NMR IV: Resonance Assignment Strategies for Proteins

1. $^1$H NMR: No isotope enrichment

   2D TOCSY and NOESY $\leq 8-10$ kDa

2. $^1$H/$^{15}$N NMR: Uniform $^{15}$N-enrichment

   3D $^{15}$N-resolved TOCSY and NOESY $\leq 12-15$ kDa

3. $^1$H/$^{15}$N/$^{13}$C NMR: Uniform $^{13}$C/$^{15}$N-enrichment

   3D triple-resonance NMR $\leq 18-25$ kDa

4. $^1$H/$^{15}$N/$^{13}$C/$^2$H NMR: Uniform $^2$H/$^{13}$C/$^{15}$N-enrichment

   3D TROSY-based quadruple-resonance NMR $\leq 40-100$ kDa
Assignments are the key prerequisite for characterizing proteins (and nucleic acids) by NMR

Sequence-specific Resonance Assignments ($^1$H, $^{13}$C, $^{15}$N)

↓

2°/3° structures in solution

Internal mobility in solution

Protein-ligand or protein-protein interactions

Charge state of ionizable groups (pKₐ values)

Conformational exchange rates and equilibria

Protein hydration
**Resonance Assignments**

Figure 1.1. Information content of $^1$H–$^1$H NOE's in a polypeptide chain with and without sequence-specific resonance assignments. Open circles represent hydrogen atoms of the polypeptide. The polypeptide chain is represented by the horizontal line in the center.

(Wüthrich, 1986)
NMR RESONANCE ASSIGNMENT STRATEGIES
FOR PROTEINS

Strategy 1: No isotope enrichment
2-D TOCSY and NOESY
- uses \(^1\text{H}\) only
- unenriched protein (as is)

Strategy 2: Uniform \(^{15}\text{N}\)-enrichment
3D \(^{15}\text{N}\)-resolved TOCSY and NOESY
- uses \(^1\text{H}\) and \(^{15}\text{N}\) NMR
- \([\text{U-99\%} ^{15}\text{N}]\) protein sample

Strategy 3: Uniform \(^{13}\text{C}\) and \(^{15}\text{N}\)-enrichment
3D/4D Triple-resonance NMR
- uses \(^1\text{H}\), \(^{13}\text{C}\), and \(^{15}\text{N}\) NMR
- \([\text{U-99\%} ^{13}\text{C}, ^{15}\text{N}]\) protein sample

Strategy 4: Uniform \(^{13}\text{C}\) and \(^{15}\text{N}\)-enrichment
\(^2\text{H}\) enrichment with amide and methyl protonation
3D/4D Quadruple-resonance NMR
TROSY
- uses \(^1\text{H}\), \(^2\text{H}\), \(^{13}\text{C}\), \(^{15}\text{N}\) NMR
- \([\text{80-90\%} ^2\text{H},
\text{99\%} ^{13}\text{C}, ^{15}\text{N}]\) protein sample
Strategy 1 for Determining Sequence-Specific Resonance Assignments

2-D TOCSY

\[ \geq 3 \] (covalent bonds)

Through-bond connections

Intra-residue connections (defines residue type)

\[ \leq 5 \text{ Å} \]

2-D NOESY

Through-space connections

Inter-residue connections (defines neighboring residues)

We are NOT sequencing the protein by NMR!!

Clusters of peptide (NMR peaks corresponding to...)

Peptide segments

\[ ^{1}H \]

Sequence-specific resonance assignments

Sequence

(only)
Strategy 1: 
$^1$H 2-D NMR

Val  Ala

2D TROSY $\Rightarrow$ through-bond, intra-residue
(dots) $(\leq 3$ covalent bonds$)$

2D NOESY $\Rightarrow$ through-space, intra- and inter-residue
(solid lines) arrows $(\leq 5\text{ Å})$
2-D TOCSY  
(Total Correlation Spectroscopy)

90°  
$t_1$  
Spin-lock  
$t_2$

\[ \text{FT}(t_2) \]
\[ \text{Transpose} \]
\[ \text{Interferogram} \]
\[ \text{FT}(t_1) \]

Diagonal  
$F_1 = F_2$

Gives total spin system correlation along one line
Shows correlations for one Val residue

2D TOCSY: STRIP PLOTS
(real data)
(30-residue peptide)

