg and Lys and the sulfur atoms in Cys and Met. The average or random coil chemical shifts for all the protons in these amino acids are given in the figure below

Amino acid	HN	H α	H β	other
Ala	8.15	4.24	1.32	-
Arg	8.20	4.28	1.71	γ 1.54; δ 3.13
Asn	8.29	4.73	2.82	δ 7.48
Asp	8.31	4.65	2.78	-
Cys	8.25	4.64	3.03	-
Gln	8.28	4.43	2.01	γ 2.32
Glu	8.22	4.34	2.01	γ 2.31
Gly	8.31	3.96	-	-
His	8.28	4.54	3.10	δ 2 6.9 ; ϵ 1 8.10
Ile	8.26	4.13	1.74	γ 1 1.15; δ 10.69
Leu	8.19	4.25	1.66	γ 1.51; δ 0.75
Lys	8.28	4.23	1.79	γ 1.33; δ 1.56; ϵ 2.94
Met	8.10	4.41	1.96	γ 2.58; ϵ 1.98
Phe	8.49	4.69	3.01	δ 7.12; ϵ 7.17; ζ 7.08
Pro	-	4.48	2.03	γ 1.97; δ 3.69
Ser	8.48	4.50	3.81	-
Thr	8.30	4.53	4.17	γ 21.15
Tyr	8.57	4.64	2.92	δ 7.08; ϵ 6.70
Trp	8.43	4.29	3.24	δ 1 7.18 ; ϵ 110.15
Val	8.20	4.16	2.02	γ 0.82

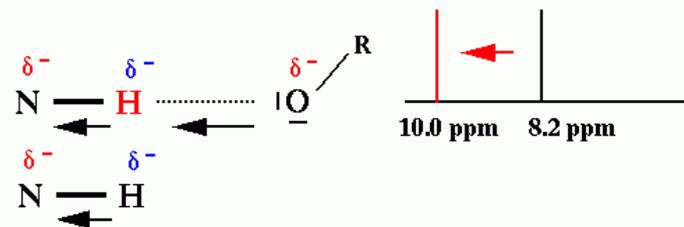
As can be seen the chemical shifts of the same type of proton are always at the same chemical shift (more or less). E.g. the α -proton is always around 4 ppm while the aromatic protons are around 7 ppm and the backbone amides at 8 ppm. In this way the appearance of NMR spectra of proteins is rather uniform.

What factors affect σ and hence δ ?

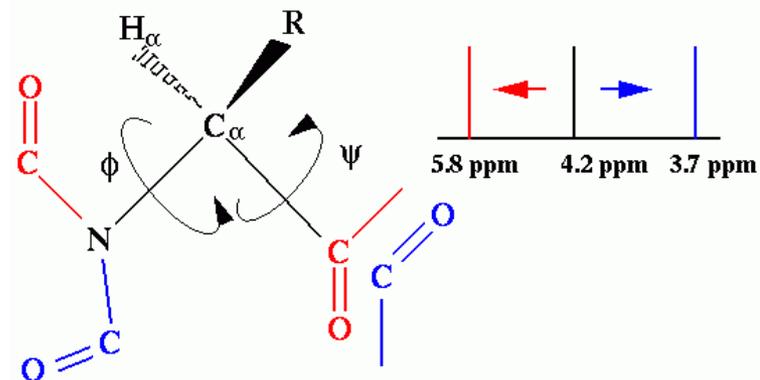
- Intramolecular shielding
 - nearby moving electrons produce a B field opposite to the applied external B (diamagnetic effect – follows Lenz's law) – usually a major effect when ring systems are nearby due to large delocalized π electrons – depends on relative location with respect to ring
- Tertiary structure effects



H-bonds shift peak downfield – very common in β -sheets



Rotational angles in backbone of protein



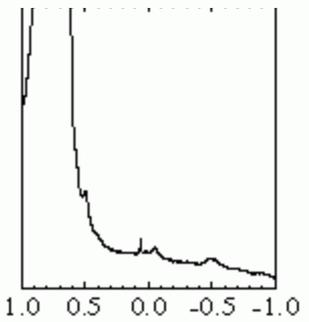
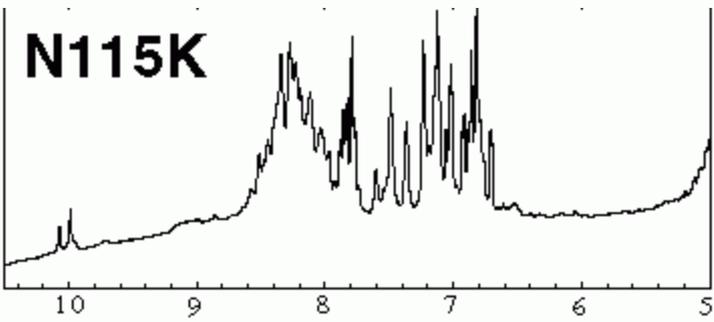
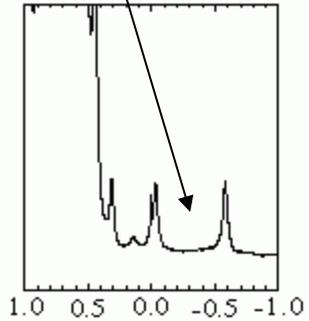
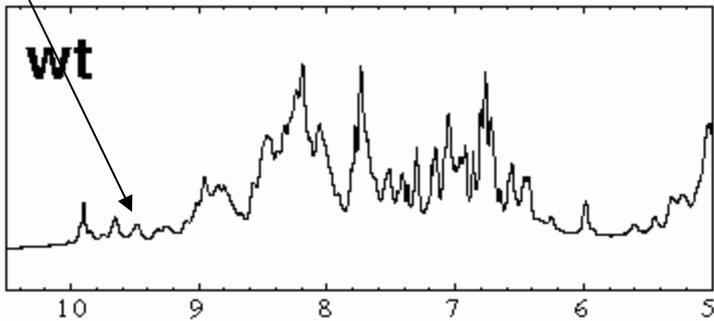
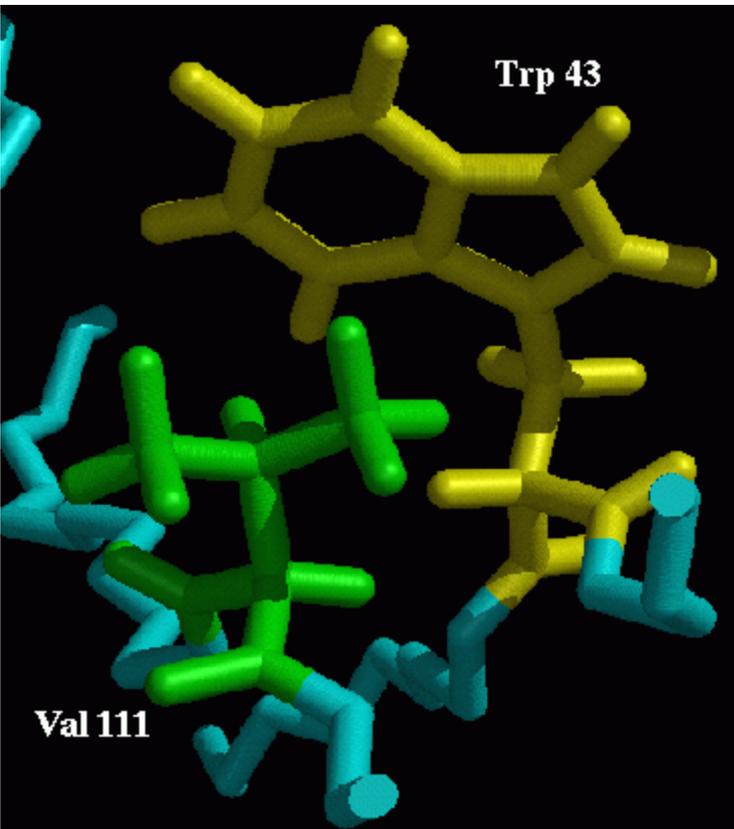
Other factors

3. Nearby unpaired electrons – paramagnetic effect – larger shift 20 – 30 ppm downfield due to magnetic moment of free electron – (can be up to 10Å from the proton) – the shift is proportional to $1/r^3$ and so can serve as a “yardstick”
4. Chemical exchange – if slow exchange occurs, see two peaks from different populations; if rapid exchange, peaks broaden and merge to reflect average environment

Example Spectrum

Low-field peaks probably indicate β sheet – not present in mutant

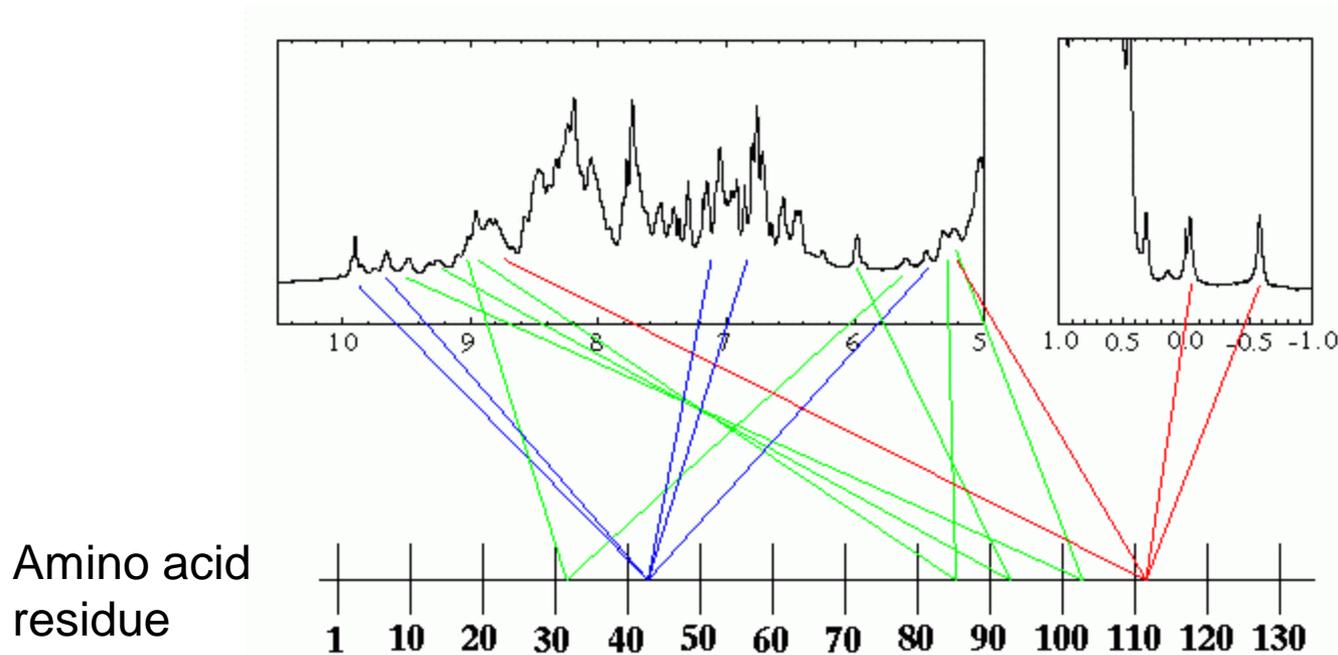
Peaks from Trp-val interaction missing in mutant



