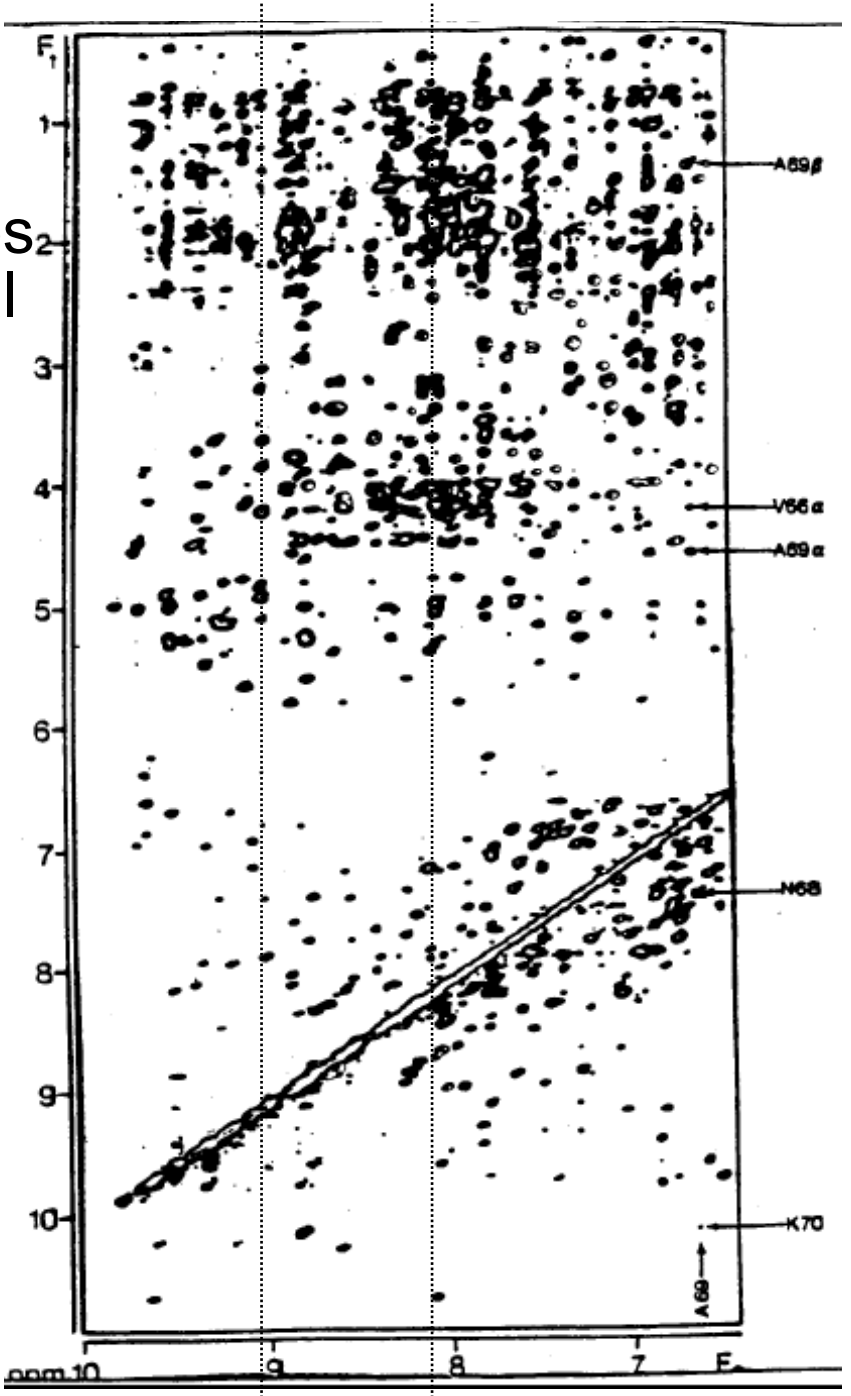
Extensions to 3D
and
Improving Efficiency with Pulsed Field Gradients

BCMB/CHEM 8190

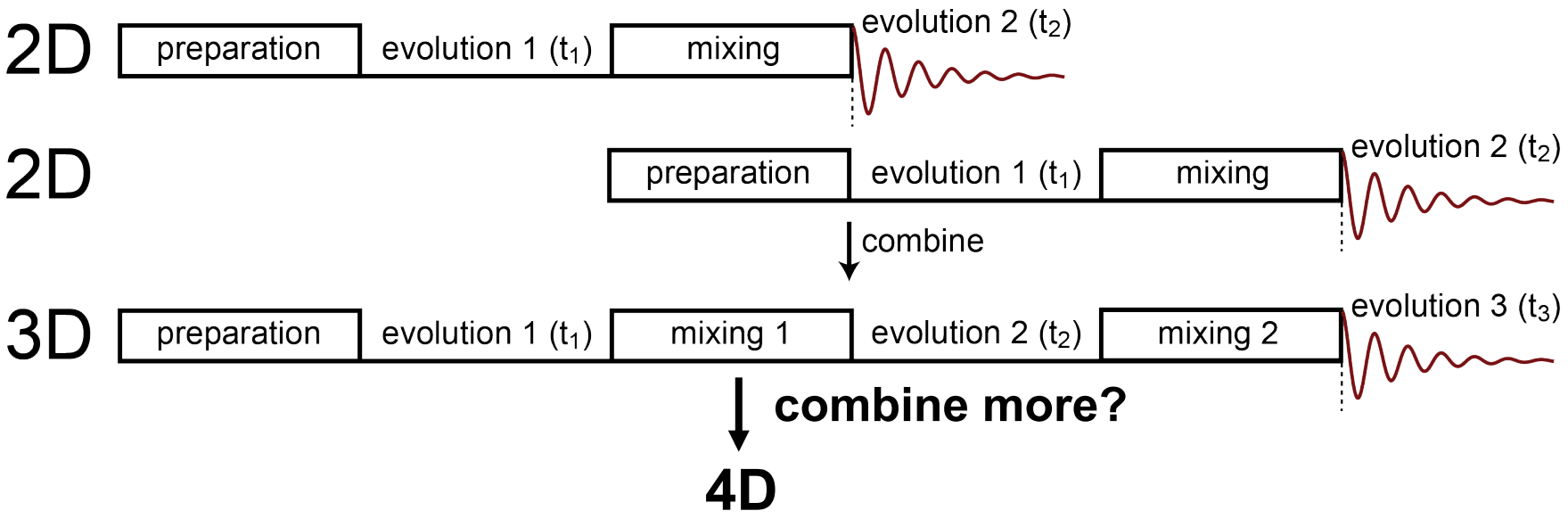
Crowding in 2D NMR Spectra

- 2D NMR spectra of biomolecules are subject to problematic signal overlap
  - here (right) is the amide region of a 2D NOESY spectrum (Staph nuclease, 156 amino acids)
  - each peak represents a short ^{1}H-^{1}H distance between an amide ^{1}H and some other ^{1}H
  - a single 'column' represents all ^{1}H nuclei within ~ 5Å of a particular amide ^{1}H nucleus (dashed lines)
  - possible to unambiguously identify (assign) a small percentage of these in 2D spectra, but impossible to unambiguously assign a majority
  - this is the least crowded region (aliphatic region much worse!)
- Solution: 3D



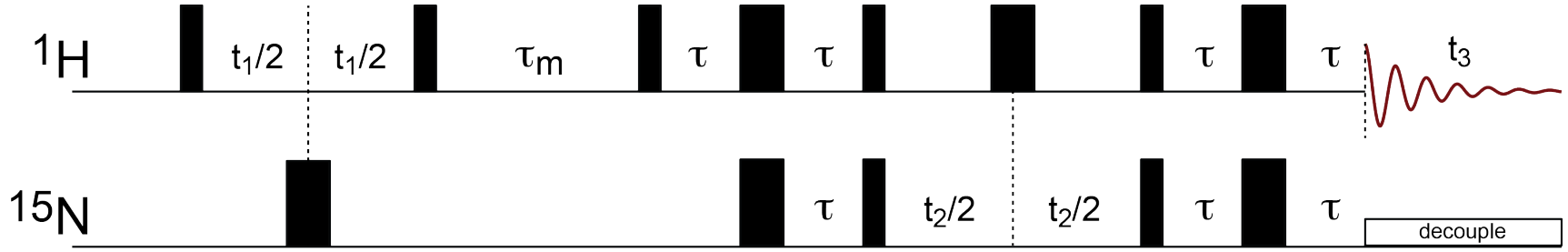
One Strategy to Alleviate Signal Overlap: Heteronuclear Edited 3D Experiments

