Basic NMR Operation for Beginners

Steps for NMR Experiments – larger molecule (biomolecules)

by Wen-Jin Wu, 09/06/2004
winston@ibms.sinica.edu.tw
Outline

• introduction of biomolecular NMR
• water suppression techniques
• brief introduction of pulsed field gradients
• 1H,15N-HSQC
• HNCO
Information Content

1H-1H COSY (2-, 3-bond)
- DQF-COSY, E. COSY
- Dihedral angle constraints

Hetero-nuclear COSY (one-bond)
- 1H,15N or 1H,13C-HSQC, HMQC

TOCSY (2-, 3-bond)
(1-bond for 13C-13C)
- Total Correlation

1H-1H NOESY
(through space, <5 Å)
- Intensity & 1/r6
- Distance constraints

Figures from Bruker’s NMR Guides
Some Basic NMR Pulse Sequences

figures from Bruker's NMR Guides
Peptide/Protein NMR Studies

**Peptide/Protein NMR:**

- NMR experiments are usually performed in H$_2$O (with 5-10% D$_2$O):
- Signals detected are pH dependent (N-H protons exchange with water).
- Water suppression is required.

\[
\text{N-H} + \text{H}_2\text{O} \rightleftharpoons \text{N-*H} + \text{H-O-H}
\]

- Higher molecular weight (NOESY V.S. ROESY), shorter $T_2$, three-bond $^1$H-$^1$H J-coupling is less efficient with increasing molecular weight (correlation time), isotope labeling ($^{15}$N/$^{13}$C/$^2$H) become necessary when #A.A.>50.

A $^1$H, $^{15}$N-HSQC ($^1$J$_{NH}$=90 Hz) or a $^1$H, $^{13}$C-HSQC ($^1$J$_{CH}$=140) is much more sensitive than a $^1$H-$^1$H COSY ($^3$J$_{HH}$<15) experiment.

Typical J-coupling constant values in peptide
Cross Relaxation (NOE) Depends on Tumbling Rates

- ROESY for small molecules (1-2 kDa).
- NOESY for sizeable molecules (>2 kDa)

![Diagram showing the dependence of NOE on molecular weight and tumbling rates.](figure from p37 of “The Nuclear Overhauser Effect” by D. Neuhaus and M. Williamson)
# Strategies for Protein NMR Studies

## Table 1: Strategies for protein studies

<table>
<thead>
<tr>
<th>Protein/Size</th>
<th>Experiment</th>
<th>Information obtained</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled/ less than 50.a.a.</td>
<td>2D Homonuclear</td>
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<td></td>
<td>COSY, TOCSY</td>
<td>intra-residue assignments</td>
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<td></td>
<td>NOESY</td>
<td>sequential connectivities</td>
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<td>NOE distance constraints</td>
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<td></td>
<td></td>
<td>$^3J_{N-H}$ coupling constants</td>
<td></td>
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<tr>
<td>$^{15}$N-labeled/ ~ 50-80.a.a.</td>
<td>3D</td>
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<tr>
<td></td>
<td>$^{15}$N-TOCSY</td>
<td>intra-residue assignments</td>
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<td></td>
<td>$^{15}$N-HNHA</td>
<td>$^3J_{N-H}$ coupling constants</td>
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<td></td>
<td>or 2D HMQC-J</td>
<td>$^3J_{N-H}$ coupling constants</td>
<td></td>
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<tr>
<td></td>
<td>$^{15}$N-HNHB</td>
<td>$^3J_{H-H}$ coupling constants</td>
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</tr>
</tbody>
</table>

*Typical J-coupling constant values

*Table taken from Bruker’s Avance 3D/ Triple Resonance manual*
From Bruker's Avance 3D/ Triple Resonance manual

The experiments presented in this manual are denoted by an asterisk.
Water Suppression

Why water suppression?

- $[\text{H}_2\text{O}] \approx 110 \text{ M}$, $[\text{Protein}] \approx 1 \text{ mM}$
- $110 \text{ M} / 1 \text{ mM} = 110,000$
- Suppression of the strong solvent signal is necessary in order to obtain high signal to noise for the protein peaks.
Water Suppression: Some Consideration

\[
\text{N-H} + \text{H}_2\text{O} \rightleftharpoons \text{N-*H} + \text{*H-O-H}
\]

**Saturation transfer**: saturation of water also reduce the intensity of the resonances that are in exchange with water.

