Why do we care about biomolecules?
Why do we use NMR?
Q: How do we take a spectrum and derive structure and function from it?

A: Take signal features, and relate them back to the originating spin
What is an assignment?

Associate a given signal/frequency back to the originating spin
Every atom with spin has a Larmor frequency

Fourier Transform (FT)
Time → Frequency

\[ \delta \text{ (ppm)} = \frac{(\nu_S - \nu_R)}{\nu_R} \times 10^6 \]

This frequency is a physical property of that atom – it is the same value no matter what experiment is run!
The exact resonance frequency (chemical shift) is determined by the electronic environment of the nucleus.
Critical features influence spin frequencies

Spins start at a given frequency based on its gyromagnetic ratio

• Local chemical bonds influence resonance frequency
• Sample solvent conditions influence frequency
• Tertiary structure leads to increased dispersion of resonances.
• Ligand binding changes spin properties

Every atom in a protein is chemically unique, thus every atom has a unique assignment!
Each atom has a unique chemical environment

![Leucine (L) diagram]

- Backbone: H — N — Cα — C —
- Sidechain: Cβ — H
  - Cγ
  - Cδ₁
  - Cδ₂

Leucine (L)
Regions of the $^1$H NMR Spectrum

What would the unfolded protein look like?
Proteins Have Too Many Signals!

$^1H$ NMR Spectrum of Ubiquitin

Resolve resonances by multi-dimensional experiments
Solutions to the Challenges of Too Many Signals

Increase dimensionality of spectra to better resolve signals: $1 \rightarrow 2 \rightarrow 3 \rightarrow 4$

Higher dimensions link spins to one another
Scalar and Dipolar Coupling

Coupling of nuclei gives information on structure through bonds and through space.
2D NMR: Coupling is the Key

Transfers magnetization between coupled spins

90° pulse

Same as 1D experiment
The 2D NMR Spectrum

Pulse Sequence

Spectrum

Before mixing

After mixing

Coupled spins
The Power of 2D NMR: Resolving Overlapping Signals

1D

2 signals overlapped

2D

2 cross peaks resolved
Basic Strategy to Assign Resonances

1. Identify resonances for each residue (scalar)
   [T G L S S R G]

2. Put residues in order
   1 2 3 4 5 6 7
   R - G - S - T - L - G - S
Homonuclear 2D Expts
COSY: One coupling
R-COSY: Add A 2nd Coupling

**Diagram:**

- **COSY**
  - $\alpha$ (A)
  - $\beta$ (C)
  - $H^N$ (B)

- **R-COSY**
  - $\alpha$ (A)
  - $\beta$ (C)
  - $H^N$ (B)

**Chemical Shifts:**

- **A:** $N-C-C$
- **B:** $N-C-C$
- **C:** $N-C-CH_3$
DR-COSY: Add A 3rd Coupling

COSY

R-COSY

DR-COSY

H \ H \ H
| | | I
A N—C—C
| H

H \ H \ H
| | | I
B N—C—C
| H

H \ H
| I
C N—C—CH₃
TOCSY: All Coupled Spins

A \[
\text{N—C—C—C—C—COOH}
\]

B \[
\text{N—C—C—C—C—C—NH}_3
\]

C \[
\text{N—C—CH}_3
\]
Homonuclear 2D Expts

COSY

R-COSY

DR-COSY

TOCSY

NOESY

$F_1$ chemical shift

$F_2$ chemical shift

A   C   B

H$_N$  α  α  α  α  α  α  α  α

β  β  β  β  β  β  β  β  β

δ  δ  δ  δ  δ  δ  δ  δ  δ

γ  γ  γ  γ  γ  γ  γ  γ  γ

α  α  α  α  α  α  α  α  α

α  α  α  α  α  α  α  α  α
Limitations of Homonuclear NMR

Nuclei are not all mutually coupled

Each amino acid gives rise to an independent NMR sub-spectrum, which is much simpler than the complete protein spectrum
Basic Strategy to Assign Resonances

1. Identify resonances for each residue (scalar)
   
   1 2 3 4 5 6 7
   R - G - S - T - L - G - S

2. Put residues in order
   
   1 2 3 4 5 6 7
   R - G - S - T - L - G - S
Solutions to the Challenges

- Most abundant nitrogens, carbons, and oxygens do not provide NMR-viable signal
- Detect signals from heteronuclei (non-hydrogens)
- Isotopically enrich protein sample with $^{13}$C, $^{15}$N, or $^2$H to overcome low natural abundance
# Intrinsic Sensitivity of Nuclei