- 3D experiments can be built by combining 2D experiments
  - a 2D experiment can be used as the preparation element of another 2D experiment to create a 3D experiment



- Resolve signals in third dimension by correlations to ^{15}N or ^{13}C
  - example: for NOESY signals involving amide ^{1}H nuclei add another dimension correlating the amide ^{15}N
  - signals are separated in the 3rd dimension based on this ^{15}N chemical shift
  - "NOESY-HSQC"
- Resolution and S/N great, but acquisition is long (hours to days)

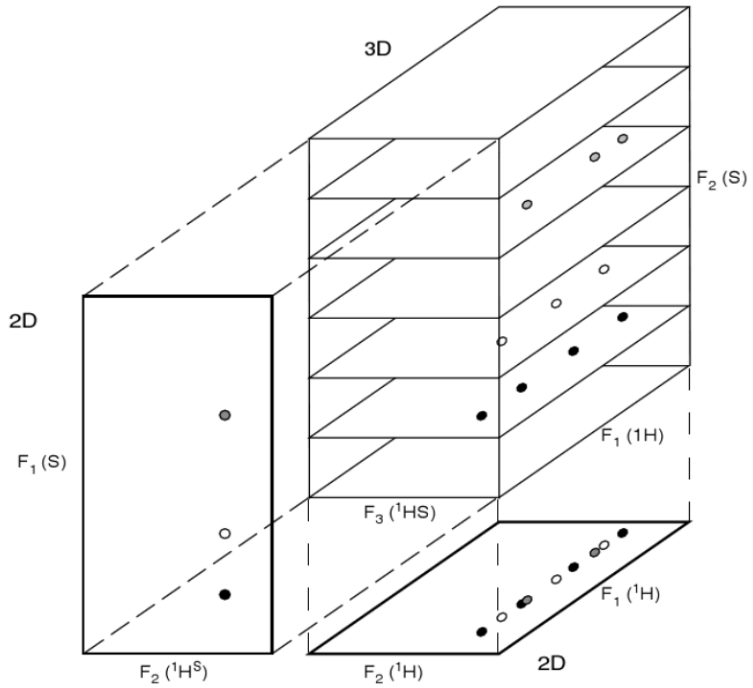
3D NOESY-HSQC

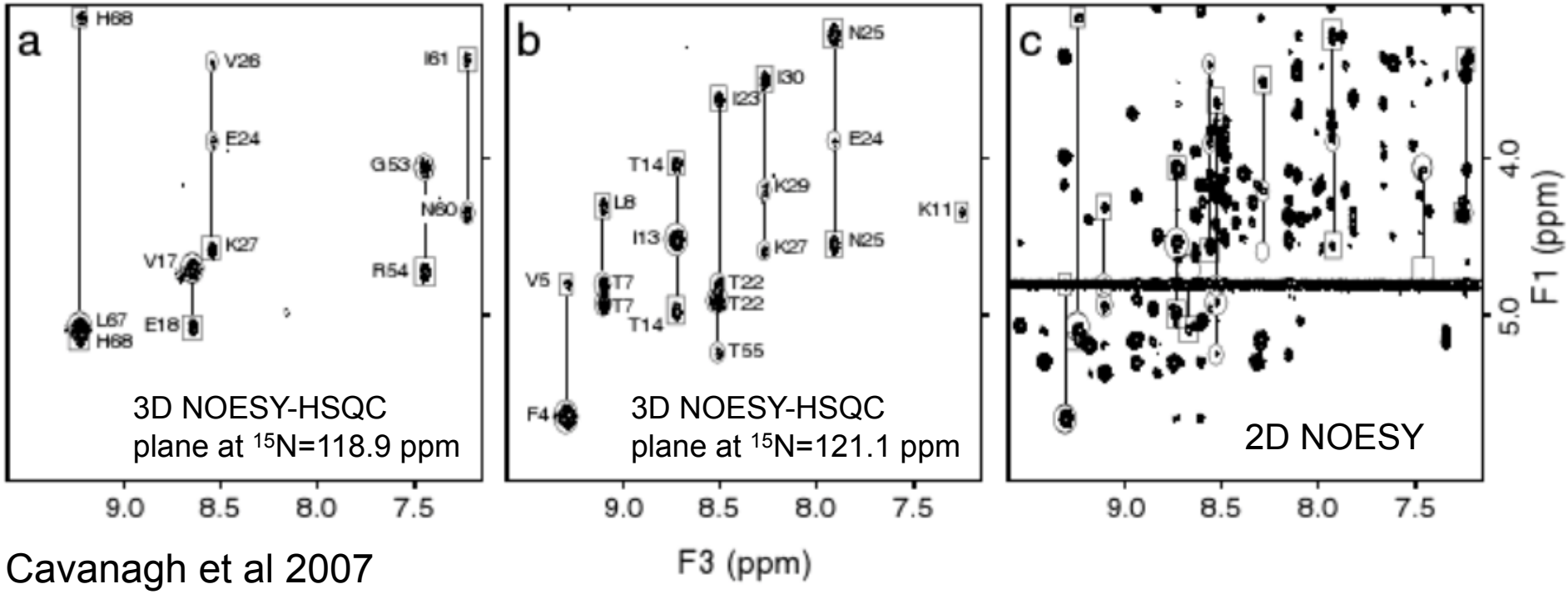


- NOESY sequence serves as preparation period for HSQC
  - the final pulse in the NOESY experiment generates the transverse magnetization for the initial INEPT of the HSQC experiment
  - only ^{1}H NOE crosspeaks where one of the ^{1}H nuclei is directly bonded to a ^{15}N nucleus are observed
- Prerequisites
  - need uniform ^{15}N (or ^{13}C) labeling (more on this in later lectures)
- Importance
  - NOE-based distance restraints are the most important restraints for traditional studies for determining high resolution protein structures by NMR
  - It is critical to be able to unambiguously assign the NOE crosspeaks, so 3D (and higher dimension) experiments are essential
- Time consuming
  - if each 2D NOESY experiment takes 1 hour, and you need 64 complex points in the ^{15}N dimension, that's 128 2D experiments (128 hours)!

3D NOESY-HSQC

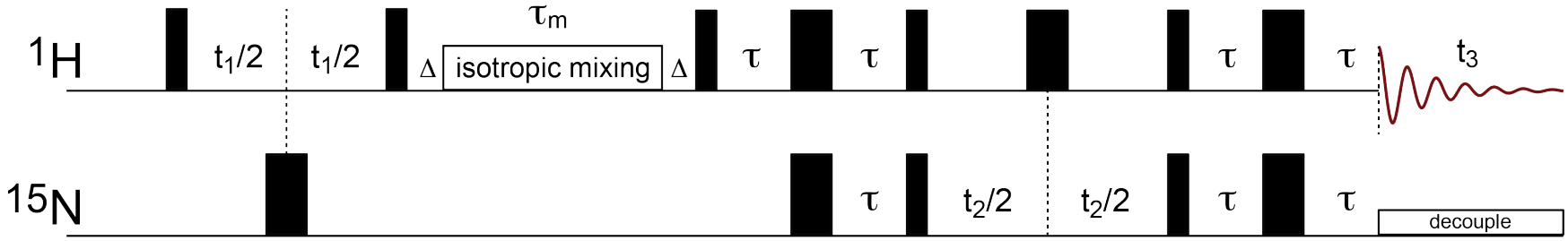
- 2D planes of 3D NOESY-HSQC much easier to analyze
  - data shown are for the very small (76 amino acids) protein ubiquitin
  - significant reduction in peak overlap in the planes compared to the 2D spectrum