NH
8.10

Hα
5.10

Hβ
1.50

Hγ
0.00

Entire 2D spectrum

F2 (ppm)
5.0

F1 (ppm)
8.01
1.9
0.9
"Ballpark" Chemical Shift Values ($^1$H) Used for the Assignment Process

2.1 SPIN SYSTEMS IN AMINO ACID RESIDUES

<table>
<thead>
<tr>
<th>Residue</th>
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<th>δH</th>
<th>βH</th>
<th>Others</th>
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<td>4H  7.14</td>
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</table>

* Data for the nonterminal residues X in tetrapeptides GAXA, pH 7.0, 35°C (from Bundi and Wüthrich (1979a), except that more precise data were obtained for Leu, Pro, Lys, Arg, Met, and Phe using nmr measurements at 500 MHz).  
$^b$ Data for trans-Pro.  

Biopolymer 18, 285-298  
(Wüthrich, 1986)
2-D NOESY

(Nuclear Overhauser and Exchange Spectroscopy)

NOE transfer returns spins to xy plane for detection

\[ \begin{align*}
\text{selective inversion to} & \\
\text{mixing time (fixed)} & \\
\text{allows transfer of magnetization} & \\
\text{between protons nearby in space} & \\
\text{(within \( \pm 5\AA \))} & \\
\end{align*} \]

\[ \text{2D FT} \]

\[ F_1 = F_2 \]

Off-diagonal cross peak indicates through-space correlation
region a $\Rightarrow$ amide 'H's, aromatic 'H's
  (amide$\leftrightarrow$amide amide$\leftrightarrow$aromatic aromatic$\leftrightarrow$aromatic correlations)

b $\Rightarrow$ amide$\leftrightarrow$alpha, aromatic$\leftrightarrow$alpha

c $\Rightarrow$ amide$\leftrightarrow$aliphatic side chain

d $\Rightarrow$ alpha$\leftrightarrow$alpha

(Wüthrich, 1986)

e $\Rightarrow$ alpha$\leftrightarrow$aliphatic side chain

f $\Rightarrow$ side-chain$\leftrightarrow$side-chain
Example: "NOESY Walk" for Zinc-finger peptide

Three-Dimensional Structure of a Zinc Finger
Figure 8.2. Unique amino acid residues (arrows) and selected unique dipeptide segments (large letters) in the lac headpiece, which are suitable references for obtaining sequence-specific $^1H$ NMR assignments.

### TABLE 8.2. Probability That a Predetermined Di-, Tri-, or Tetrapeptide Segment Is Unique in a Protein with Less Than 200 Residues

<table>
<thead>
<tr>
<th>Number of Amino Acid Types</th>
<th>Probability of Uniqueness (%)</th>
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<tbody>
<tr>
<td></td>
<td>Dipeptide</td>
<td>Tripeptide</td>
<td>Tetrapeptide</td>
</tr>
<tr>
<td>18$^b$</td>
<td>56</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>8$^c$</td>
<td>15</td>
<td>53</td>
<td>83</td>
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<td>13$^d$</td>
<td>33</td>
<td>78</td>
<td>95</td>
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<td>15$^e$</td>
<td>45</td>
<td>91</td>
<td>98</td>
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* Statistical data on 1905 amino acid sequences of globular proteins shorter than 200 residues taken from the protein-sequence data base of the National Biomedical Research Foundation, Washington, D.C., USA.

$^b$ Common L-amino acids, with Asx = Asp + Asn, Glx = Glu + Gln, since these are often listed as Asx or Glx in the data base.

$^c$ Gly, Ala, Val, Leu, Ile, Thr, □ = all AMX spin systems of $\alpha$CH--$\beta$CH$_2$, ■ = all others (long side chains).

$^d$ Same as footnote c, except that the eight AMX species are further distinguished as follows: Phe, Tyr, Trp, His, Ser, □ = Cys, Asp, and Asn.

$^e$ Same as footnote d, except that the following three subgroups of long side chains are distinguished: Pro; ▽ = Lys and Arg; ▲ = Met, Glu, and Gln.