A 130 residue fragment of cardiac myosin binding protein C. On the top is the wild type protein, on the bottom is a mutant related to a heart disease (familial hypertrophic cardiomyopathy)

Overcrowding of 1D proton spectra (and guess why the region between 1 and 5 ppm is not shown) is a problem of protein NMR and does not come entirely unexpected. If we take the 130 residue protein from above - modest size that it is - we will find it contains just over 1000 protons. Squeezing all the corresponding peaks into the narrow spectral region accessible to protons will inevitably lead to substantial overlap. In fact, in most parts of the spectrum, individual resonance lines will be impossible to distinguish. Only at both ends of a spectrum will it be possible to see individual peaks

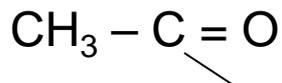
Assignment Problem



- Impossible from 1-d NMR – impetus to use multi-dimensional NMR – discussed soon

Multiplicity

- Third general feature – at high resolution many peaks are split
- Example: splitting due to spin-spin interactions between protons that are on covalently bonded C only – acetaldehyde

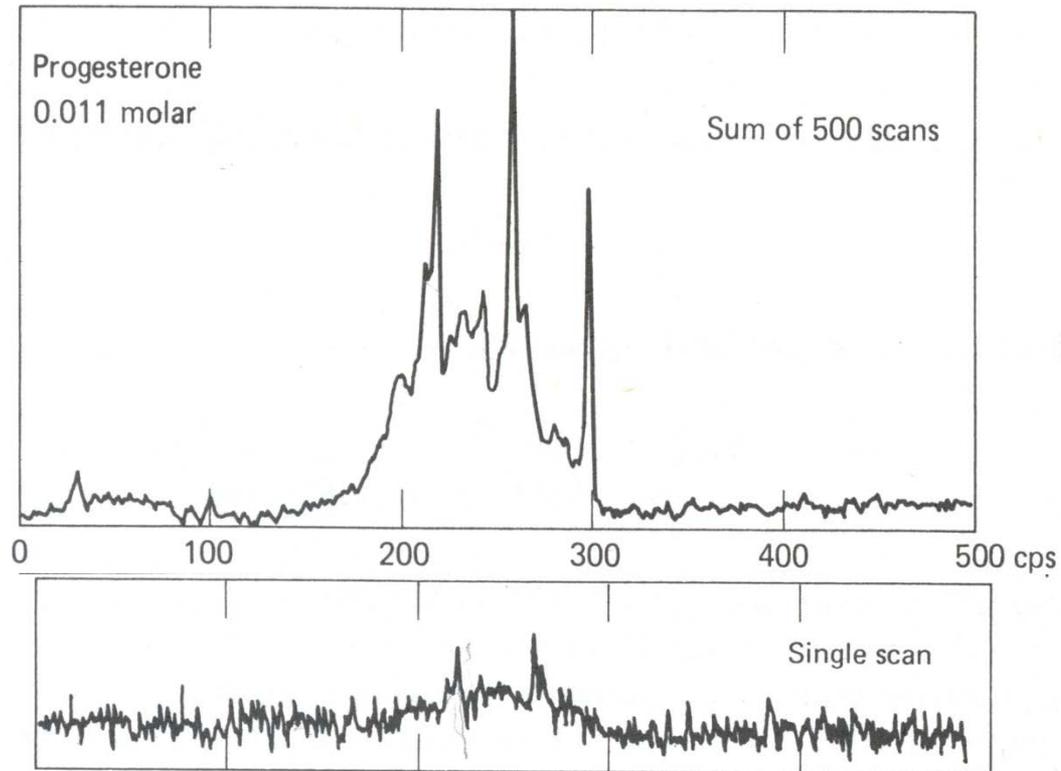


3 equivalent H's on CH₃ and one other – all spins have ~ equal probability of pointing up or down with respect to B so:

Line Shape

- Two effects here:
 - Spin-lattice relaxation time = T_1 – spins exchange energy with neighboring lattice (in a solid or solvent in a liquid) – returns net population to ground state – can measure this directly in FT-NMR
 - Spin-spin relaxation time = T_2 – spins exchange energy with neighboring nuclear spins (of same type)
 - no net change in number of excited state nuclei
 - Times are inversely related to line broadening since $\Delta E \Delta t > h$ – shorter Δt – more rapid movement - gives larger ΔE (more broadening)
 - In general $T_2 \ll T_1$, and so intrinsic linewidths are determined by T_2

Signal Averaging



1D NMR applications for proteins

Description	Application
Check for folded proteins	Simply record 1D spectrum of sample and analyze
Melting temperature	Record spectra at increasing temperatures and follow peak position & intensity of well resolved peaks in the high-field region by recording 1D NMR spectra. The resulting plot of peak intensity and/or chemical shift of the monitored peak will then allow an estimation of the melting temperature
Diffusion Coefficients	A modification of the simple 1D NMR experiment in which the magnetic field homogeneity is changed in a controlled manner can be used to estimate diffusion coefficients, which can in turn be used to measure molecular weights and thus test oligomerization states and aggregation.
Ligand Titration	If a proton that is involved in ligand binding is also in a well resolved part of the spectrum the chemical shift can be monitored as a function of ligand concentration

Videos

- <http://www.foothill.edu/psme/armstrong/nmr.shtml>
- <http://www.irtutor.com/nmrtutor/>
(look at 1, 2, 3, T1)

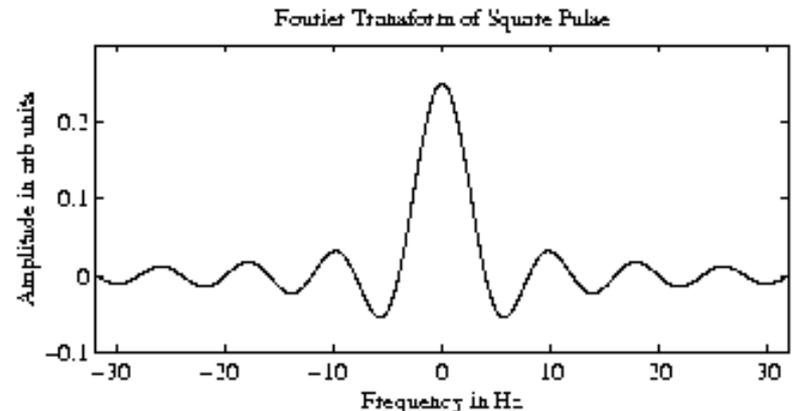
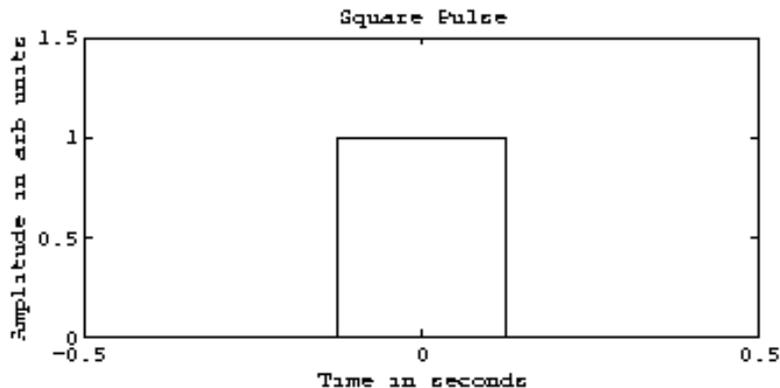
FT NMR

- The FT method can be compared to the tuning of a bell. In principle, you could measure each of the tones which make up the sound of a bell in a 'cw experiment': Excite the bell with all frequencies, one at a time, from the deepest tones to the edge of ultrasound and measure the reaction of the bell with a microphone. But this method is extremely complicated and every bell founder knows a much faster way: Take a little hammer (or perhaps a bigger one) and - **BOIIINGGGG**.....