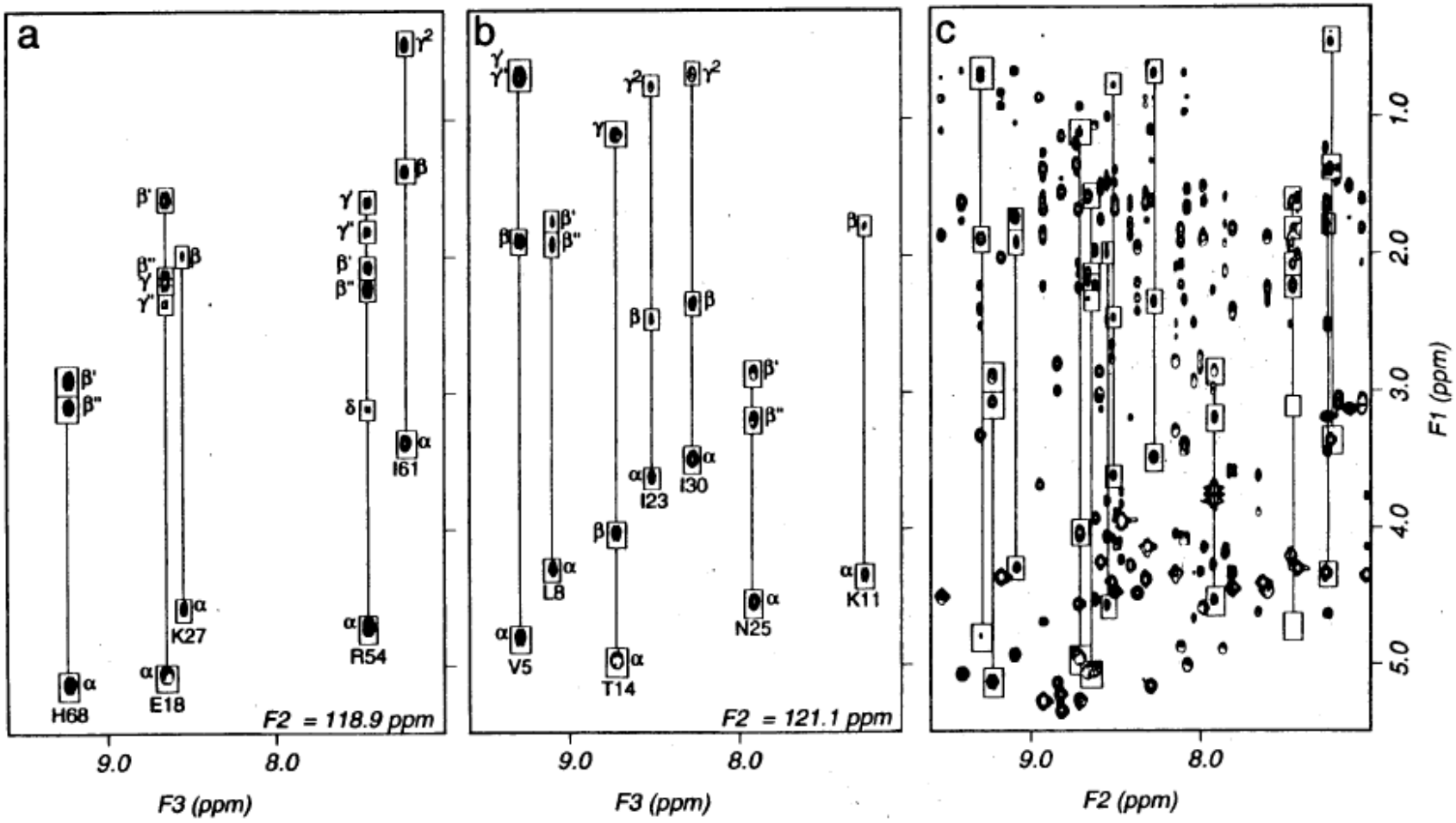
Cavanagh et al 2007

3D TOCSY-HSQC



- TOCSY sequence serves as preparation period for HSQC
  - the final pulse in the TOCSY experiment generates the transverse magnetization for the initial INEPT of the HSQC experiment
  - only TOCSY spin systems where one of the ^1H nuclei is directly bonded to a ^{15}N nucleus are observed
- Prerequisites
  - need uniform ^{15}N (or ^{13}C) labeling
- Importance
  - identifying spin systems and correlating all chemical shifts in each
  - helpful in identifying amino acid type (side chain chemical shifts are often diagnostic of the amino acid type)
- Time consuming
  - like NOESY-HSQC

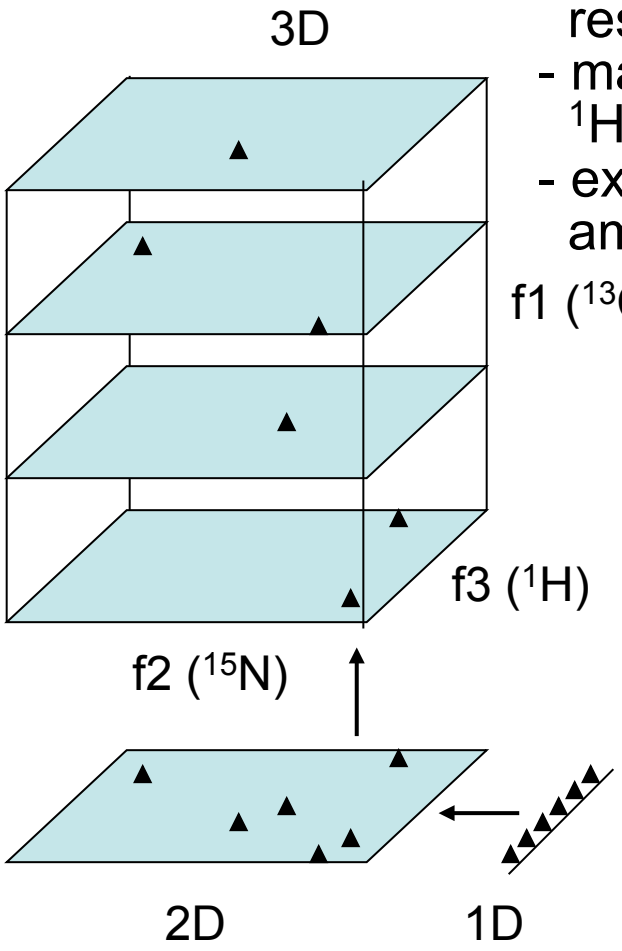
3D TOCSY-HSQC for ^{15}N-Labeled Ubiquitin

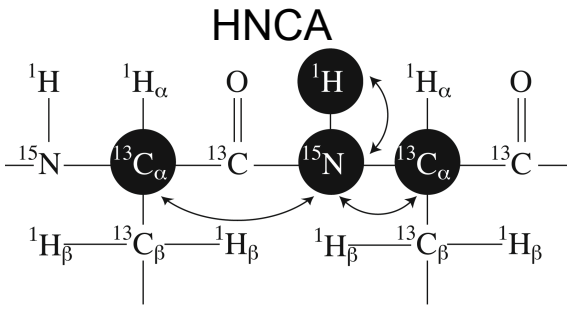


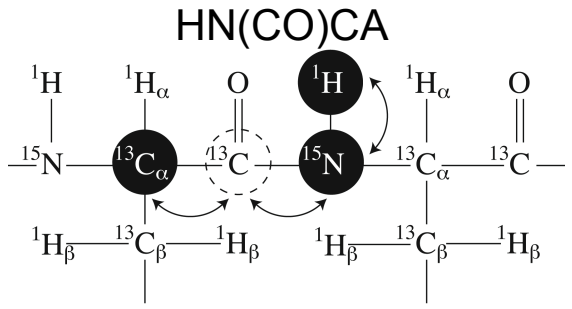
Cavanagh et al 1996

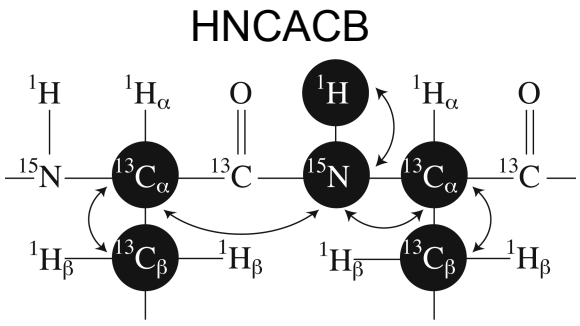
3D Triple Resonance Experiments

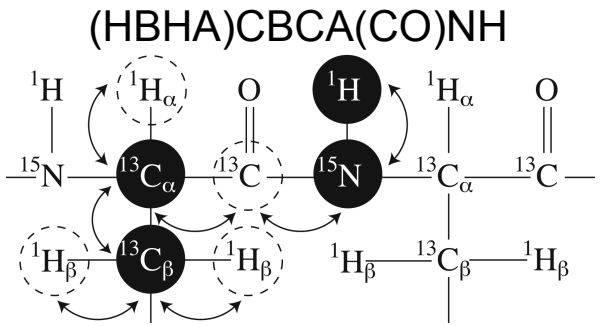
- Triple resonance experiments correlate multiple nuclear types (typically ^{1}H, ^{13}C and ^{15}N, each in its own dimension)
  - these aren't necessarily simple combinations of 2D experiments, but they employ similar pulse sequence elements (INEPT, COSY, etc.)
    - are dozens of these experiments, critical for assigning resonances of biomolcules
    - magnetization transferred via scalar couplings, with ^{1}H detection (inverse detect experiments)
    - examples: (names have meaning, HNCA correlates amide ^{1}H, amide ^{15}N and alpha carbon ^{13}C)











