Basics of NMR Spectroscopy

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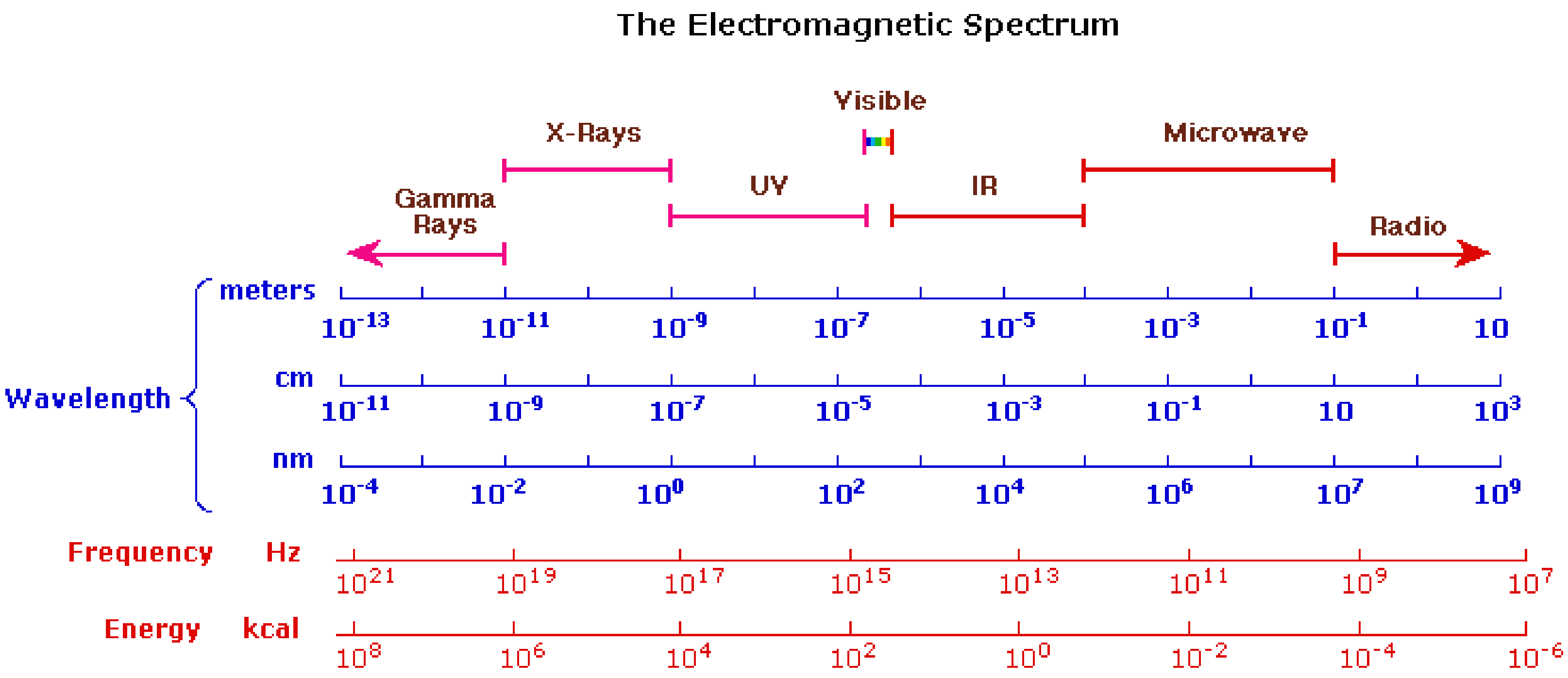
What is Spectroscopy?

Spectroscopy is the study of the interaction of electromagnetic radiation (light) with matter.



NMR uses electromagnetic radiation in the radio frequency range

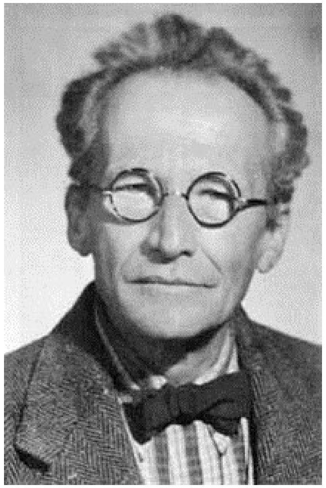
- Long wavelength, very low energy
  - Low energy has significant consequences:
    - **Sharp signals (Good)**
    - **Poor sensitivity (Bad)**
    - **Longer experiment time (Bad)**

Dirac equation

Applied relativity to quantum mechanics and derived the *Dirac* equation

- Predicted the existence of anti-matter
- Provided the theoretical basis for the quantum principle of "***spin***"

Schrödinger



$$i\hbar \frac{\partial}{\partial t}\psi = H\psi$$

Dirac

Einstein

$$E^2 = c^2\mathbf{p}^2 + m^2c^4$$

Nuclear Spin

$$E\psi = \left(\frac{\mathbf{p}^2}{2m} - \frac{\mathbf{p}^4}{8m^3c^2} + V - \frac{\hbar^2}{4m^2c^2}\frac{dV}{\partial r}\frac{\partial}{\partial r} + \frac{1}{2m^2c^2}\frac{1}{r}\frac{dV}{dr}\mathbf{S}\cdot\mathbf{L} \right)\psi$$

NMR Nobel Prize Laureates

- **Otto Stern, USA:** Nobel Prize in Physics 1943, "for his contribution to the development of molecular ray method and his discovery of the magnetic moment of the proton"

- **Isidor I. Rabi, USA:** Nobel Prize in Physics 1944, "for his resonance method for recording the magnetic properties of atomic nuclei"

- **Felix Bloch, USA and Edward M. Purcell, USA:** Nobel Prize in Physics 1952, "for their discovery of new methods for nuclear magnetic precision measurements and discoveries in connection therewith"

- **Richard R. Ernst, Switzerland:** Nobel Prize in Chemistry 1991, "for his contributions to the development of the methodology of high resolution nuclear magnetic resonance (NMR) spectroscopy

- **Kurt Wüthrich, Switzerland:** Nobel Prize in Chemistry 2002, "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution"

- **Paul C. Lauterbur, USA and Peter Mansfield, United Kingdom:** Nobel Prize in Physiology or Medicine 2003, "for their discoveries concerning magnetic resonance imaging"

Fundamentals of Spin

Properties of Spin

Spin is a fundamental property of nature

- Any unpaired electron, proton, or neutron will possess a spin of ½
- Atomic nuclei, which are composed of protons and neutrons, may also possess spin

The spin of an atomic nucleus is determined by the number of protons and neutrons

- Atoms with odd number of protons will have spin
- Atoms with odd number of neutrons will have spin
- Atoms with EVEN number of protons and neutrons will not have spin

The value of the nuclear spin is defined by `I`, the ***nuclear spin quantum number*** and can have values of (`I = 0, 1/2, 1, 3/2, 2, 5/2,` …)

A nucleus of spin `I` can exist in (`2I+1`) spin states. We will primarily deal with spin ½ nuclei

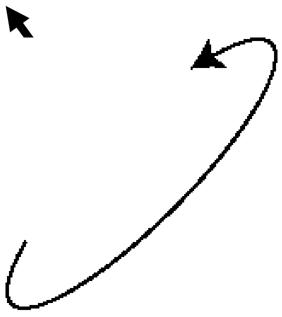
Levitt

"Spin is a highly abstract concept, which may never be entirely 'grasped' beyond knowing how to manipulate the quantum mechanical equations."

-**Spin Dynamics. Basics of Nuclear Magnetic Resonance. (2002)**

Magnetic properties of atomic nuclei

$$\mathbf{L} \propto \mathbf{I}$$

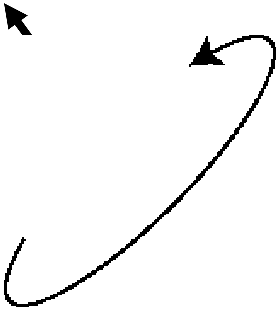


- The nuclear spin quantum number (`I`) will have a corresponding angular momentum (`L`) and a set of **quantized** spin states.
- The magnitude of the spin angular momentum is give by:

$$L = \hbar\sqrt{I(I+1)}$$

Magnetic properties of atomic nuclei

$\mathbf{L} \propto \mathbf{I}$



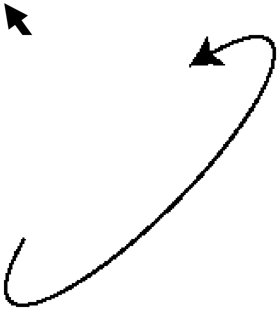
- The nuclear spin quantum number (**I**) will have a corresponding angular momentum (**L**) and a set of quantized spin states.
- The magnitude of the **spin angular momentum** is give by:

$$L = \hbar\sqrt{I(I+1)}$$

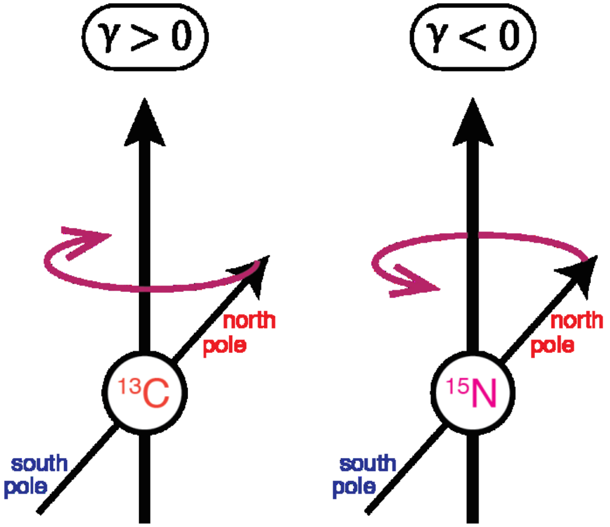
- A nucleus has an inherent charge due to the presence of protons
- Any spinning charge will generate a magnetic field
- The magnitude of the **magnetic moment** (**μ**) is given by:

$$\mu_m = \gamma\mathbf{L}$$

$\boldsymbol{\mu} \propto \mathbf{L}$

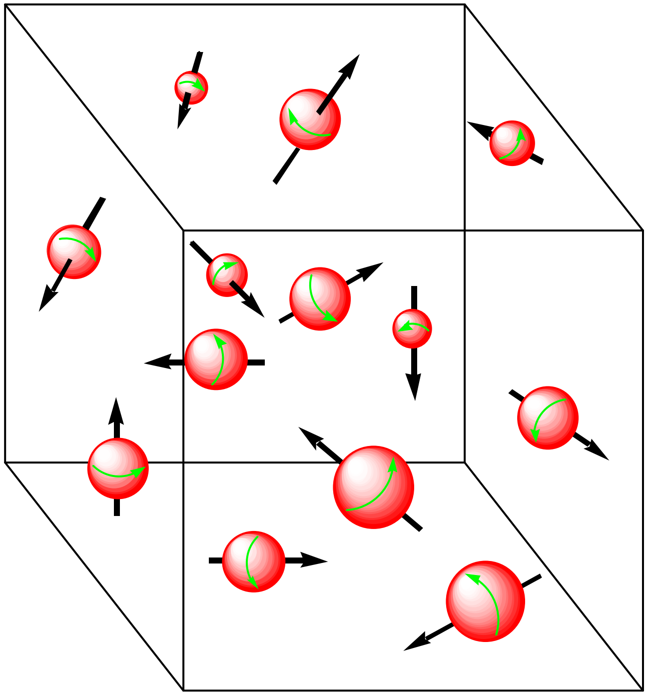


- A nuclei with spin can be thought of as if it were a tiny bar magnet.
- The gyromagnetic ratio (γ) is an *empirically* determined constant that is unique for each nucleus type.
- Values of γ can be positive or negative and determine the sense of precession and thus the direction of the magnetic moment.



Magnetic properties of atomic nuclei

- The magnetic moment (**μ**) is a vector quantity that has both magnitude and direction
- In the absence of an external magnetic field the magnetic moments (**μ**) are randomly orientated.



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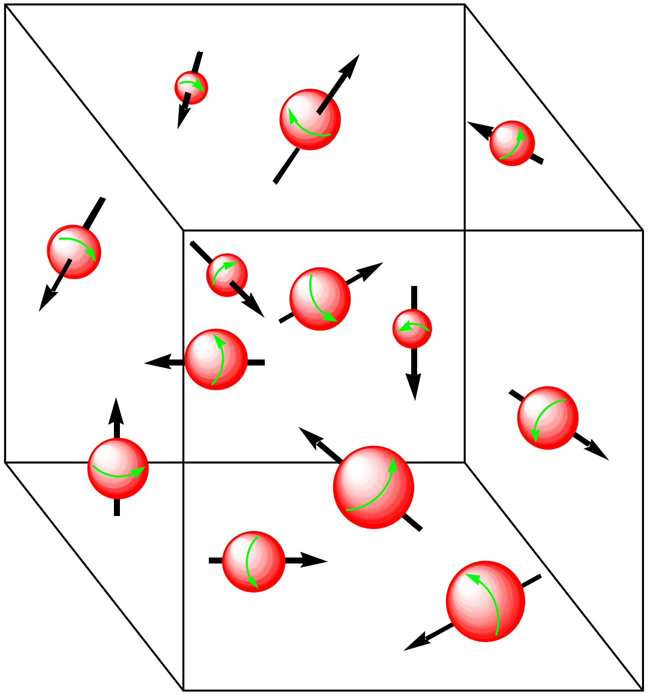
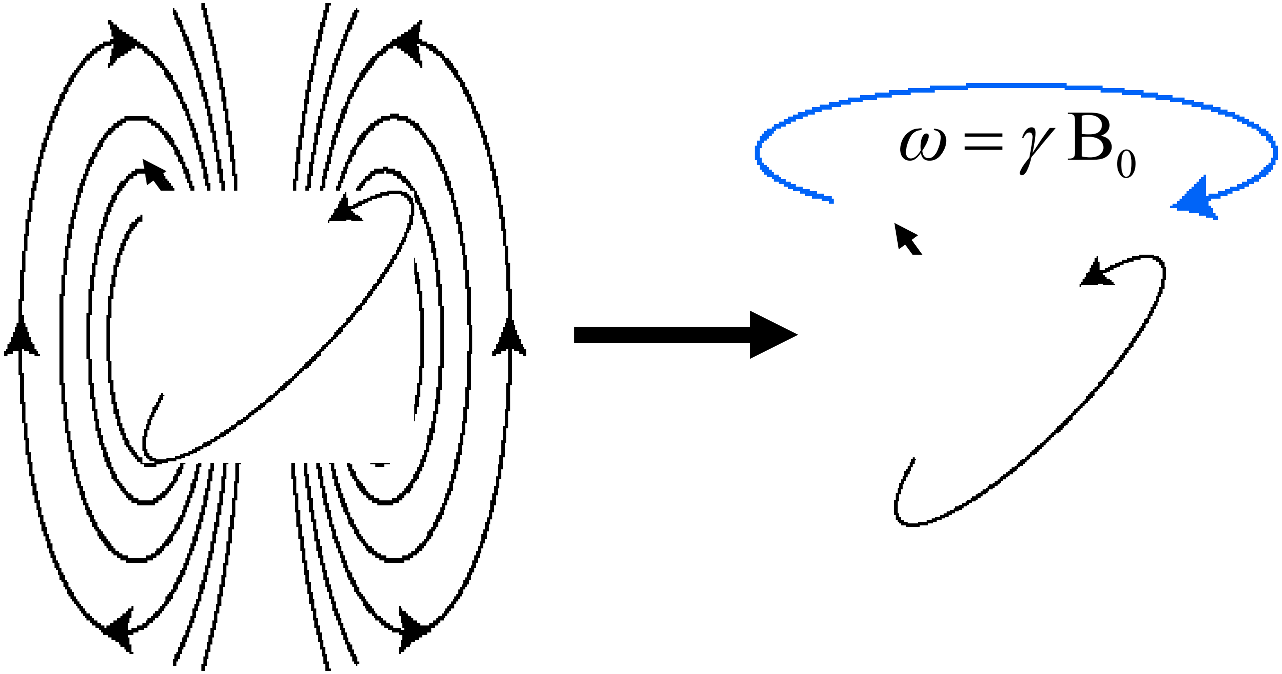


Lets put the sample in a strong external magnetic field

Nuclear Precession in a Magnetic Field

Semi-Classical Description



$$\omega = \gamma B_0$$

Magnetic Field B_0

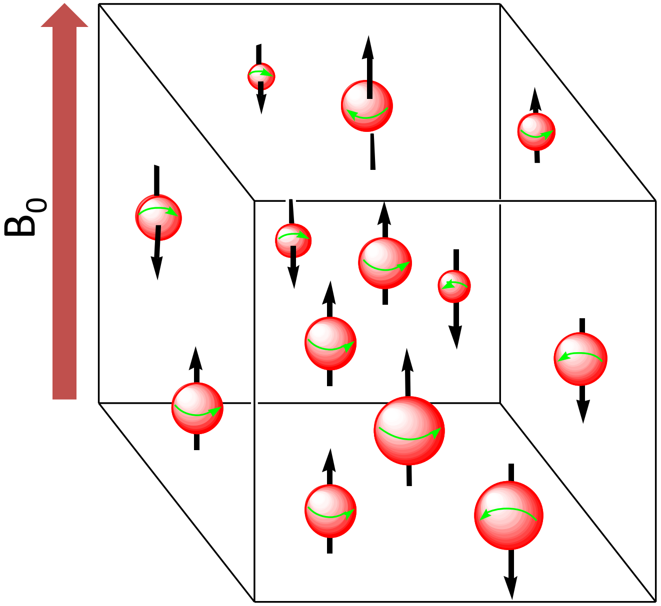
Example:

For ^{1}H nuclei (protons)
(γ=2.68x10^8 rad T^{-1} s^{-1})
in a magnetic field of
11.74 Tesla
ω=3.15 x 10^9 rad s^{-1}
or
$\nu=\omega/2\pi$=5x10^8 s^{-1}
= 500 MHz

The Magnetic Field (**B_0**) exerts torque on angular momentum (**L**) and causes Nuclear Precession, analogous to precession of spinning top. The frequency of the precession (**ω**), often called the Larmor frequency, is proportional to the gyromagnetic ration (γ) and the strength of the external magnetic field (**B_0**).

Nuclear Precession in a Magnetic Field

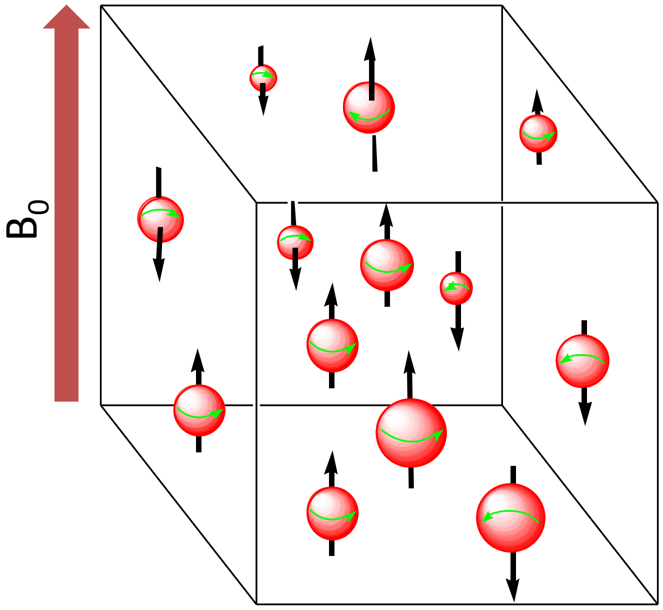
Quantum Mechanical Description



In the macroscopic world the two magnets can be aligned in an infinite number of orientations. At the atomic level, these alignments are **quantized** and the number of orientations (spin states) are equal to `2I+1`. We will only deal with spin ½ nuclei (i.e. two states)

Nuclear Precession in a Magnetic Field

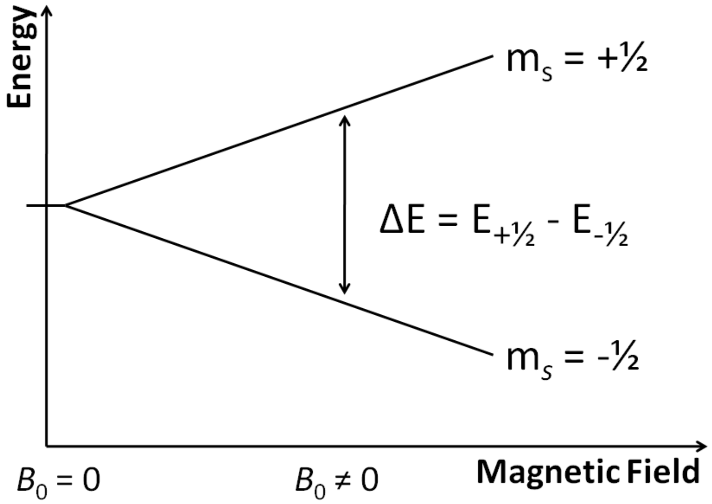
Quantum Mechanical Description



In the macroscopic world the two magnets can be aligned in an infinite number of orientations. At the atomic level, these alignments are **quantized** and the number of orientations (spin states) are equal to **`2I+1`**. We will only deal with spin ½ nuclei.

