**Sequential Assignment Strategies:**

- Need to know the sequence of the protein that you are working on.

- Several strategies can be employed but the goal is to assign all $^1$H, $^{15}$N and $^{13}$C chemical shifts.

- Most assignments are obtained using through bond experiments although some NOE experiments will help.

- Attempts are being made to automate this and different methods use different sets of experiments.
Triple Resonance Experiments

Homonuclear experiments are useful up to a maximum of 8-10 KDa

Basic limitation of 1D and 2D Experiments:

1) Experiments suffer from spectral overlap and degeneracy problems as the size of the protein increases.

2) Sharp decrease in the efficiency with which magnetization can be transferred through the small three-bond $^1\text{H} - ^1\text{H}$ $J$ couplings as the linewidths become larger than the couplings.
Homonuclear 2D NOESY
General Scheme 1D/2D/3D NMR

1D NMR
- preparation
- pulse
- detection
- t

2D NMR
- preparation
- evolution
- evolution
- detection
- t1
- t2

3D NMR
- preparation
- evolution
- evolution
- detection
- t1
- t2
- t3
3D Homonuclear NMR

- first example for a protein was in 1988

3D NOESY-TOCSY

- This experiment was useful for proteins that were not labeled. It greatly increased the complexity of the 2D spectrum but it also contained a lot of data.

For 76 amino acid protein Ubiquitin:
  2D: 2-3,000 crosspeaks
  3D: ~10,000 crosspeaks

* Heterocuclear NMR simplifies the 2D NMR spectrum and has far superior resolving powers *
Schematic of Homonuclear 3D
Heteronuclear NMR

- resolves crosspeaks between $^1$H's by correlating to the frequency of an attached $^{13}$C or $^{15}$N.

For example: We can concatenate the NOESY or the TOCSY to an HSQC (HMQC)

$^1$H $\leftrightarrow$ $^1$H ----- $^{15}$N

NOESY   HSQC

nDimensional NMR is not a perfect world:

- relaxation during incremental periods.

- square-root of two loss in signal/noise for each dimension.

- you need a labeled sample.
Examples of building blocks

**HMOC:**

\[ \text{HMOC:} \]

**HSQC:**

\[ \text{HSQC:} \]

**TROSY:**

\[ \text{TROSY:} \]

\[ \phi_2 = \pi - \Delta \psi_{\text{rec}} = \pi - \phi_1 \]

E/\theta \text{ selection: } \psi = y/\psi_2 = -\psi_1; \phi_1 = +10^\circ - 10^\circ. \]
Schematic of Heteronuclear 3D
Labeling Strategy

Isotopes Used: $^{13}$C, $^{15}$N and $^2$H

- the labels are incorporated by expressing the proteins in recombinant systems.

- the cheapest system is bacteria in which you use a bacterial strain that is able to make all its components.

- use $^{13}$C-labeled glucose and $^{15}$N-labeled ammonium chloride.

- incorporate the isotopes to $>98\%$ so essentially the molecules are completely labeled.
Labeled Samples

$^{15}\text{N}$-labeled sample:

$^{15}\text{N}\text{C\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C}}}}}}}}$  
$\text{H\text{-H\text{-H\text{-H\text{-H\text{-H\text{-H\text{-H}}}}}}}}$

$^{13}\text{C} / ^{15}\text{N}$-labeled sample:

$^{15}\text{N}\text{C\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C}}}}}}}$  
$^{13}\text{CH\text{O\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C}}}}}}}$  
$^{13}\text{CH\text{O\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C}}}}}}}$  
$^{13}\text{CH\text{O\text{-C\text{-C\text{-C\text{-C\text{-C\text{-C}}}}}}}$  
$\text{H\text{-H\text{-H\text{-H\text{-H\text{-H\text{-H\text{-H}}}}}}}$
Important \( ^1J \) and \( ^2J \) couplings
$^1$H Chemical Shifts in Proteins