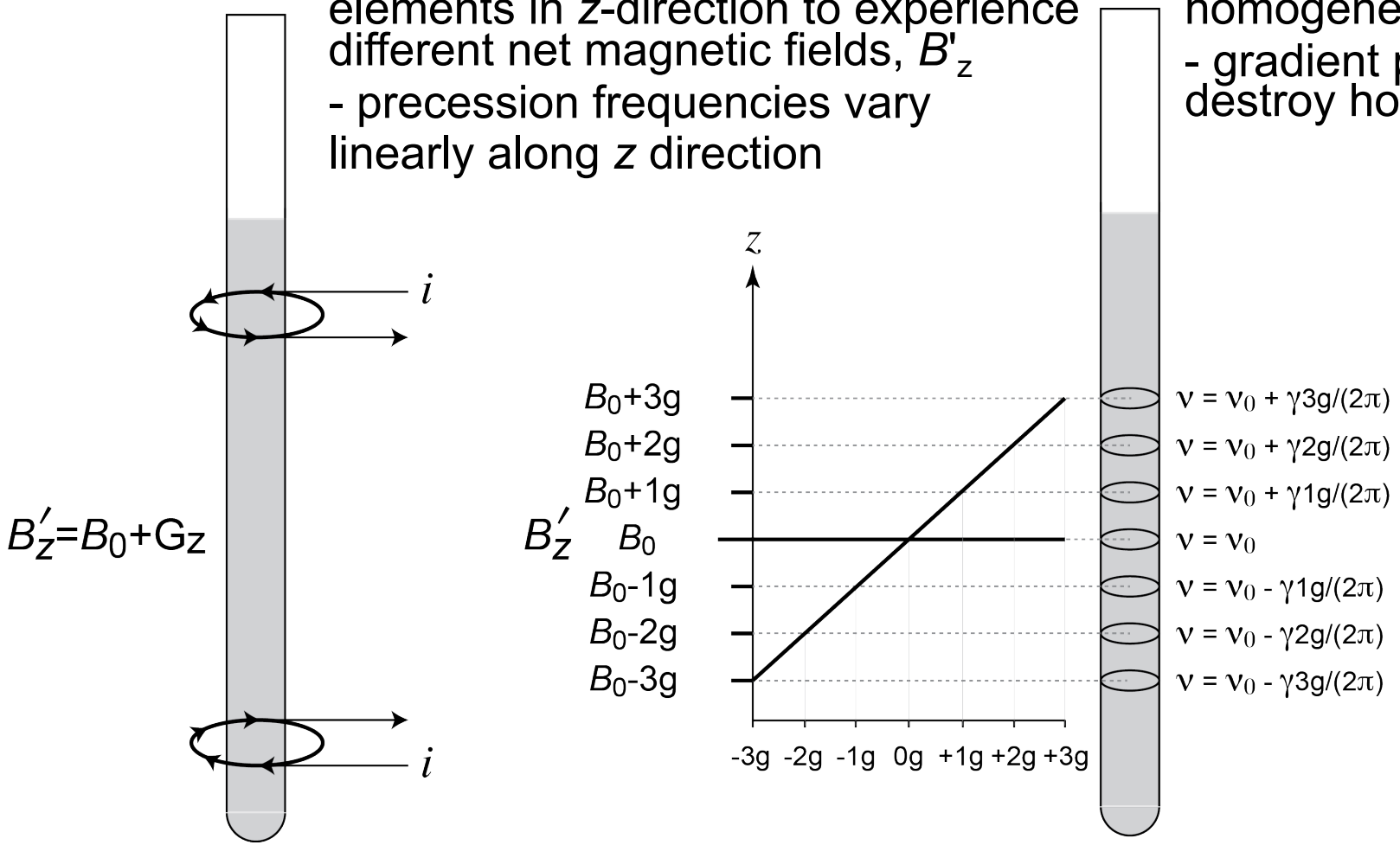
Pulsed Field Gradients: Improved Efficiency in Multidimensional Spectra

- Pulsed field gradients are used in many important ways in modern biomolecular NMR experiments
  - they can be used in a very simple way to remove spurious signals and unwanted magnetization
  - they can be used to reduce phase cycling (thus significantly decreasing overall time necessary to acquire an experiment)
  - they can be used to suppress water signals (and other signals as well)
  - they can be used for coherence selection
  - they can be to measure diffusion

- Coherence selection using pulsed field gradients. J. R. Tolman & J. H. Prestegard, *Concepts in Magnetic Resonance,* **7**, 247-262 (1995).
- Water suppression (WATERGATE), M. Piotto, V. Saudek & V. Sklenar, *J. Biomol. NMR,* **2,** 661-665 (1992).
- Diffusion measurements. Altieri, Hinton & Byrd, *J. Am. Chem. Soc.,* **117,** 7566-7567 (1995).

Pulsed Field Gradients – How they Work

- Similar to a shim coil
  - simplest 'Z' shim coil, two loops of wire with current running in opposite directions, sets up a linear magnetic field gradient along the *z*-axis
  - Z gradient coil is similar, but used to apply transient current (pulses)
  - Z-gradient pulse causes volume elements in *z*-direction to experience different net magnetic fields, B'_z
  - precession frequencies vary linearly along *z* direction
  - shim coils: improve homogeneity
  - gradient pulses, destroy homogeneity