Labile, exchange with water (pH-, structure-, temperature-dependent)
Amide Proton Exchange Rate

\[ \text{N-H} + \text{H}_2\text{O} \rightleftharpoons \text{N}^*\text{H} + \text{H-O-H} \]

~330 times faster (pH 7 VS pH 5)

In practice: the pH value for a protein sample for NMR studies is kept below 7.5

Figure modified from p151 by Cavanagh et. al “Protein NMR Spectroscopy”
Water Suppression

- Presaturation (‘zgpr’)
- Watergate (‘zggpwg’)
- Water flipback (‘*fp*’)
- Jump and return, 1-1, 1331
- Coherence pathway rejection (Echo/anti echo)
Usually during the relaxation delay, apply a low power continued wave (C.W.) irradiation before the first 90 degree pulse.

Pulprog: zgpr

**Power level:**
- *pl9*: for weak saturation on channel 1
- *pl1*: high power level for channel 1
Drawback of Presaturation

• Saturation transfer to the exchangeable NH protons

• Bleaching of signals near water

• Large dispersive tail of water signal: tilted baseline
A field-gradient pulse is a pulse or a period during which the magnetic field is made deliberately inhomogeneous.

\[ B = B_0 + B_G(z) \]

The magnetic field, generated by a gradient pulse, \( B_G(z) \) varies linearly along the Z-axis

\[ B_G(z) = zG_z \]

where

\( G_z \): gradient strength (G/cm), \( Z \): z-axis position

Viewing on the rotating frame, spins at different z-position acquire different phase (Larmor frequencies):

\[ \phi(z) = \gamma z G_z t \]

where \( \gamma \): gyromagnetic ratio, \( t \): gradient duration

A coherence can be dephased by a strong pulse field gradient. A dephased coherence can be refocused by a refocus-gradient providing the “overall phase change “ is zero.

\[ \phi_i + \phi_f = 0 \]; Coherence refocused.

\[ \phi_i + \phi_f \neq 0 \]; Coherence dephased.
Pulsed Field Gradient (PFG)

Viewing from the Z-axis:

• **Z-gradient destroy transverse magnetization.**

• The destroyed magnetization can be refocused by another z-gradient pulse of the same amplitude but of opposite phase. (or use a 180 pulse in between two identical z-gradient pulse).

• **H_2O:** the two extra selective 90 pulse on water makes the 2nd z-gradient pulse act as another defocus gradient pulse. Water is suppressed.

• **Protein signals:** the 180 pulse makes the 2nd Z-gradient act as a refocus gradient. Protein signals are observed.

Ref: M. Piotto, V. Saudek & V. Sklenar, *J. Biomol. NMR* 2, 661 - 666 (1992)
**Watergate**

- **Parameter adjustment, Pulprog=zggpwg**
- **p11**: pulse length for 90 degree shaped pulse
- **sp1**: power level for 90 degree shaped pulse
- **spnam1**: type of shaped pulse

**For example**: set spnam1=Sinc1.1000, p11=1.5-2 msec,
Minimize the fid (less water signal) by adjusting the power level “sp1” under the “gs” utility.
3-9-19 Watergate

- Off resonance DANTE excitation technique.
- **3-9-19**: $3\alpha - \tau - 9\alpha - \tau - 19\alpha - \tau - 19\alpha - \tau - 3\alpha$, where $\alpha = 180/26$-degree hard pulse, $\tau =$ delay.

![Graph showing the center of maximal intensity and nulls](null)

**Delay $\tau = 1/(4 \Delta \nu_{\text{max}})$, where $2\Delta \nu_{\text{max}} =$ distance of next null (Hz). (The delay $\tau$ is field-dependent !)**

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**Parameter adjustment:** Pulprog=p3919
Set pl18=pl1, p27=p1, p0=p1
;d19: delay for binomial water suppression
;d19 = $1/2*d$, $d =$ distance of next null (in Hz)

Adjust d19 according to the magnetic field strength and where you want the center of maxima excitation to be.

**For example:** Have the center of NH region (i.e. 8.2 ppm) to be the center of maximal excitation region:

t=$1/[4*(8.2-4.75)*600.13] = 121$ usec @600 MHz machine

t=$1/[4*(8.2-4.75)*500.13] = 145$ usec @500 MHz machine
(Carrier frequency on H$_2$O at 4.75 ppm)
**Water Flip-back Watergate**

- Water is aligned along the z axis before any Z-gradient pulse point “a”.
- Water is not destroyed by the z-gradient pulse; this reduces the signal loss of exchangeable protons due to attenuation of water Signal.

Parameter adjustment:

**Pulprog=fp***, i.e. “hsqetf3gp” calibrate the shape pulse as describe in WATERGATE.
Pulse Sequence for Observing Fast-Exchanging Protons

\[
\text{N-H + } ^*\text{H}_2\text{O} \leftrightarrow \text{N-}^*\text{H} + ^*\text{H-O-H}
\]

Imino protons in DNA, hydroxyl protons (-OH), Histidine side chain protons in proteins are usually in a fast exchange process with water.

- Flip-back WATERGATE (marginal performance)
- Jump and return 1-1
- 1-3-3-1
Jump and Return: 1-1

![Diagram of pulse sequence with notation and graph](image)


- **Water signal**: “on resonance”, aligned to the “z” axis, null signal.
- **Protein signals**: free to precess on the transverse plan
- **Peak Intensity**: \( I_x \sin(\Omega \tau) \)
- **Delay** \( \tau = 1/(4\Delta \nu_{\text{max}}), \Delta \nu_{\text{max}}=\text{distance of maxima intensity} \)

**Parameter adjustment**: Pulprog=p11

- **p1**: 90 pulse, **p0**: 90 degree “return” pulse, adjust p0 to be 0.1-0.2 usec less than p1.
- **d19**: \( d19 = 1/2*d \), \( d = \text{distance of next null (in Hz)}=2*\text{distance of maxima intensity} \)

*The delay time is magnetic field-strength dependent!*

**For example**: To observe a peak at 14 ppm (o1p on water at 4.75 ppm)

- \( d19=1/[4*(14-4.75)*600.13]=45 \text{ usec (at 600 MHz)} \)
- \( d19=1/[4*(14-4.75)*500.13]=54 \text{ usec (at 500 MHz)} \)
Binominal: 1-3-3-1

\[ a^{*}P_{1} b^{*}P_{1} a^{*}P_{1} \]

\( d_{1} \) \( \tau \) \( \tau \) \( \tau \) Acqu

\( \tau = \frac{1}{2\Delta \nu_{\text{max}}} = \frac{1}{d} \), 
\( \Delta \nu_{\text{max}} \) = distance of maximal intensity 
\( d \) = distance of next null

Parameter adjustment:
- **Pulpro**=p1331
- \( d_{19} \): delay for binominal water suppression
- \( d_{19} = \frac{1}{d} \), \( d \) = distance of next null (in Hz)=2*distance of maximal intensity
- \( d_{19} = \tau \) as defined above

**For example:** To observe a peak at 14 ppm (o1p on water at 4.75 ppm)
\[ d_{19} = \frac{1}{2 \times (14-4.75) \times 600.13} = 90 \text{ usec at 600 MHz} \]
\[ d_{19} = \frac{1}{2 \times (14-4.75) \times 500.13} = 108 \text{ usec at 500 MHz} \]

Binomial excitation profiles of 1-1 and 1-3-3-1.

Both are for observing fast exchanging protons.

- **1-3-3-1**: Better water suppression (higher receiver gain), but with offset-dependent phase distortion (unsuitable for 2D)

- **1-1**: low receiver gain, the dispersive tail of water interferes with the signals of interest.
What are you trying to detect?

- p1331
- Presat
- Watergate
Water Suppression via Coherence Pathway Rejection

Coherence pathway selected by gradients:

In a gradient selection experiment (echo/antiecho), the water coherence is not “refocused” by the refocus gradient (therefore, is not selected), this naturally suppresses the water signal.

Example: *cosydfetgp.1*, *hsqcetf3gp*

*Figure from John Cavanagh et al., “Protein NMR Spectroscopy: Principles and Practice”, Academic Press (1995)*
Practical Implementation: 1D, 2D and 3D

- **Exchangeable NH**: Water-flip-back HSQC, Fast-HSQC, echo/anti echo WATERGATE HSQC (compare sensitivity with real samples).

- **Signals near water (i.e. H_α)**: (i.e. TOCSY, COSY, NOESY) WATERGATE with selective pulse, echo/anti echo PFG.

- **Fast exchangeable proton (His sidechain, -OH)**: 1-1 (good for 2D), 1331 (not suitable for 2D, 3D).
Standard Configuration of NMR Channels in IBMS

1H: F1 (channel 1)
13C: F2 (channel 2)
15N: F3 (channel 3)
2H: F4 (channel 4)

The pulse sequence “hsqcetf3gpsi” is by default 15N-HSQC
The pulse sequence “hsqcetgpsi” is by default: 13C-HSQC
Power level and limitation

- Bruker uses “attenuation level” (dB) to represent power level. So, the larger dB number, the more attenuation and therefore, weaker power.