(Wüthrich, 1986)
End Result of Stage I (Strategy 1):

'H Resonance Assignment Table

Table 1: Proton Resonance Assignments of the Enhancer Binding Protein Zinc Finger Peptide at 6 °C and pH 5.8*

<table>
<thead>
<tr>
<th>residue</th>
<th>NH</th>
<th>C^H</th>
<th>C^OH</th>
<th>chemical shift (ppm)</th>
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<tr>
<td>R1</td>
<td>3.84</td>
<td>1.58*</td>
<td>1.00</td>
<td>C^H 1.75, 1.65; C^H 2.69, 2.58</td>
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<tr>
<td>P2</td>
<td>4.28</td>
<td>2.04*</td>
<td>1.22</td>
<td>C^H 2.16, 2.04; C^H 3.70, 3.70</td>
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<tr>
<td>Y3</td>
<td>7.42</td>
<td>2.95*</td>
<td>2.71</td>
<td>C^H 6.91; C^H 6.81</td>
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<tr>
<td>H4</td>
<td>9.56</td>
<td>3.26*</td>
<td>2.97</td>
<td>C^H 6.76; C^H 7.91</td>
</tr>
<tr>
<td>C5</td>
<td>8.34</td>
<td>3.40*</td>
<td>2.71</td>
<td>C^H 6.88; C^H 6.74</td>
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<tr>
<td>S6</td>
<td>9.04</td>
<td>3.51*</td>
<td>3.30</td>
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<td>Y7</td>
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<td>2.72*</td>
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<td>K15</td>
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<td>1.16</td>
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*Chemical shifts are reported with respect to 4,4-dimethyl-4-silapentane-1-sulfonate. Stereosepecific assignments are denoted as follows: for the C^H methylene protons, the asterisk indicates the H^D proton; for the NH_3 protons of Asn, the asterisk indicates the H^D proton that is cis to C^H; for the methyl protons of Leu, the asterisk refers to the C^H methyl group. +The chemical shifts of the C^H protons at 25 °C are 2.88 and 2.82 ppm, with the downfield resonance corresponding to the H^D proton.
As proteins \( \uparrow \) in MW, more peaks occupy the "same-size box".

Figure 2: A 2D NOESY spectrum of 5mM lysozyme in 90\% H\textsubscript{2}O, acquired on a UNITY 500 with a PFG triple resonance probe.

Two Problems

1. Poor resolution: severe peak overlap
2. Low information content
To overcome limitations of strategy 1:

**Strategy 2:**

Uniformly $^{15}$N-enriched protein
$^{15}$N-resolved 3-D TOCSY and NOESY

( utilizes $^3J_{HH}$, $^1J_{NH}$ and $'H-'H$ NOE)

2D HSQC: $'H-'^N$ correlation ($^1J_{NH}$)
2D TOCSY: $'H-'H$ correlation ($^3J_{HH}$)
2D NOESY: $'H-'H$ correlation ($'H-'H$ NOE)

Combine the above to produce:

3D TOCSY-HSQC
3D NOESY-HSQC
HSQC Apo I-BABP

Mostly βb NH's

"Fingerprint" of protein backbone

1^N (ppm)

1^H (ppm)

NH

Heteronuclear Single Quantum Correlation
a) \[ ^1H \quad t_1 \quad 90 \quad t_{mix} \quad 90 \quad t_2 \quad \text{ACQ.} \quad 20 \text{ NOESY} \]

b) \[ ^1H \quad 90 \quad 180 \quad 90 \quad 180 \quad 90 \quad 180 \quad t_2 \quad \text{ACQ.} \]

\[ ^{15}N \quad 180 \quad 90 \quad 90 \quad 180 \quad \text{DECOPUELLE} \]

\[ 20 \text{ HSQC} \]

c) \[ ^1H \quad t_1 \quad 90 \quad t_{mix} \quad 90 \quad 180 \quad 90 \quad 180 \quad 180 \quad t_2 \quad \text{ACQ.} \]

\[ ^{15}N \quad \text{DECOPUELLE} \quad 180 \quad 90 \quad 90 \quad 180 \quad \text{DECOPUELLE} \]

3D \text{ NOESY-HSQC}

a.k.a. 3D \[ ^{15}N \text{-resolved NOESY} \]

(Con do same thing with 3D TOCSY-HSQC)

(Marion et al., 1989)
FIGURE 2: Schematic representation of the relationship between the 3D heteronuclear spectra recorded in this paper and conventional homonuclear 2D spectra. The 3D spectrum is viewed as a series of $F_1^{(1H)}-F_3^{(15N)}$ slices edited by the chemical shift of the directly bonded $^{15N}$ atom along the $F_2$ axis.