The different **quantized** orientations will each have an energy level determined by the Zeeman splitting

Zeeman Splitting

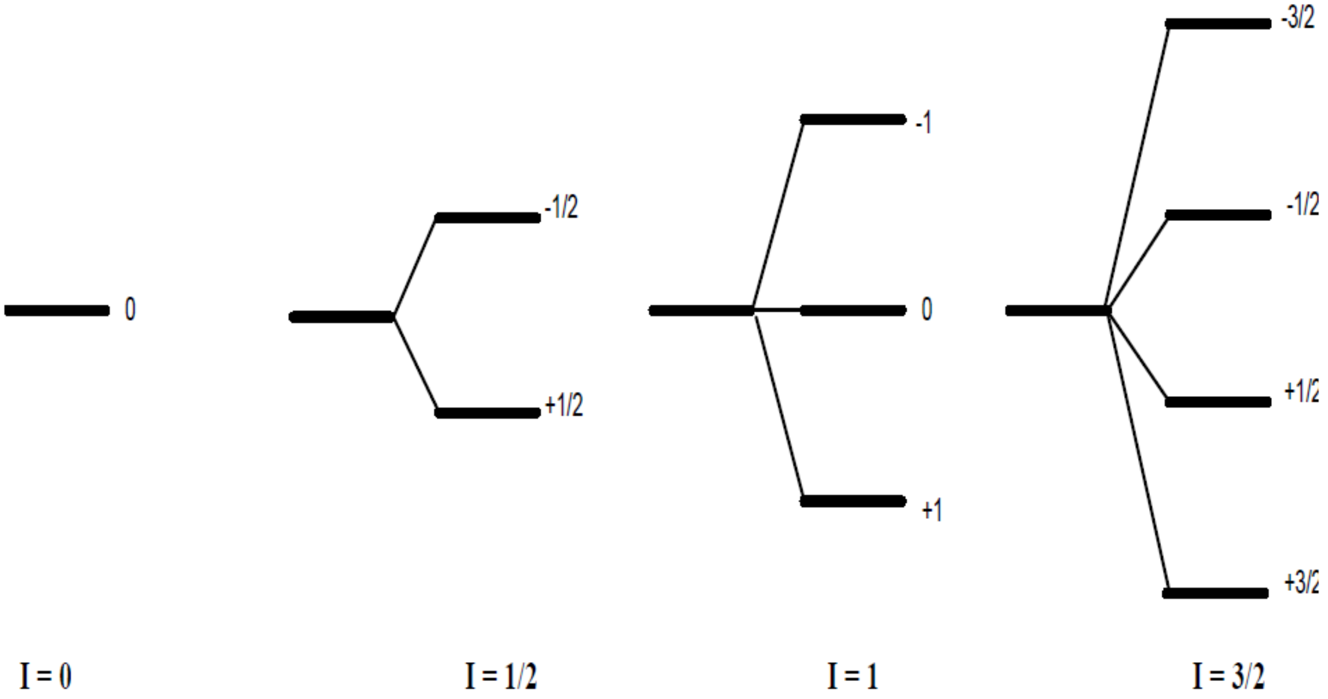


Nuclear Precession in a Magnetic Field

Quantum Mechanical Description

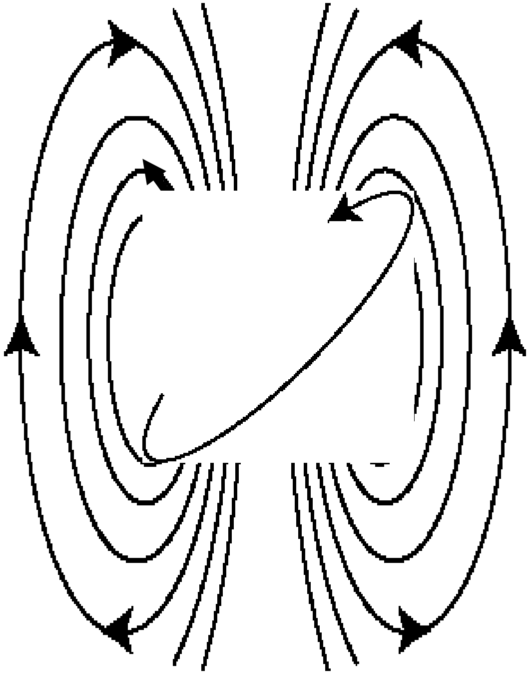
The energy levels are more complicated for **`I`** > 1/2

Zeeman Splitting

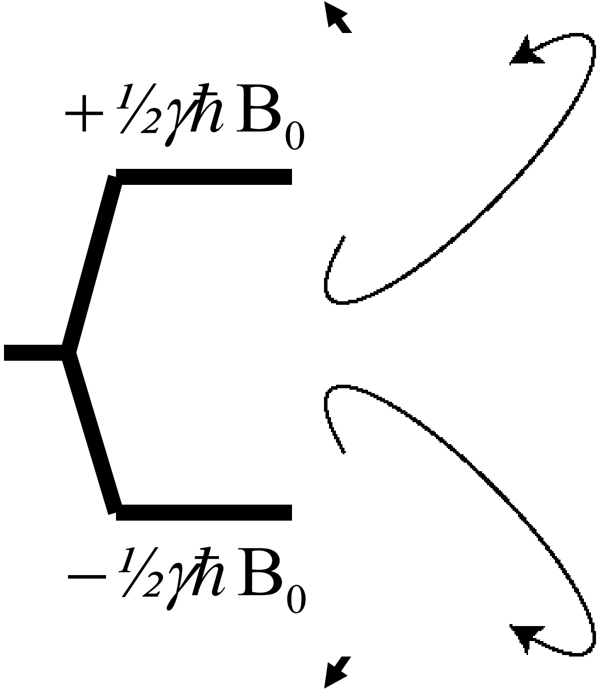


Nuclear Precession in a Magnetic Field

Quantum Mechanical Description



Magnetic Field B_0



$+\frac{1}{2}\gamma\hbar B_0$

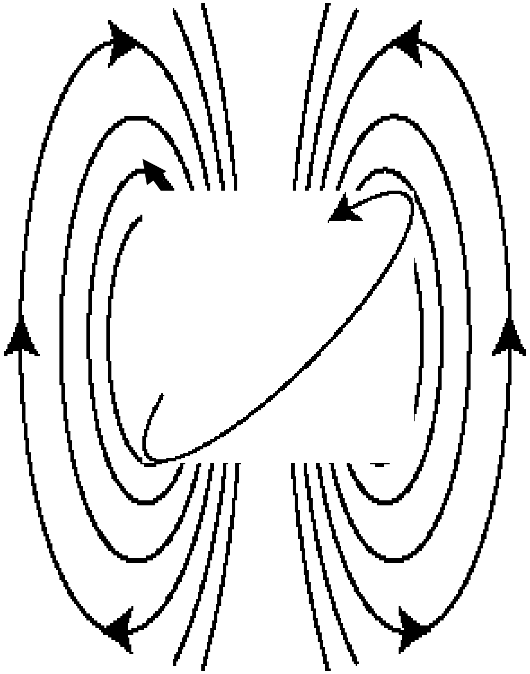
$-\frac{1}{2}\gamma\hbar B_0$

Zeeman Splitting

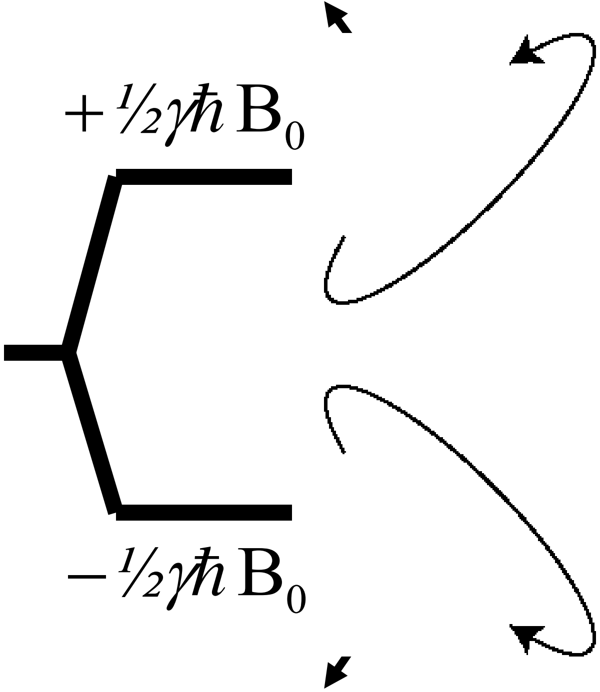
$$\Delta E = \gamma\hbar B_0$$

Nuclear Precession in a Magnetic Field

Quantum Mechanical Description



Magnetic Field B_0



Zeeman Splitting

$$\Delta E = \gamma\hbar B_0$$

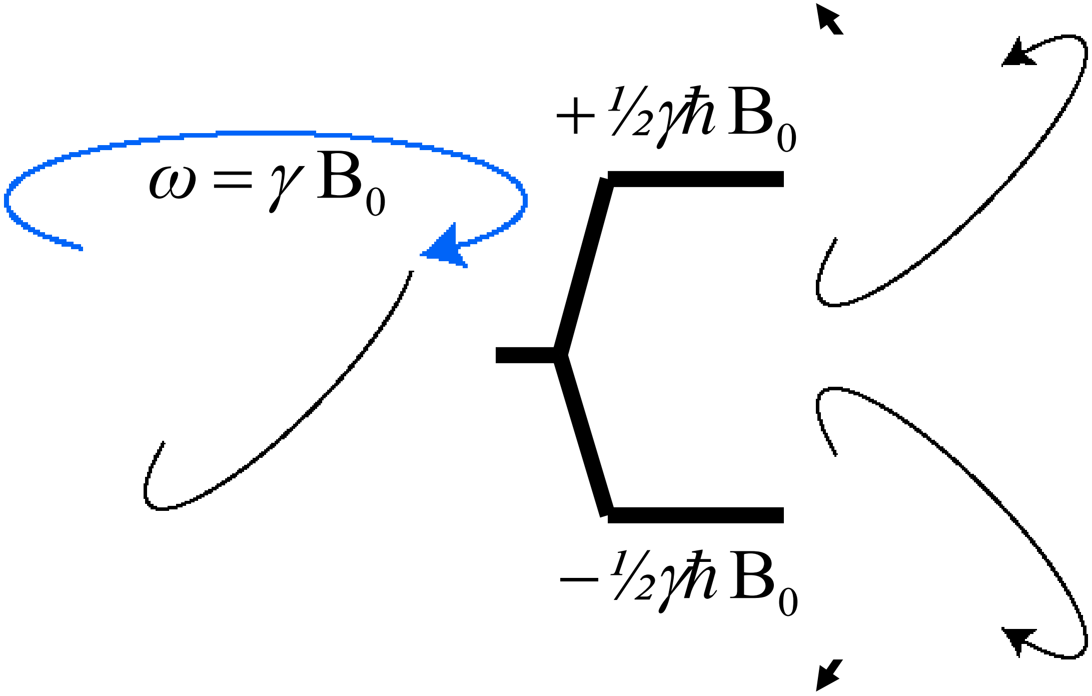
Knowing ΔE, we can stimulate the transition between these two states by applying an RF field such that:

$$E = h\nu = \hbar\omega$$

In NMR spectroscopy we are going to perturb the spin states by stimulating transitions between the energy levels.

Nuclear Precession in a Magnetic Field

Quantum-Classical Correspondence

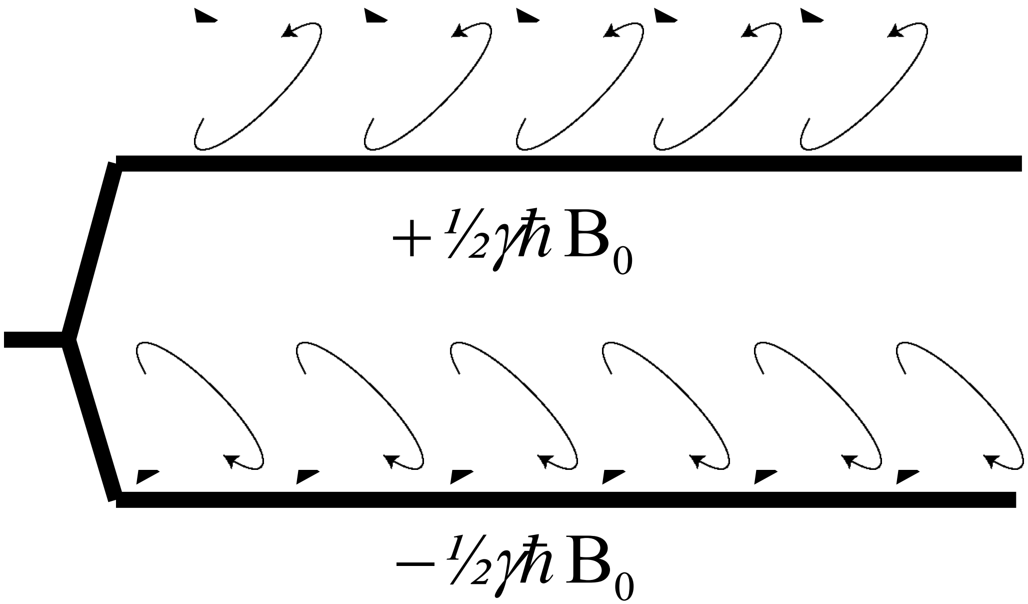


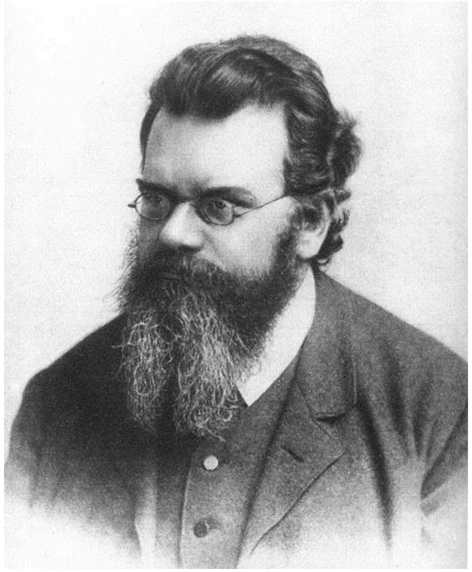
$$\Delta E = \gamma\hbar B_0$$

Knowing ΔE, we can stimulate the transition between these two states by applying an RF field which satisfies the resonance condition:

$$E = \hbar\omega = \gamma\hbar B_0$$

Boltzmann Distribution





$$\frac{N_\alpha}{N_\beta} = e^{\frac{\Delta E}{kT}}$$

For ^{1}H at 25°C, B_0 = 14 Tesla, $\Delta E = 4 \times 10^{-25}$ J
At that ΔE there is one extra low energy hydrogen nucleus for every 20,000 nuclei.

Due to its lower gyromagnetic ratio, nitrogen has a $\Delta E = 4 \times 10^{-26}$ J, yielding one extra low energy nucleus for every 200,000 nuclei.

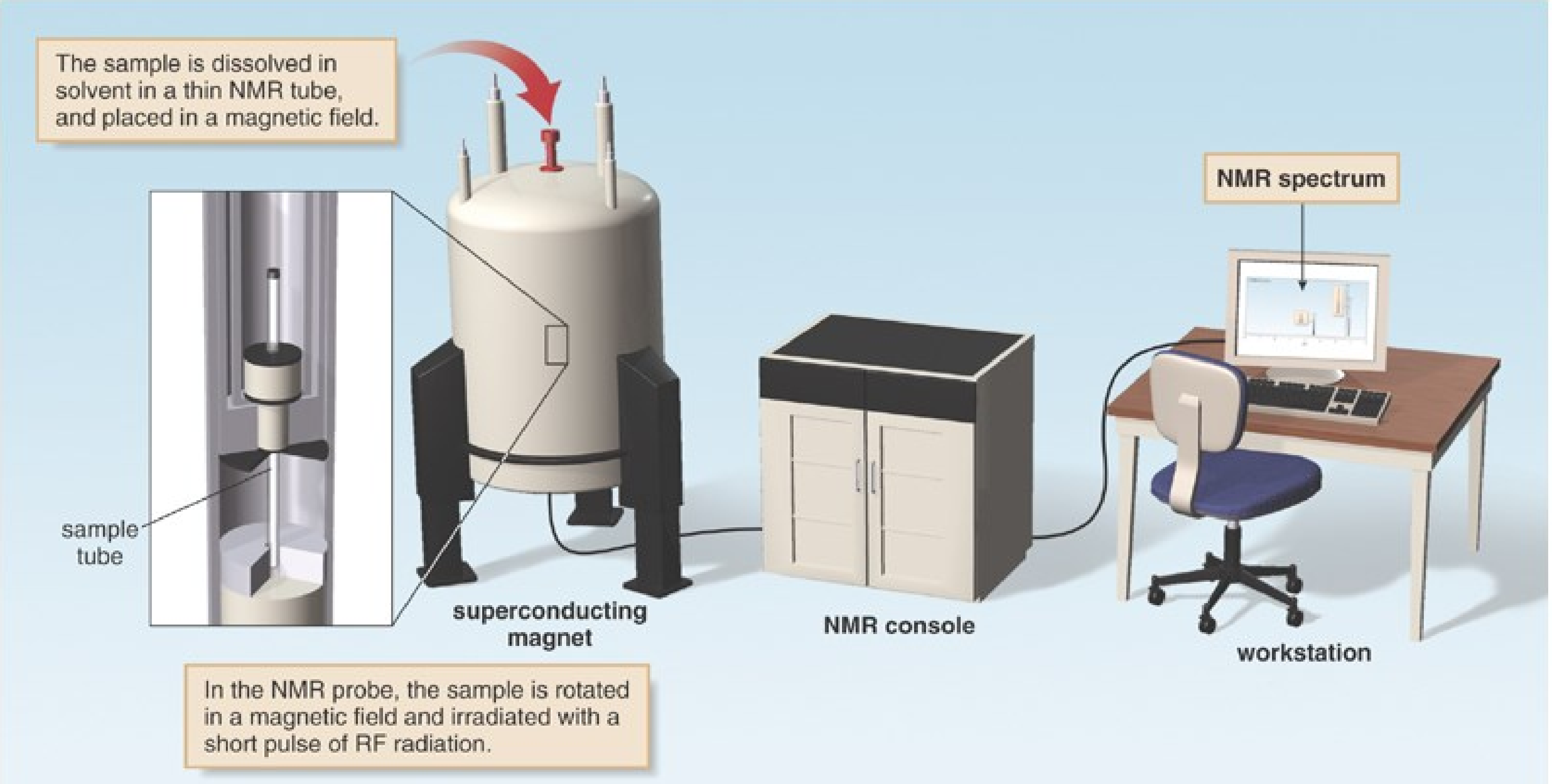
NMR is INSENSITIVE

Manipulating Magnetization

RF Excitation, Relaxation

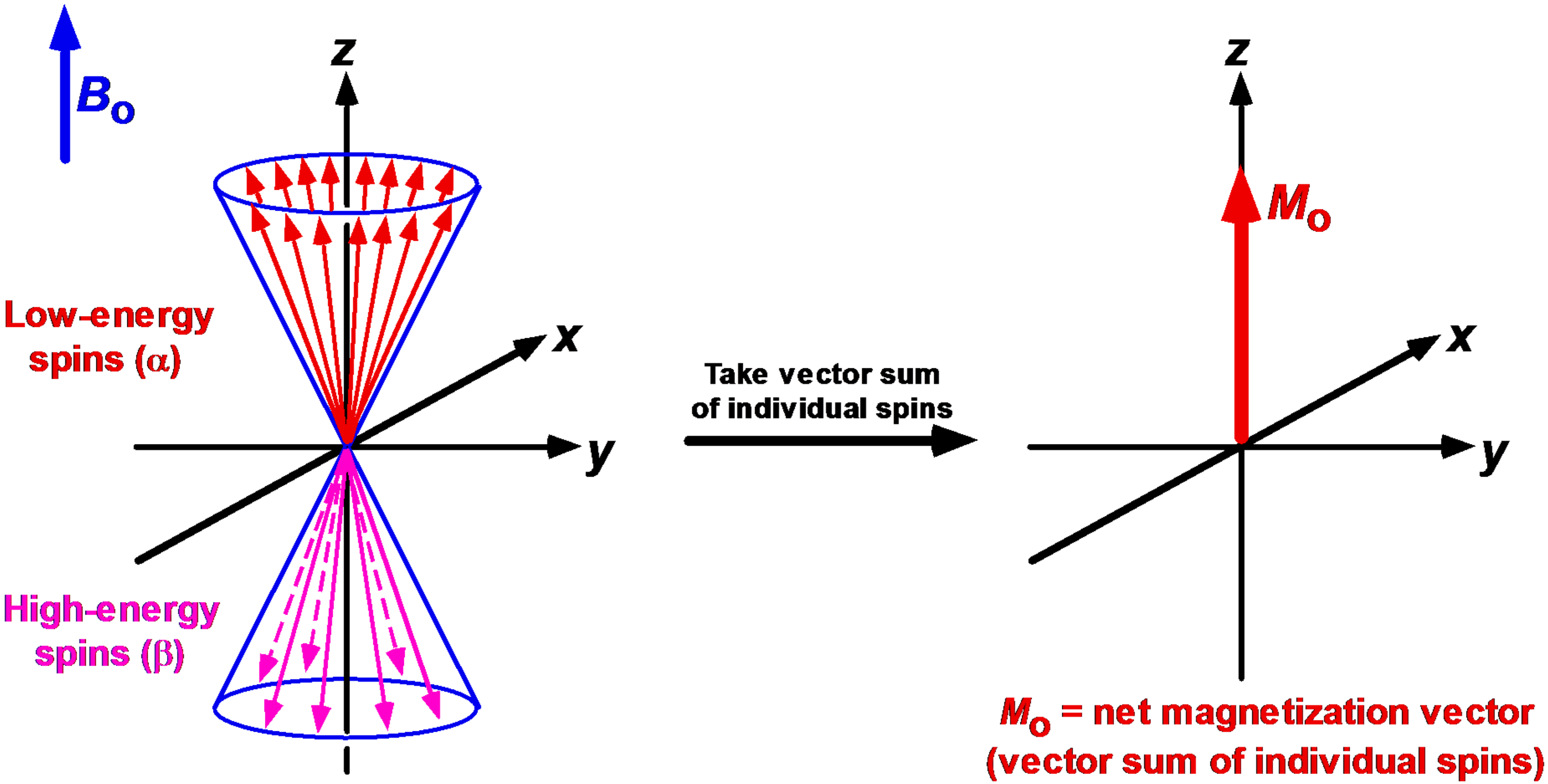
- Assuming spin ½ nuclei there will be two energy states (lower and upper)
- The ΔE determines the RF frequency that is applied in order to stimulate excitation from the lower energy level to the upper energy level.
- The Boltzmann distribution is then re-established by a process called relaxation.

NMR Spectrometer



- NMR spectrometers are akin to both a radio station and recording studio. RF pulses at specific frequencies and durations are pulsed at high energy into the sample (Radio station) which sits inside a probe inside the magnet. Tiny currents are then picked up by the receiver coil, amplified, and digitized into a signal (Recording studio) ready for post-collection processing.

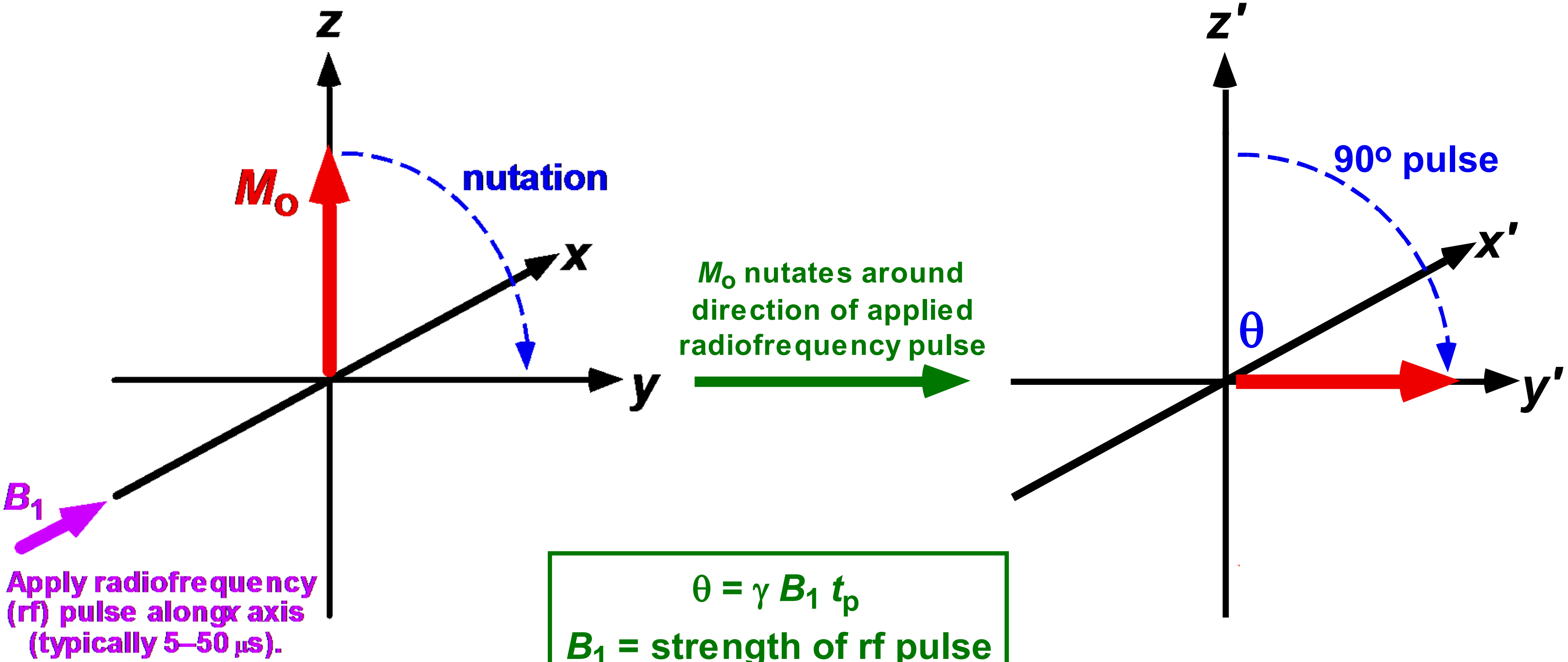
Bulk Magnetization M_0



M_o = net magnetization vector (vector sum of individual spins)

For the remainder of the lecture we will deal with bulk magnetization

Manipulation of M_0 with radiofrequency pulses



Apply radiofrequency (rf) pulse along *x* axis (typically 5–50 μs).

M_o nutates around direction of applied radiofrequency pulse

$\theta = \gamma B_1 t_p$

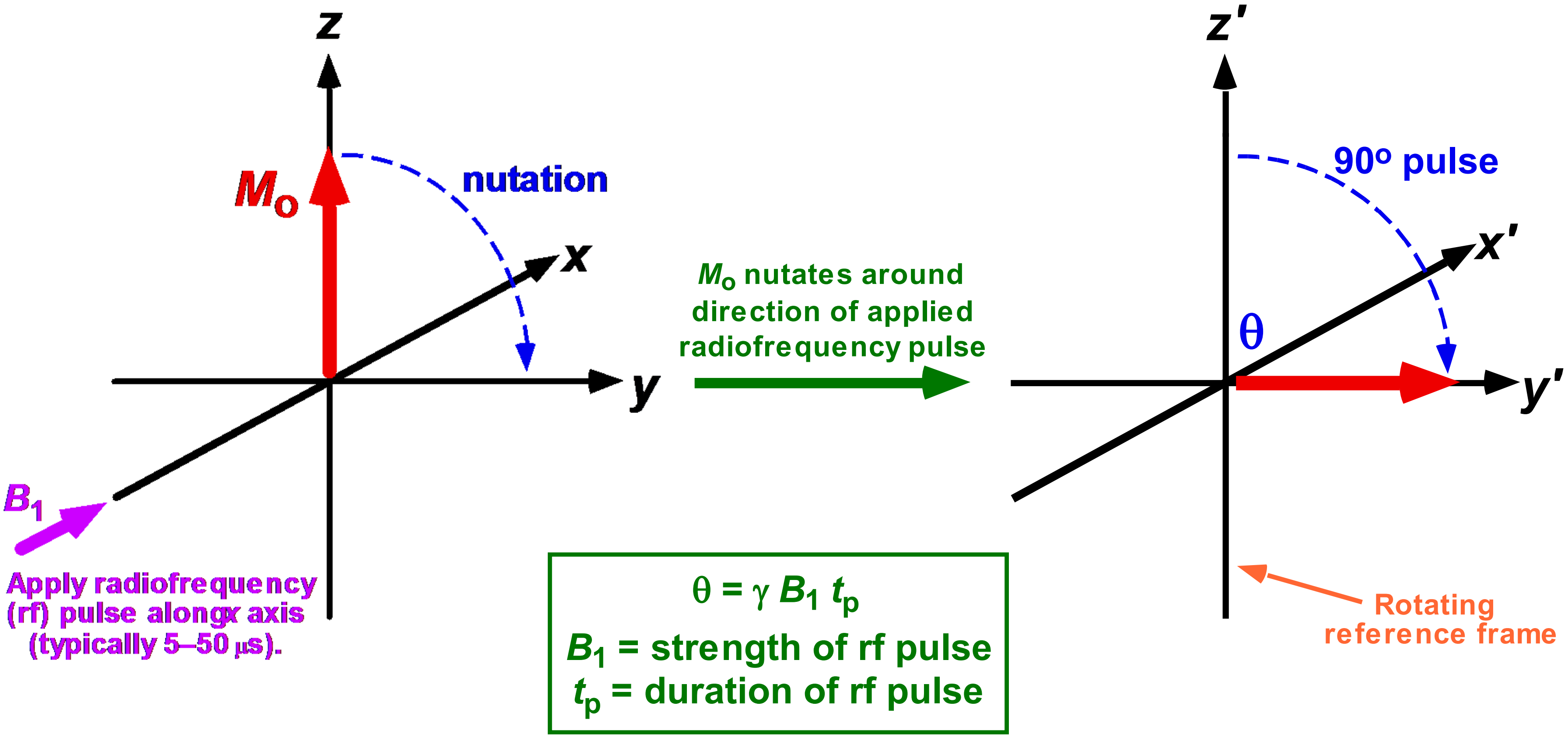
B_1 = strength of rf pulse

t_p = duration of rf pulse

A 90° pulse along the *x* axis will nutate the magnetization onto the *y* axis.

- We can flip the magnetization an arbitrary number of degrees

Manipulation of M_0 with radiofrequency pulses



$$\theta = \gamma B_1 t_p$$

B_1 = strength of rf pulse

t_p = duration of rf pulse

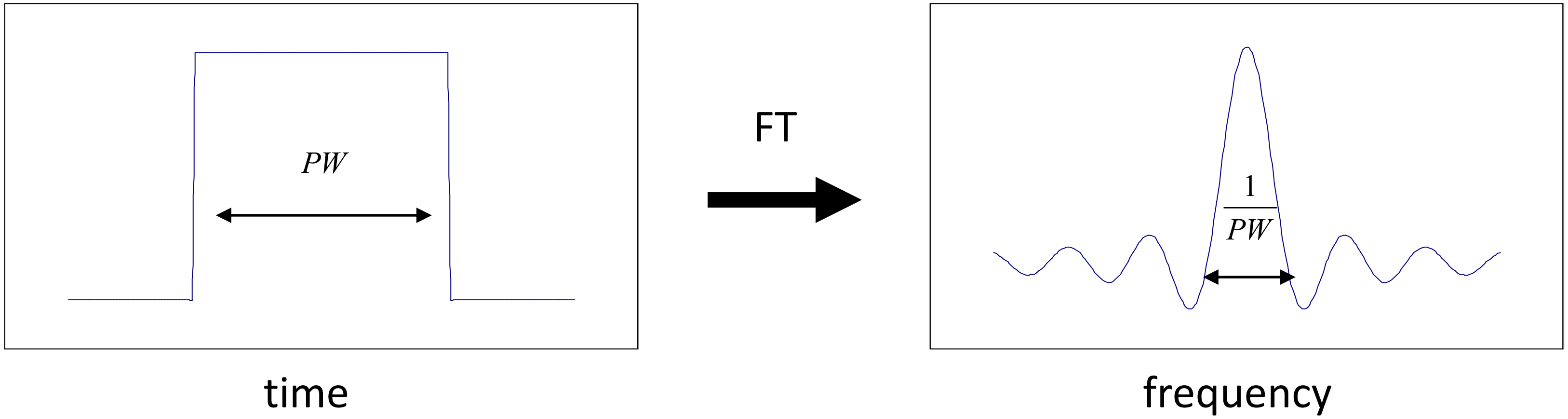
A 90° pulse along the *x* axis will nutate the magnetization onto the *y* axis.

- **Rotating reference frame** - The x/y plane is rotating at the RF frequency of the excitation pulse. Think about watching someone on a merry-go-round when you are standing on the merry-go-round versus standing on the ground next to the merry-go-round.
- All further discussion will assume we are in the rotating reference frame.

Fourier Excitation

Fourier’s theorem dictates that if we apply a square radio frequency pulse of finite duration, it is equivalent to applying a large series of pure frequencies.

- Note: *PW* = pulse width = t_p from previous slide



In essence, we can excite all NMR frequencies simultaneously.

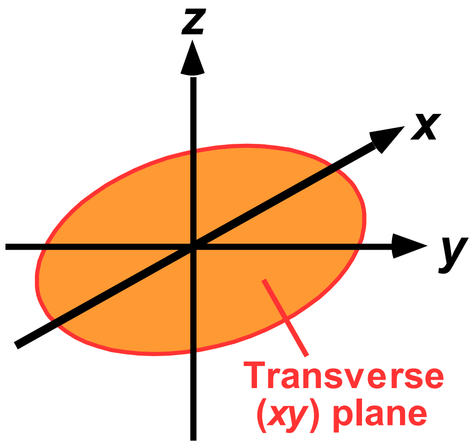
<u>Caveat:</u> The frequency spread is inversely proportional to the duration of the pulse. To cover a large range of frequencies we need a short pulse and hence lots of power.

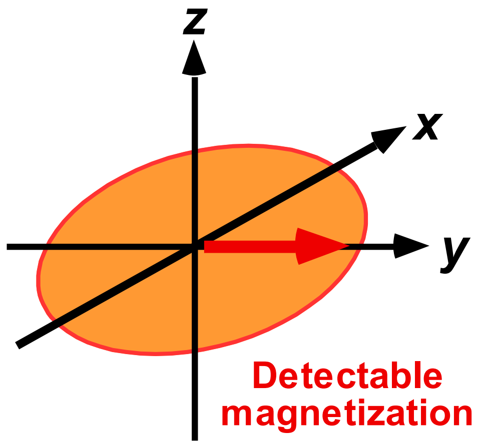
$$\theta = \gamma B_1 \mathrm{PW}$$

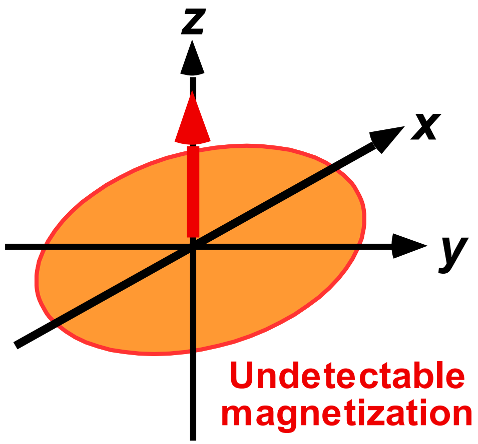
Note: Old NMR instruments (pre 1980s) were continuous wave machines and worked in a different manner to modern pulse NMR described here.

Receiving the Signal

Detection in NMR machines is along the xy plane





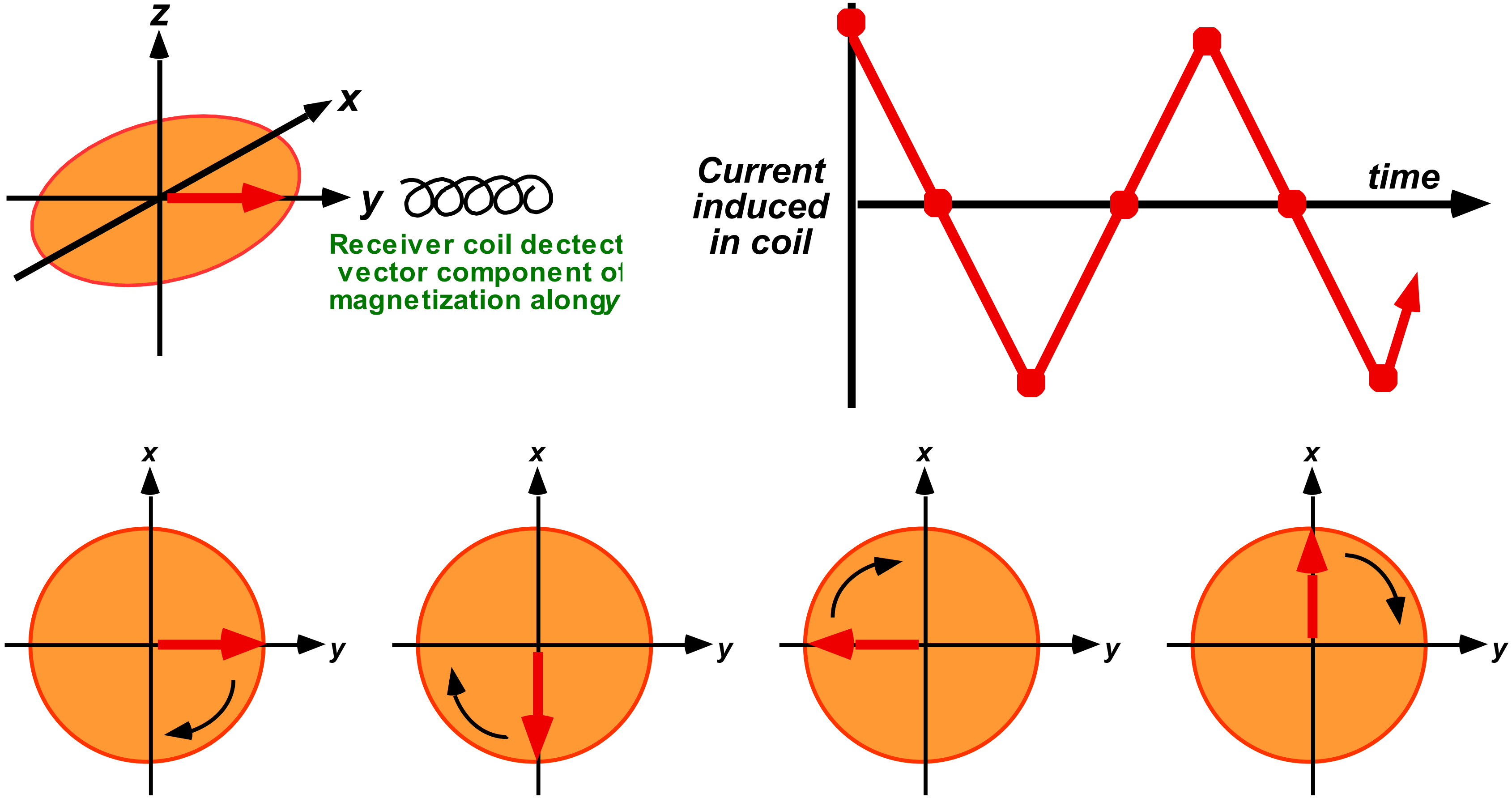


- The net magnetization vector is undetectable when at thermal equilibrium along the z-axis (the longitudinal axis) and must be perturbed by an RF pulse and tipped into the transverse (xy) plane to be detected.

- The signal is detected along the x and y dimension simultaneously and is often referred to as having a real and imaginary component.

$$\mathrm{FID} \sim M_x + i\,M_y$$

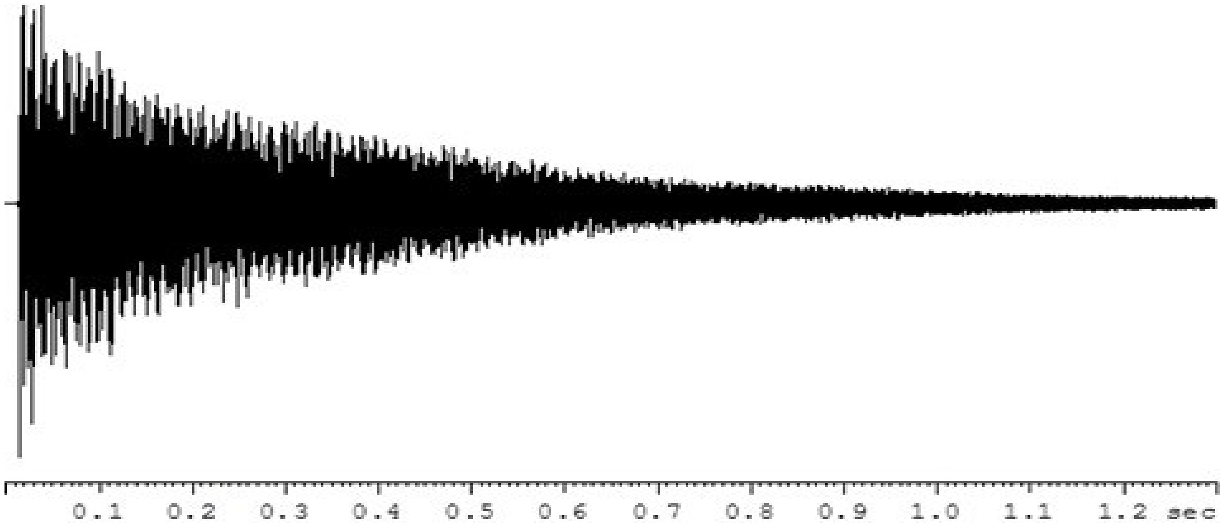
Precessing Magnetization Induces a Current in Receiver



Receiver coil dectect vector component of magnetization along *y*

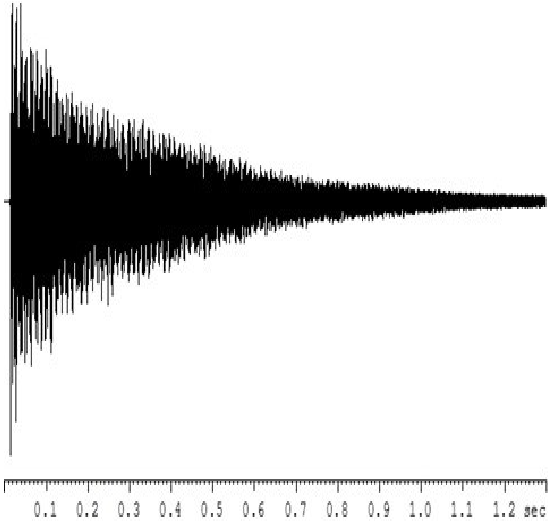
- Not all nuclear spins will have the exact same frequency, but they are very close (differences are between zero and a few thousand Hz) and have measured differences in parts-per-million (ppm) compared to the excitation frequency (MHz range)
- The excitation frequency is subtracted from the signal that the receiver detects to leave a signal in the audio frequency range (rotating frame)

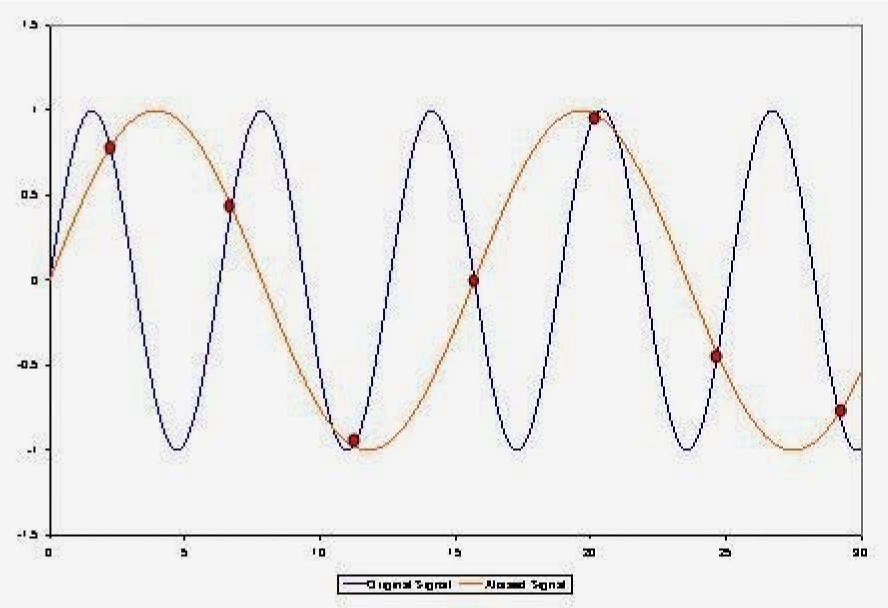
Free Induction Decay



- Points are digitized at discrete time points separated by a fixed dwell time
- The bandwidth (frequency range we will observe) is inversely proportional to the dwell time
  - Need high frequency sampling to observe high frequency signals.

Free Induction Decay – Nyquist frequency





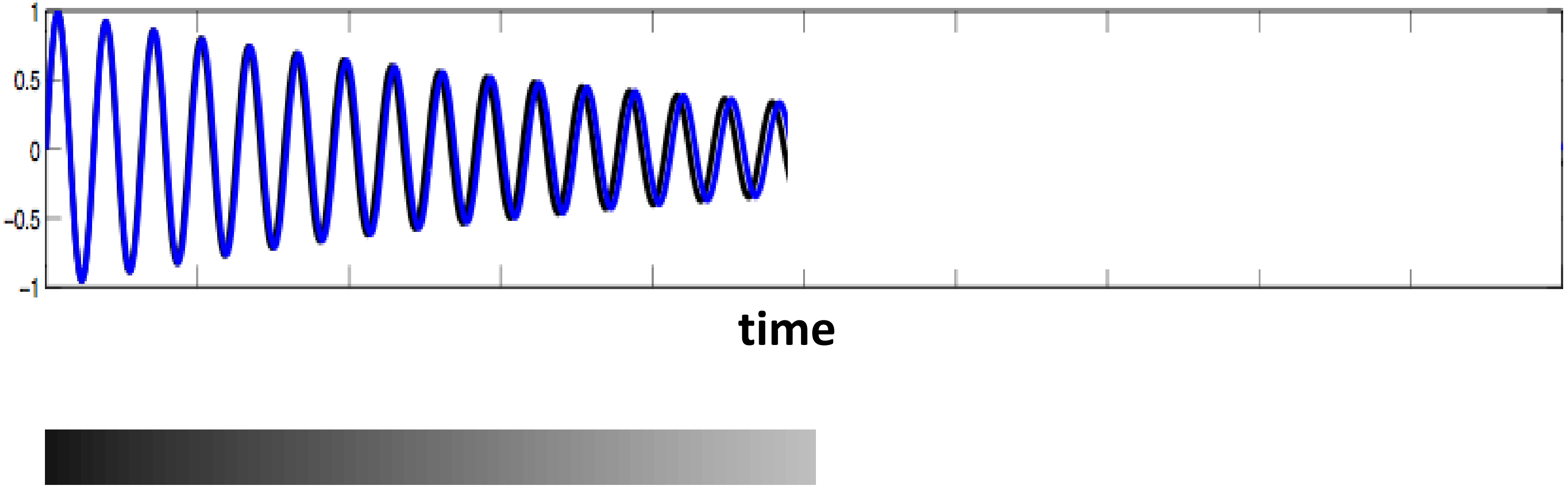
- Frequencies that are outside the bandwidth (i.e. have higher frequencies than the sampling rate) MAY be aliased (frequencies outside the bandwidth are often filtered and may not be aliased)

- In the figure above on the right the two sine waves would have the identical frequencies if sampled at the red points.

- The Nyquist Theorem states that we must digitize at a rate not less than two data points per cycle for the highest frequency of interest.

Free Induction Decay – Sensitivity vs Resolution

Two decaying sinusoids close in frequency

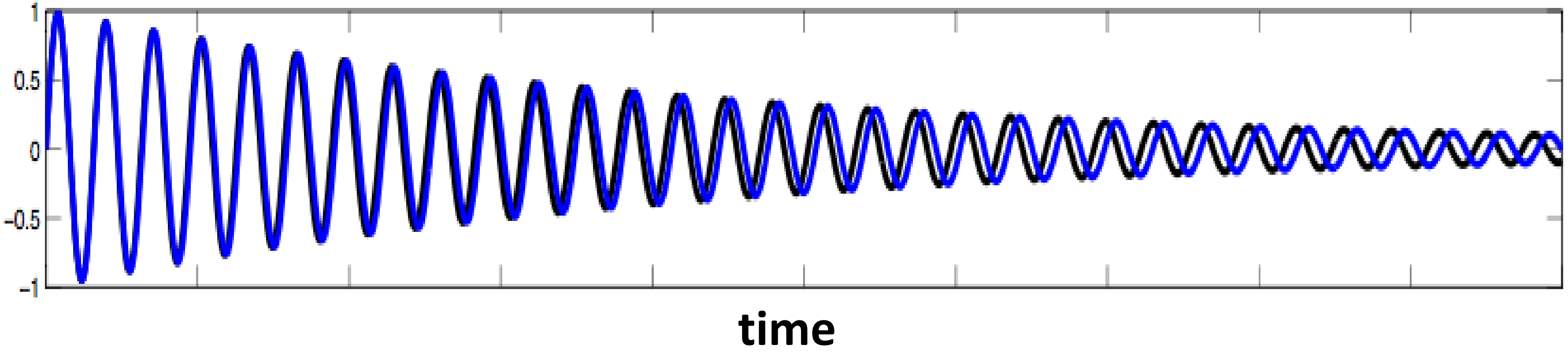


short evolution times

- **high sensitivity**
- **low resolution**

Free Induction Decay – Sensitivity vs Resolution

Two decaying sinusoids close in frequency



short evolution times
- **high sensitivity**
- **low resolution**

long evolution times
- **low sensitivity**
- **high resolution**

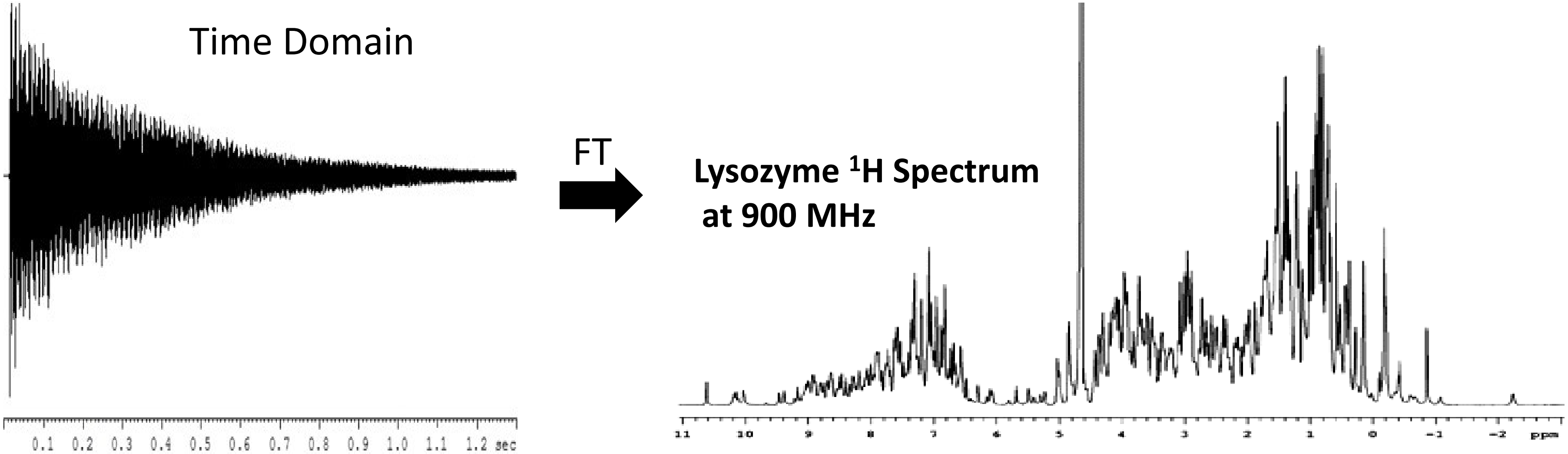
Converting Time Domain Data to Frequency Domain Data

Fourier Transform (FT)

- FT is the mathematical transformation that converts time-domain signal to frequency domain spectrum.