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>$\gamma$</th>
<th>% Natural Abundance</th>
<th>Relative Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>$2.7 \times 10^8$</td>
<td>99.98</td>
<td>1.0</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>$6.7 \times 10^7$</td>
<td>1.11</td>
<td>0.004</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>$-2.7 \times 10^7$</td>
<td>0.36</td>
<td>0.0004</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>$1.1 \times 10^8$</td>
<td>100</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Prepare samples enriched in these nuclei.
Double-Resonance Experiments
Increases Resolution/Information Content

\[ ^{15}\text{N}-^1\text{H} \text{ HSQC} \]
## Acronyms For Basic Experiments

### Differ Only By The Nature Of Mixing

<table>
<thead>
<tr>
<th>Scalar Coupling</th>
<th><strong>Homonuclear</strong></th>
<th><strong>Heteronuclear</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Heteronuclear" /></td>
<td>COSY</td>
<td>HSQC</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Hetero-TOCSY</td>
<td></td>
</tr>
<tr>
<td>Multiple Quantum</td>
<td>HMQC</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dipolar Coupling</th>
<th><strong>Homonuclear</strong></th>
<th><strong>Heteronuclear</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image2.png" alt="Heteronuclear" /></td>
<td>NOESY</td>
<td>NOESY-HSQC</td>
</tr>
<tr>
<td>NOESY-HMQC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: The acronyms represent different spectroscopic techniques used in nuclear magnetic resonance (NMR) to study molecular structures.*
Higher Dimensional NMR: Built on the 2D Principle

3D NMR Pulse Sequence

3D- detect signals 3 times

90° pulse

exitation

90° pulse

excitation

Same as 1D experiment

3D NMR Pulse Sequence
TOCSY: All Coupled Spins

A \[ \text{N--C--C--C--COOH} \]

B \[ \text{N--C--C--C--C--C--NH}_3 \]

C \[ \text{N--C--CH}_3 \]
$^{15}$N Dispersed $^1$H-$^1$H TOCSY

3 overlapped NH resonances

Same NH, different $^{15}$N

$^1$H-1H 2D spectrum

$^1$H shifts

$^{15}$N shifts

$^1$H$^N$ shifts

$^{15}$N-edited 3D spectrum

$^1$H-$^{15}$N 2D spectrum

TOCSY  HSQC

$^1$H  $^1$H  $^{15}$N

t$_3$  t$_2$  t$_1$
Heteronuclear Side Chain Experiments

HBHA(CBCA)NH / HBHA(CBCACO)NH

HCCH-COSY / HCCH-TOCSY

Multiple redundancies increase reliability
Basic Strategy to Assign Resonances in a Protein

1. Identify resonances for each residue (scalar)
   T G L S S R G

2. Put residues in order
   1 2 3 4 5 6 7
   R - G - S - T - L - G - S

Need a coupling to cross the carbonyl!
Heteronuclear Triple-Resonance Backbone Experiments

Names of scalar experiments based on atoms detected

HNCA / HN(CO)CA

HN(CA)CO / HNCO

HNCACB / HN(CO)CACB
Pairs of Experiments

*Distinguishes Intra-residue from Inter-residue*

**HNCA**
- $H(t_3), N(t_2), CA(t_1)$
- 2 peaks- Intra + Inter
- Mixing from $N \rightarrow Ca$ occurs over 1 bond to same Ca and over 2 bonds to adjacent Ca

**HN(CO)CA**
- $H(t_3), N(t_2), CA(t_1)$
- 1 peak- Inter only
- Mixing from $N \rightarrow Ca$ occurs ONLY over 2 bonds to adjacent Ca
Equivalent Experiments
Can be run in either direction

HNCACB
- H(t₃), N(t₂), CA and CB(t₁)
- Mixing from N→Ca occurs over 1 bond to same Ca and over 2 bonds to adjacent Ca

CBCANH
- CB and CA(t₁), N(t₂), H(t₃)
- Mixing from N→Ca occurs over 1 bond to same Ca and over 2 bonds to adjacent Ca