(Marion et al., 1989)
Figure 3: (A) Fingerprint regions of the homonuclear $^{15}$N-edited 2D NOESY (upper) and $^{15}$N-decoupled HOHAHA (lower) spectra of a uniformly $^{15}$N-labeled (~95%) sample of 2 mM interleukin 1β recorded at 27 °C and pH 7.5. (B) Corresponding regions of a slice from the 3D heteronuclear NOESY-HMQC (upper) and HOHAHA-HMQC (lower) spectra of the protein under identical conditions. The slice shown corresponds to a $^{15}$N chemical shift δ = 123.7 ppm downfield of liquid ammonia.

Marion et al., (1989) Biochemistry 28, 6150-6
Strategy 3: Uniform $^{13}$C and $^{15}$N enrichment
Triple-resonance 3-0 NMR
Takes advantage of $J_{CH}$, $J_{NH}$, $J_{C}=C$
through bond couplings

The larger the J value, the more efficient the "pipeline"

Circumvents need to use NOESY for inter-residue correlations
Strategy 3:
Uniform $^{13}$C and $^{15}$N enrichment
Triple-resonance 3-0 NMR

"Classic" Strategy 3:
1. 3D HNCO
2. 30 HNCA
3. 30 HCACO
4. 30 H CA(CO)N
5. 30 $^{15}$N-resolved TOCSY

through-bond correlations only:
No NOESY!
Schematic of "Classic Strategy 3"

2D Planes from 3D spectra

3-D ASSIGNMENT STRATEGY

for backbone assignments

for side-chain assignments

Hodsdon et al. (1995) J. Biomol. NMR 6, 198-210
Real Data: I-FABP (15.4 kDa)

Shows one cycle from CO \((i-1)\), through residue \(i\), to \(N(i+1)\)

(Hodson et al., 1995)
"Modern Version"

**Strategy 3:**

Uniform $^{13}$C and $^{15}$N enrichment

Triple-resonance 3-D NMR

- eliminates 3D $^{15}$N-resolved TOCSY (weak link)
- adds $\beta\beta$ correlations (more info content)

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1. 30 CBCA(CD)NNH
2. 30 HNCACB
3. 30 HNCO
4. 30 CBCA CO(CA) HA
Strategy 4:

"Uniform" $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ enrichment

Quadrupole-resonance NMR

Similar to Strategy 3 except non-amide 'H's replaced with $^2\text{H}$ (oz D)

\[
\begin{array}{c}
\text{CD}_3 \\
\text{C-D} \\
N - C_\alpha - C \\
H \\
\text{etc.}
\end{array}
\quad
\begin{array}{c}
\text{CD}_3 \\
\text{C-D} \\
N - C_\alpha - C \\
H \\
\text{etc.}
\end{array}
\]

$^2\text{H}$ "invisible" in $^1\text{H}$ NMR

(used to eliminate $^1\text{H}$ nuclei)
Transverse Relaxation of NMR Resonances

\[ I_2 \xrightarrow{\gamma_0 x} -I_y \xrightarrow{MS-S} \phi \]

Exponential decay rate

\[ \sqrt{R^*_2} \]

\[ \uparrow R_2 \Rightarrow \text{broader NMR resonances} \]

FT

\[ \text{Fast decay rate gives short, broad NMR peaks} \]

\[ \text{Slow decay rate gives tall, narrow NMR peaks} \]

\[ \downarrow \text{resolution} \]

\[ \downarrow \text{sensitivity} \]
Contributions to $R_2$ (e.g. amide group)

\[
\begin{align*}
  & R_2(\text{H}) = R_2^{\text{DD}}(\text{H-H}) + R_2^{\text{DD}}(\text{H-}^{15}\text{N}) + R_2^{\text{CSA}}(\text{H}) + R_2^{\text{EXCH}}(\text{H}) \\
  & \quad \quad \quad \text{\textsuperscript{2}H suppresses TROSY partially cancels these} \\
  & R_2(\text{N}) = R_2^{\text{DD}}(\text{H-N}) + R_2^{\text{CSA}}(\text{N}) + R_2^{\text{EXCH}}(\text{N}) \\
\end{align*}
\]

\text{\textsuperscript{2}H enrichment $\Rightarrow$ suppresses $R_2^{\text{DD}}(\text{H-H})$ terms}

\text{TROSY $\Rightarrow$ transverse relaxation optimized spectroscopy}

\text{partially cancels $R_2^{\text{CSA}}(\text{H})$, $R_2^{\text{CSA}}(\text{N})$ terms}

**TROSY - HSQC**

**HSQC (non-TROSY)**

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**TROSY Effect Maximized at ~ 900 MHz**

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(Wüthrich, 1998)