Fourier

$$S(\omega) = \int_{0}^{\infty} s(t)\exp(-i\omega t)dt$$

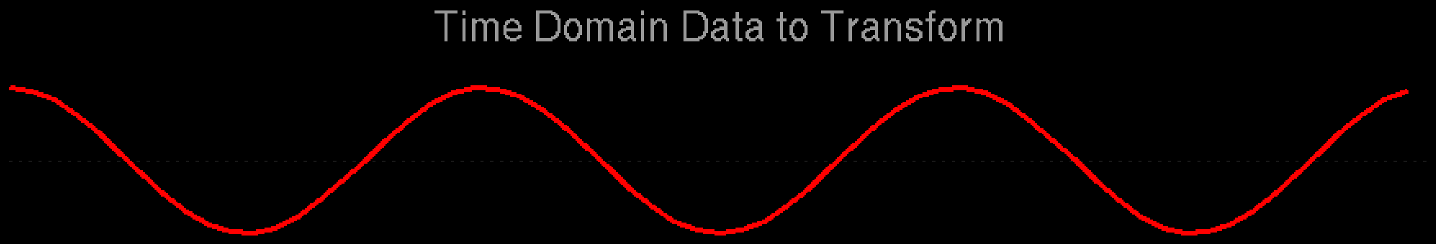

Time Domain

FT

Frequency Domain

Lysozyme ^{1}H Spectrum at 900 MHz

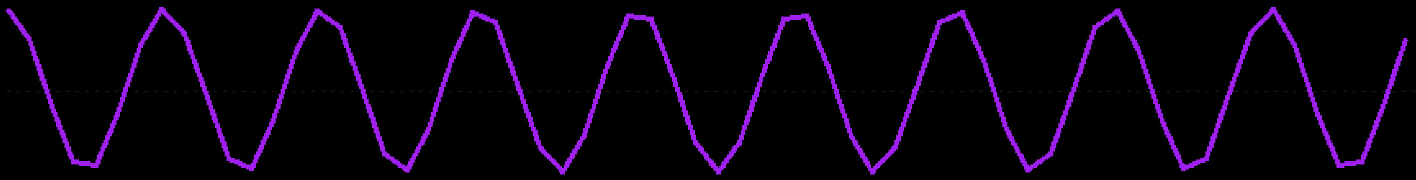
Fourier Transform Animated



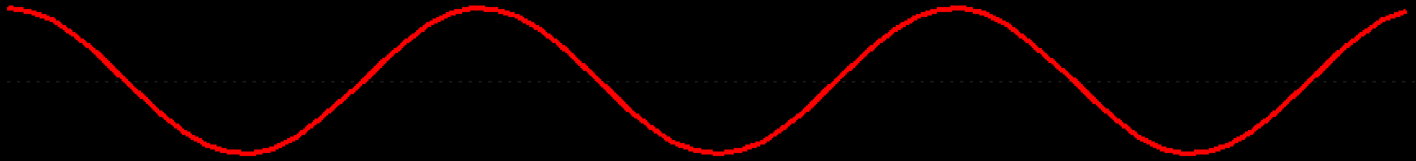
Time Domain Data to Transform

Time Domain (Real Part)

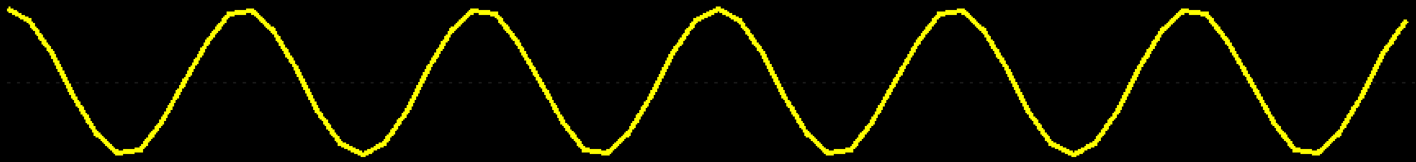
Fourier Term



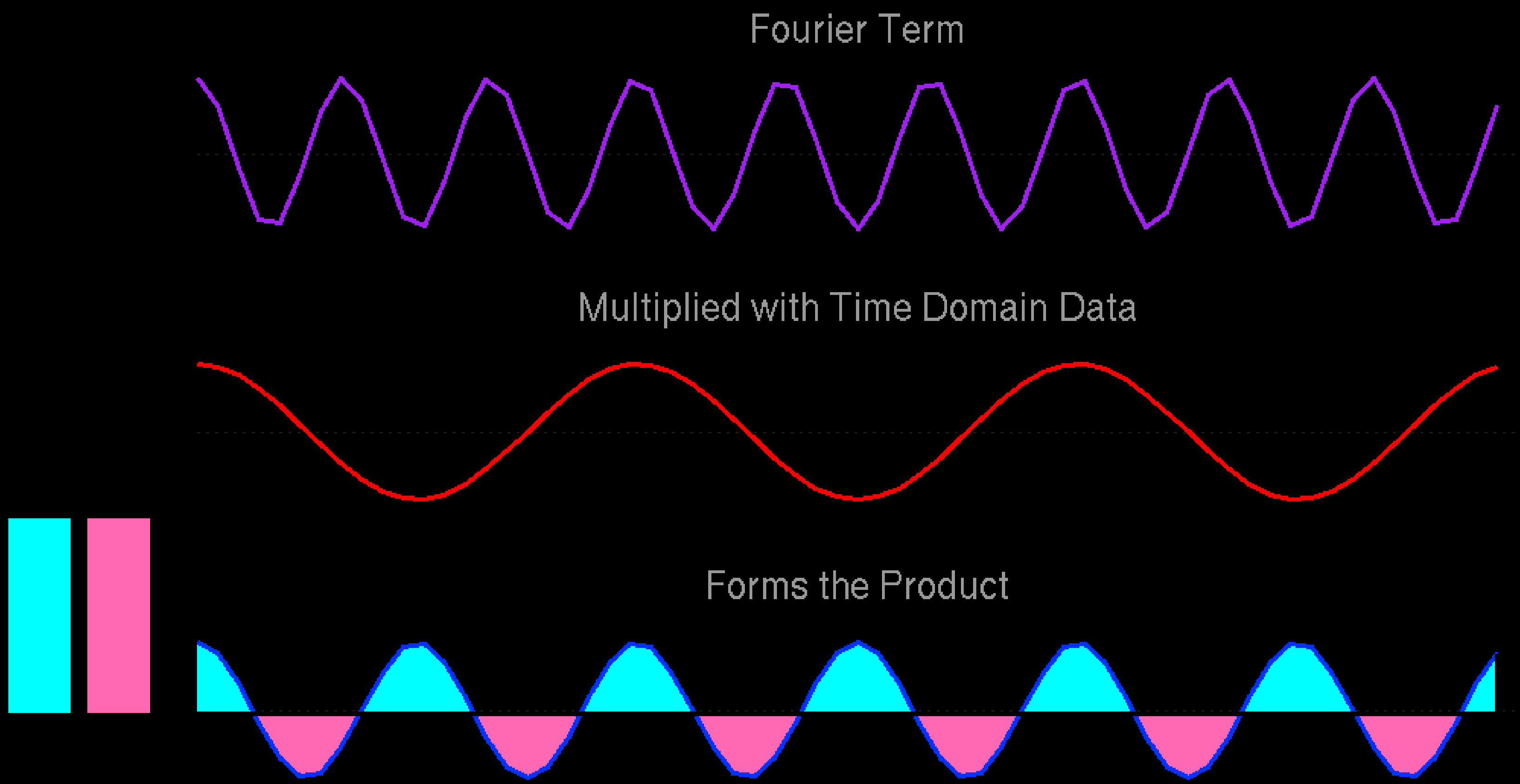
Multiplied with Time Domain Data



Forms the Product



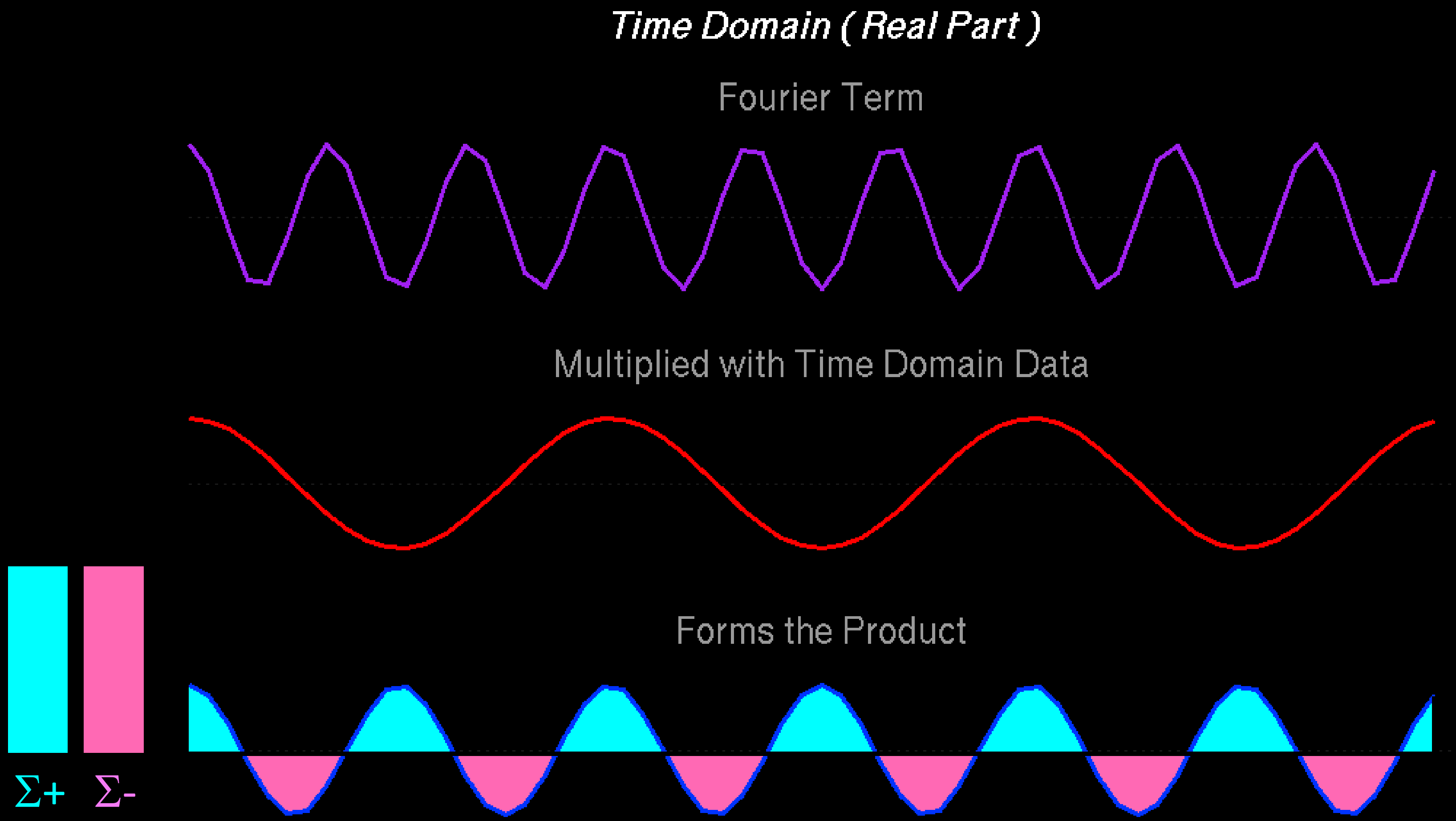
Time Domain (Real Part)



Fourier Term

Multiplied with Time Domain Data

Forms the Product

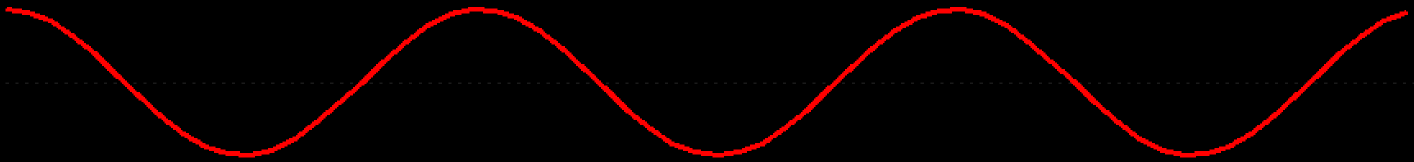


When the Fourier term does not match any frequency in the data, the product has balanced amounts of positive and negative intensity, and sums to zero.

Time Domain (Real Part)

Fourier Term

Multiplied with Time Domain Data



Forms the Product

$\Sigma+$ $\Sigma-$

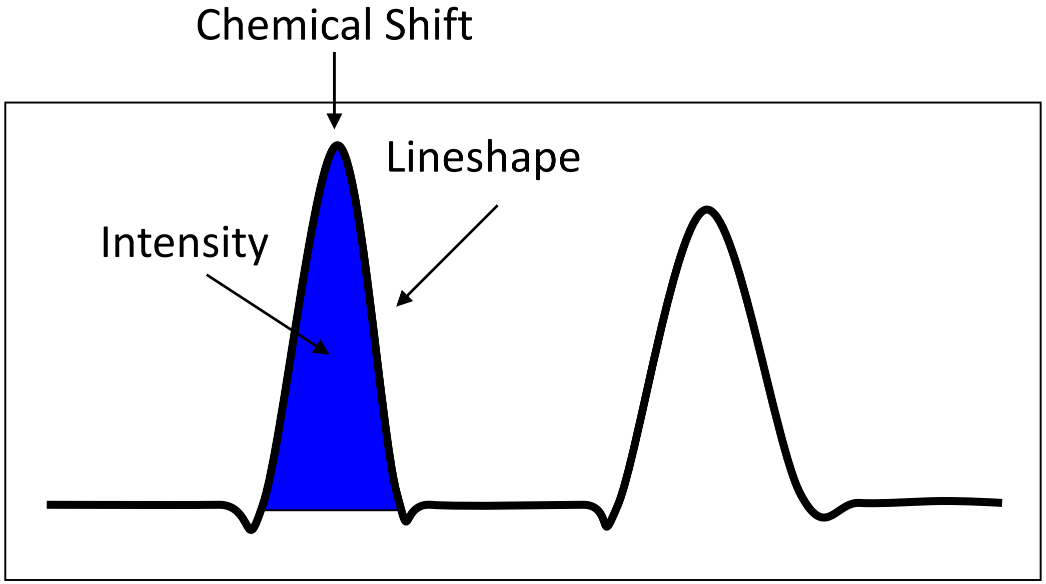
Sum Over Product to Form a Frequency Point:

Frequency Domain (Real Part)

NMR Observables

In general NMR experiments yield the following information about a population of spins:

- Chemical shift – Frequency of signal
- Intensity – Population of spins (i.e. C**H**$_3$ gives a stronger signal than C**H**)
- Lineshape – Inhomogeneity of the population
- Spin-spin splitting – Not shown in this figure, but would provide information about nuclei which are one to three bonds away.



The Chemical Shift (δ)

The Chemical Shift

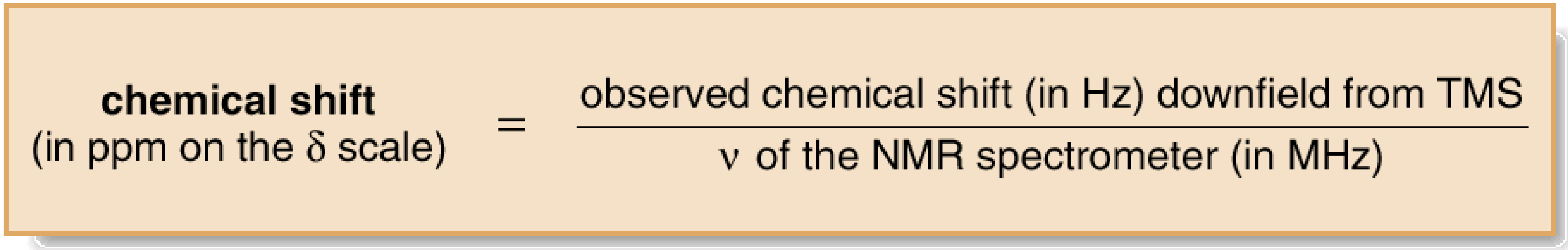
The following discussion is about protons, but applies to any NMR active nuclei.

- Since all protons have the same magnetic moment it might be expected that all hydrogen atoms will resonate at the same frequency and only give rise to a single signal.

- Fortunately this is not the case and different protons will give rise to slightly (ppm) different frequencies.

- The reason is that the effective magnetic field B_{eff} that a nuclei experiences is equal to the very large static magnetic field (B_0) plus a much smaller shielding magnetic field which arises from electrons surrounding the nucleus.

Note: The chemical shift is normalized along a ppm scale such that the spectrometer frequency is independent in the ppm value



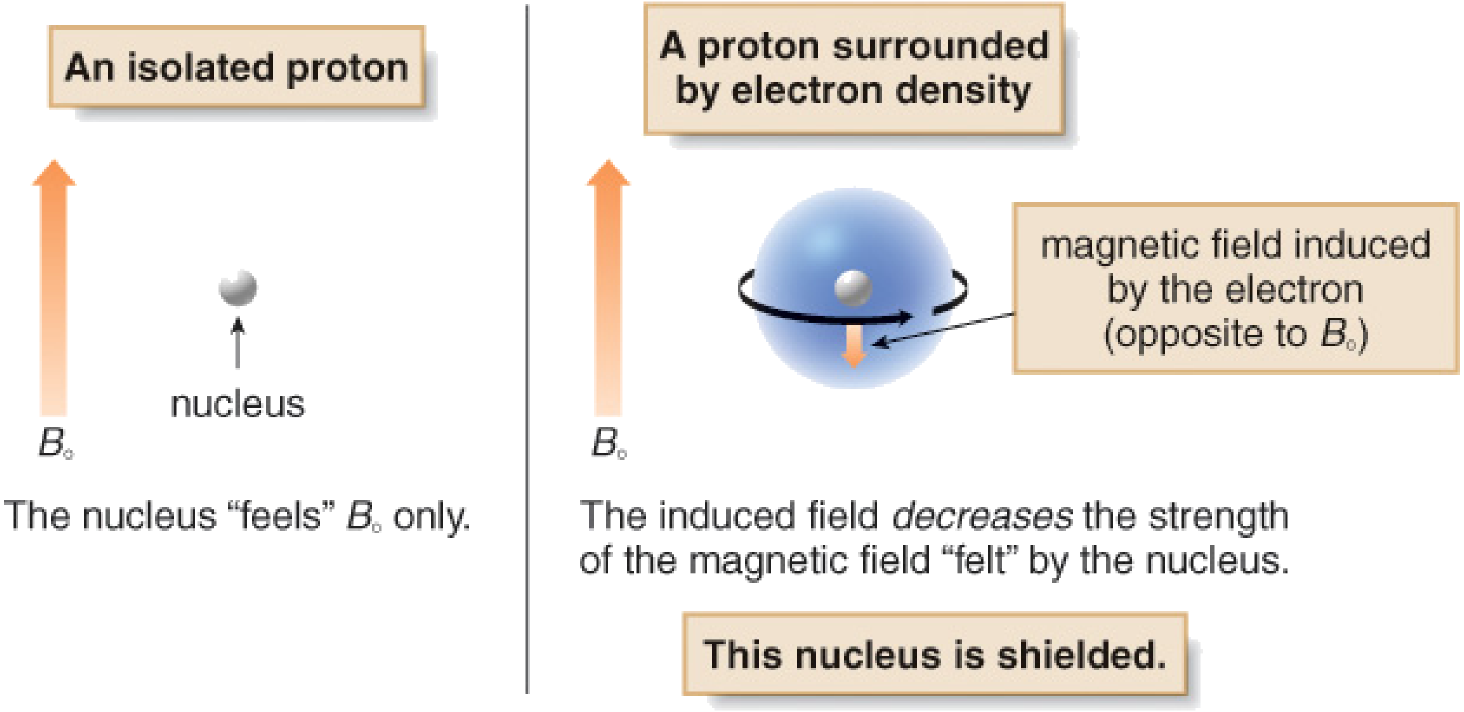
$$\begin{array}{c}\textbf{chemical shift}\\ \text{(in ppm on the } \delta \text{ scale)}\end{array} = \frac{\text{observed chemical shift (in Hz) downfield from TMS}}{\nu \text{ of the NMR spectrometer (in MHz)}}$$

The Chemical Shift

Diamagnetic Shielding

- Electrons are charged particles that will move in an external magnetic field (B_0) to generate a secondary field that will oppose "shield" or reinforce "de-shield" the B_0 field.

- The **s** orbital electrons circulate about the direction of the applied magnetic field (B_0) and thus oppose the external magnetic field reducing the effective field (B_{eff})



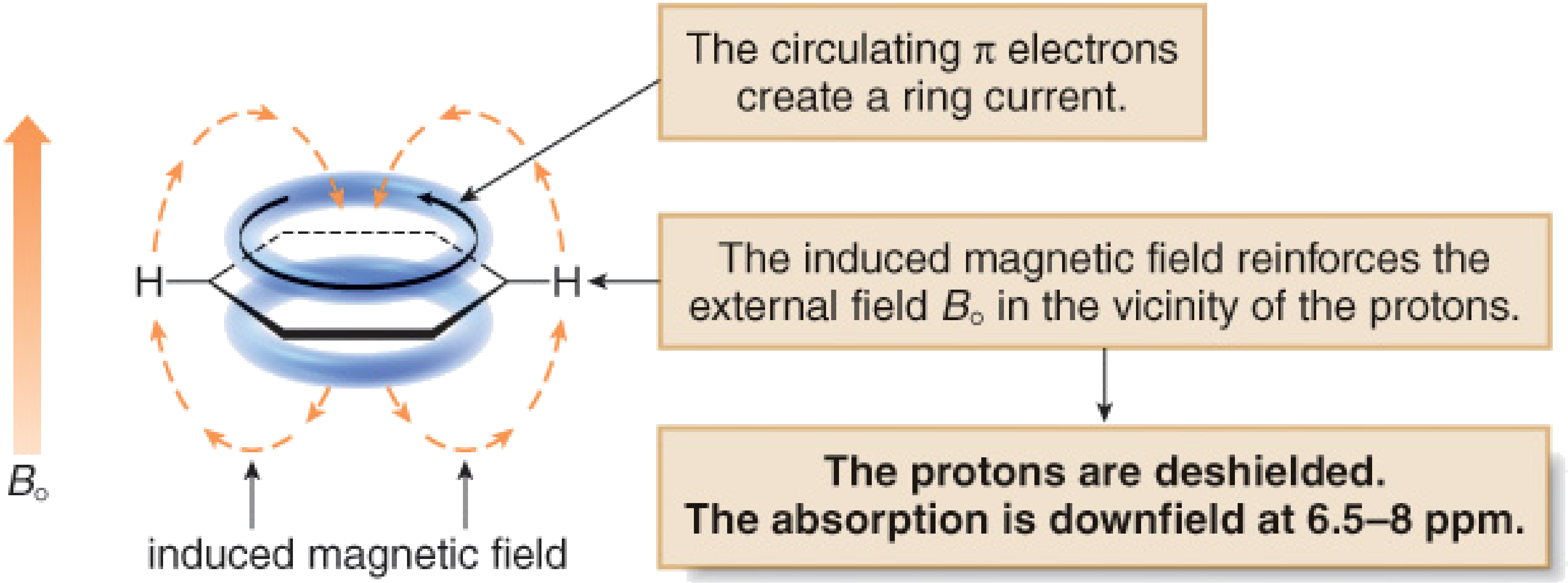
The nucleus "feels" B_0 only.

The induced field *decreases* the strength of the magnetic field "felt" by the nucleus.

The Chemical Shift

Ring Current Shifts

- The six π electrons in an aromatic benzene ring circulate in the external magnetic field creating a ring current
- The magnetic field reinforces the applied magnetic field for the bonded protons
- Thus the protons feel a stronger magnetic field and resonates at a higher frequency. They are said to be de-shielded and are shifted downfield.



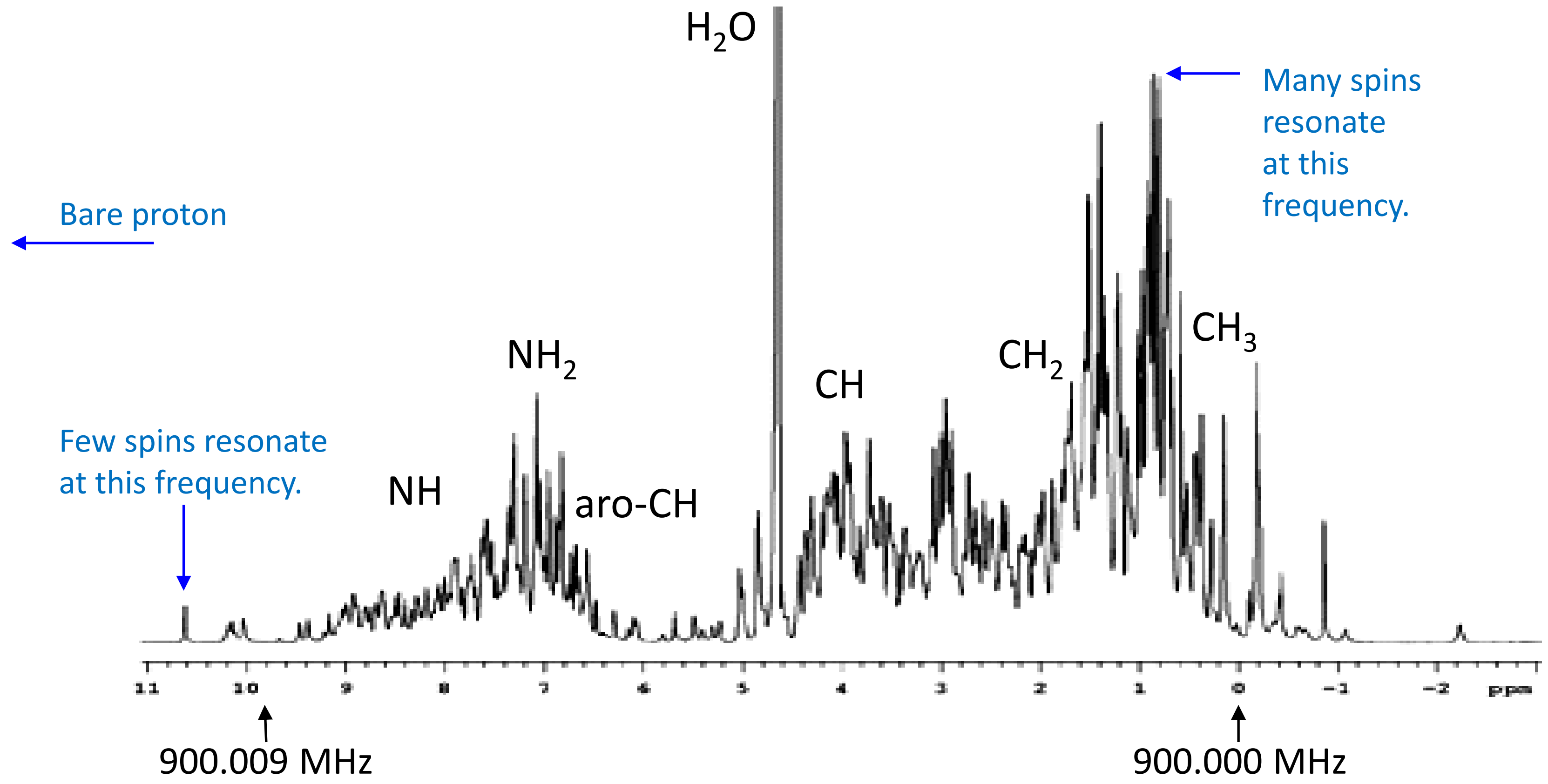
The circulating π electrons create a ring current.

The induced magnetic field reinforces the external field B_o in the vicinity of the protons.

The protons are deshielded.
The absorption is downfield at 6.5–8 ppm.

- The chemical shift will then be the product of the shielding and de-shielding effects, but the shielding effects will always be greater in magnitude than the de-shielding effects.

The Chemical Shift



Bare proton

Few spins resonate at this frequency.

Many spins resonate at this frequency.

Increasing shielding by electrons

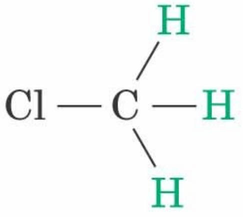
Downfield ⟷ Upfield

NMR spectra are plotted backwards with decreasing frequency.

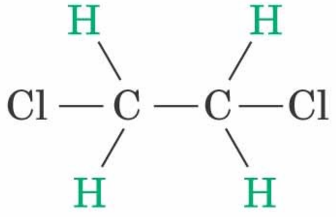
Spin-Spin Couplings
J Couplings
Scalar Coupling

Rules for Spin-Spin Splitting (*J*, or Scalar couplings)

- Equivalent protons do not split each other

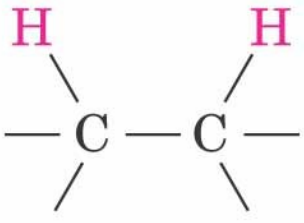


Three C–H protons are chemically equivalent; no splitting occurs.

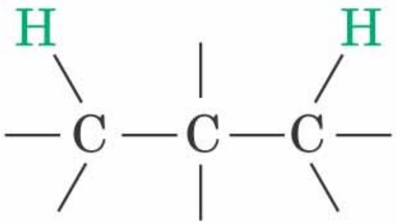


Four C–H protons are chemically equivalent; no splitting occurs.

- Protons that are farther away than three bonds generally do not split each other



Splitting observed

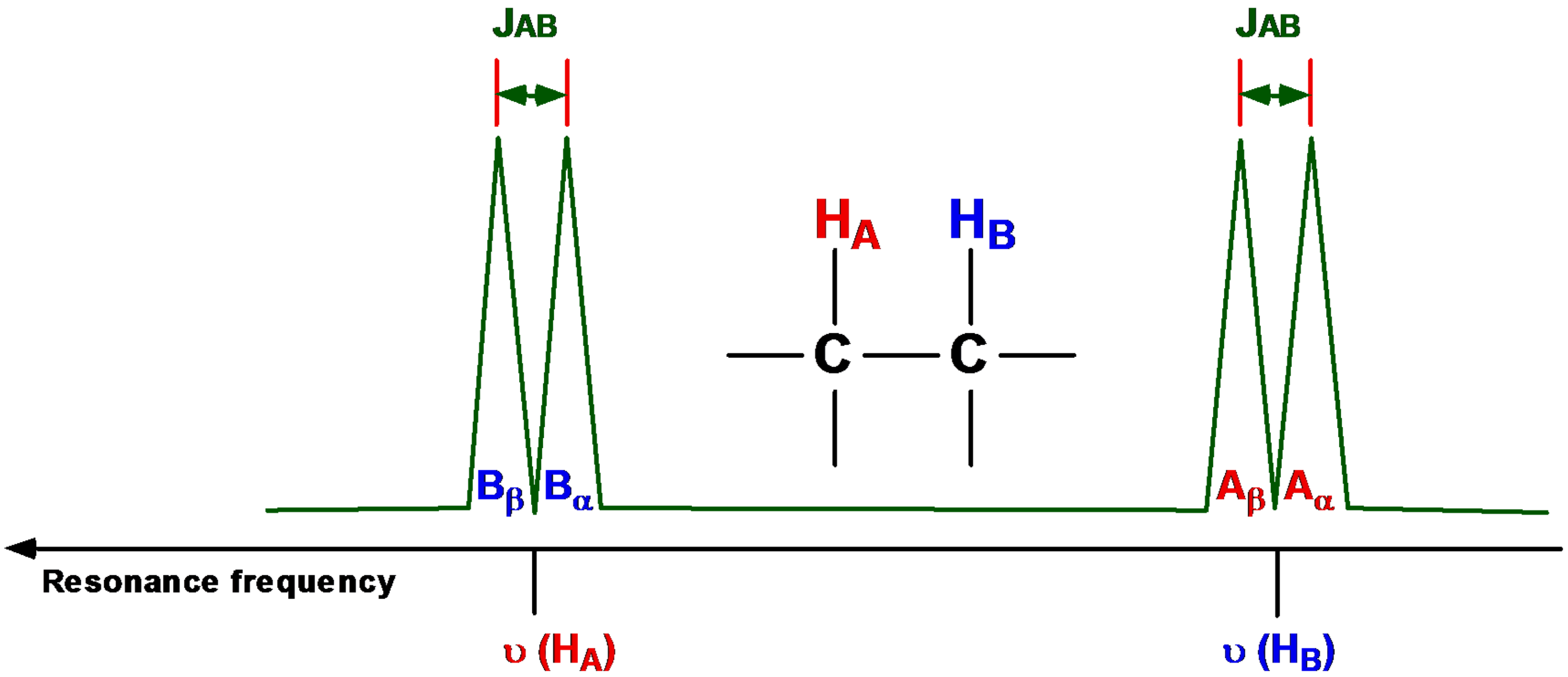


Splitting not usually observed

- Generally, one bond couplings >> two bond couplings > three bond couplings

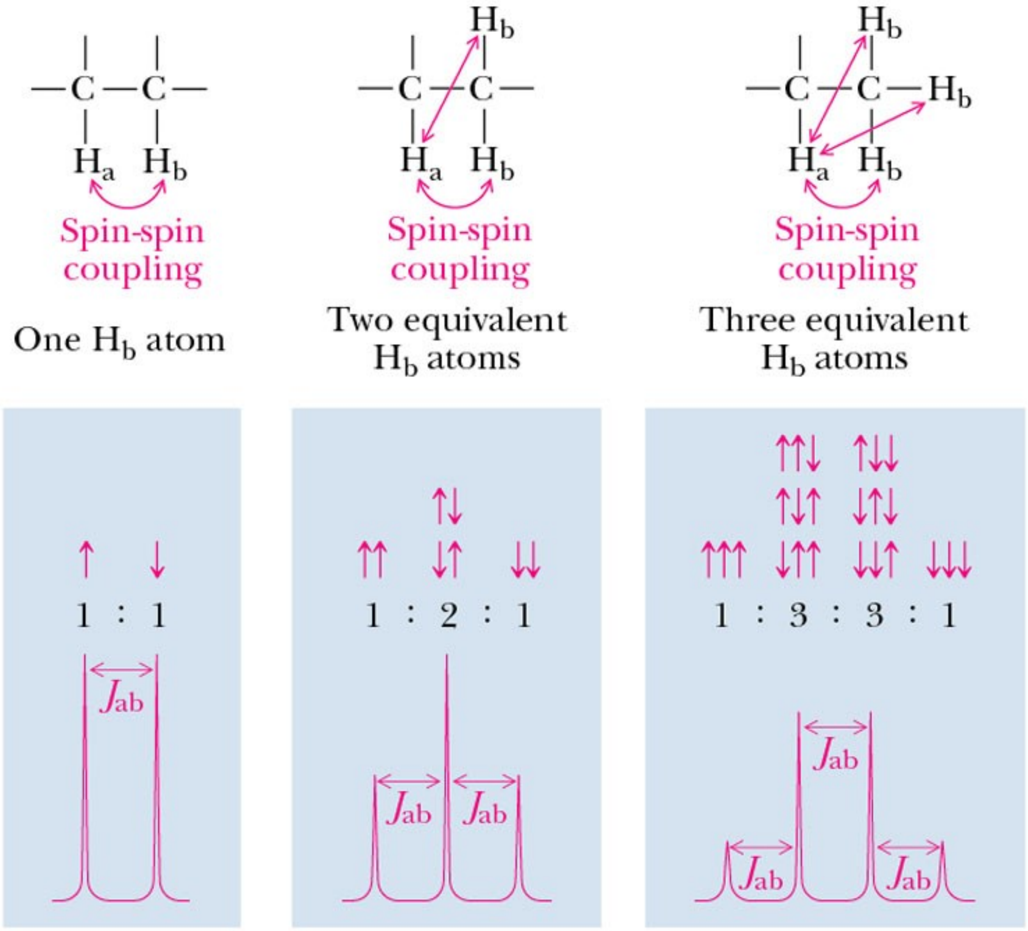
The Origin of Spin-Spin Splitting

- Spin-spin splitting occurs between nonequivalent nuclei and arises due to the fact that each nuclear spin can be in two states (assuming spin ½ nuclei), aligned with the magnetic field and opposed to the magnetic field.
- As we saw from the Boltzmann distribution earlier there is essentially a 50/50 chance of being in either of the two states.
- Thus, a neighboring nuclear spin will cause the spin to experience two states and thus be split into two signals.



The Origin of Spin-Spin Splitting

Different numbers of **equivalent** adjacent nuclei will cause different splitting patterns and the pattern follows Pascal’s triangle



Observed splitting in signal of H_a

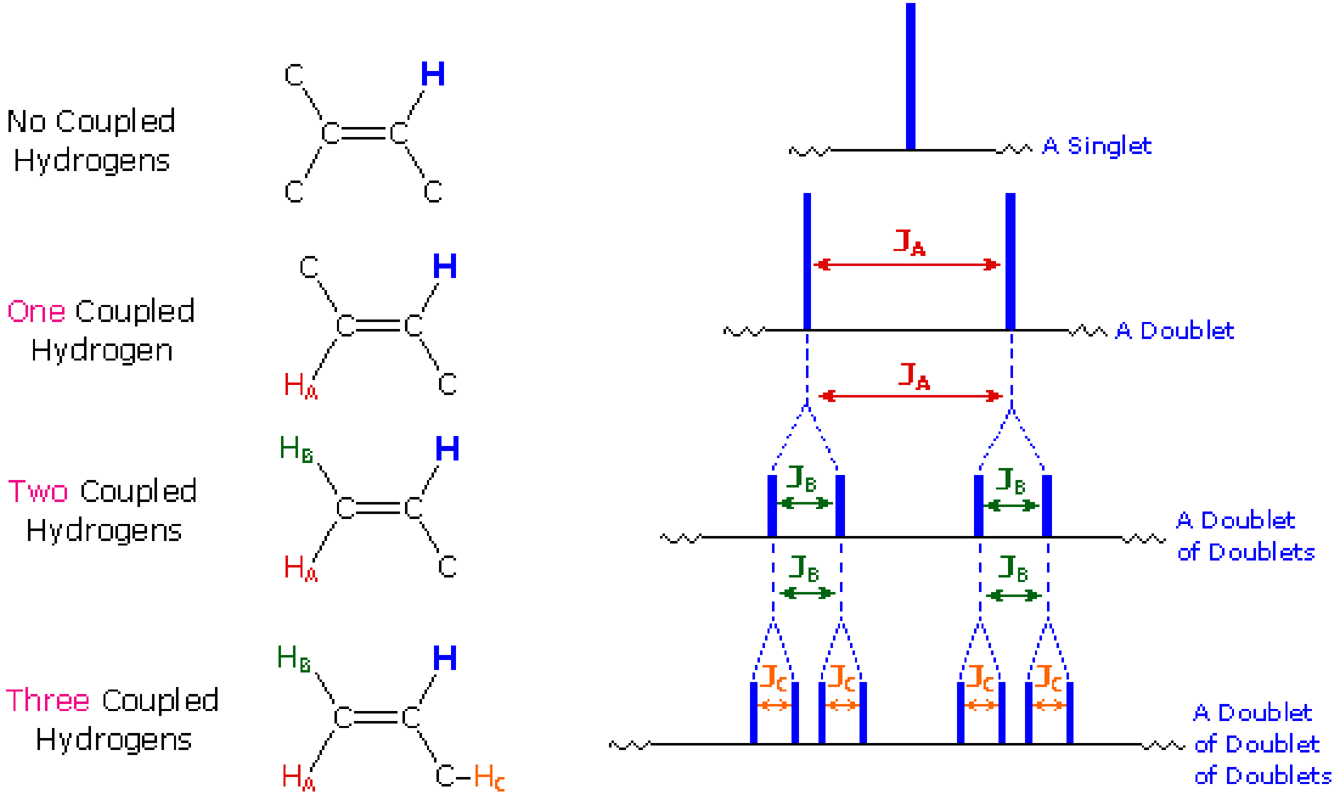
Pascal’s triangle

$n = 0$	**1**
$n = 1$	**1 1**
$n = 2$	**1 2 1**
$n = 3$	**1 3 3 1**
$n = 4$	**1 4 6 4 1**

The Origin of Spin-Spin Splitting

What if there are more than one **non-equivalent** nuclei that cause splitting's?

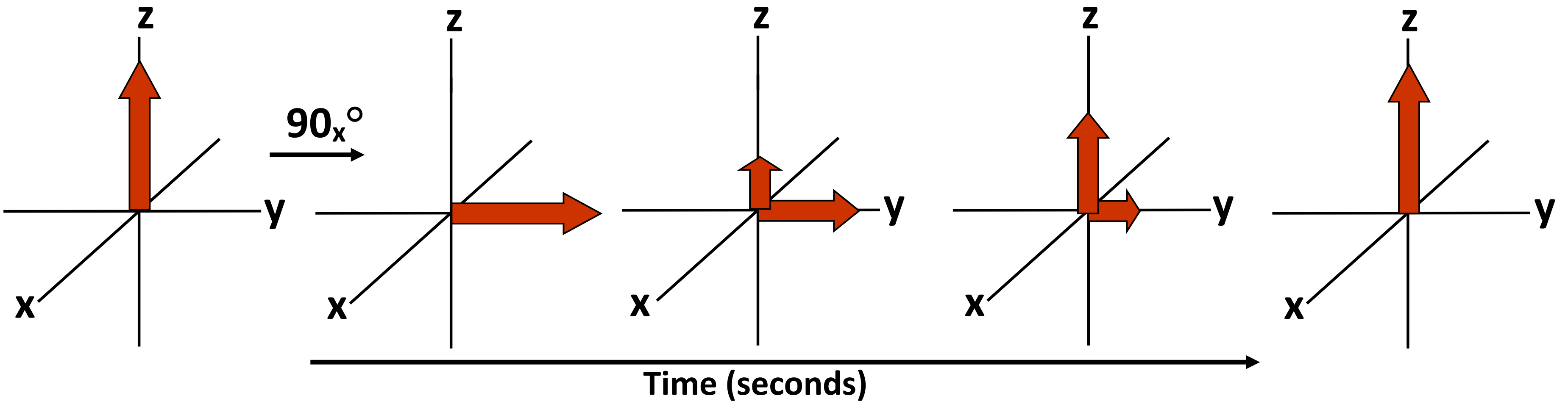
- Below are examples for one, two and three coupled hydrogens where all the hydrogens are non equivalent and have different J splitting's to the proton highlighted in Blue.
  - Note: If the smaller J values were a multiple of a larger J value then some of the peaks would be degenerate and a more complicated pattern would emerge.



NMR Relaxation

NMR Relaxation – Return to Thermal Equillibrium

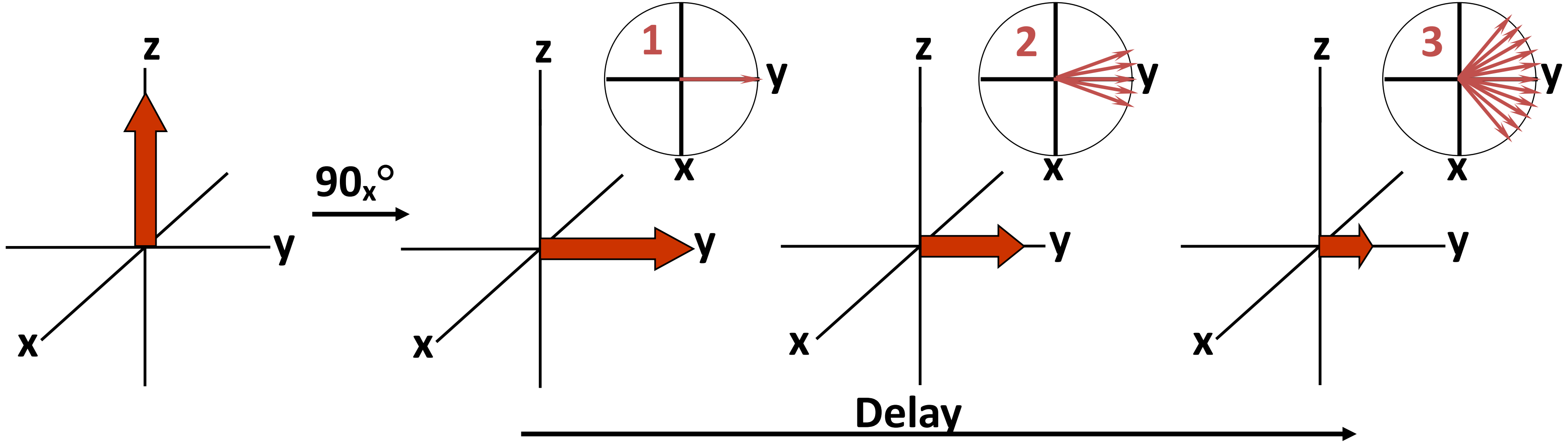
- After a pulse we must wait for the magnetization to return to thermal equilibrium (Boltzmann distribution) before we can pulse again.
- The rate that the magnetization returns to the +z axis is called T_1 (longitudinal) relaxation



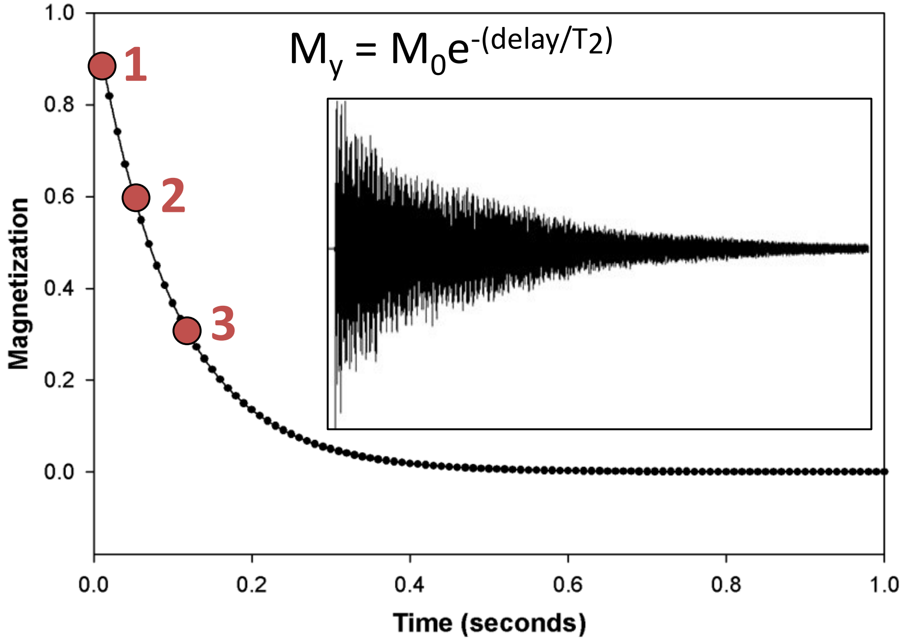
Why are T_1 values so long?

- Dissipating the tiny energies to the thermal energy of the sample should be easy so it is not lack of where the energy can go, but rather the means to move it there.
- For the closely spaced energy levels in NMR (ΔE) the probability of spontaneous emission is ~ 10^{-25} per second (on the order of the age of the universe)
- Therefore, relaxation must occur through **stimulated emission** and since T_1's are long means that there is not a suitable means to stimulate the relaxation.