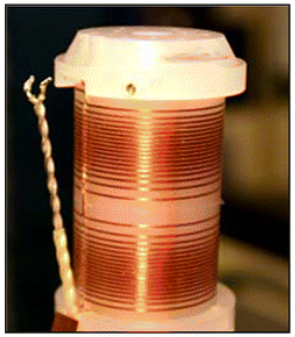


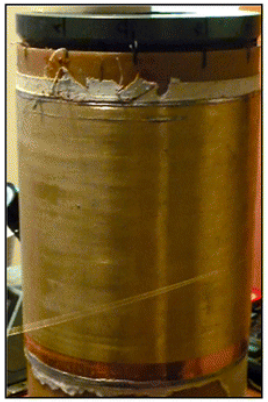
Shim Coils and Gradient Coils

Dewar

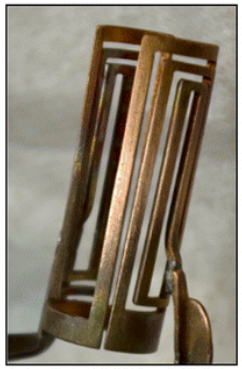
room temperature
shim coils

gradients





superconducting
magnet + shims

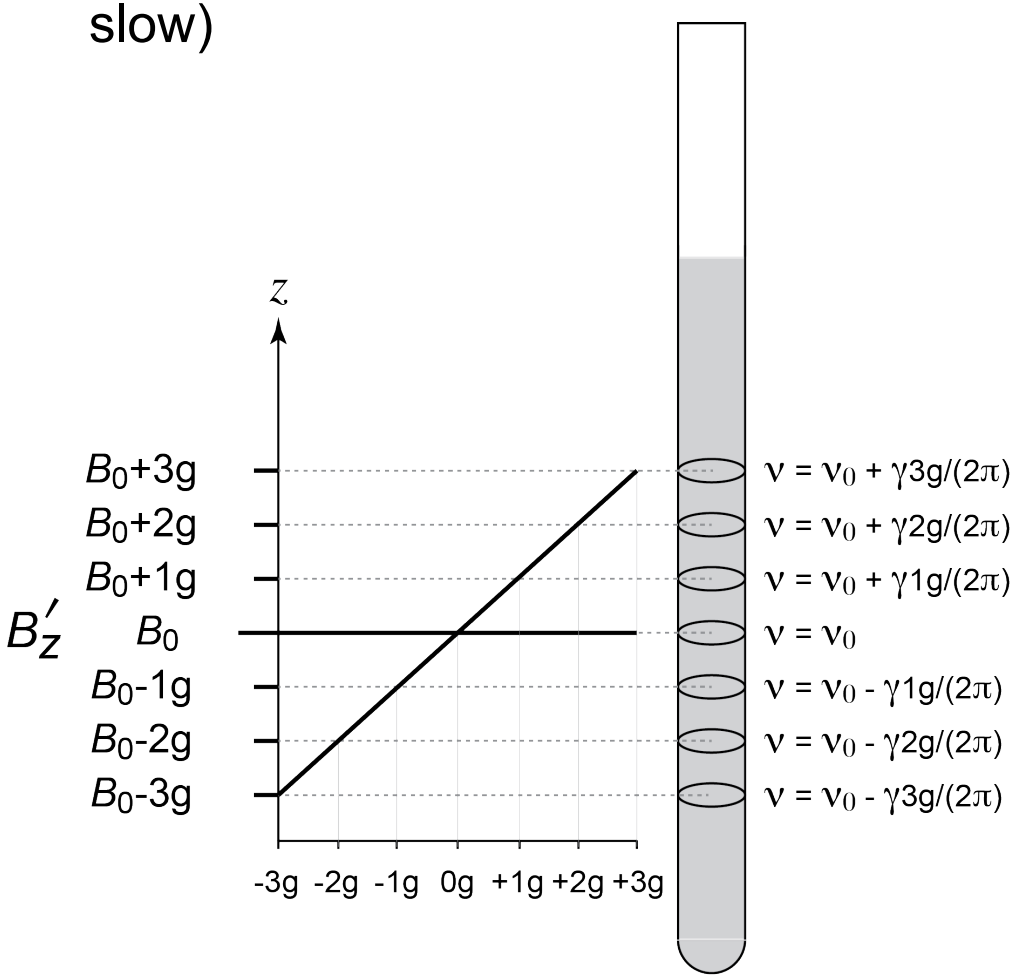


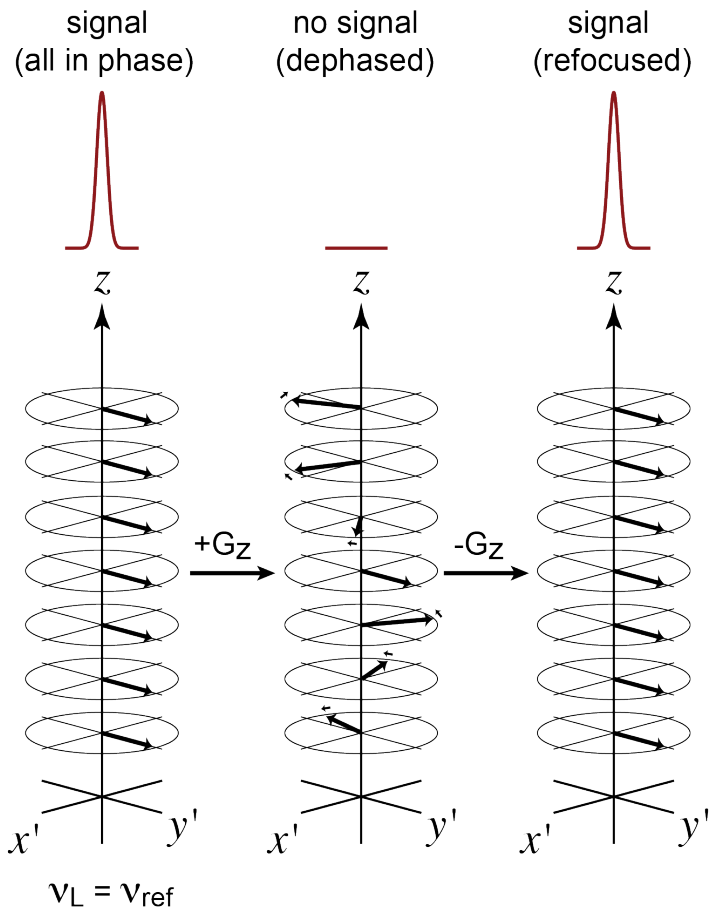
RF coil

http://pubs.rsc.org/services/images/RSCpubs.ePlatform.Service.FreeContent.ImageService.svc/ImageService/ChapterImage/bk9781782623595/BK9781782623595-00001/bk9781782623595-00001-f2_hi-res.gif

Pulsed Field Gradients – How they Work

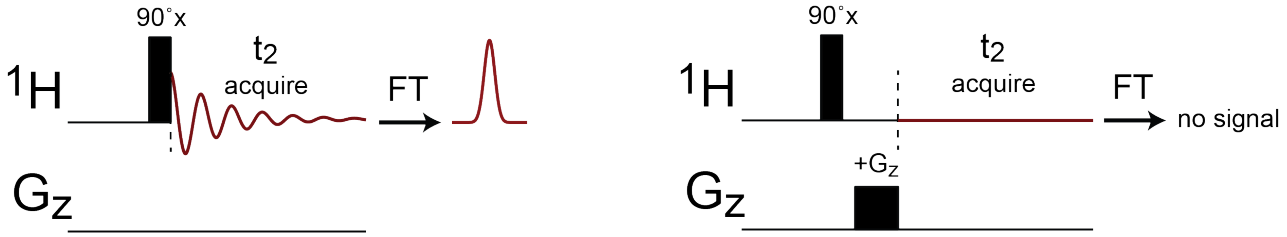
- Gradient pulses defocus and refocus magnetization
  - applying a Z gradient pulse on coherent transverse magnetization causes defocusing (dephasing) of magnetization in the transverse plane
  - however, the magnetization can be refocused by applying a second, identical gradient pulse with opposite polarity (works because diffusion is slow)



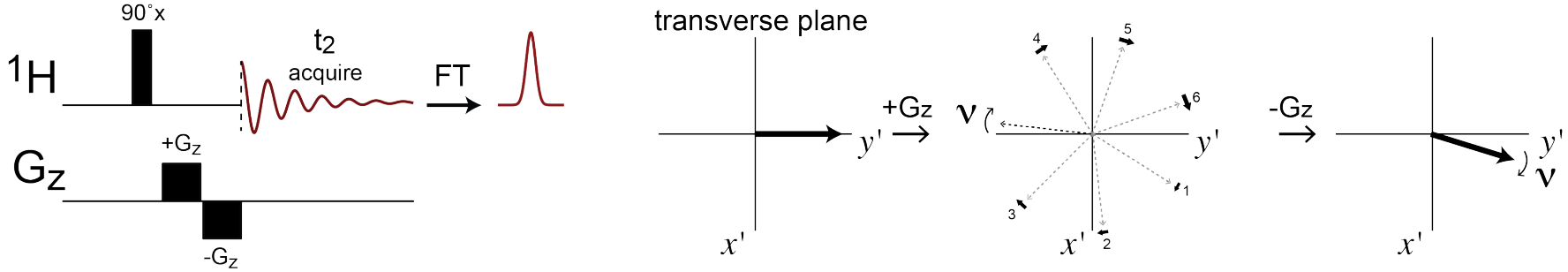


Defocusing/Refocusing, Encoding/Decoding

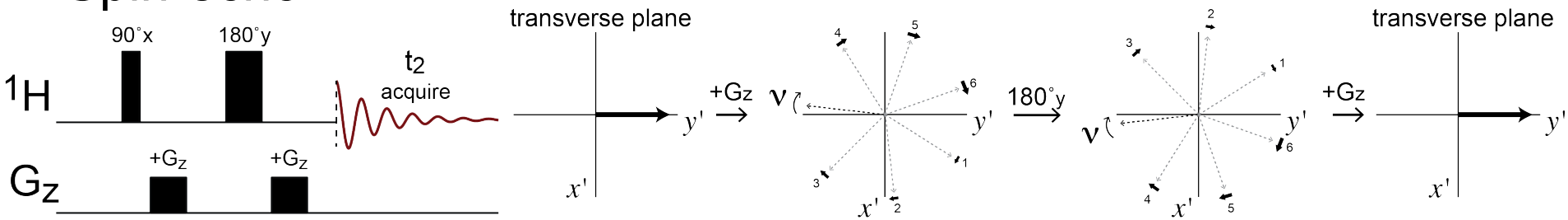
- Field gradient pulses encode a spatially dependent phase, that can be decoded by another gradient pulse
  - as on the previous page, a simple example is here, whereby application of a gradient pulse encodes a spatially dependent phase, which is decoded by the second gradient pulse of opposite polarity