- backbone $H^N$
- aromatic
- side-chain $H^N$
- $H_\alpha$
- methyl
- aliphatic
$^{13}$C Chemical shifts in proteins
Typical Carbon Chemical Shifts in Amino Acids
Carbon Chemical Shifts in Aromatic Amino Acids
Labeled Samples

$^{15}N$-labeled sample:

\[ \text{CH} \ O \quad \text{CH} \ O \quad \text{CH} \ O \]
\[ ^{15}N-C-C \quad ^{15}N-C-C \quad ^{15}N-C-C \]
\[ H \quad H \quad H \quad H \quad H \quad H \quad H \]

$^{13}C / ^{15}N$-labeled sample:

\[ \text{CH} \ O \quad \text{CH} \ O \quad \text{CH} \ O \]
\[ ^{15}N-^{13}C-C \quad ^{15}N-^{13}C-C \quad ^{15}N-^{13}C-C \]
\[ H \quad H \quad H \quad H \quad H \quad H \quad H \]
$^{1}H - ^{15}N$-HSQC
3D View of HSQC-NOESY
View of Slices from 3D $^{15}\text{N}$-NOESY
Examples of experiments
Relative sensitivity of Triple resonance experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Assignment</th>
<th>Comment</th>
<th>Relative S/N [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNCO</td>
<td>H(i), N(i), C'(i-1)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>100</td>
</tr>
<tr>
<td>HNCA</td>
<td>H(i), N(i), C_\alpha(i), C_\alpha(i-1)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>50/15</td>
</tr>
<tr>
<td>HN(CO)CA</td>
<td>H(i), N(i), C_\alpha(i-1)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>71</td>
</tr>
<tr>
<td>HN(CA)CO</td>
<td>H(i), N(i), C'(i)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>13/4</td>
</tr>
<tr>
<td>CBCA(CO)NH</td>
<td>H(i), N(i), C_\alpha(i-1), C_\beta(i-1)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>13/9 (\alpha/\beta)</td>
</tr>
<tr>
<td>HBHA(CO)NH</td>
<td>H(i), N(i), H_\alpha(i-1), H_\beta(i-1)</td>
<td>&lt;20 kD, above use (^1^H) labeling</td>
<td>13/9 (\alpha/\beta)</td>
</tr>
<tr>
<td>CBCANH,</td>
<td>H(i), N(i), C_\alpha(i), C_\beta(i), C_\alpha(i-1), C_\beta(i-1)</td>
<td>&lt;15 kD, above use (^1^H) labeling</td>
<td>4/1.7 (\alpha/\beta(i)) (1.3/0.5 \alpha/\beta(i-1))</td>
</tr>
<tr>
<td>HNCACB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H)CC(CO)NH-TOCSY</td>
<td>H(i), N(i), Caliph.(i-1)</td>
<td>&lt;15-20 kD, above use (^1^H) labeling</td>
<td></td>
</tr>
<tr>
<td>H(CC)(CO)NH-TOCSY</td>
<td>H(i), N(i), Haliph.(i-1)</td>
<td>&lt;15-20 kD, above use (^1^H) labeling</td>
<td></td>
</tr>
<tr>
<td>HCCCH-TOCSY</td>
<td>Haliph., Caliph.</td>
<td>&lt;25 kD, - sensitive, but tedious to analyze, combine with HCCONH type experiments</td>
<td></td>
</tr>
</tbody>
</table>
The 3D HNCO

\[ \begin{align*}
& H - C - H \\
& C - N - C - C - O \\
& Rest_{i-1} \quad Rest_i
\end{align*} \]

\[ \begin{align*}
& \text{\( T_a = 2.3\, \text{ms} \)} \\
& \text{\( T_b = 5.5\, \text{ms} \)} \\
& \text{\( T_c = 2.3\, \text{ms} \)} \\
& \text{\( T_d = 12.4\, \text{ms} \)} \\
& \text{\( T_e = 12.4\, \text{ms} \)} \\
& \text{\( T_f = 1/6.25\, \text{ms} \)} \\
& \text{\( T_g = \frac{1}{65\, \text{ms} \cdot 4.5} \)}
\]
3D HNCO

\[ J_{\text{NH}} \quad J_{\text{NC'}} \quad J_{\text{NC'}} \quad J_{\text{NH}} \]

\[ \text{NH} \Rightarrow ^{15}\text{N} \Rightarrow ^{13}\text{C}'(t_1) \Rightarrow ^{15}\text{N}(t_2) \Rightarrow \text{NH}(t_3) \]

1. \( \text{NH} \Rightarrow ^{15}\text{N} \) one-bond INEPT \( ^1J \sim 90 \) Hz.