The sound of the bell contains each tone at the same time and every person can analyze it directly with his or her ears (which are a cleverly 'constructed' instrument for FT). The advantages of this 'pulse FT method' over the 'cw method' are clearly obvious

$$\Delta t \Delta f \geq 1 \quad \text{or} \quad \Delta t \Delta E \geq (h/2\pi)$$

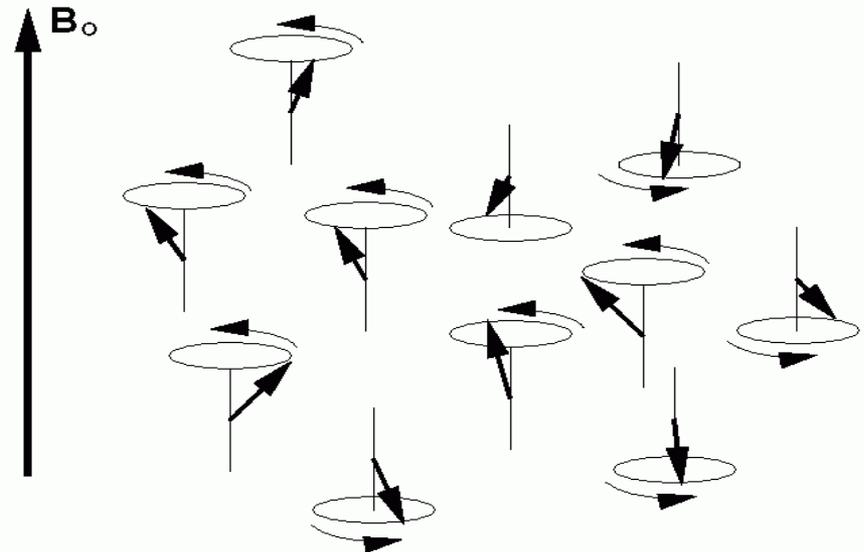
<http://www.youtube.com/watch?v=Mk25uY197K4>



Classical Picture of FT NMR

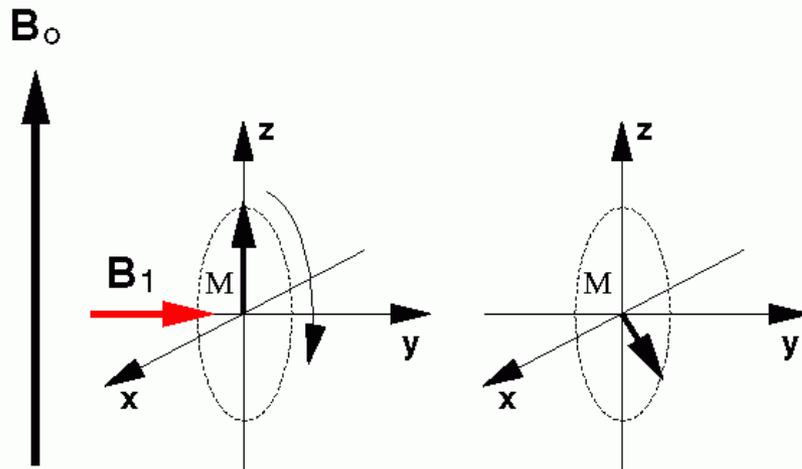
- For a collection of magnetic moments, in the absence of a B field – they orient randomly so that the net Magnetization = M = average total dipole/volume = 0;
- But in the presence of a B they precess around the B field direction and there is a net M along B so there's a B_z , but $B_x=B_y = 0$

Dipole moments precess around B at an angular frequency called the Larmor frequency $hf = \mu_z B$



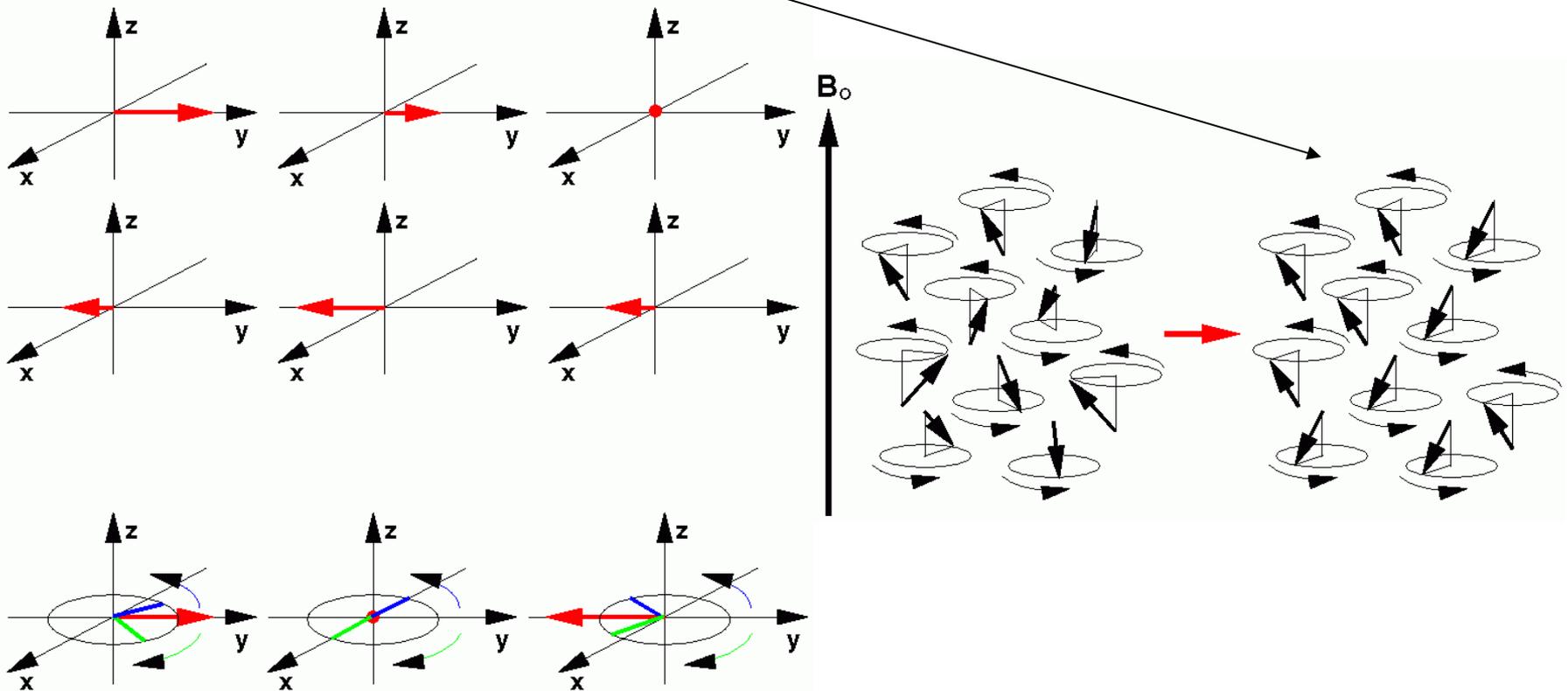
Classical Picture II

- Now, we apply an RF pulse – corresponding to an applied B field in the x-y plane rotating at the same Larmor frequency - $B_{xy} = B_1$ in figure – for a short duration, which exerts a torque on M and rotates the Magnetization vector and gives rise to an M_{xy} component, decreasing M_z
- But B_{xy} is oscillating and so M is “torqued” to rotate to the x-y plane at the Larmor frequency



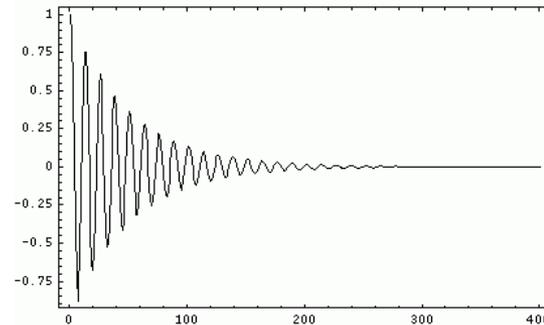
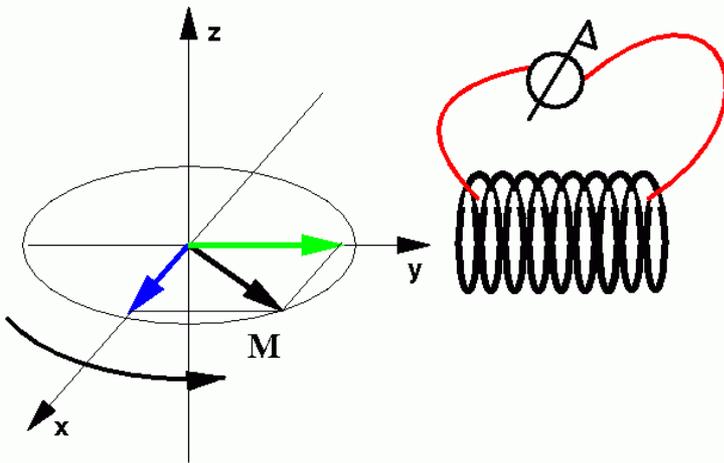
Classical Picture III

- The oscillating B_{xy} (red) can be thought of as two rotating B fields in the x-y plane (blue and green) rotating in opposite directions at the Larmor frequency
- This leads to a torque on M rotating it into the x-y plane (so that the net M in the z-direction = 0) with equal numbers of spin up and down nuclei locked in phase (coherent) and rotating around the z-axis at the Larmor frequency



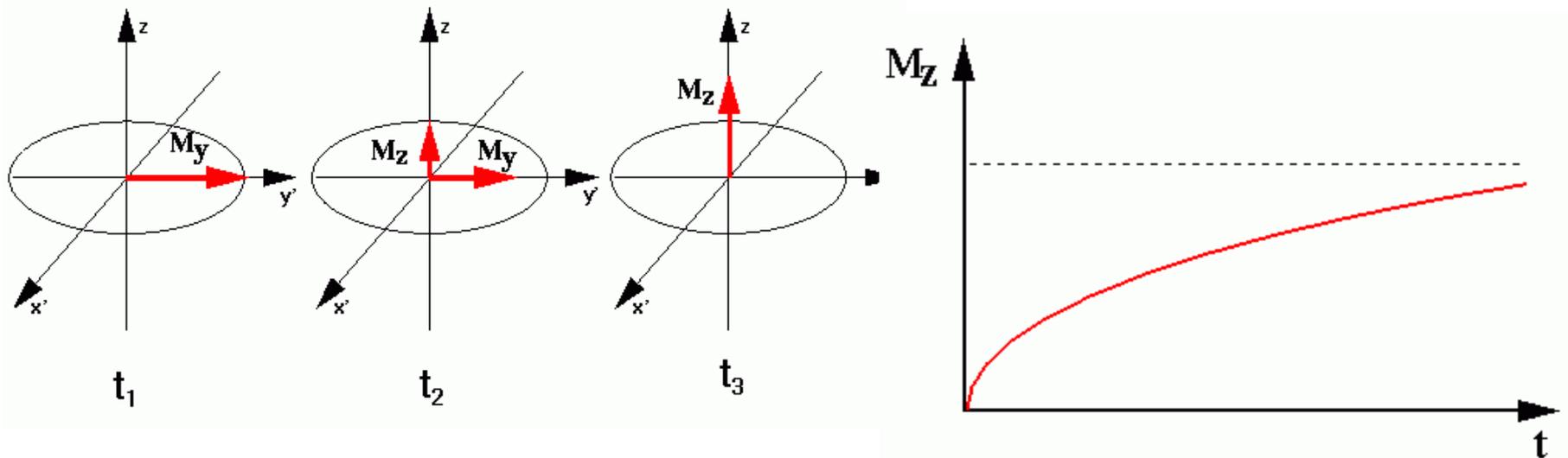
Classical Picture IV

- As M rotates in the x - y plane it generates an induced current in the detector coil that is measured as the “free induction decay”
- Why does the signal decay away?
- Two types of “relaxation” phenomena



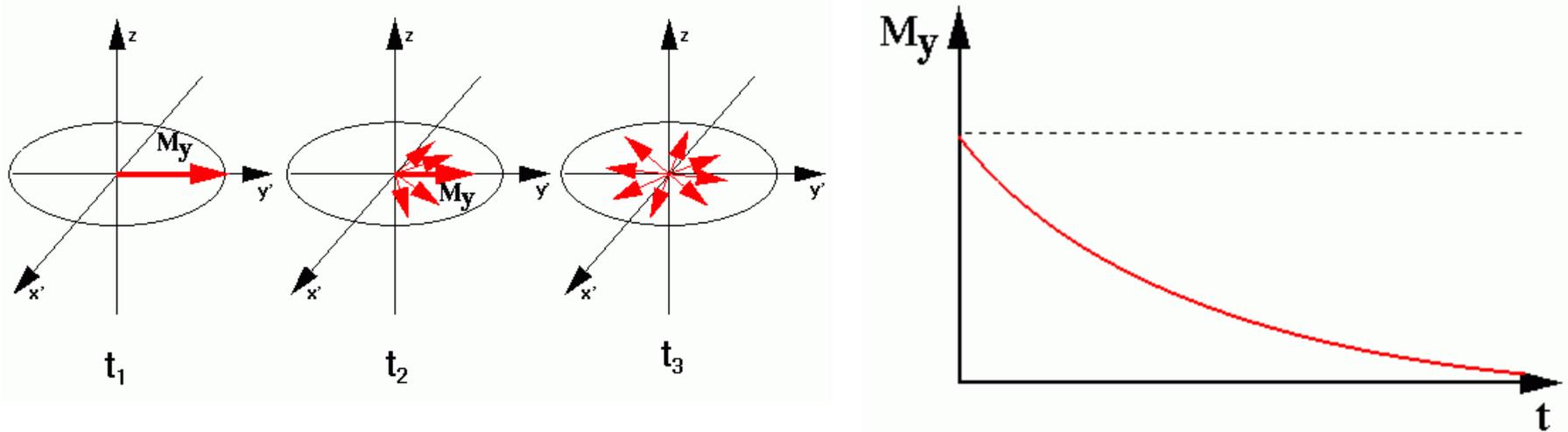
Classical Picture V

- Longitudinal relaxation = T_1 characteristic time (also called spin-lattice relax. t): the system can lose energy to the surrounding solvent molecules and slowly relax back to its “ground state” with a net M along the z -axis – this is a thermal relaxation process



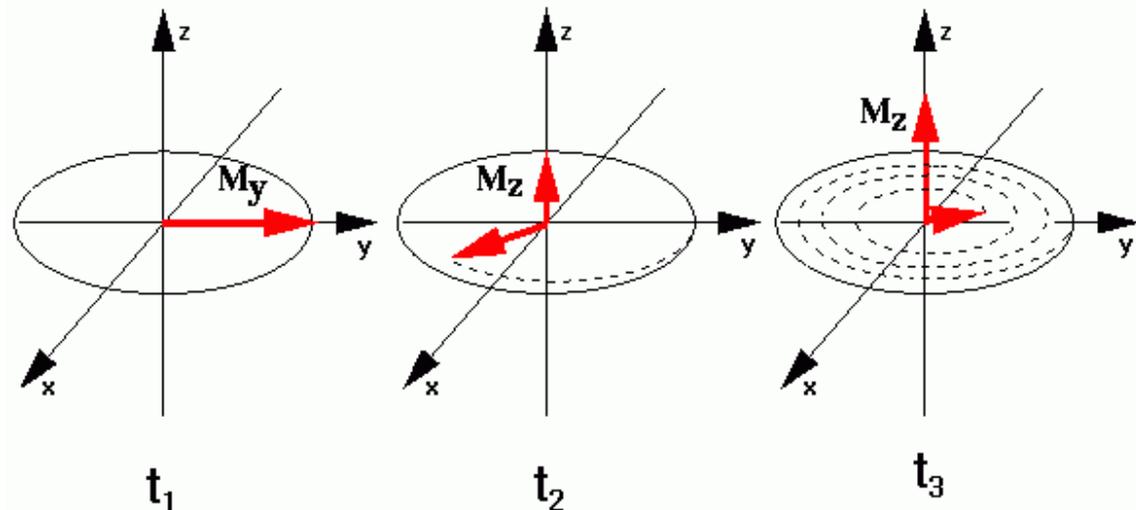
Classical Picture VI

- Transversal Relaxation = T_2 characteristic time (also called spin-spin relaxation) – the x-y M is slowly dispersed by inhomogeneities in the B to cancel away to zero – back to equilibrium value (T_2 is always $< T_1$)

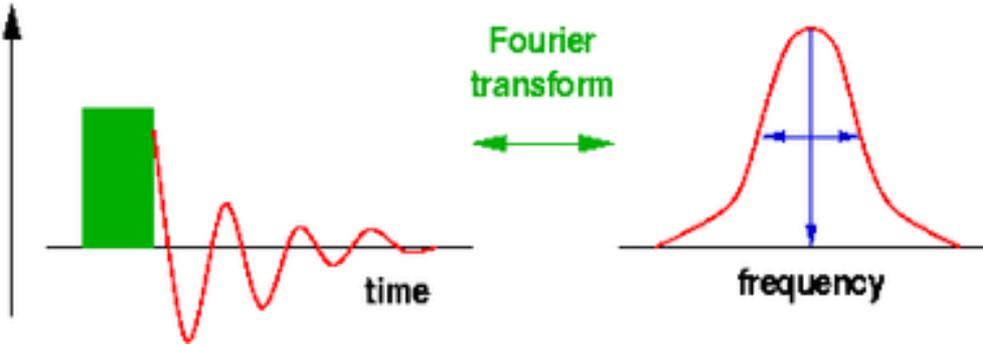


Classical Picture VII

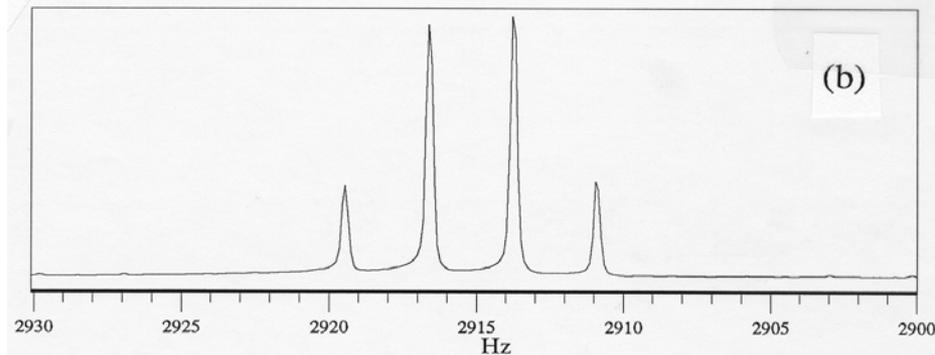
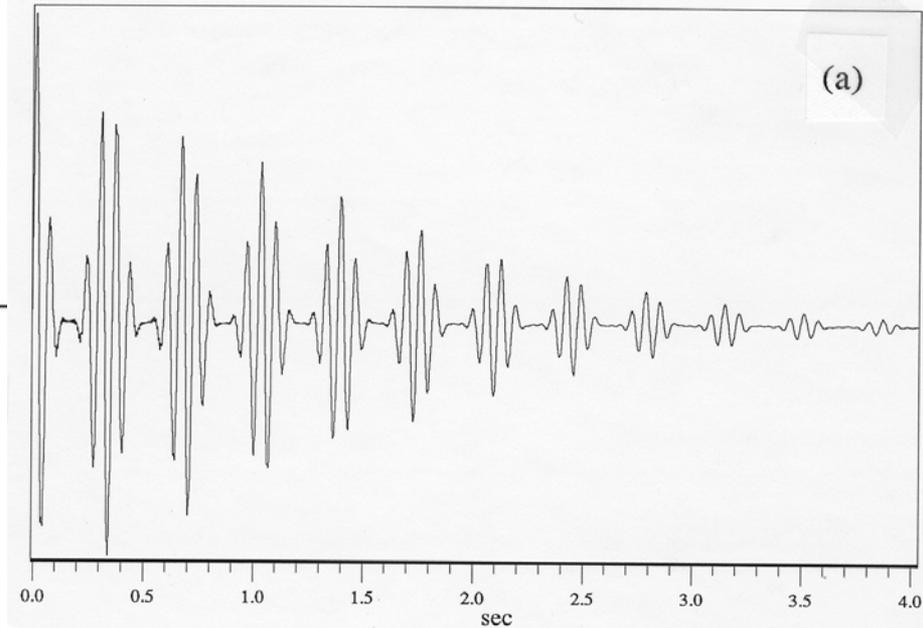
- Net relaxation picture – sum of T_1 and T_2 mixture – Bloch equations describe dM/dt
- Contributions to T_1 and T_2 processes from spin-spin coupling interactions - transfer of energy between neighboring spins directly [dipolar coupling] or via electron cloud intermediary [scalar coupling]