- Simple defocus/refocus (spatial encoding/decoding)



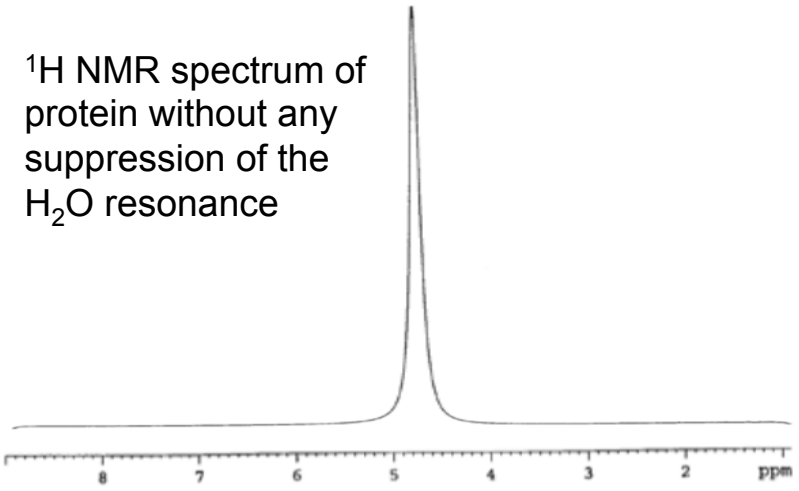
- Spin-echo



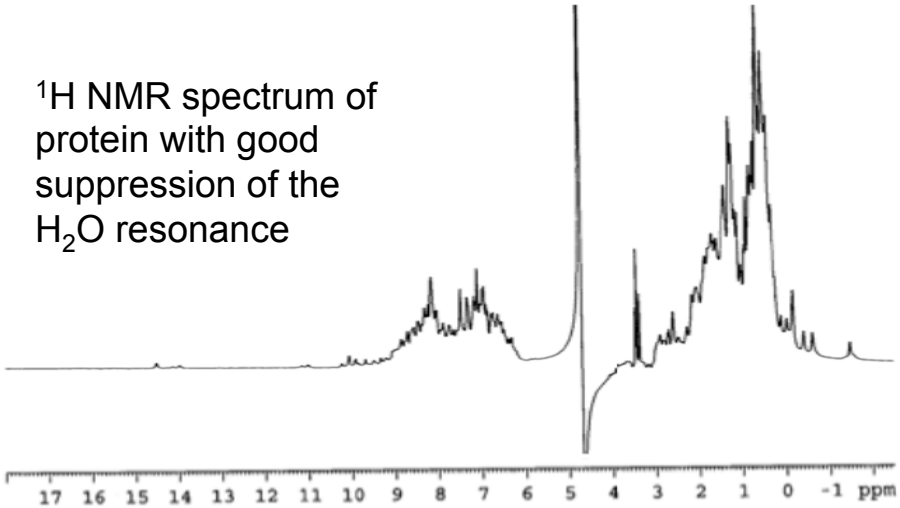
Field Gradients Application: Water Suppression

- Much of biomolecular NMR is done in H_2O
  - this is the natural milieu for proteins, nucleic acids, etc.
  - replacing with D_2O can cause some subtle effects on stability, dynamics, etc.
  - amide ^1H hydrogens exchange with solvent: if D replaces H, can't detect amide ^1H
- Many experiments detect amide ^1H magnetization
  - very convenient: signals are further downfield than most other ^1H signals in proteins, well resolved, 1 per amino acid residue
  - so, NOESY-HSQC, TOCSY-HSQC, and many, many triple resonance experiments detect amide ^1H magnetization
- Problem: protein in H_2O only observable with good suppression of large H_2O signal
  - H_2O is 55 M H_2O (110 M protons), protein 1 mM (110,000 fold difference)
  - with good H_2O suppression, protein signals easily observed

^1H NMR spectrum of protein without any suppression of the H_2O resonance

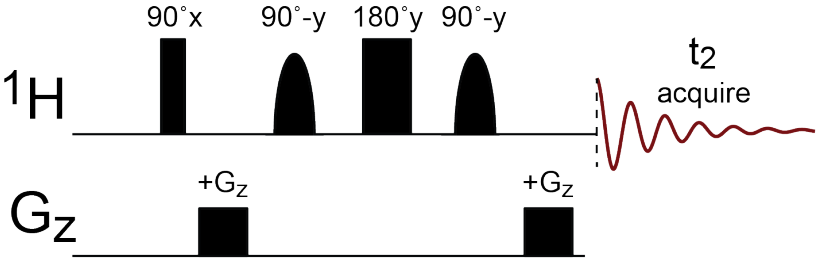


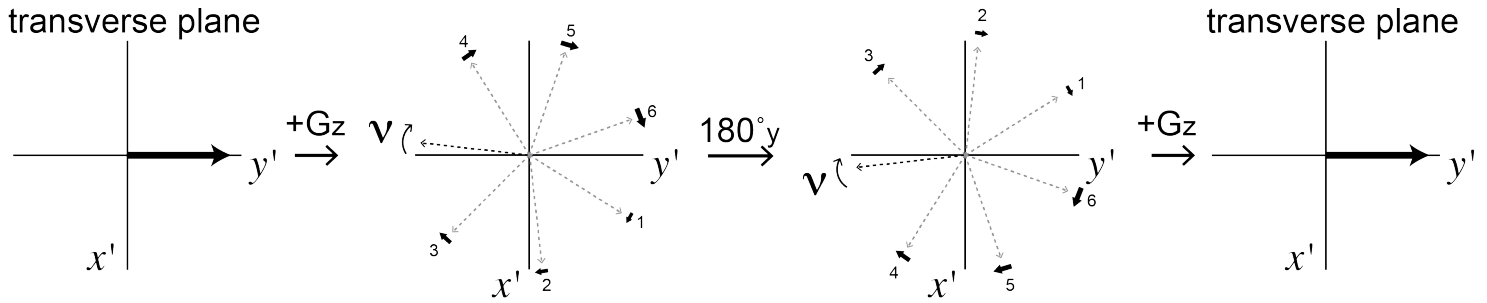
^1H NMR spectrum of protein with good suppression of the H_2O resonance



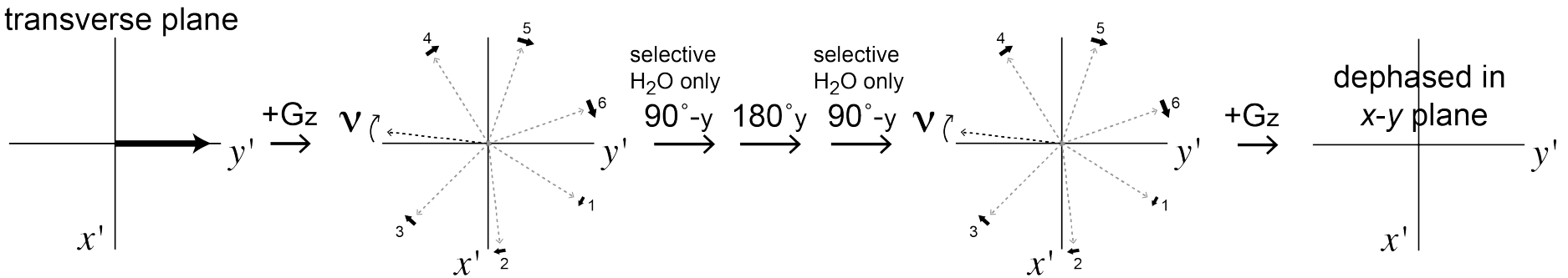
Field Gradients Application: Water Suppression

- This application uses field gradient pulses, and frequency selective pulses (selective for H_2O) to reduce in great measure the signal from H_2O
- First, consider all signals other than H_2O
  - these will not be affected by the pulses selective for H_2O, so the result is just the spin-echo we saw previously



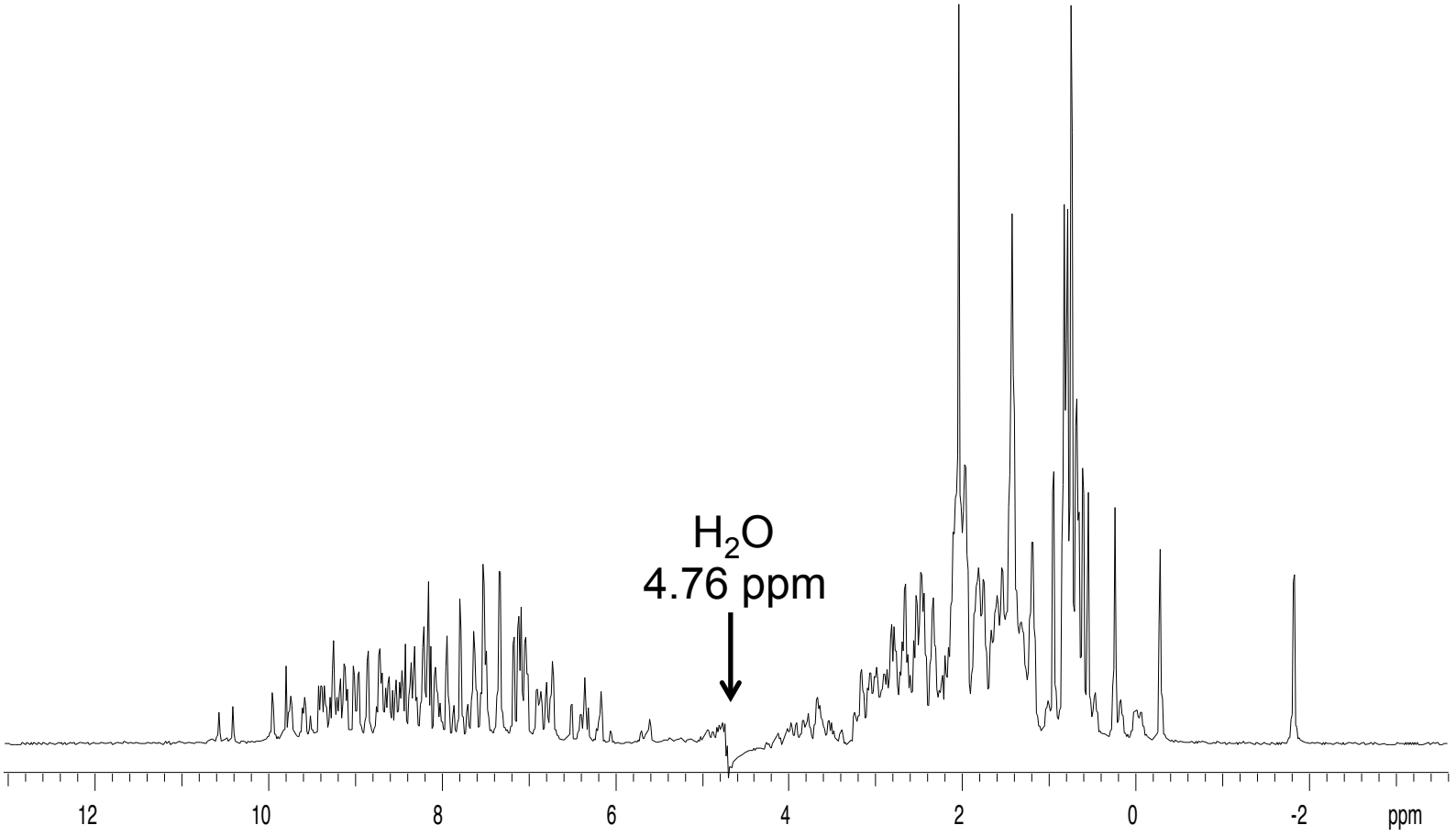


- For H_2O, the 90°-*y*, 180°*y*, 90°-y pulses move all H_2O magnetization back to where it started (dephased)
  - the second gradient pulse then just continues dephasing the H_2O (so, not observed)



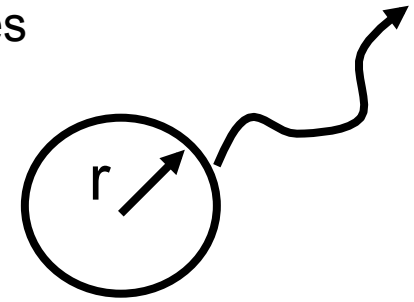
1D ^{1}H Water-Suppressed Spectrum *Pf*-Rubredoxin in $^{1}H_2O$

- Works remarkably well (combined with very good shimming, good phase cycling, very accurate pulse angle calibrations, etcetera)



Translational Diffusion Constants for (Macro)molecules

- NMR methods employing pulsed field gradients (PFG) are commonly used to study molecular diffusion
  - the PFG experiments we've seen thus far depend on the fact the diffusion is very slow on the NMR timescale
  - however, it is not insignificant, and PFG methods can be employed to measure diffusion constants and relative translational diffusion rates



- Diffusion studies can be used for many purposes:
  - measure the effective size of a protein
  - study protein complexes or aggregates
  - analyze/detect protein aggregation
  - identify and study protein-protein interactions
  - screen for ligand binding and otherwise study interactions of proteins with ligands
- The average displacement (squared) is proportional to the diffusion constant
  - the diffusion constant, D, is dependent on viscosity (η) and molecular size (r, radius)

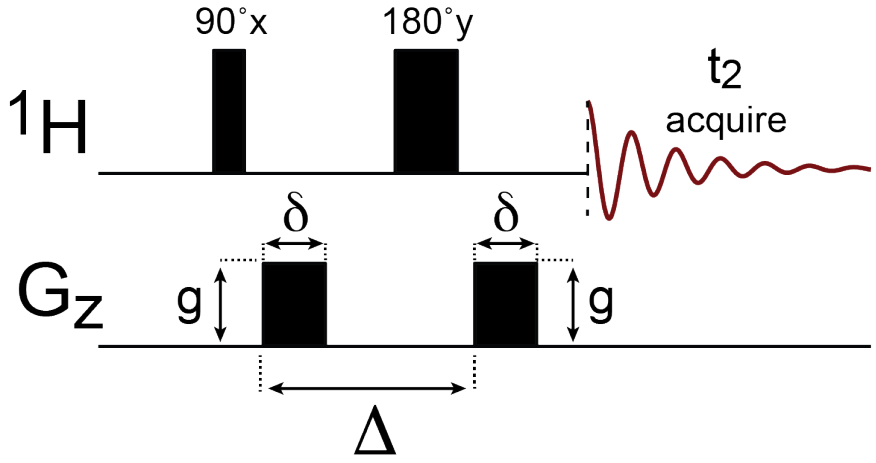
$$(Z_1 - Z_0)^2 / N = Dt \qquad D = kT / (6\pi\eta r) \text{ (Stokes formula)}$$

  - $D \propto \sqrt[3]{(MW)}$ ($MW \propto r^3$)

- PFG methods for measuring diffusion rely on the fact that magnetization dephased with a gradient pulse will not rephase fully if the molecule moves (remember, phases are spatially encoded)

Measuring Diffusion Using Field Gradient Pulses

- Stejskal and Tanner pulse sequence for diffusion measurement



- This is just the gradient 'spin-echo' experiment we saw previously
  - if the molecule moves significantly (translationally, z-direction) during the time D (time between beginning of one gradient pulse and beginning of second), then the magnetization is not refocused fully and the signal decreases
  - thus, diffusion is detected by signal intensity decrease
- Intensity of signal decreases as molecule diffuses according to:

$$I = I_0 e^{\left[(-2\tau/T_2)-\gamma^2 g^2 \delta^2 D(\Delta-\delta/3)\right]}$$

linear form, $\ln[I/I_0] = \left(-2\tau/T_2\right) - \gamma^2 g^2 \delta^2 D(\Delta - \delta/3)$