NMR Relaxation – Dephasing in the xy plane



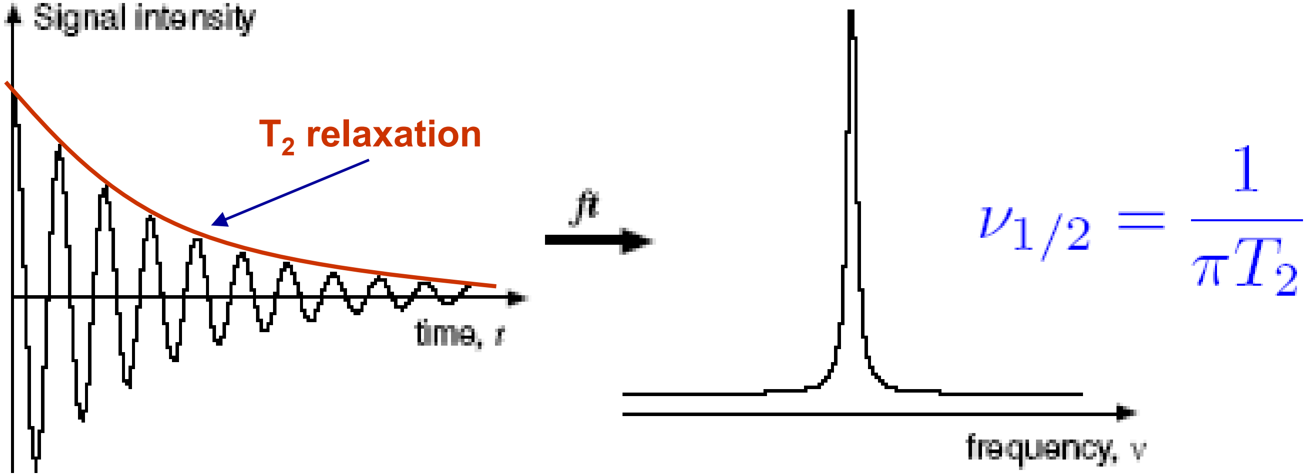
- Even ignoring T_1 relaxation the net magnetization in the x-y plane will disappear with time due to T_2 (transverse) relaxation.
- Loss of net magnetization (signal) is due to de-phasing in the x-y plane.



$M_y = M_0 e^{-(\text{delay}/T_2)}$

NMR Relaxation – Dephasing in the xy plane

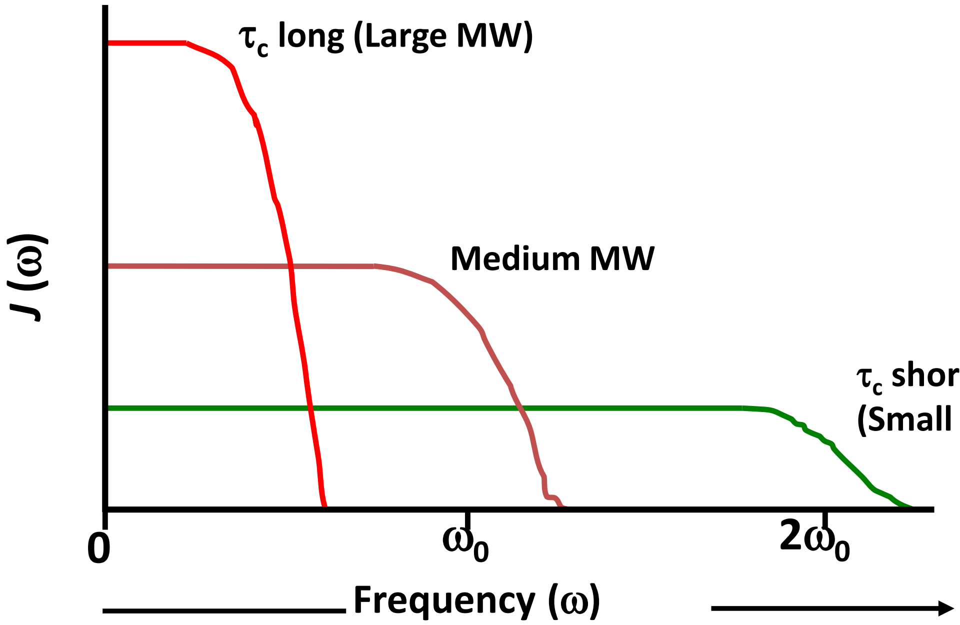
- T_2 relaxation causes the FID to decay and the faster the decay the broader the NMR signal.
- For larger systems, such as proteins, the linewidths become broader than the ^{1}H-^{1}H spin-spin couplings



$$\nu_{1/2} = \frac{1}{\pi T_2}$$

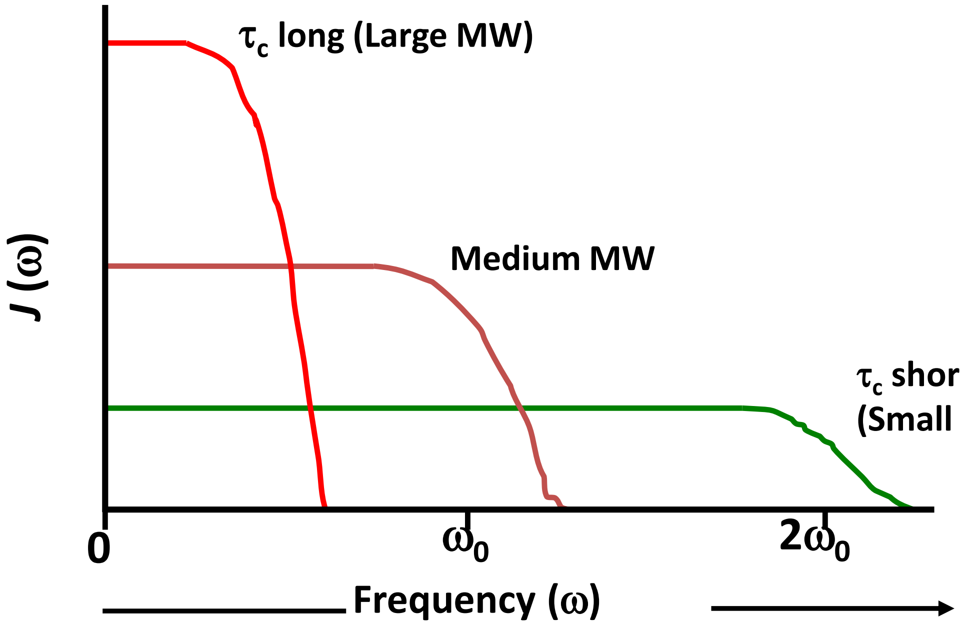
- Peak shape is also affected by the magnetic field inhomogeneity

Relaxation – The Spectral Density Function (*J*ω)



- Spectral density function is a measure of the amplitude (or number) of fluctuating fields at different frequencies.
    - **The fluctuating fields are generated by the dynamics of the molecule itself as it re-orientates in the applied magnetic field.**

Relaxation – The Spectral Density Function (*J*ω)

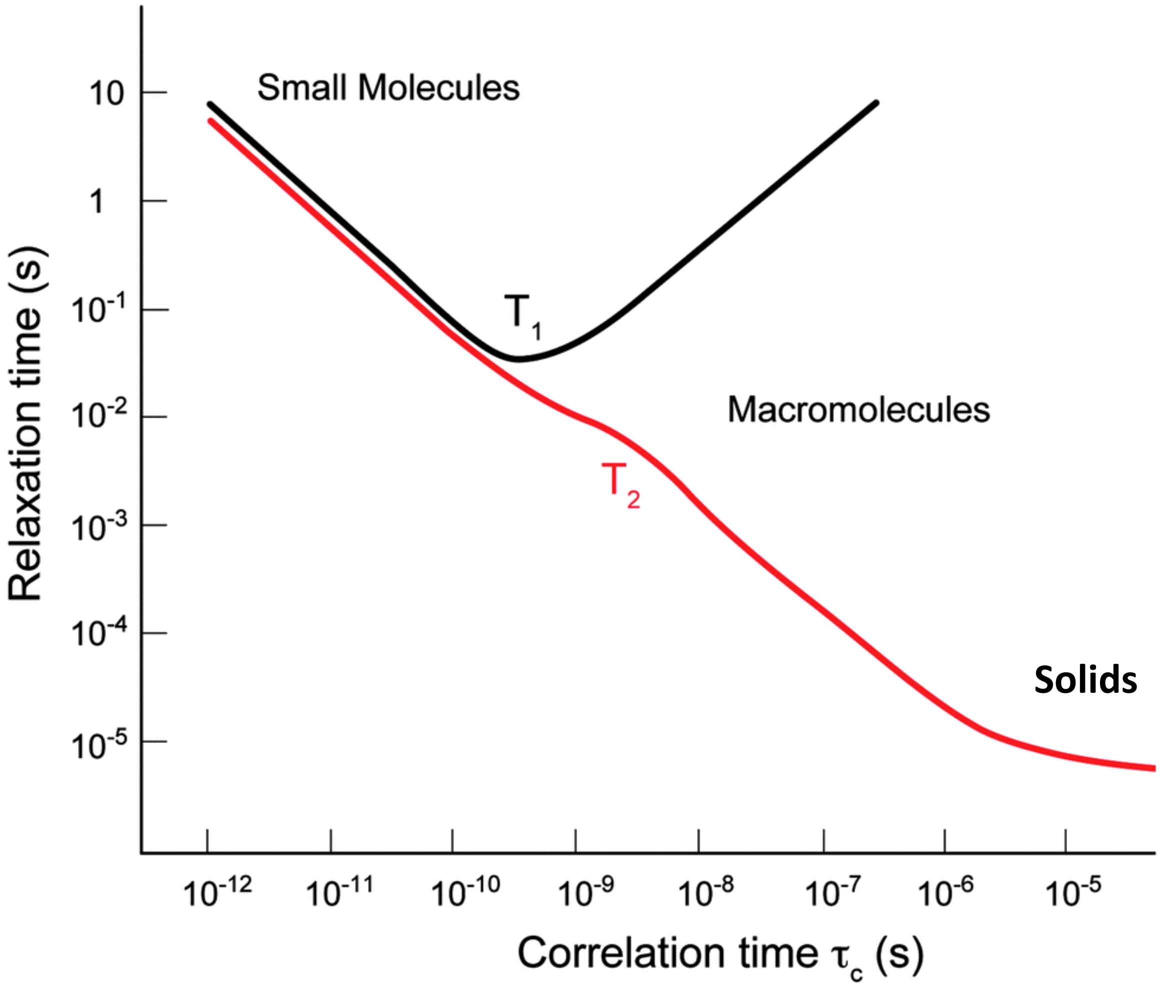


- T_1 relaxation is dominated by stimulated transitions near ω_0 and $2\omega_0$
- T_2 relaxation is dominated by stimulated transitions near 0 frequency

Therefore,

- Expect T_1 and T_2 times to be similar for small molecules
- Expect T_2 times to get smaller as the molecular size increases (slower tumbling)
  - This will broaden lines, remember linewidth = πT_2^{-1}
- Expect T_1 times to get smaller, than larger, as molecular size increases.

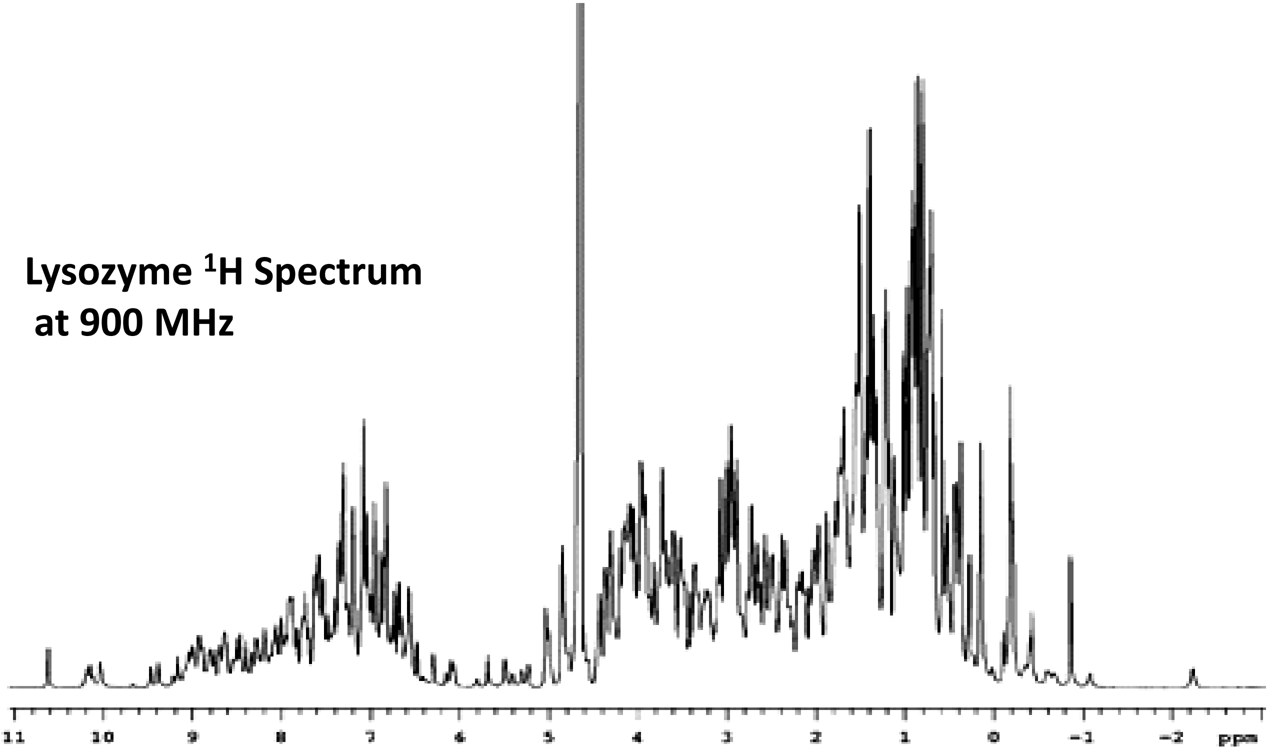
Relaxation as a Function of Molecular Tumbling (size)



Multi-dimensional NMR

Why do Multi-dimensional NMR?

For macromolecules the spectral overlap is too significant. We need better spatial resolution.

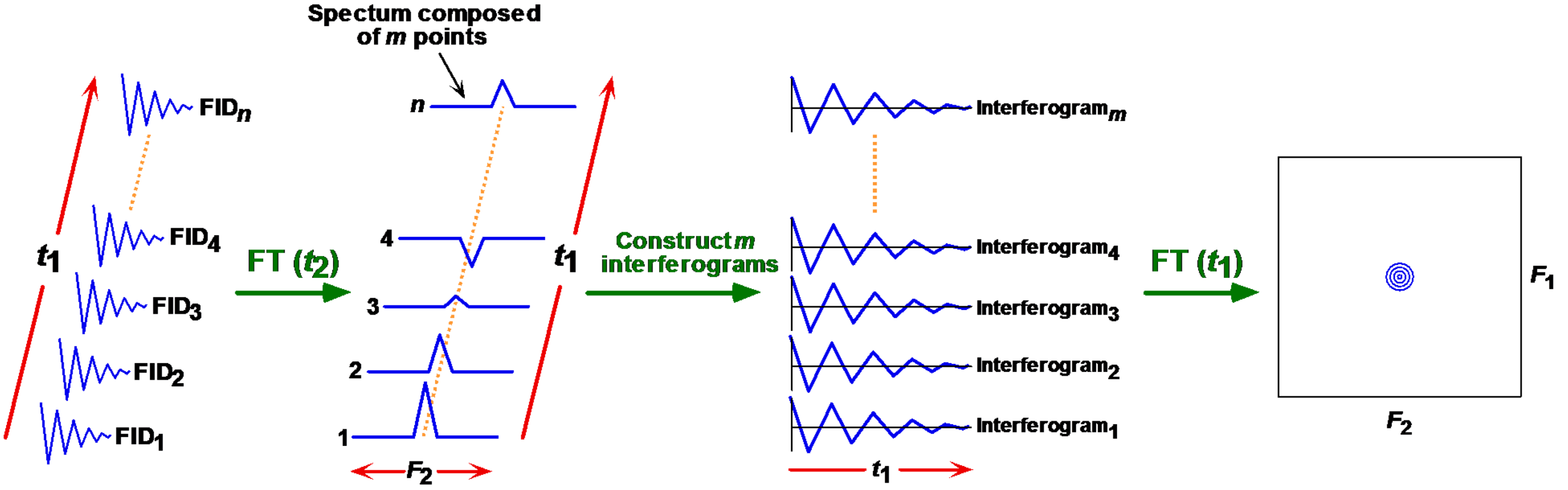


Lysozyme ^{1}H Spectrum at 900 MHz

Advantages also include the ability to manipulate magnetization in ways that produce additional information along the indirect dimensions.

Multi-dimensional NMR

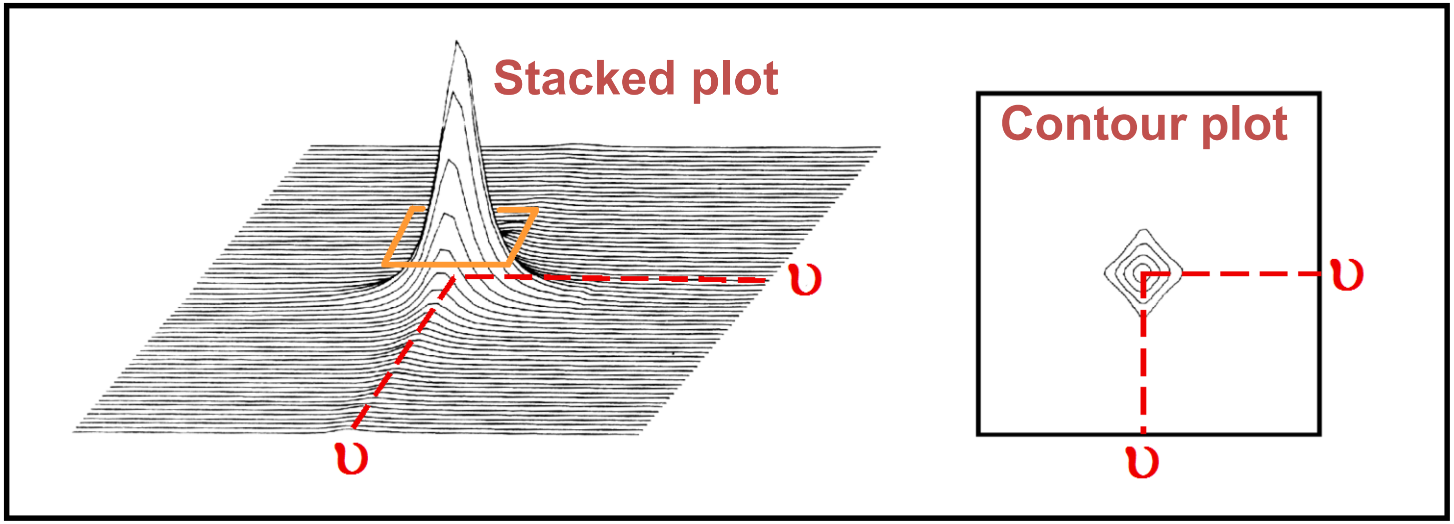
Consider an experiment where multiple 1D FIDs were collected and each FID was collected with an increasing time delay t_1 which modulates the signal by another frequency, say an attached ^{15}N nucleus. How the modulation comes about is well understood, but we are treating it as a "black box" for this lecture.



The concept can be extended to *n*-dimensions, but is only practical to 3 or 4 dimensions due to T_2 relaxation – we can only have so many delays before the signal vanishes.

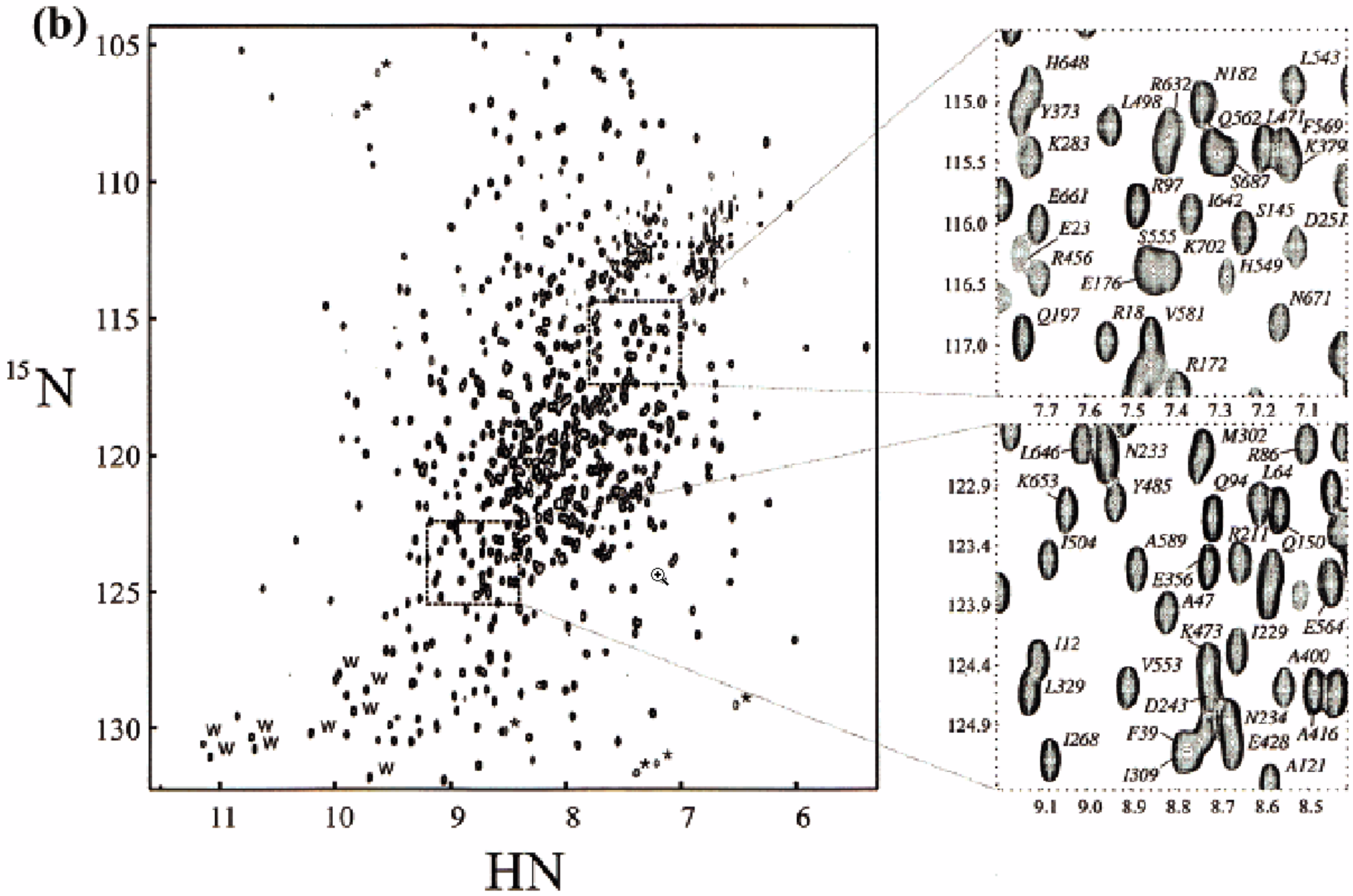
Multi-dimensional NMR

- 2D spectra are actually stacked plots which are typically viewed as contour plots.
- 3D and higher dimensional spectra are viewed as contour plots of slices taken from the higher dimensional space.