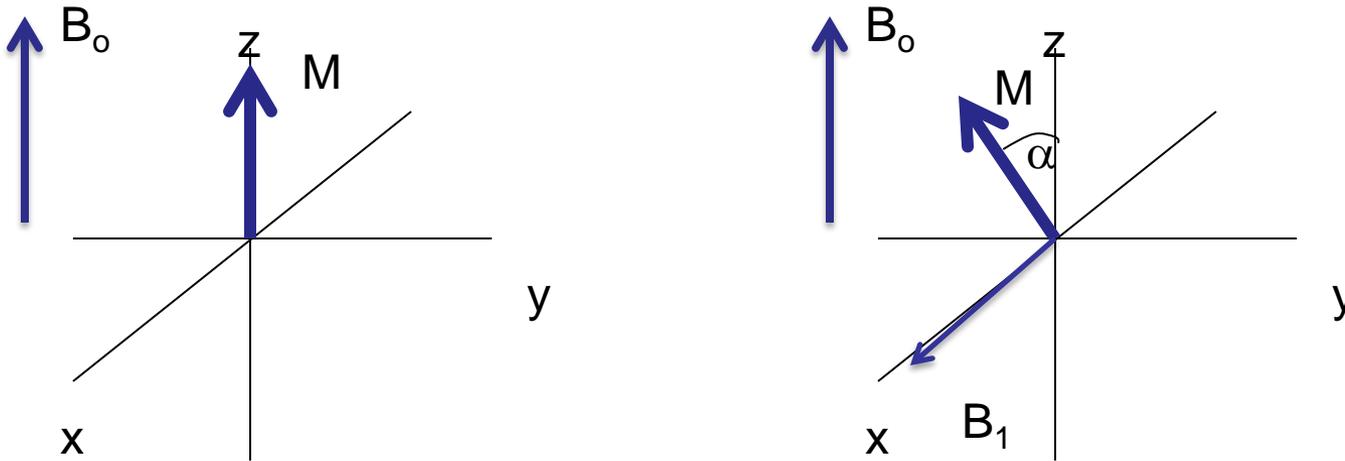
FT Single Pulse Expt I



A Single FT pulse of some duration T is applied and the detected signal is recorded for long enough for it to decay away This is called the Free Induction Decay or FID. A FT is then calculated to see the NMR frequency spectrum



FT single pulse expt II



B_0 orients magnetic dipoles to produce M along z axis

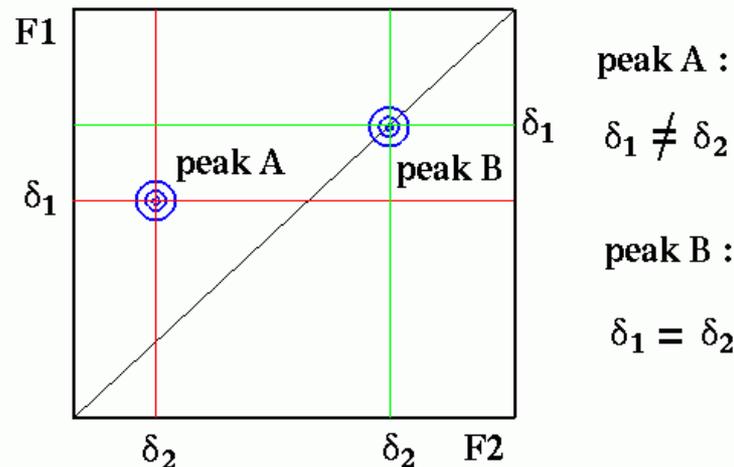
An RF pulse of duration T creates B_1 along the x -axis which produces a torque on M causing it to rotate in the y - z plane towards $-y$ through an angle α where $\alpha = g\beta B_1 T$

A 90° pulse is designed to rotate the M vector through 90° so it points along the $-y$ axis; A typical $T \sim 10^{-5}$ s so $B_1 \sim 6 \times 10^{-4}$ T $\ll B_0$

A 180° pulse is applied for twice as long and results in an inversion of M

Multi-dimensional NMR

- Example: a 2D plot against frequency shows peaks that are off the diagonal – having different f 's on both axes – are able to link two spins together through an interaction
- How are these spectra obtained?

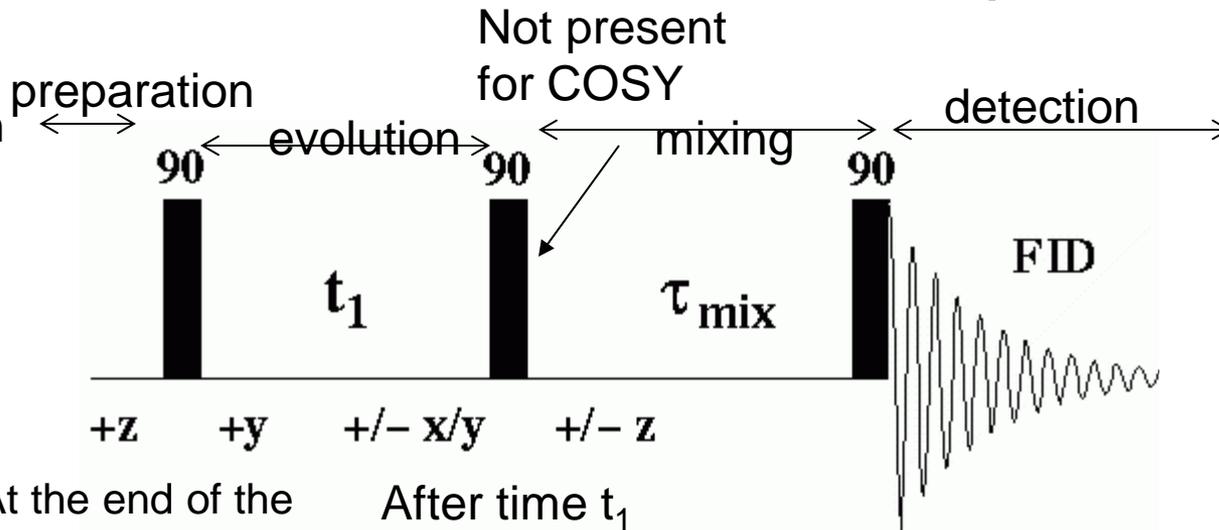


2D – COSY & NOESY experiments

COSY = **C**ORrelated **S**pectroscop**Y**

NOESY = **N**uclear **O**verhauser **E**ffect **S**pectroscop**Y**

B1 pulse will rotate the z-magnetization on to the x-y plane



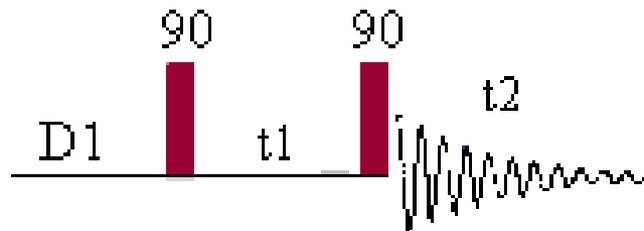
At the end of the pulse the B1 field is switched off and M starts to precess about the z-axis according to the chemical shift of the corresponding spin

After time t_1 another 90° B1 pulse follows that rotates part of M back to the $\pm z$ -axis. Here **M** will spend τ_{mix} . For COSY τ_{mix} occurs during the next pulse time

At the end of τ_{mix} a third (2nd for COSY) 90° B1 pulse which rotates M back to the x-y plane. As depicted at this stage the receiver of the spectrometer gets switched on and starts recording the FID as with any 1D experiment.

2D COSY – first 2D method

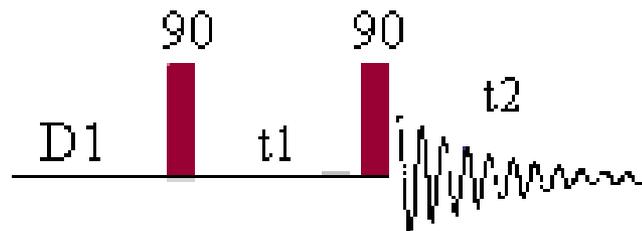
- Pulse sequence is 90° - t_1 - 90° -data acquisition where $t_1 = kt$ and $k = 0, 1, 2, \dots, 2^n$



- Scans are repeated for each k and FID are measured and plotted as $s(t_1, t_2)$
- FT then computes $FT\{s\}(f_1, f_2)$
- A 2D topographic plot showing $(x,y) = (f_1, f_2)$ and $(z) =$ peak strength will show a set of peaks along the diagonal (where $f_1 = f_2$) and also peaks at cross-correlated locations due to spin-spin interactions that transfer coherence

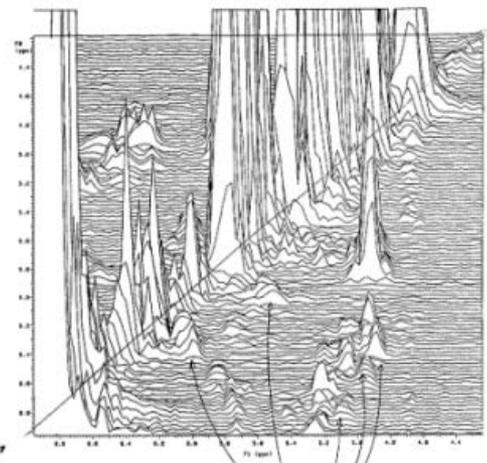
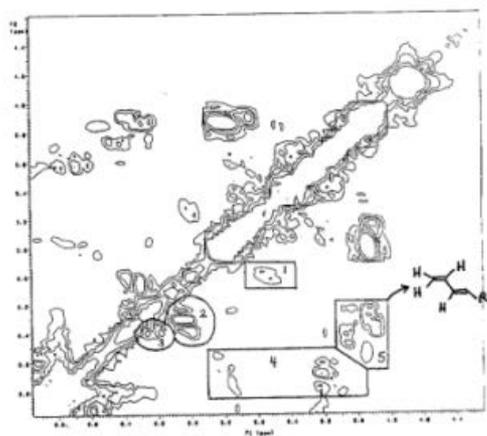
2D COSY - 2

- Cross-peaks in the spectrum come from spin-spin interactions (short-range) between nuclei attached through bonds
- Data are recorded by fixing t_1 at various (say 512) different times from 0 to some long time (~ 100 ms) and at each t_1 recording a signal-averaged FID (Free-Induction Decay) that lasts for some time t_2 – then for each of the 512 t_1 values, the FID is Fourier-transformed into a 1D spectrum vs f_2 – the one for $t_1 = 0$ is the normal 1D spectrum

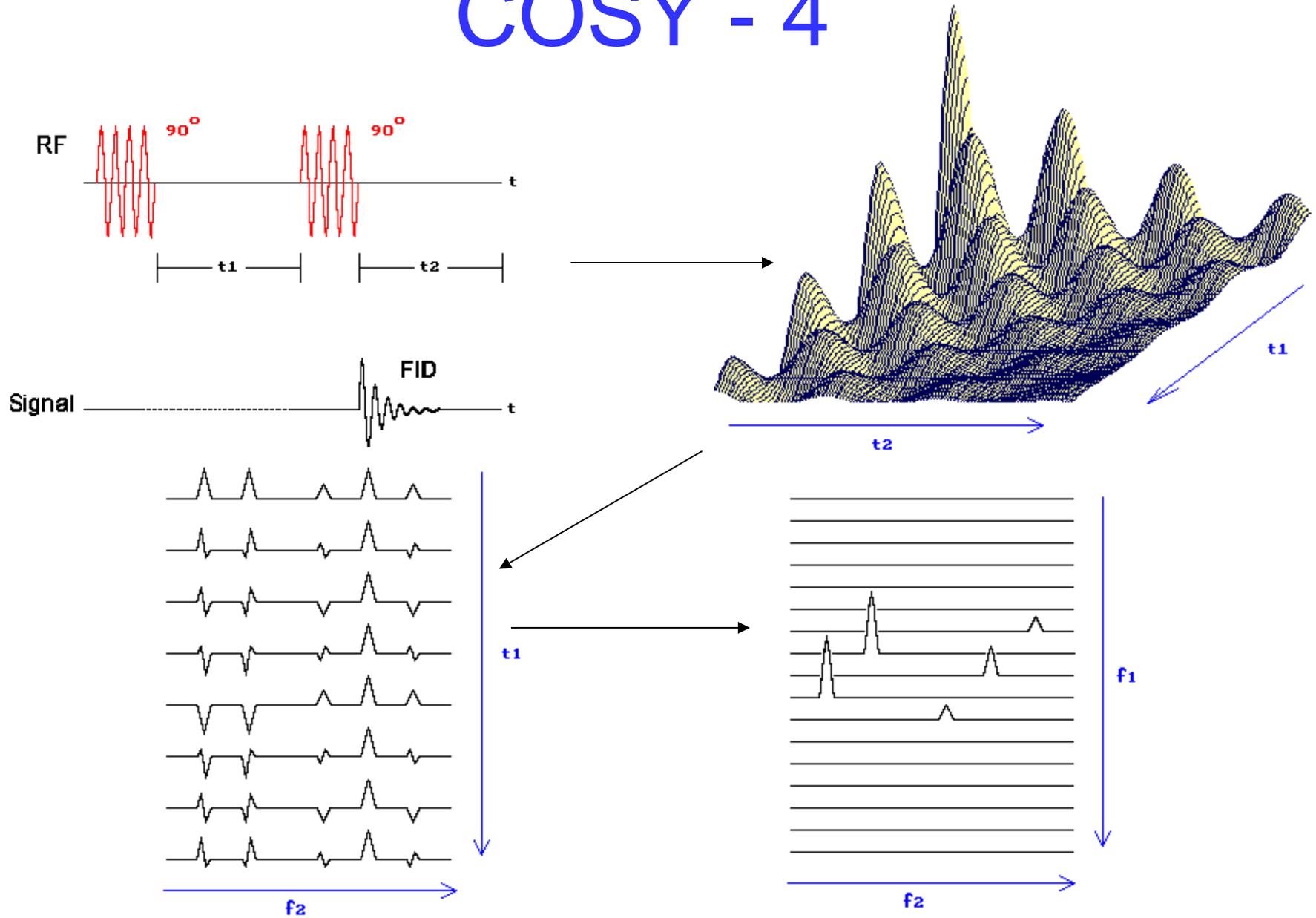


COSY - 3

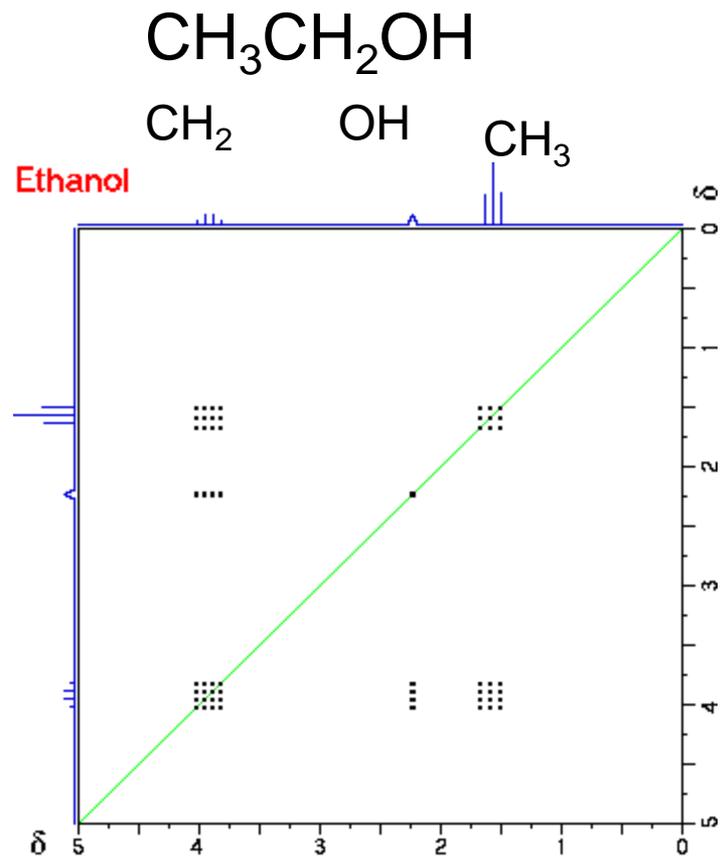
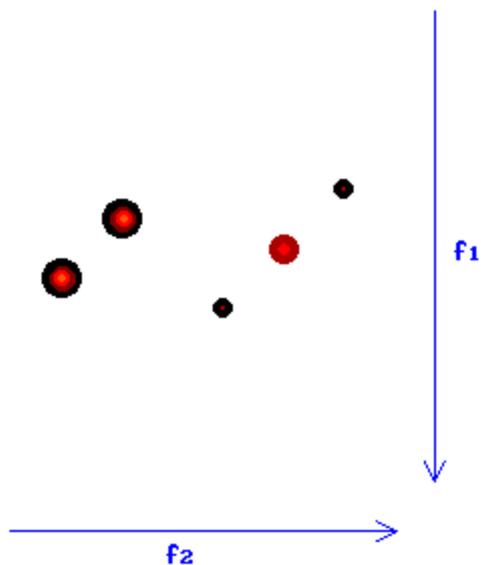
- Then for each one of 512 different f_2 values, these 512 1D spectra select 512 data points for intensities vs t_1 which can be Fourier-transformed into 1D spectra vs f_1 and both sets of spectra can be plotted on a 2D array (with axes f_1 and f_2), plotting the intensity strength as a contour plot
- The diagonal has the same 1D information, while the off-diagonal cross-peak intensities show the spin-spin coupled nuclei that are covalently bonded



COSY - 4

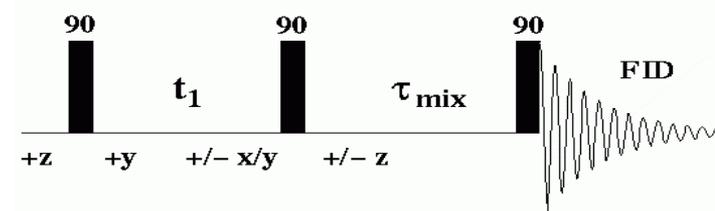


COSY - 5

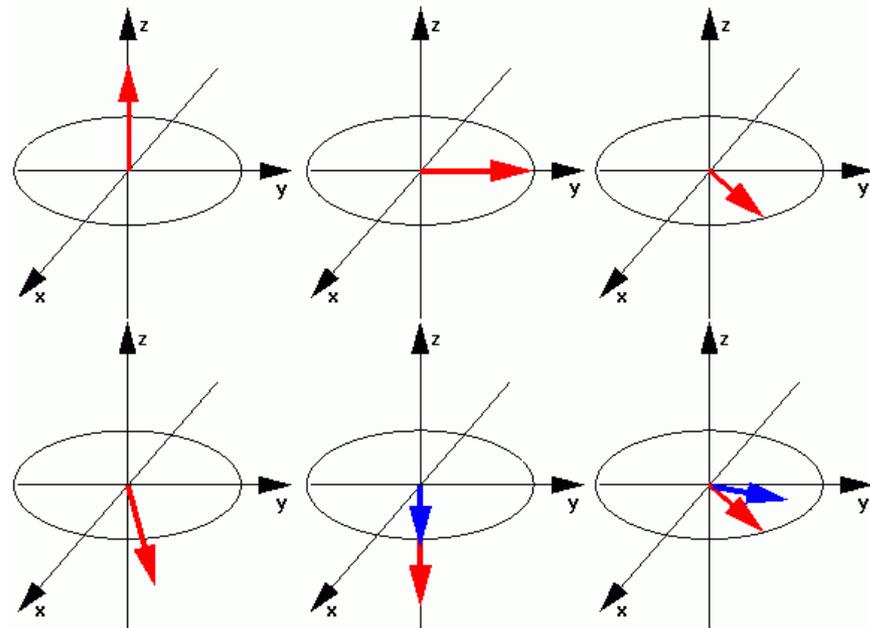


Note – OH does not couple to CH₃ – while all other cross-peaks are present

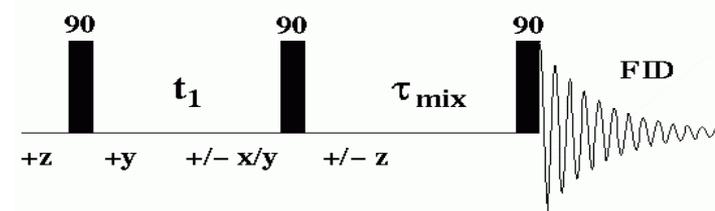
NOESY I



- What is the sequence of M for NOESY?
 - Rotates to xy ; precess for time t_1 ; second pulse rotates some to $-z$, others to $+z$; those in $-z$ are in excited state and during the mixing time (constant $\sim 30 - 300$ ms) some will transfer energy via spin interactions and return to the ground state ($+z$ M); final pulse needed to proce xy M to detect
 - But how? Via spin interactions – the longer the mixing time, the more time to transfer energy to neighboring spins.

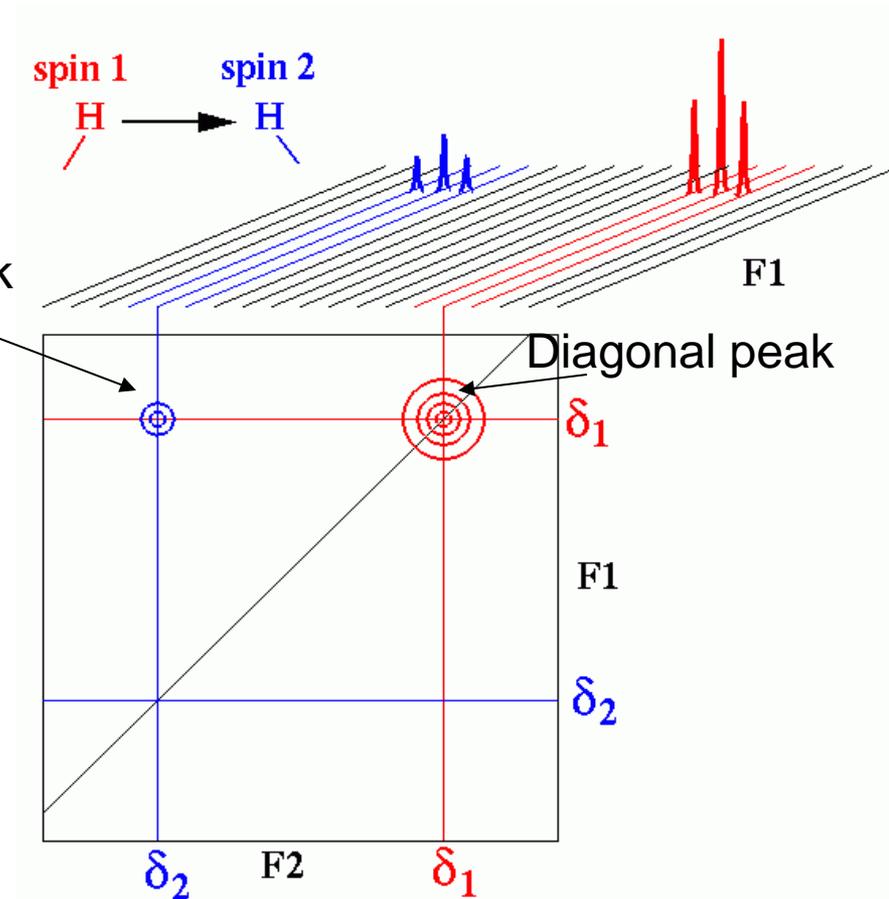
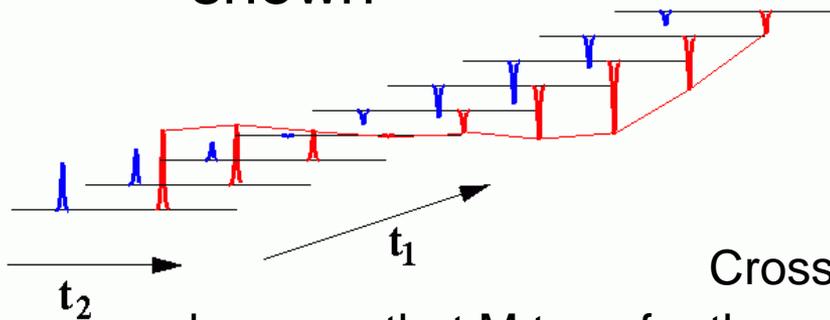


NOESY 2



- Repeat scans as function of both t_1 and $t_2 = t_{mix}$; as a function of t_1 for fixed t_2 we see an oscillating FID just as in 1D FT NMR;

FT analysis as a function of t_2 and t_1 gives a 2D plot as shown

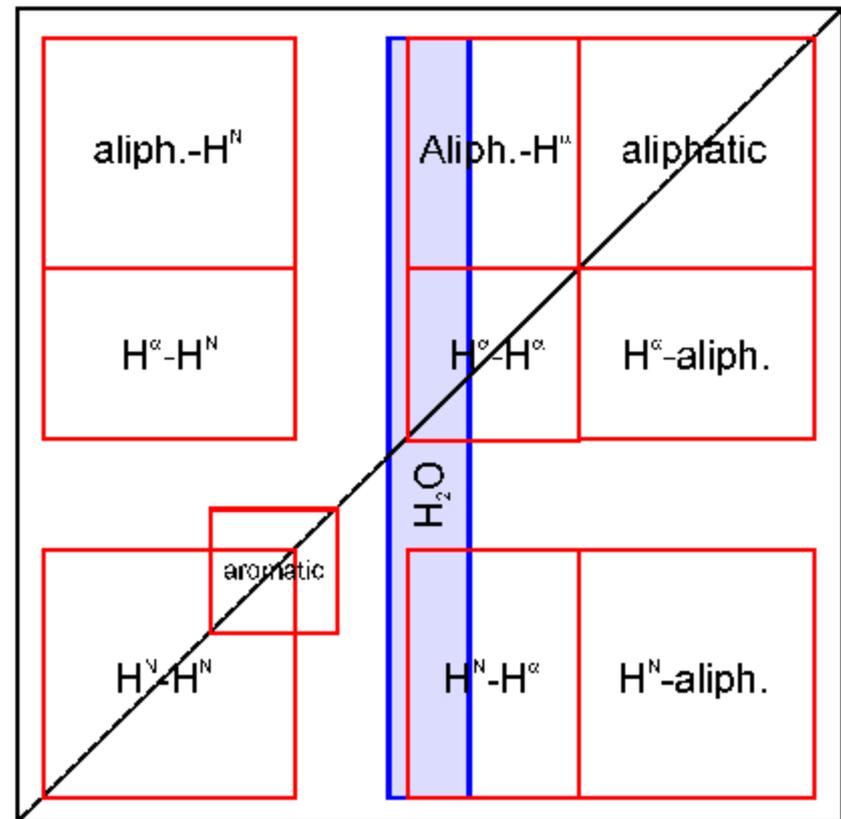
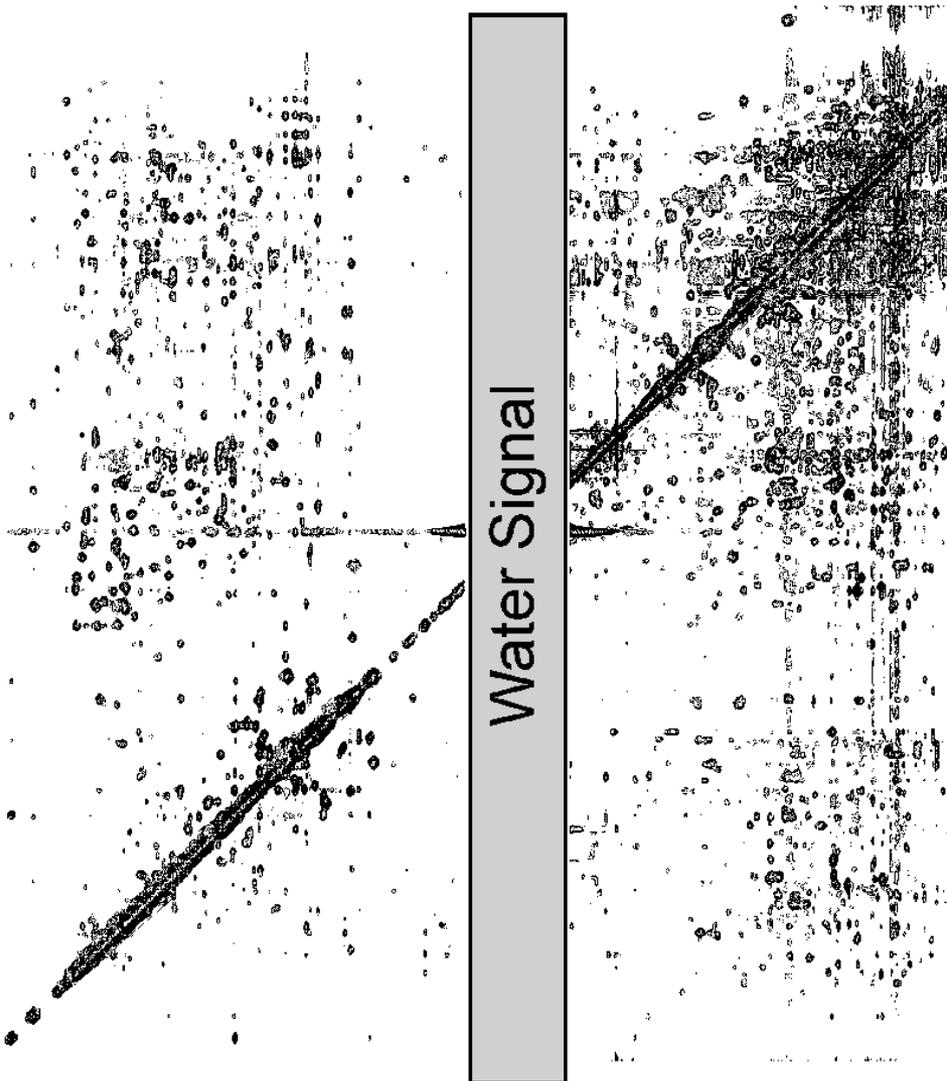


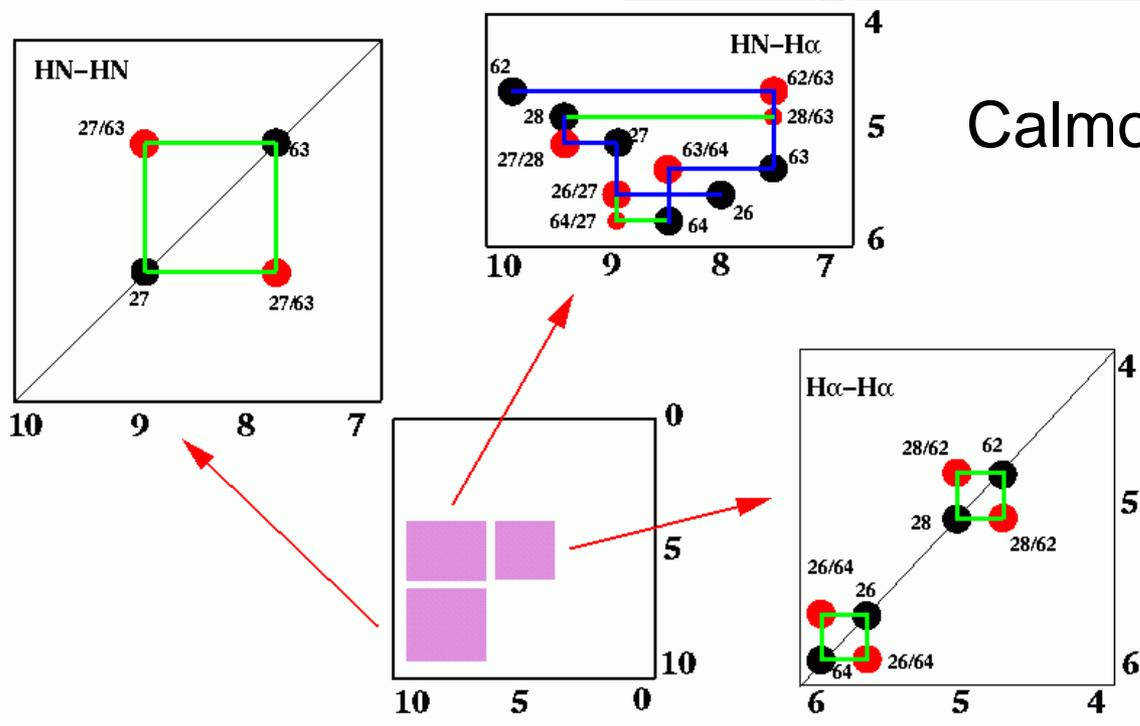
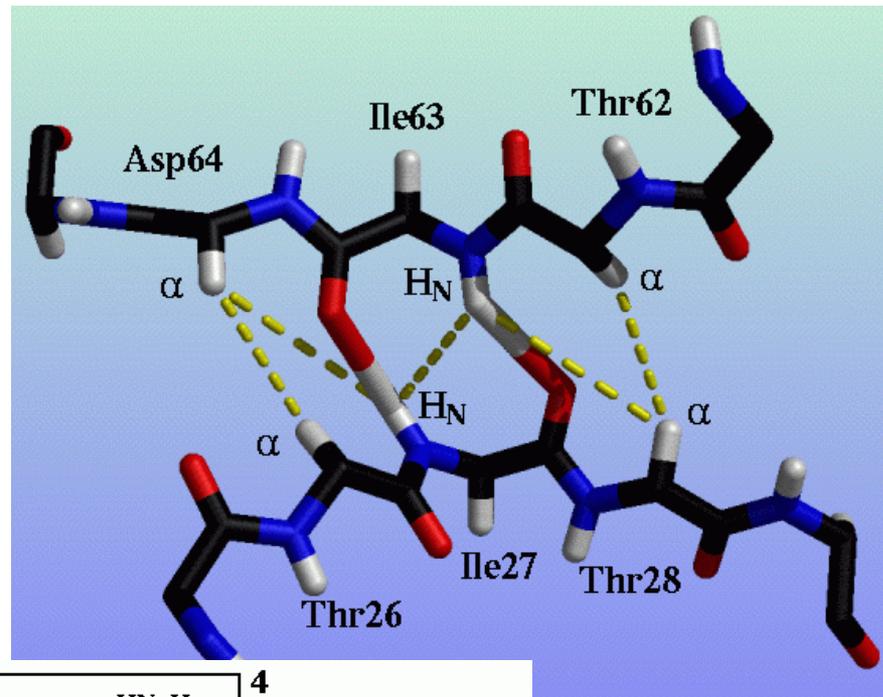
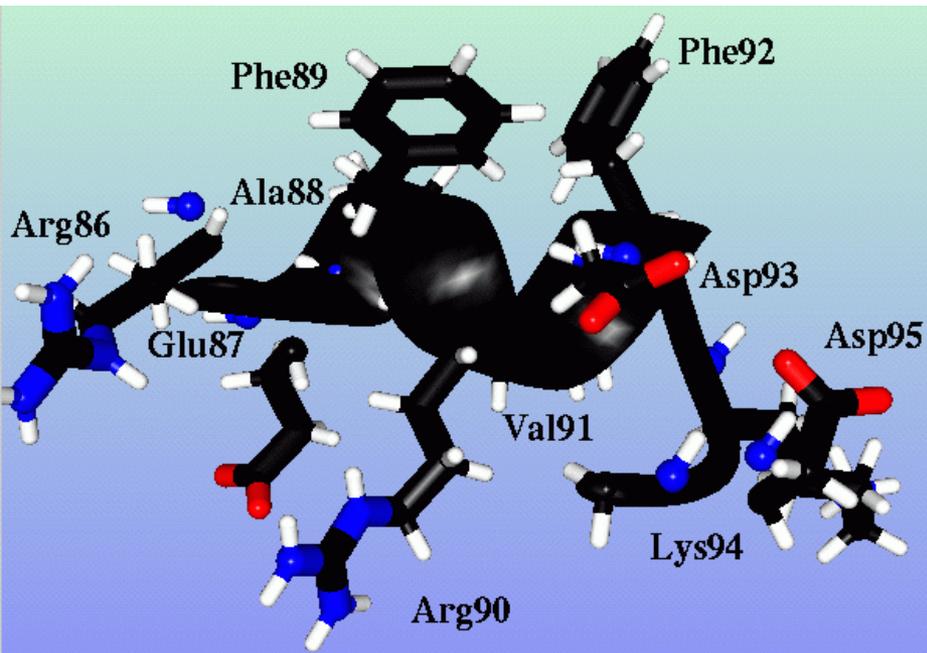
A cross peak means that M transfer through dipolar interactions between two neighboring spins must have taken place during the mixing time

The difference between NOESY and COSY is that the two spins here need not be co-valent neighbors

The NOE depends on spin separation distance d as $1/d^6$ and is a good yardstick measurement of spin separation up to $\sim 5 \text{ \AA}$

NOESY 3





Calmodulin example

Limitations of 2D homonuclear NMR

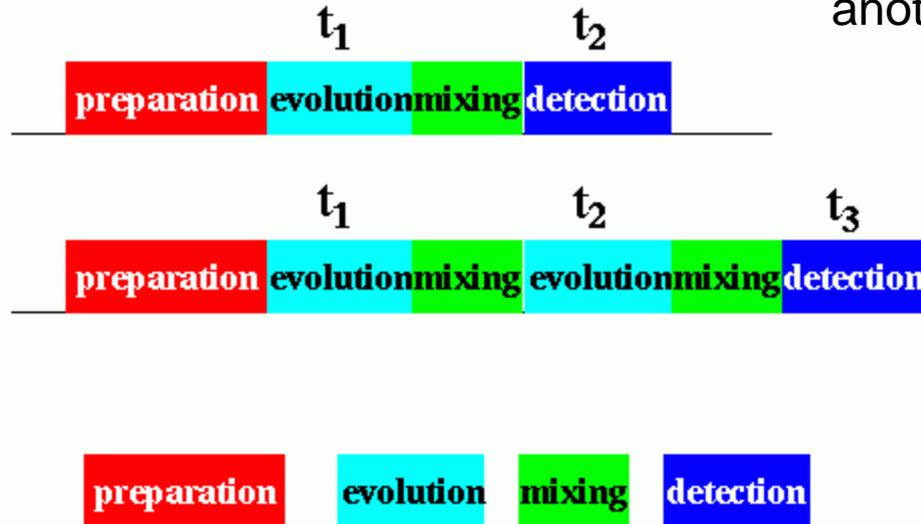
- The preceding techniques involved 2D methods using only protons
- Spectra limit usefulness to smaller macromolecules (e.g., for proteins < 100 amino acids) – why? Larger macromolecules have longer rotational diffusion times leading to shorter T_2 and hence to broader frequency peaks that start to overlap
- For larger macromolecules at least two additional methods have been developed:
multi-dimensional and heteronuclear expts

General multi-D NMR Scheme

preparation period
which leads to the
production of M to start
the experiment can be
as simple as a single
90° pulse

evolution period
allows M to precess
or evolve to create
one of the frequency
axes in our multiD
experiment

mixing period is
designed to
transfer M or
coherence from
one spin to
another



detection period
where we normally
have our M back on
protons whose
signal is detected in
the receiver coil of
the spectrometer

Heteronuclear NMR

- Use other nuclei with nuclear spin – e.g. ^{15}N or ^{13}C and induce spin coupling between protons and these
- Adds new features to spectra which help in determining structure of larger macromolecules
- One commonly used method (1980) is called HSQC = heteronuclear single quantum correlation – it involves cross-correlating an FID signal from protons and another nucleus in a 2D plot
- Since many of these isotopes are not present at high concentrations, isotope enrichment and transfer of magnetization from protons to these isotopes is used

MRI ideas



[animation](#)