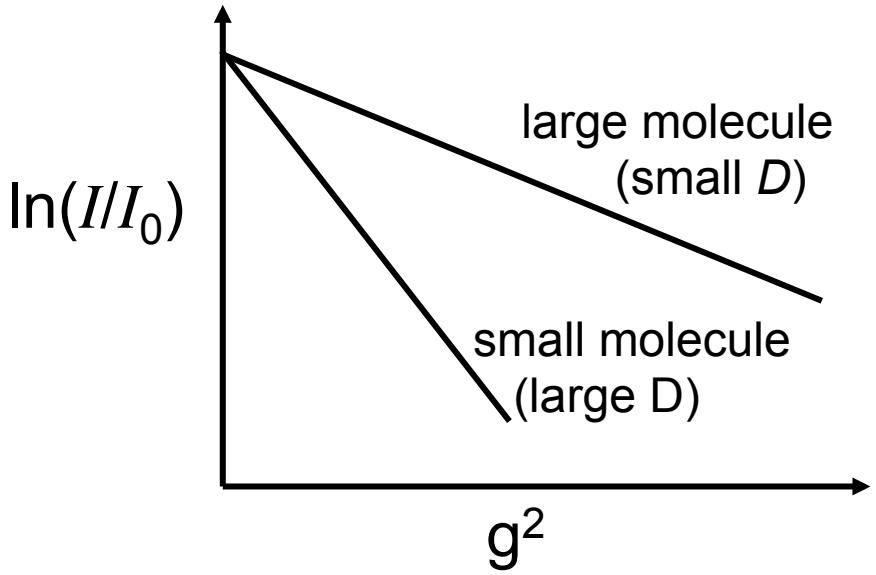
  - can measure change in intensity as a function of either δ, Δ, or g
  - changing δ or Δ introduces additional complications due to signal loss from relaxation, so the nearly universal practice is to change g

Measuring Diffusion Using Field Gradient Pulses

$$I = I_0 e^{\left[(-2\tau/T_2)-\gamma^2 g^2 \delta^2 D(\Delta-\delta/3)\right]}$$

linear form, $\ln[I/I_0] = (-2\tau/T_2) - \gamma^2 g^2 \delta^2 D(\Delta - \delta/3)$

- In linear form, the slope of ln (I/I_0) versus g^2 is proportional to *D*
  - units: g (gauss/cm), γ ($s^{-1}gauss^{-1}$), *D* (cm^2s^{-1})
- Small molecules diffuse fast, so plot drops off fast with g^2, not as fast for large molecules

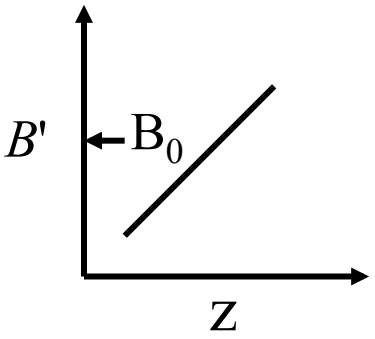


- Diffusing measurements are limited by T_2
  - the simple Stejskal and Tanner pulse sequence is prone to T_2 related problems when working with large molecules
  - newer, improved methods perform better overall, in particularly for larger molecules (for instance, Alteiri et al. (1995) *J. Am. Chem. Soc*. 117, 7566-7567)

Coherence Selection Using Pulse Field Gradients

- Consider the simple Hamiltonian (neglecting chemical shift) including B_z (field due to gradient pulse)

$$B_z' = B_0 + B_z \qquad \hat{\boldsymbol{H}}(r) = -\sum_k \gamma_k [B_0 + B_z(r)] \hat{\boldsymbol{I}}_{kz}$$



- In order to understand how to select coherence using field gradient pulses, it is convenient to demonstrate their effects on our product operators
  - first, recall that we can write operators in terms of raising and lowering operators

$$\hat{\boldsymbol{I}}_x = (\hat{\boldsymbol{I}}_+ + \hat{\boldsymbol{I}}_-)/2 \qquad \hat{\boldsymbol{I}}_{kx} = (\hat{\boldsymbol{I}}_{k+} + \hat{\boldsymbol{I}}_{k-})/2$$

  - consider the + and - operators as counter-rotating magnetization components

$$\mathbf{I}_{kz} \xrightarrow{-\gamma_k B_z(z)\mathbf{I}_{kz}\tau} \mathbf{I}_{kz}$$

z-magnetization doesn't precess, isn't in transverse plane, so isn't defocused (affected) by gradient pulse

$$\mathbf{I}_k^+ \xrightarrow{-\gamma_k B_z(z)\mathbf{I}_{kz}\tau} \exp(\mathrm{i}\gamma_k B_z(z)\tau)\mathbf{I}_k^+$$

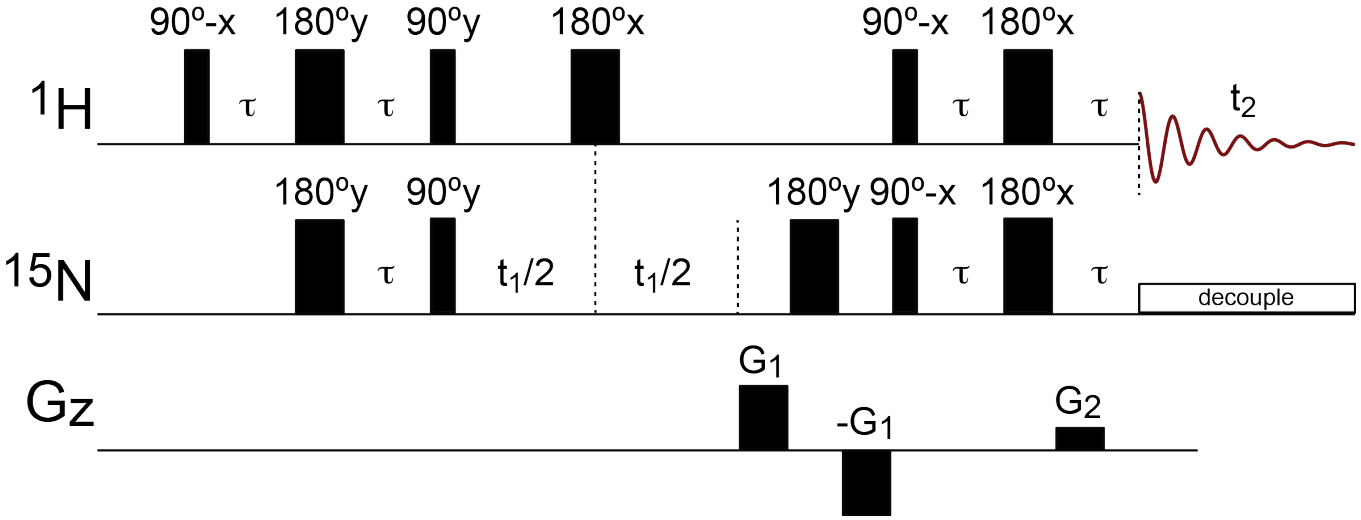
precession due to the field gradient in one direction (i.e. gradient increases precession frequency)

$$\mathbf{I}_k^- \xrightarrow{-\gamma_k B_z(z)\mathbf{I}_{kz}\tau} \exp(-\mathrm{i}\gamma_k B_z(z)\tau)\mathbf{I}_k^-$$

precession due to the field gradient in the other direction (i.e. gradient decreases precession frequency)

- For linear (z) gradients, we know $B_z(z)$ (the gradient strength, G_z)
- Observables (M_x, M_y) are integrals over z
  - these are zero: proportional to sum of I_k^+ and I_k^- (why signals go away, no net difference in + and - components, so no net transverse magnetization in any direction

Gradient Selected HSQC



- At the end of the t_1 evolution period, the ^{15}N magnetization is dephased (spatially encoded) twice by G_1 (G_1, 180, $-G_1$) (i.e. phase encoded by $\gamma_N 2G_1$)
- In order to decode (in order to get observable magnetization in t_2), after transferring to ^1H, $\gamma_N 2G_1$ must equal $\gamma_H G_2$ (for maximal signal)

$$I^+(t_2) \propto \int_z \{S^+(t_1) \exp[i\gamma_N 2G_1 z] \exp[-i\gamma_H G_2 z]\}$$
$$\propto \int_z \{S^+(t_1) \exp[i(\gamma_N 2G_1 z - \gamma_H G_2 z)]\}$$

- the only magnetization observed is that defocused by $G_1/-G_1$ and refocused by G_2
- all spurious magnetization components are defocused by the gradients and never refocused (so, spectra are very clean, no spurious signals, without phase cycling)
- quadrature achieved by cycling gradients, or final 90° ^{15}N pulse (+x/-x) and G_2 (+/-)