2. \( ^{15}\text{N} \Rightarrow ^{13}\text{C}' \) one-bond INEPT \( ^1J \sim 15\)Hz.
   (Reduce the delay to balance between transfer and relaxation.)

3. \(^{13}\text{C}' \) \( t_1 \) evolution; decouples \(^1\text{H}, ^{15}\text{N}, \text{C}' \) couplings.

4. Constant time evolution of \(^{15}\text{N} \) in \( t_2 \).
   Simultaneous evolution of \(^{15}\text{N} - ^{13}\text{C}' \) scalar coupling.

5. \( ^{15}\text{N} \Rightarrow \text{NH} \) one-bond reversed INEPT.
Basic Experiments for Backbone Assignments

HNCACB

(HB)CBCA(CO)NH
HNCACB 3D View
The 3D HNCACB
3D HNCACB

\[ J_{\text{NH}} \quad J_{\text{NC}} \quad J_{\text{CC}} \quad t_{1} \quad J_{\text{CC}} \quad J_{\text{CN}} \]

\[ \text{NH} \rightarrow ^{15}\text{N} \rightarrow C^{\alpha} \rightarrow C^{\alpha}C^{\beta} \rightarrow C^{\alpha}C^{\beta} \rightarrow C^{\alpha} \rightarrow \]

\[ J_{\text{NH}} \]

\[ ^{15}\text{N}(t_{2}) \rightarrow \text{NH}(t_{3}) \]

1. \( \text{NH} \rightarrow ^{15}\text{N} \) one-bond INEPT.

2. \( ^{15}\text{N} \rightarrow C^{\alpha} \) one- or two-bond INEPT.
   Also, \( C^{\alpha} \rightarrow C^{\beta} \) so T is chosen for max. transfer.

3/5. \( C^{\alpha}/C^{\beta} \rightarrow C^{\beta}/C^{\alpha} \) one-bond INEPT.

4. \( t_{1} \) evolution of both \( C^{\alpha} \) and \( C^{\beta} \).

6. \( C^{\alpha} \rightarrow ^{15}\text{N} \) one- or two-bond reverse INEPT.
The 3D (HB)CBCA(CO)NH
3D (H)C(CCTOCSY)(CO)NH
3D (H)C(CCTOCSY)(CO)NH
Deuteration Reduces Relaxation

- maximum sensitivity with 100% deuteration
Experiments with deuteration

- useful for both backbone and side-chain assignments.
Fractional Deuteration

- What is optimal %?
Automated analysis of NMR Assignments:

• Most programs use a general analysis scheme

1. Filter peaks (filtering) and relate resonances from different spectra (referencing).

2. Group resonances into spin systems.

3. Identify the amino acid type of spin system

4. Identify and link sequential spin systems into segments.

5. Map spin-system segments onto the primary sequence.

AutoAssign Program as an example:

- can analyze data from eight triple resonance experiments although five or six are required.

HNCO, HNCACB, HN(CO)CACB, HNCA, HN(CO)CA are required.

HN(CA)CO, HNHA and HN(CO)HA are not required.

Pitfalls in automated assignment protocols:

- The number of possible solutions increases exponentially with the length of the sequence.

- Accurate chemical shift referencing between experiments, they can be very sensitive to temperature and other conditions.

- They must be able to deal with chemical shift degeneracy and incomplete data sets.

- They may not detect conformational/chemical heterogeneity.

- Most procedure require some human intervention to be successful.
References:

HNHA:

3D $^{15}$N-NOESY:

Triple Resonance Experiments:

Deuteration Experiments: