# BIOC530 Protein NMR Sessions

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**BIOC530: NMR Unit, Part 2**  
**Protein NMR: What can it do?**

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**Protein NMR... definitely NOT your one-trick pony**

1. CHEMICAL SHIFTS (i.e., frequency at which a given nucleus resonates)

Benefits: easy to measure (2D spectra), with good S/N, high accuracy, and high precision

Information content:
- Protein backbone shifts ("H\textsubscript{15}N, \textsuperscript{13}C\textsubscript{\alpha}, \textsuperscript{13}C") → secondary structure info
- \textsuperscript{13}CH\textsubscript{3} (Ile) → distinguish \(\alpha\)-helix and \(\beta\)-strand in very large proteins/complex
- As \(f(\text{pH})\), \(pK_R\) values of individual sidechains in protein
  - \(pH\)-dependent conformational change
  - tautomeric states of histidines
- As \(f([\text{ligand}])\), binding site identification (at residue-level resolution)
  - estimate exchange rate/lifetime of bound species
  - estimate \(K_D\) (but not very well!)
Protein NMR...  
definitely NOT your one-trick pony

2. Nuclear Overhauser Effects (NOE), interaction bet. Nuclei w/ \( r^6 \) dependence

Benefits:
- under optimal conditions, can measure hundreds – thousands of pairwise distances that can define a 3D structure \textit{de novo}.
- Even limited NOE information can guide structure determination using sparse constraints.
- Heteronuclear NOEs (hNOEs) such as \(^1\text{H},\, ^{15}\text{N}\) provide information about dynamics in the ps - ns timescale on a residue level. (great for segmental motions)

Limitations:
- Low sensitivity experiment (not hNOE).
- Difficult/tedious to assign unambiguously to specific pairs
- Not strictly \( r^6 \), so have to use distance bins rather than explicit distances
- Practically speaking, limited to protein systems with fewer than 200 residues.

Protein NMR...  
definitely NOT your one-trick pony

3. Paramagnetic Relaxation Enhancement (PRE), effect of “spin label” on NMR resonances.

Benefits:
- Measurements are made using robust 2D spectra (\(^{15}\text{N} \text{ or } ^{13}\text{C}\) )
- Can be either intramolecular (especially useful for IDPs) or intermolecular
- Can be used in highly flexible systems (IDPs, fuzzy complexes, etc.)
- Effects go out to longer distances than NOEs

Limitations:
- Need to attach a paramagnetic label via a single Cys residue.
- Label is long and flexible, so data is often used qualitatively.
Protein NMR... definitely NOT your one-trick pony

4. Residual Dipolar Couplings (RDCs), information on bond vector orientation relative to external frame (external magnetic field)

Benefits:
• Measurements are made using robust 2D spectra ($^{15}$N or $^{13}$C)
• Very useful for structure determination using sparse constraints.
• Can provide long-range information, so good for defining domain-domain orientations, subunit-subunit orientation.

Limitations:
• Requires partial alignment of sample in the magnetic field. Alignment media include polyacrylamide gels, phage, organic solvents.
• Spectrum can degrade due to peak broadening.

Protein NMR... definitely NOT your one-trick pony

5. Dynamics! A whole lecture of its own...

NMR Timescales

Transport, catalysis, many interesting biological processes.

Relaxation $T_1$, $T_2$, HETNOE

Saturation transfer $Z$-$Z$-exchange, NOESY

Line-shape analysis

H/D exchange
SCOTT’S SLIDES

Practical Considerations for Protein NMR

NMR SAMPLES

• Proteins < 25kDa
  reduced signal from relaxation
  spectral crowding
  methods to overcome this limitation (later!)
• 100-500μM
  >90% pure, structurally homogenous
  concentrated samples required for low sensitivity experiments
  some data can be collected at lower concentrations (20μM)
• 300-500μL
Solution Conditions

• Buffers “invisible” in experiments
  Phosphate is preferred, Tris is not

• Optimize conditions for data collection
  seeking the strongest signal
  Vary pH, ionic strength, temperature, and [protein] mutagenesis

• For binding experiments, material must be in matching solutions

1D $^1$H Spectra

• “Quick” experiments ~15 minutes
• Natural abundance
• Is your protein folded?
• Mutagenesis
• New Constructs
• Sample quality
• Degradation can often be observed
**THIS PROTEIN IS FOLDED**

NH Dispersed

Shifted Methyl

**THIS PROTEIN IS NOT**

10 watergate
100um Shave
NaF, 150nm NaCl pH 6
300K
Isotopic Labeling

- Multi-dimensional NMR (2D, 3D) has many advantages over 1D NMR
- $^{15}\text{N}$, $^{13}\text{C}$, proteins
  Purified from bacteria grown in minimal media with $^{15}\text{N-NH}_4\text{Cl}$ (20$/L) and/or $^{13}\text{C-glucose}$ (100-200$/L)
  Other metabolic precursors may be used
- Deuteration
  Reduced relaxation (more robust signal)
  grown in $\text{D}_2\text{O}$ media (up to 1000$/L)$
Add one dimension for better resolution and more information

1D

Stacked Plot

2D

Add one dimension for better resolution and more information

1D

Stacked Plot

2D

intensity

$^1$H (ppm)  $^{15}$N (ppm)
Add one dimension for better resolution and more information

1D

2D

$(^1\text{H}, ^{15}\text{N})$-HSQC: the simplest heteronuclear 2D spectrum

- Essentially, 1 peak/residue:
  1. amide NH
  2. Asn & Gln NH$_2$
  3. Trp indole NH
  4. His imidazole NH
  5. (Arg NH, Lys NH$_3$)

- “fingerprint” of protein

- BUT...chemical shifts of NH resonances are sensitive to:
  1. pH
  2. temperature
  3. (ionic strength)
  4. other (ligand binding, etc.)
\[(^1H^{15}N)\)-HSQC vs. \[(^1H^{13}C)\)-HSQC\]
3D spectra: increased dispersion and information

"triple resonance"

3D NMR data set

“walk” through $^{15}\text{N}$ dimension
NMR Assignments – A simple example

34 Residue peptide: **STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD**

Backbone triple resonance experiments (need $^1$H, $^{13}$C, $^{15}$N sample)

- HNCA
- HN(CO)CA
- HN(CA)CO
- HNCO
- HNCA CB
- HN(CO)CAB

**Intra/Inter-residue**

- $i$ and $i-1$ peaks

**Inter-residue**

- $i-1$ peaks
3D spectra for backbone assignments

Backbone Assignments – Step 1: Pick the peaks

34 Residue peptide: STDST PMFEY ENLED NSAFW MLFA TDIPV TTDD
34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD
Backbone Assignments

HN(CO)CA  HNCA

(pk #4)  (pk #5)

(possibly) C-term D134

Look for strip with Cα peak at this shift

Have to start somewhere ...

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments

HN(CO)CA  HNCA

(pk #6)  (pk #7)  (pk #8)

Close but i-1 not i peak

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD
34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD
Backbone Assignments

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Chain stops here

Backbone Assignments

Alanines have distinctive Cβ shifts

Look for i-1 peaks

So do Thr & Ser

Peak is A118 if the previous strip looks like a Ser

Peak is A125 if the next strip looks like a Thr

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD
Backbone Assignments

Keep finding the connections

Repeat for remaining sections ...

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments: HN, N, Ca, Cb, C'

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone amides all assigned
Also know: Ca & Cb shifts

Trivial to add the C' shifts:
HNCO
Side chain assignments

$^{13}$C-HSQC

- $\text{Ca}$ & $\text{Cb}$ are known
- Don't know Ha, Hb, ...

$\beta/\gamma$ CH$_2$

$\text{Cβ (Ser & Thr)}$

$\text{CH}_3$

$\text{Ca}$ & Cb are known

$\text{HN}$

$\text{15N-TOCSY (flattened)}$

- Methyls
- $\text{Hβ/γ}$
- $\text{Ha}$
- Amides on diagonal
- Side chain protons
Side chain assignments

**HNCACB**

**15N-TOCSY**

![Graph showing side chain assignments and NMR spectra](image)

**13C-CHSQC**

**T102**

**Ca**

**Hb**

**Ha**

**Side chain assignments**

**HNCACB**

**15N-TOCSY**

![Graph showing side chain assignments and NMR spectra](image)
Side chain assignments

**Don’t explicitly have Cg but Hg shift is enough to assign for this peptide**

**Cβ’s would be sufficient to assign the alanines for this peptide**
Side chain assignments: Ha, Ca, Hb, Cb, Hg, Hd ... Cg, Cd inferred

For this peptide:
Can unambiguously assign pretty much everything except some CH2γ groups & the aromatics (not shown)

More Experiments required for larger systems:

$^{13}$C-NOESY
HCCH-TOCSY & HCCH-COSY
CmCgCbCaHN .... And other tricks as necessary