Introduction to 3D NMR

Obstacles to complete 2D NMR analysis of proteins > 50 residues: spectral overlap & sensitivity

- Linewidth increases with size ($\Delta v_{\text{w}} \sim 1 \text{e}^{3*\text{MW}}$)
- Number of resonances in same spectral region increases linearly with size
- Efficiency of magnetization transfer goes down with decreasing $T_2$

A partial solution: increase resolution by going from 2D experiments to 3D experiments

- Analogous to going from 1D to 2D experiments
- First 3D experiment was homonuclear NOESY-TOCSY
  - Oschkinat et al., Nature 332, 374 (1988)
- Often used for carbohydrates

Worked, but:
- Poor signal to noise
- Limited experimental options
- Dizzying number of cross peaks
- If limited dispersion in $^1\text{H}$ dimension then going into three $^1\text{H}$ dimensions does not always resolve overlap.

![Diagram of 1D, 2D, and 3D NMR experiments](image-url)
Heteronuclear 3D experiments

Within the year (1989), two labs developed 3D methods based on the amide $^1\text{H}^\text{N}$-$^\text{15N}$ coupling

Bax @NIH - JACS 111, 1515 (1989) & Biochemistry 28, 6150 (1989)
Fesik & Zuiderweg @Abbott Labs - Biochemistry 28, 2387 (1989)

Rationale for the 3D $^1\text{H}$-$^\text{15N}$ methodology:
Need uniformly $^\text{15N}$ labeled protein (e.g. from growth of *E. coli* on $^\text{15NH}_4\text{Cl}$ as the only nitrogen source)
Resolve the $^\text{1H}^\text{N}$ by the frequency of the attached $^\text{15N}$ (amide $^\text{15N}$ chemical shift range is ~35 ppm, from 100 ppm to 135 ppm = a good spread)

Assignment strategy is the same as for unlabeled proteins/peptides:
Use [$^\text{1H}^\text{N}$ to all $^\text{1H}$ TOCSY] separated by $^\text{15N}$ for type/spin system assignments
Use [$^\text{1H}^\text{N}$ to all $^\text{1H}$ NOESY] separated by $^\text{15N}$ for sequential assignments

*Overlapped peaks in a 2D NOESY experiment are resolved by adding the amide $^\text{15N}$ chemical shift as a third dimension*
Basis for these 3D experiments is the HSQC - Heteronuclear Single Quantum Coherence experiment:

It consists of an INEPT transfer from $^1$H to $^{15}$N, followed by $t_1$ evolution on $^{15}$N (removing the $^1$H-$^{15}$N coupling with a 180° pulse on $^1$H during the $^{15}$N evolution), ending with transfer from $^{15}$N back to $^1$H, using a reverse INEPT, for detection during the acquisition, $t_2$, with broadband (GARP) $^{15}$N decoupling during acquisition to remove the one bond $^1$H-$^{15}$N coupling.

For a protein you expect to see 1 peak for every backbone amide proton and nitrogen pair.

1 cross peaks for each residue except:
- Proline (no $^{14}$N)
- N-terminal residue

Additional cross peaks:
- Trp indole $^{14}$N
- Side chain NH$_2$ groups of Asn/Gln
- Side chain $^{14}$N ε Arg (NH$_2$ side chain groups of Arg/Lys are not normally present)

76 amino acid protein:
- 73 backbone $^{14}$N, 4 Arg-$^{14}$ε and 8 sidechain NH$_2$.

Random coil position of Q/N NH$_2$ groups is 6.8-7.6ppm ($^1$H) and 110-114ppm ($^{15}$N) (main red box).

In ubiquitin one NH$_2$ is at 104ppm ($^{15}$N) because it is involved in a hydrogen bond.

$^{1}H,^{15}N$ HSQC spectrum of Ubiquitin
On to 3D:
3D pulse sequence is obtained by concatenating two 2D sequences. Combination of TOCSY with HSQC gives the TOCSY-HSQC, whereas NOESY with HSQC yields the NOESY-HSQC (see also Cavanagh, pp. 447-457):

What does the TOCSY-HSQC do?
1. Magnetization transfer pathway:
2. All $^1$H excited, their chemical shift is labeled in $t_1$ evolution
3. TOCSY-transfer, all protons to all protons
4. Magnetization transfer from $^{15}$N-attached protons to nitrogen via INEPT-transfer – selection of H$^N$ protons only
5. $^{15}$N chemical shift labeled during $t_2$ evolution
6. Reverse inept back to H$^N$
7. Detection $t_3$ of H$^N$ protons only

$t_1$ and $t_2$ are the incremented delays, giving rise to the 2nd and 3rd dimensions – one can increment both (full 3D), either (gives 2D planes equivalent to a $^1$H,$^1$H TOCSY or $^1$H,$^{15}$N HSQC), or neither (1D spectrum of only those protons selected in the sequence, so will only have protons attached to nitrogens, i.e. amide proton).
NOESY-HSQC:

Magnetization transfer pathway:
All $^1$H excited, their chemical shift is labeled in $t_1$ evolution
NOESY-transfer, all protons to all protons
Magnetization transfer from $^{15}$N-attached protons to nitrogen via INEPT-transfer – selection of $\text{HN}$ protons only
$^{15}$N chemical shift labeled during $t_2$ evolution
Reverse inept back to $\text{HN}$
Detection $t_3$ of $\text{HN}$ protons only
**Example: Ubiquitin**

The process of sequential assignments is exactly the same as for unlabelled peptides. Use the TOCSY experiment to identify the spin system type (and in some cases the amino acid type). Then link the individual spin systems together using the NOESY experiment.

Below is an example of strips for residues I3 to K11 in Ubiquitin from the $^{15}$N TOCSY-HSQC spectrum (black) and the $^{15}$N NOESY-HSQC spectrum (red). Highlighted in blue are the sequential NOEs ($^N\text{H}-^N\text{H}$ or $^N\text{H}-^\alpha\text{H}$) that allow sequence specific assignments.

For an example of a paper where they use 15N TOCSY/NOESY-HSQC spectra to assign a protein, see B.J. Stockman *et al.*, (1993) *J. Biomolecular NMR*, 3, p133-149.
Summary of assignment procedure using $^1$H,$^{15}$N HSQC, $^{15}$N-edited TOCSY-HSQC and NOESY-HSQC:

For each cross peak in the HSQC spectrum, identify the corresponding spin system in the TOCSY-HSQC

Identification of amino acid type and assignment of spin system

Look at the same strips from NOESY-HSQC spectrum: additional cross peaks that are not present in TOCSY-HSQC are likely sequential contacts: $H^N - H^N$ (and $H^\beta_{i-1} - H^N_i$) in $\alpha$-helices, and $H^\alpha - H^N$ in extended conformations.

Identify which residues these cross peaks are to (from TOCSY-HSQC spin systems), and assign adjacent amino acids.

Repeat the same process starting from the newly identified neighboring amino acid.

When the connectivity breaks down, move to a new starting point.

Once a substantial number of residues are assigned, ambiguities can be resolved and gaps filled in (hopefully)

Problems:

Identification of amino acid type is often ambiguous

Unambiguous assignment of all side chain protons is difficult using only a TOCSY spectrum

As the protein size increases so does the linewidths and the efficiency of the TOCSY transfer gets worse so that you do not see all sidechain proton correlations

Decreased possible NOEs for structure determination

Structure determination of all $\alpha$-helical proteins is difficult because of low number of long range inter-helical NOEs.