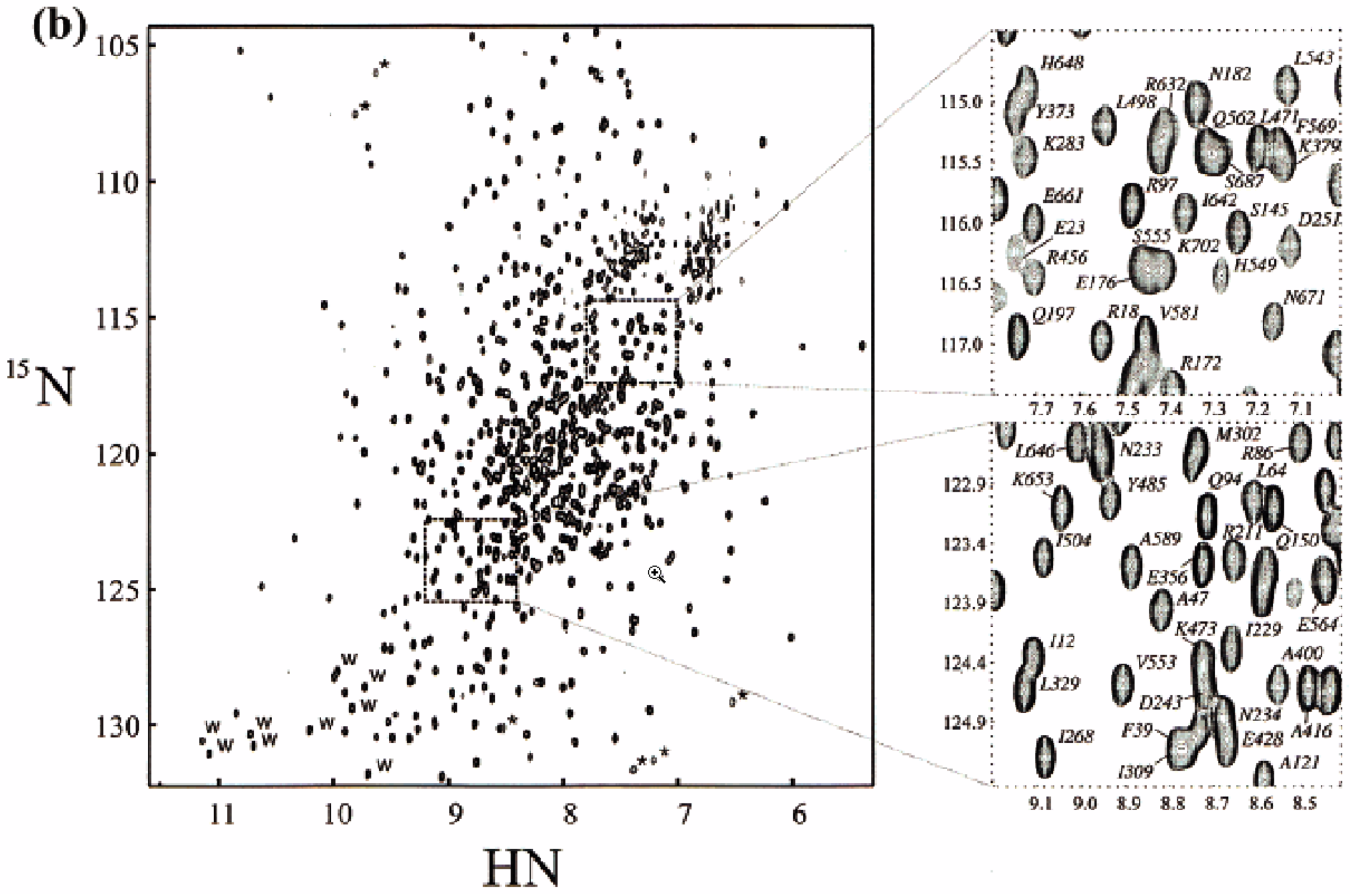
2D 1H-15N HSQC

HSQC (Heteronuclear single quantum correlation) is a 2D experiment which correlates a proton with its directly bound heteronuclei (typically ^{15}N or ^{13}C)



The 2D give tremendous resolving power as compared to the 1D spectrum. The spectrum is also simplified by only showing protons bound to nitrogen.

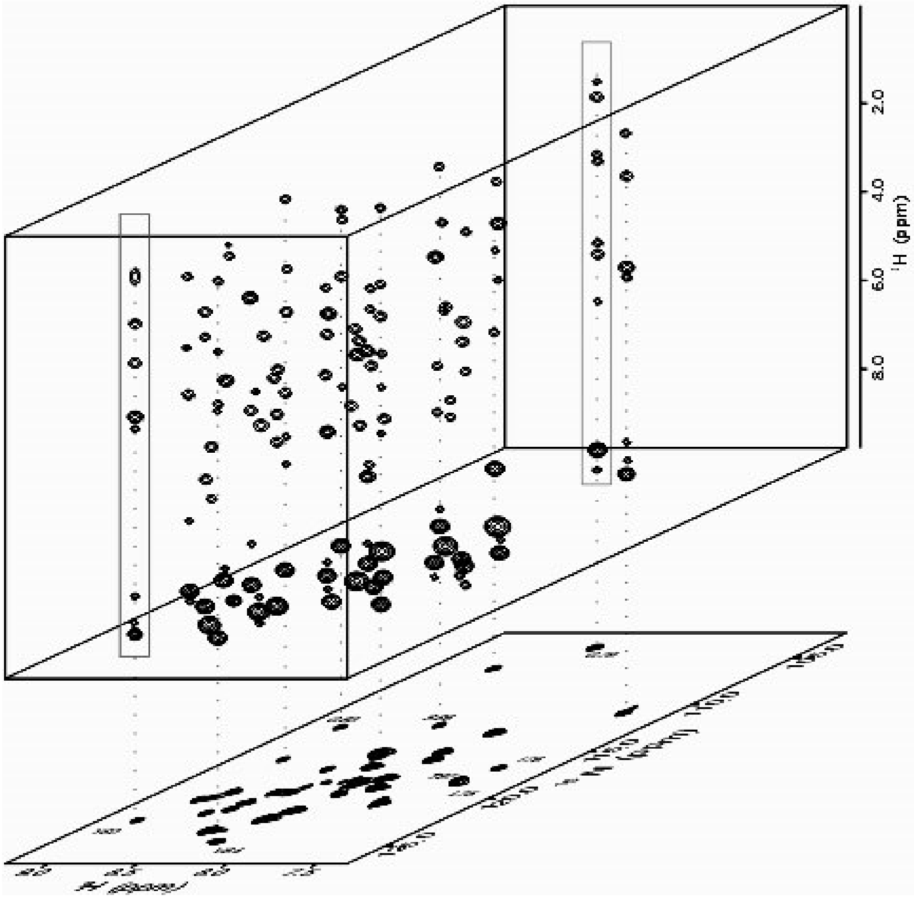
Multi-dimensional NMR



Magnetization in multi-dimensional NMR can be transferred between nuclei

- through-bonds via scalar spin-spin couplings (1 to 3 bonds)
- through space via the Nuclear Overhauser Effect (^1H-^1H < 5Å)

Protein NMR: 3D Spectrum



3D ^{1}H-^{15}N NOESY-HSQC

Correlates an amide proton (x) with its directly bound nitrogen (y) with the third dimension (z) being correlated to any proton within 5 Å of the amide proton.

- Frequencies of different types of nuclei can be correlated through bonds and through space to create a wide variety of multi-dimensional NMR spectra.

- 3D and 4D spectra are generally viewed as 2D strips through the 3D or 4D space.

NMR Sensitivity

NMR Sensitivity

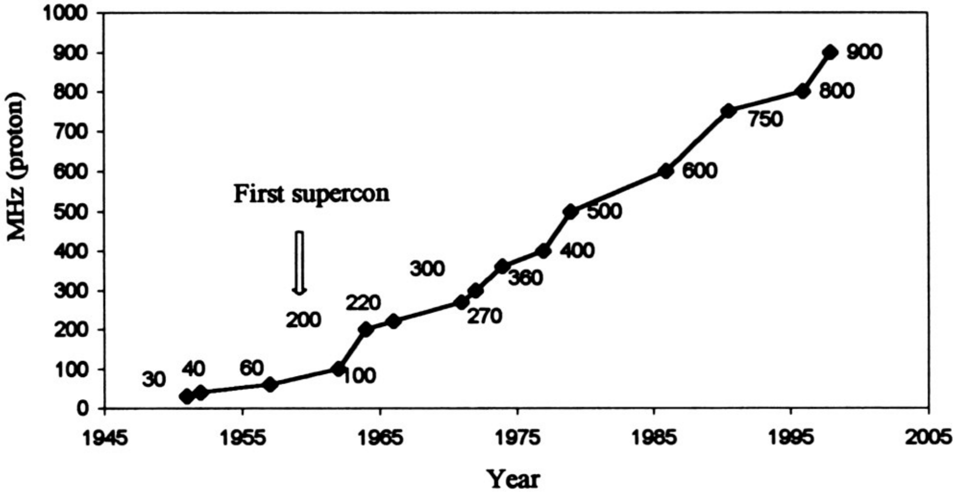
NMR is an insensitive technique

- Requires 100 μM (preferably 1 mM) sample concentrations.

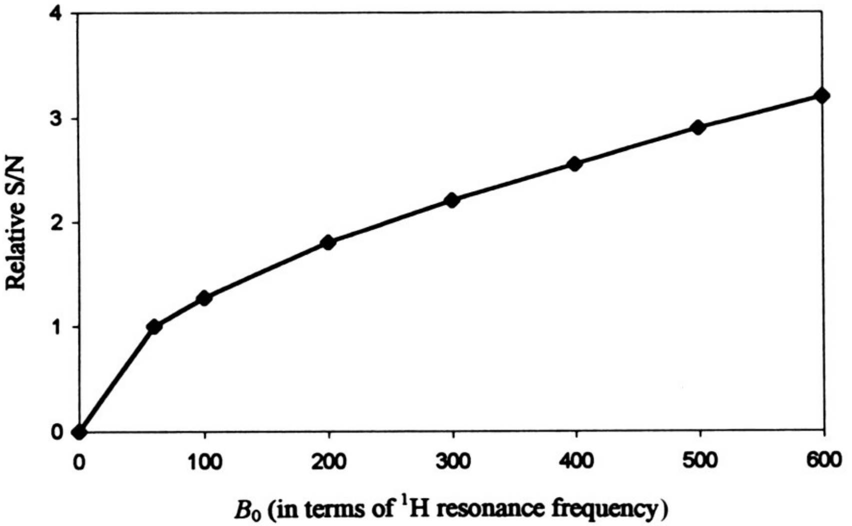
- The sample volume required is 250-600 μL

- Sample has to be enriched with NMR active nuclei or have very high sample concentrations (10-200 mM)
  - ^{1}H is found at natural abundance, while ^{13}C, ^{15}N must be incorporated

NMR Sensitivity – Bigger Magnets



Signal to noise improvement with magnetic field (sensitivity)



~$800,000

~$2,000,000

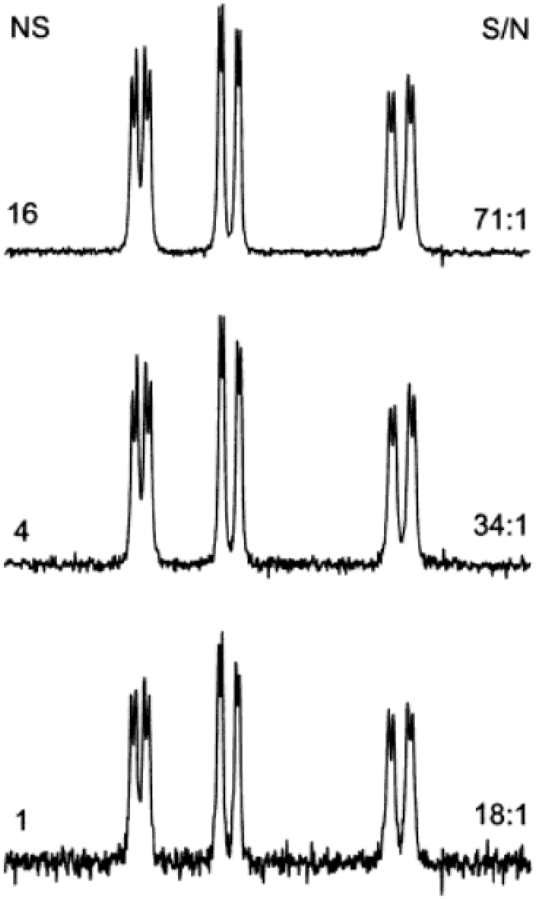
~$4,500,000

NMR Sensitivity – Signal Averaging

- An increase in the number of scans will improve the signal to noise by:

$$\mathbf{S/N} \approx (\text{number of scans})^{1/2}$$

NS	$NS^{1/2}$
1	1.00
8	2.83
16	4.00
80	8.94
800	28.28



- However, there is a practical limitation for how much signal averaging can be performed – especially for multi-dimensional experiments.

Uses of NMR

What can NMR be used for?

NMR is the most versatile scientific tool for studying matter

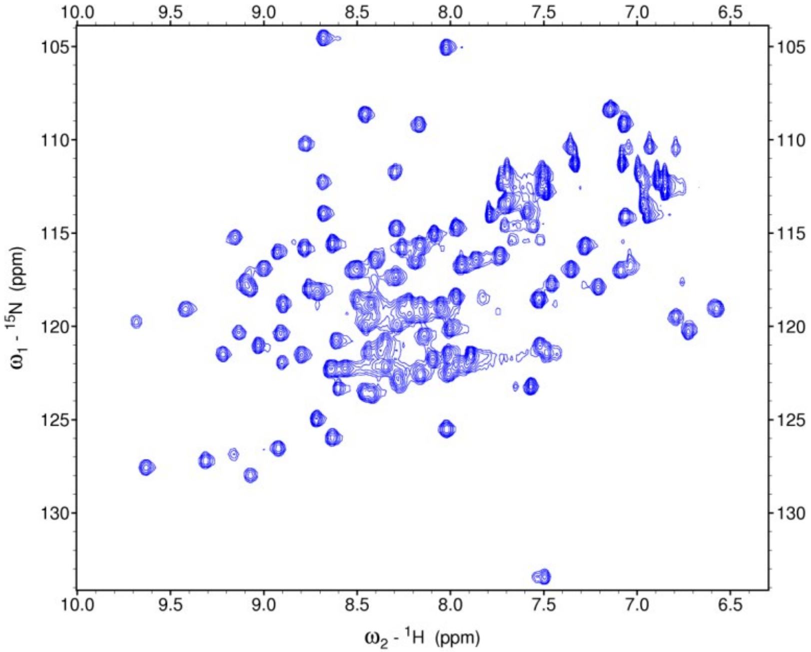
- Study gases, **solutions**, and solids

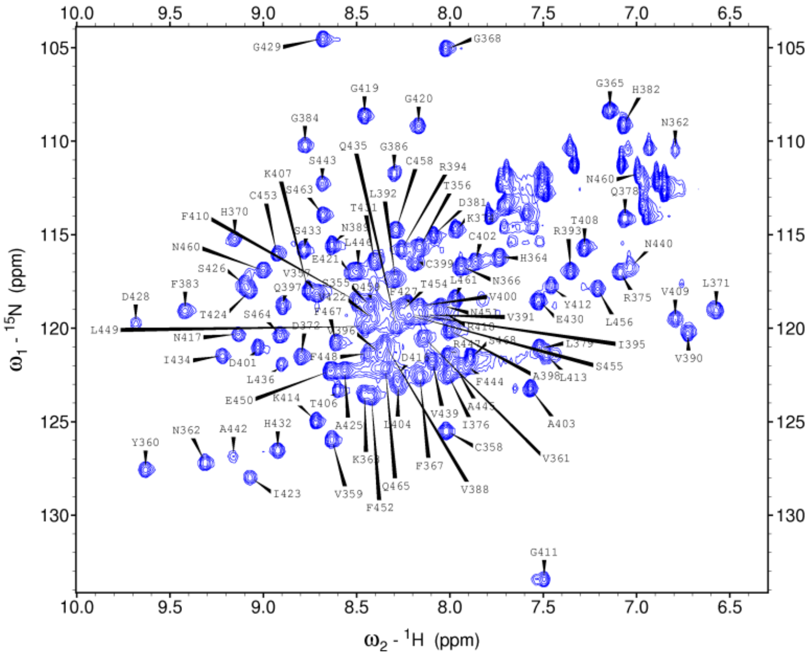
- Study small molecules, **macromolecules**, carbohydrates, organic, and inorganic, people

- Used across many industries; **research**, food, agriculture, pharmaceutical, polymer, chemical, **healthcare**

- **Small molecule identification and verification**
- **3D Structure elucidation** (small and macromolecules)
- **Monitoring perturbations from ligand binding or environmental changes**
- Measuring hydrogen bonds
- Monitoring pKa values
- **Probing molecular motions on a wide variety of time scales**
- Following enzymatic reactions
- Quality assurance and control
    - Big push in pharmaceutical companies to validate production of biologics with structural information for FDA; HSQC spectra appear to be the method of choice
- Monitoring conformation and chemical exchange processes.
- **Clinical and functional MRI**

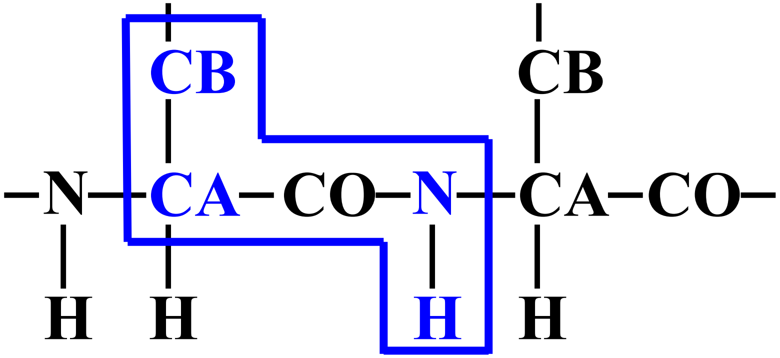
Protein NMR: Resonance Assignments





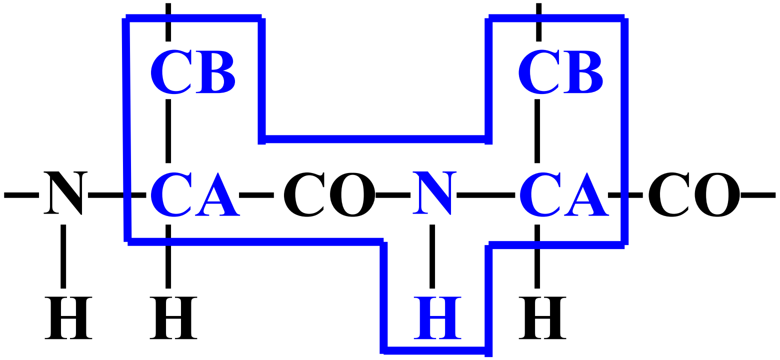
- NMR resonance assignments needed for most studies of proteins
- But, how to determine which nuclei correspond to which peak?
  - Answer: 3D triple resonance NMR experiments

Protein NMR: Resonance Assignments



3D Experiment	Connections
HNCO	HN(i), N(i), CO(i-1)
HN(CA)CO	HN(i), N(i), CO(i), CO(i-1)
HNCA	HN(i), N(i), CA(i), CA(i-1)
HN(CO)CA	HN(i), N(i), CA(i-1)
HNCACB	HN(i), N(i), CA(i), CB(i), CA(i-1), CB(i-1)
CBCA(CO)NH; HBHA(CBCACO)NH	HN(i), N(i), CA(i-1), CB(i-1) or HA(i-1), HB(i-1)
H(CC)(CO)NH-TOCSY	HN(i), N(i), sidechain H(i-1)
(H)CC(CO)NH-TOCSY	HN(i), N(i), sidechain C(i-1)
HCCH-TOCSY; (H)CCH-TOCSY	sidechain H(i), sidechain C(i)

Protein NMR: Resonance Assignments



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HN(CO)CA	HN(i), N(i), CA(i-1)
HNCACB	HN(i), N(i), CA(i), CB(i), CA(i-1), CB(i-1)
CBCA(CO)NH; HBHA(CBCACO)NH	HN(i), N(i), CA(i-1), CB(i-1) or HA(i-1), HB(i-1)
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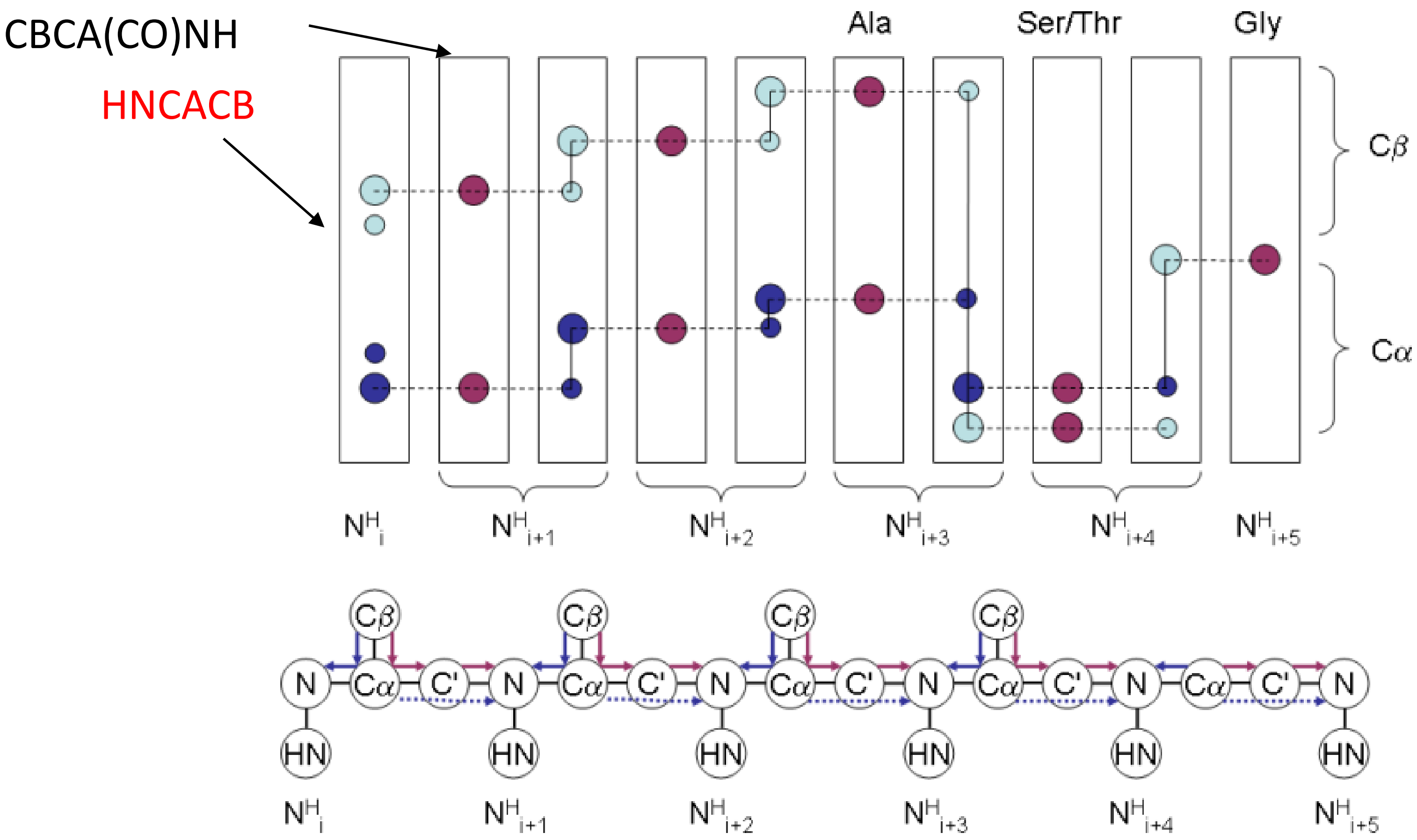
(H)CC(CO)NH-TOCSY	HN(i), N(i), sidechain C(i-1)
HCCH-TOCSY; (H)CCH-TOCSY	sidechain H(i), sidechain C(i)