For example: on 1H (F1 channel), a power level of 55 dB is stronger than 61 dB. -2 dB stronger than 0 dB. -3 dB stronger than -2 dB,

- Each channel (1H, 13C, 15N, 2H) at each probe at each spectrometer (500-800 MHz) head has its limitation on maximal power, so please DO NOT exceed the maximal power level limitation (see “pulse width calibration table” for each probe.) If the P90 of 13C is 10.2 us at -3 dB, don’t use -4 dB etc.

- After you read in our standard parameter file, you only have to change the 90-degree pulse on 1H (salt-dependent), the pulse widths for 13C and 15N are quite insensitive; use our default values, and Do Not change them.
Coherence Order

- Zero quantum coherence: \( I_z S_z \) etc
- Single quantum coherence: \( I_x S_z, I_y S_z, I_z S_y \) etc
- Double quantum coherence: \( I_x S_x, I_x S_y \) etc
- Triple quantum coherence: \( I_x K_x S_x, I_x K_x S_y \) etc

- **HSQC**: Hetero-nuclear single quantum coherence. \( I_x S_z \)
- **HMQC**: Hetero-nuclear multiple quantum coherence.
**HSQC:** Hetero-nuclear single quantum coherence

\[ d_{26} = \frac{1}{4} J_{XH} \]

- \( J_{CH} = 140 \text{ Hz (aliphatic)} \)
- \( J_{NH} = 90 \text{ Hz} \)

*figure from Bruker’s NMR Guide*
Setting up an $^{15}$N-HSQC Experiment

(1) read in the standard parameter file for **2D 1H-15N HSQC**
   (pulprog=hsqcetf3gpsi2):
   \textit{rpar 2d\_15n\_hsqc\_etsi2} (on the 800 MHz spectrometer)
   \textit{rpar CRP\_2D\_15N\_HSQC\_ETS1} (on the AV600 with a CryoProbe)
   (check the specific spectrometer manual for the exact name)

(2) change p1 (1H), o1, sw (1H) that you’ve determined from 1D, take a quick
    spectrum with a large spectral width (40 ppm for 15N for o3p at 116-120 ppm)
    Use minimal number of scans required, use small ni (i.e. ni=32).

(3) process and inspect the spectrum

(5) change o3p to the center of 15N region
under the \texttt{eda} window (not just under the command line)

(6) acquire another quick spectrum

(7) acquired spectrum with minimal spectra width, and sufficient t1 experiments in
    the 15N dimension (i.e. ni=128)
Setting up an $^{15}$N-HSQC Experiment

(A) $o_{3p} = 116$ ppm ($sw=40, ni=32$)

(B) change $o_{3p}$ to the center of the 15N region $o_{3p} = 119.5$ ppm

(c) reduced spectral width for good digital resolution $sw (15N=32$ ppm)

(D) acquire the final spectrum with sufficient NS and $t_1$ experiment ($ni=128$)
Note: If the protein is double labeled with both 13C&15N, apply adiabatic 13C inversion pulse during 15N evolution to remove the 13C-15N J-coupling.

enter “-DLABEL_CN” in the ZGOPTNS field under the EDA window.
Spread the Resonances into the Third Dimension

3D NOESY-HSQC

3D HNCO

Figure from Bruker NMR Guide
3D HNCO:
Correlation between $^1H^N_i - ^{15}N_{i-1} - ^{13}C'_{i-1}$

- $o3p$ ($^{15}N$) at ~119 ppm, $sw$~32 ppm
- $o2p$ ($^{13}C$) at ~176 ppm, $sw$~22 ppm

Look at the 1st fid to optimize parameters, and acquire a short 2D version of a 3D to optimize SW, ni, o2p, o3p, and make sure the first 2D plane look normal before a long 3D experiment.
Carbonyl (C') center ~176 ppm (15-25 ppm wide)
The aromatic resonances center at ~ 120 ppm (~ 54 ppm wide),
The aliphatic resonance ~ 10-75 ppm
C_\alpha centers at ~ 56 ppm (sw ~35 ppm)
C_\alpha-C_\beta center at ~ 39 ppm (sw~75 ppm)
Intrinsic Problems of Solution NMR of Large Molecules

- Many more signals: spectral overlap
- Slow tumbling: fast transverse relaxation rate (short $T_2$)
- Faster decay of signal: poor signal to noise (s/n)

Linewidth $\Delta \nu_{1/2} = 1/(\pi T_2)$

Figures from M. Sattler’s website
When Molecular Weight >20 KDa: TROSY at High Magnetic Field

TROSY: Transverse Relaxation-Optimized Spectroscopy

45 kDa, 750 MHz