Protein NMR: Resonance Assignments



CBCA(CO)NH

HNCACB

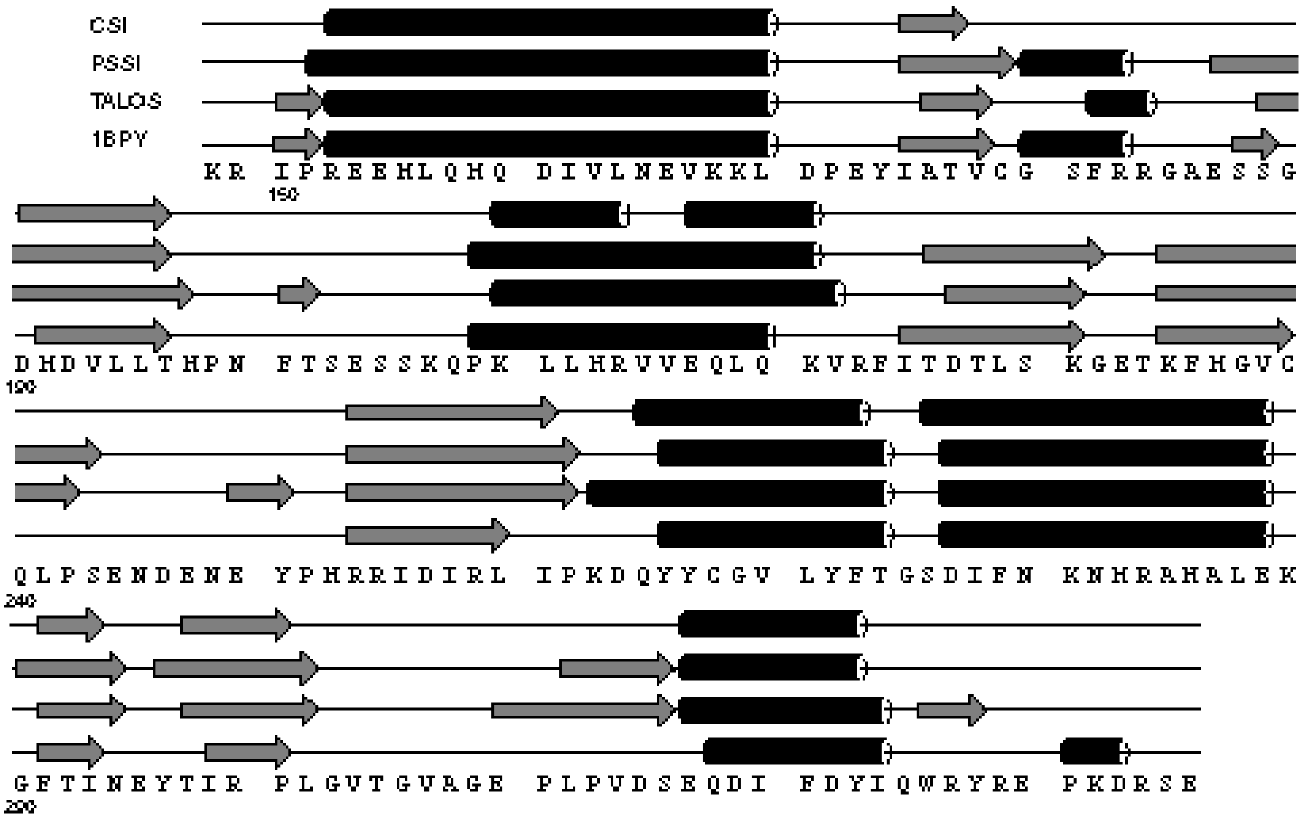
- Other combinations of experiments can also be used:
  - HNCA, HN(CO)CA
  - HNCO, HN(CA)CO

Protein NMR: Resonance Assignments

- Different class of experiments can determine the side chain resonance assignments after the backbone assignments are complete

3D Experiment	Connections
HNCO	HN(i), N(i), CO(i-1)
HN(CA)CO	HN(i), N(i), CO(i), CO(i-1)
HNCA	HN(i), N(i), CA(i), CA(i-1)
HN(CO)CA	HN(i), N(i), CA(i-1)
HNCACB	HN(i), N(i), CA(i), CB(i), CA(i-1), CB(i-1)
CBCA(CO)NH; **HBHA(CBCACO)NH**	HN(i), N(i), CA(i-1), CB(i-1) or HA(i-1), HB(i-1)
H(CC)(CO)NH-TOCSY	HN(i), N(i), sidechain H(i-1)
(H)CC(CO)NH-TOCSY	HN(i), N(i), sidechain C(i-1)
HCCH-TOCSY; (H)CCH-TOCSY	sidechain H(i), sidechain C(i)

Protein NMR: Secondary Structure Determination



The chemical shift of protein backbone resonances (HN, N, C’, C_α, H_α, C_β, H_β) is highly dependent on secondary structure.

- With this information alone, one can determine the secondary structure of the protein using the programs: CSI, PSSI or TALOS.

Protein NMR: Structure Determination

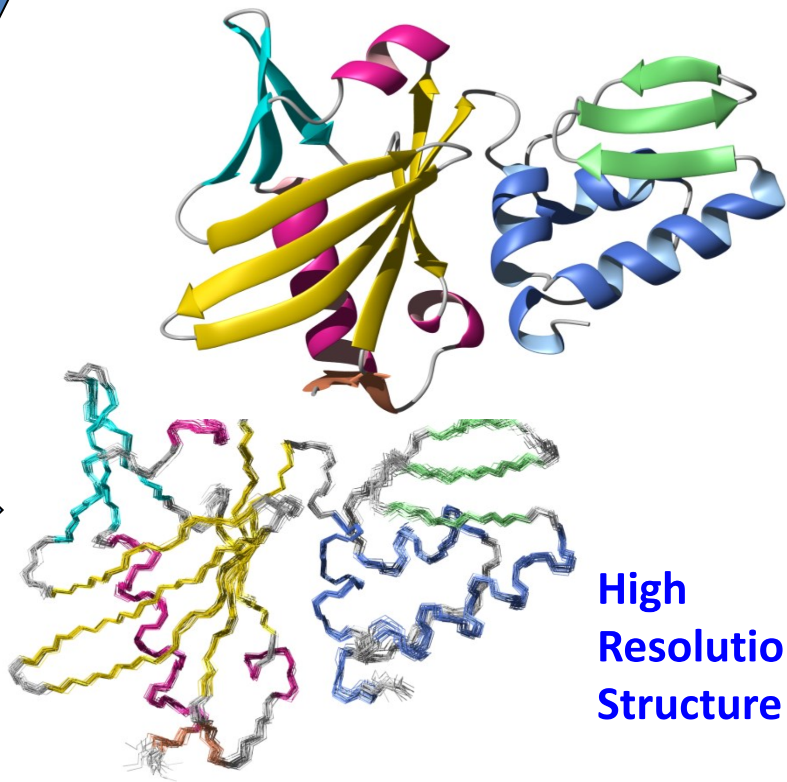
Resonance Assignments
Sequential
Side-Chain

Determine Torsion
Angle Constraints (ϕ, ψ, χ)
Based on Chemical Shift
Based on *J*-couplings

Determine Distance
Constraints (thousands)
From 2D/3D NOESY Spectra

Iterative

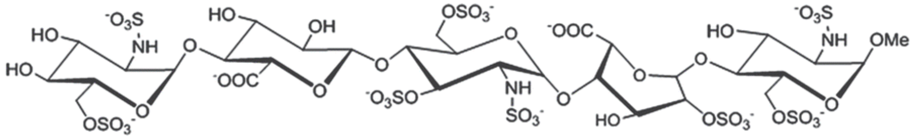
Structure Determination
Torsion Angle Dynamics
Simulated Annealing



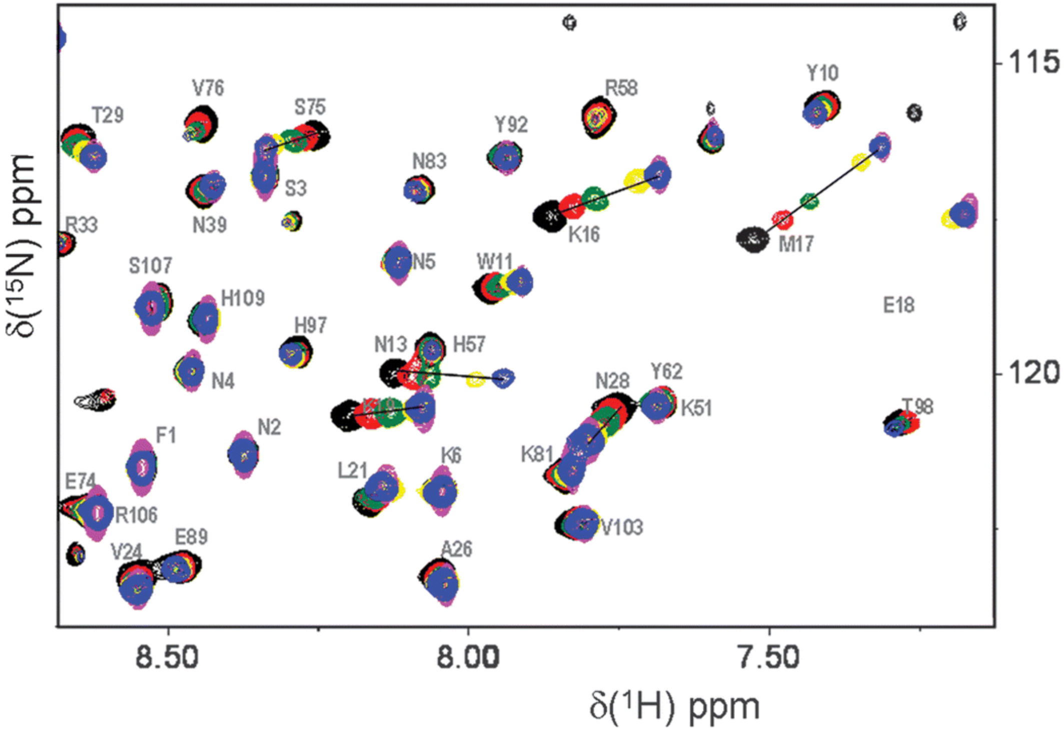
High
Resolution
Structure

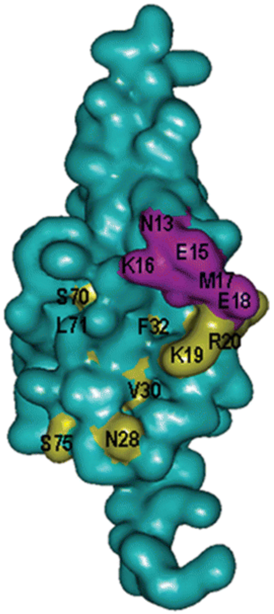
Protein NMR: Chemical Shift Mapping

- The NMR Chemical shift is one of the most sensitive probes to local environment.
- By measuring perturbations of NMR chemical shifts during a titration binding events can be mapped onto a structure and in certain cases accurate binding affinities calculated.



^{15}N-^{1}H HSQC FGFR-Ig2 domain/pentasaccharide

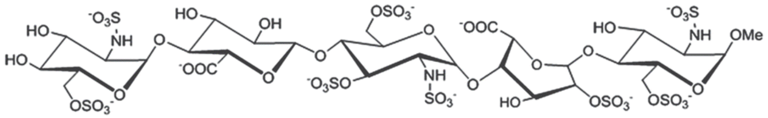


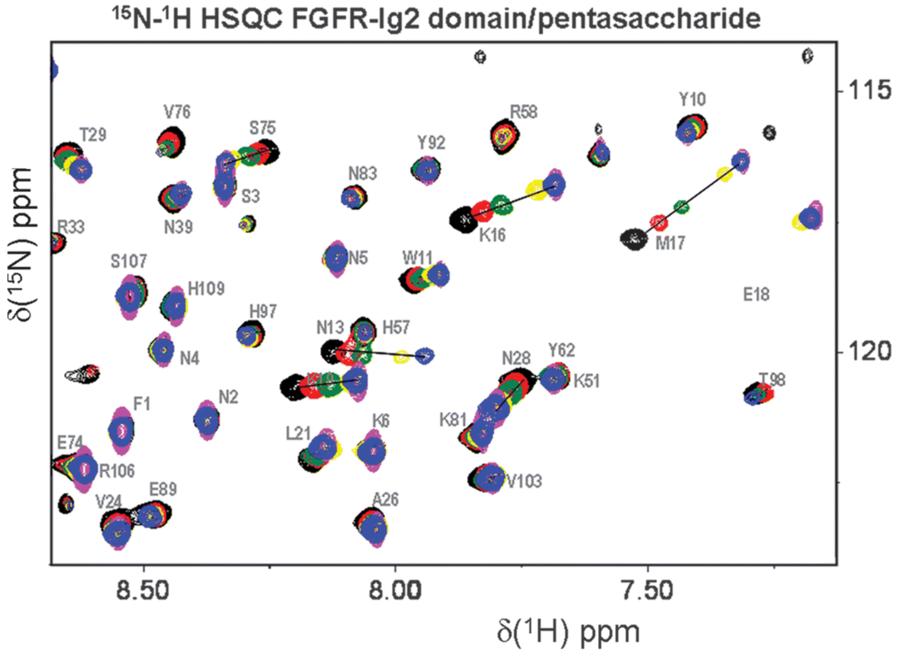


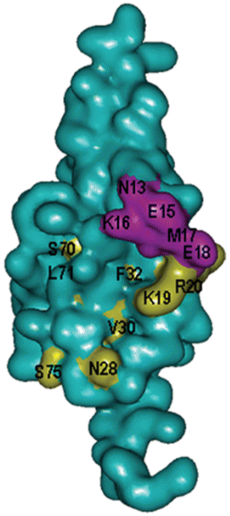
FGFR-Ig2

Protein NMR: Chemical Shift Mapping

- Chemical Shift Mapping can be performed for:
    - Ligands (small, macromolecular)
    - pH (Determine pKa values)
    - Temperature (Determine protein stability; Determine hydrogen bond strength)
    - Ionic strength
    - Enzyme catalysis

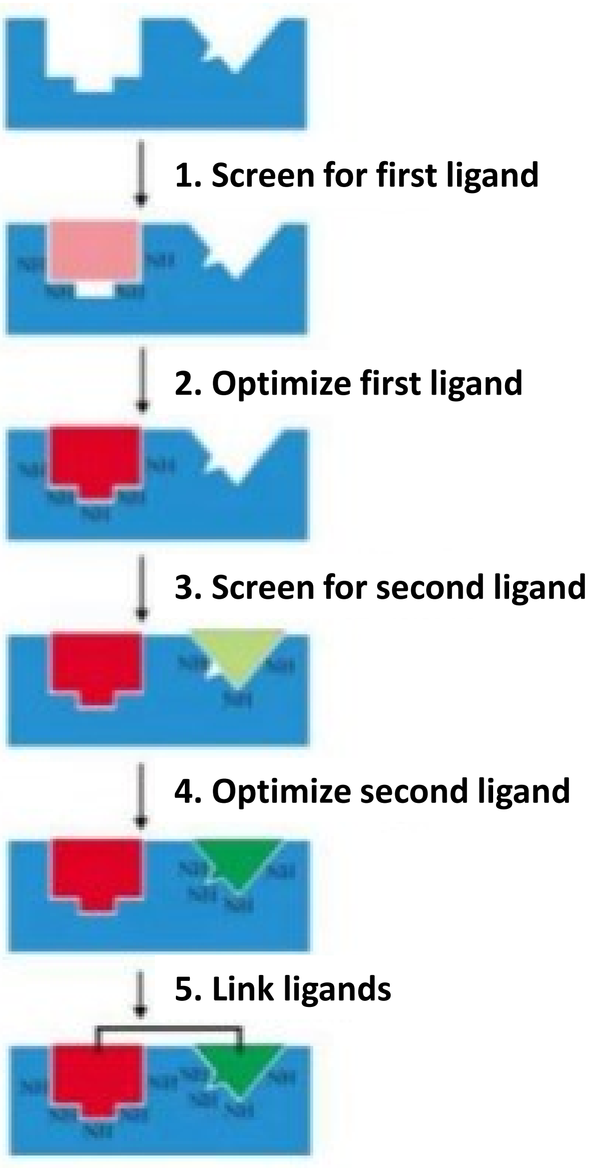






FGFR-Ig2

Protein NMR: SAR by NMR



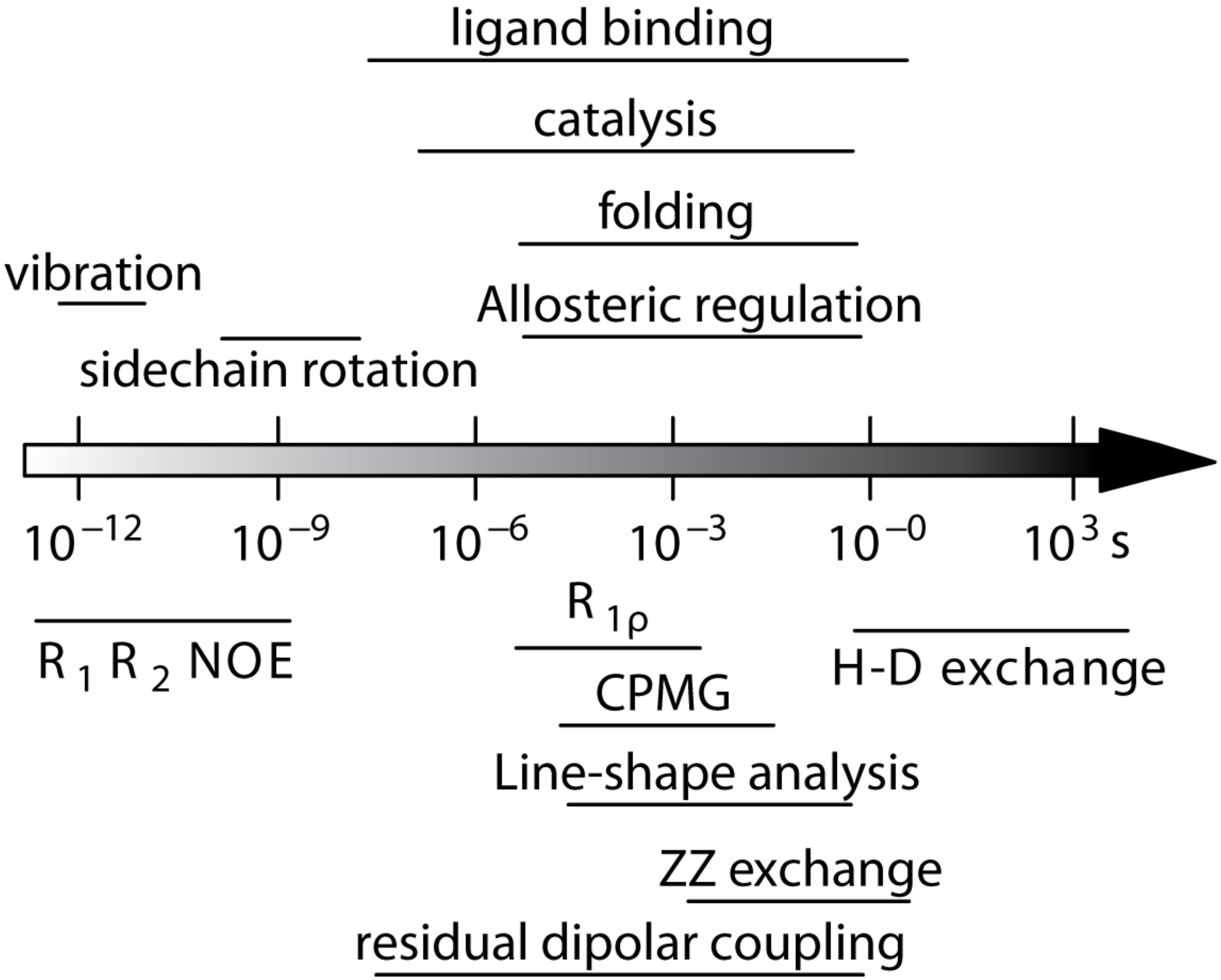
1. Screen for first ligand
2. Optimize first ligand
3. Screen for second ligand
4. Optimize second ligand
5. Link ligands

- Structure Activity Relationship (SAR) by NMR
  - Developed by Abbott to screen a library of ***small*** ligands for initial drug leads

  - Protocols have been optimized to allow screening of tens of thousands of compounds in days
  - Mixtures of compounds are used (5 to 100) in each screen with the same compound appearing in multiple screens
  - Robot for sample production and NMR data collection fully automated
  - Expensive due to large amounts of labelled protein, but many pharmaceutical companies turn to SAR by NMR when other screening procedures fail

Protein NMR: Dynamics

- NMR can probe molecular motions over time scales with 15 orders of magnitude
- In particular NMR relaxation phenomenon is tied directly to molecular motions



What Nuclei

- Nuclei with spin number of 0 have no angular momentum and are therefore not observable by NMR.
  - Examples: ^{12}C, ^{16}O, ^{32}S

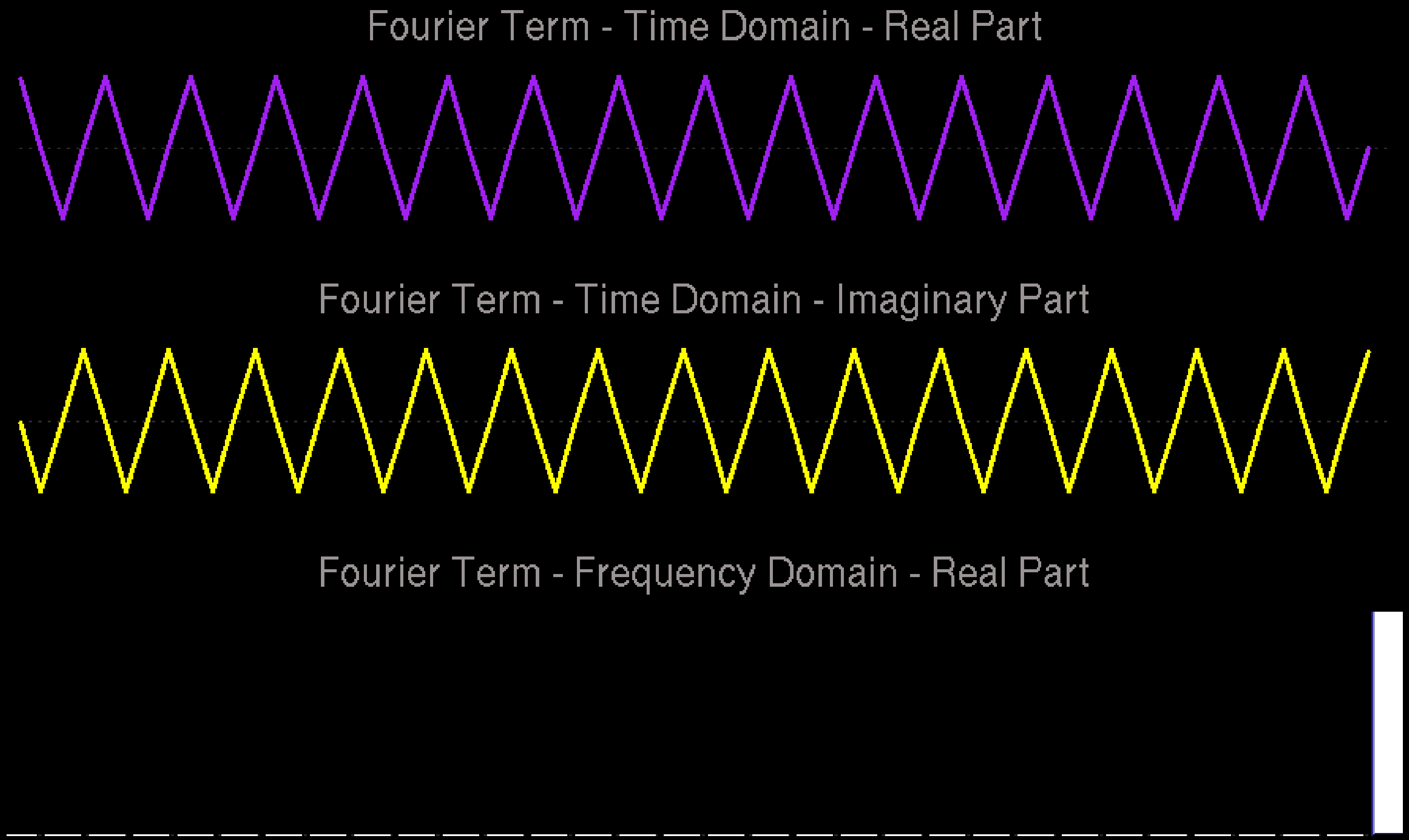
- Almost every atom has an isotope that can be studied by NMR.

- Nuclei with spin > ½ have poor magnetic properties and are not commonly studied
  - Examples: ^{2}H, ^{14}N

- The most commonly studies nuclei, especially for biological samples, are ^{1}H, **^{13}C, ^{15}N**, ^{19}F, and ^{31}P.
  - ^{13}C and ^{15}N are stable isotopes, but have low natural abundance
    - $^{13}C = 1.1\%$
    - $^{15}N = 0.36\%$

Questions?

$$\mathbf{X}(f) = \sum x(t)\,[\cos(2\pi ft/N) - i\sin(2\pi ft/N)]$$



Each Fourier term corresponds to a point in the spectrum.