Three ways to measure NOEs:

(a) *Equilibrium NOEs*:
- Saturation of a selected resonance and observation of the effect on the remaining resonances.
- Saturate a selected resonance for at least 3x longest $T_1$, apply a 90° pulse and acquire.
- Usually done using difference spectroscopy - subtract a control spectrum without saturation.

![Diagram of molecular structure with NMR spectrum](image)

(b) *Transient NOEs*:
- Selectively invert one resonance, and observe the change in intensity at other resonances.
- Difference spectroscopy
- The initial build-up rate of the NOE with $\tau_m$ is governed by $1/r^6$

![Graph showing NOE intensity vs. $\tau_m$](image)

(c) *Two Dimensional NOESY experiment*:
The transient NOE is the basis for 2D NOESY experiments. All the NOEs are observed in a single two-dimensional NMR spectrum.
Protein structure determination by NMR

Each cross-peak in a NOESY spectrum (recorded with sufficiently short mixing time to prevent spin-diffusion) indicates a proton-proton distance < 5 Å and therefore represents a distance restraint. With many hundred distance restraints, the 3D structure of a protein can be determined:

Of course, each resonance in the NMR spectrum must first be assigned to the individual proton it comes from (this is called resonance assignment). Next, each cross-peak in the NOESY spectrum must be assigned to the corresponding pair of nearby protons. In practice, the assignment of the NOESY spectrum is achieved iteratively: Initially, only well-resolved NOESY cross-peaks are assigned and an initial structure is calculated. Based on this structure, more NOEs can be assigned, leading to a refined structure. This allows the assignment of further NOEs etc. This process has been automated.

In calculating the structure, one assumes that only the dihedral angles around rotatable bonds are unknown, i.e. standard values of bond lengths and bond angles are used to define the covalent geometry of the polypeptide chain.

The final protein structure is represented by a bundle of conformers that all fulfill the experimental distance and dihedral angle restraints:

The 1D $^1$H NMR spectrum and the 2D NOESY spectrum of a protein contains so many peaks that 3D and 4D NMR spectra are used to facilitate the assignment of the peaks. 3D and 4D spectra use $^{15}$N and $^{13}$C labelled proteins and HSQC pulse sequences.
The HSQC pulse sequence is based on INEPT.

**INEPT (insensitive nuclei enhanced by polarization transfer)**

For example, \( A = ^1H \) and \( X = ^{15}N \). \(^{15}N\) NMR much less sensitive than \(^1H\) NMR, because \( \gamma(^{15}N) = 0.1 \gamma(^1H) \). The polarization transfer from \(^1H\) enhances the \(^{15}N\) magnetization well beyond equilibrium magnetization:

- **a** shows the conventional 1D \(^{15}N\) NMR spectrum, **c** shows the INEPT spectrum. The doublet in the conventional spectrum is “in-phase” (both components have the same sign); the doublet in the INEPT spectrum is “antiphase” (both components have the opposite sign).
Vector description:

The time points a-e are indicated in the pulse sequence.

a) The $^1$H NMR spectrum of the NH group is a doublet. One of the doublet components belongs to the molecules where $^{15}$N is in the state $\alpha$, the other doublet component belongs to the molecules where $^{15}$N is in the state $\beta$. The 90° pulse makes both components transverse.

b) The two components precess with different frequencies ($\pm J/2$). They are 90° out of phase after $1/(4J)$.

c) The 180°($^1$H) pulse flips the magnetization vectors by 180°. The 180°($^{15}$N) pulse flips the $^{15}$N spin-state $\alpha$ to $\beta$, and $\beta$ to $\alpha$. This changes the relative frequencies of the doublet components – the component precessing faster during the first period $1/(4J)$ is going to precess more slowly during the second period $1/(4J)$.

d) $^1$H antiphase magnetization.

e) The 90°($^1$H) pulse creates longitudinal two-spin order: the vector pointing up belongs to the molecules with $^{15}$N in one spin state (say, $\alpha$), the vector pointing down belongs to the molecules with $^{15}$N in the other spin state (say, $\beta$). The vector pointing up also corresponds to $^1$H in the state $\alpha$, while the vector pointing down corresponds to $^1$H in the state $\beta$. The $^1$H and $^{15}$N spins are thus both in $\alpha$ and $\beta$ states, in a coherent fashion. A subsequent 90°($^{15}$N) pulse generates the $^{15}$N antiphase magnetization which is detected.

Remark:
The weak interactions between the small magnetic moments of nuclear spins with their environment result in slow relaxation rates and allow the design of long and complicated pulse sequences. In contrast to optical spectroscopy, nuclear spins can access only a finite number of energy levels. This allows the accurate calculation of the outcome of pulse sequences.
**HSQC spectrum**

HSQC stands for heteronuclear single-quantum coherence (no need to remember this!).

*Pulse sequence*

1) $H_z$  (equilibrium magnetization)
2) $-H_y$  (H magnetization in-phase with respect to N)
3) $-H_xN_z$  (H magnetization antiphase with respect to N)
4) $H_yN_y$  (N magnetization antiphase with respect to H)
   (so far it’s just the INEPT)
5) $H_yN_y \cos \Omega N t_1 - H_xN_x \sin \Omega N t_1$  (frequency labelling with $\Omega_N$)
6) $-H_yN_x \cos \Omega N t_1$  (the second term has been dropped, because it will result in unobservable 2-spin coherence, $H_yN_x$)
7) $H_x \cos \Omega N t_1$
   (between time points 5 and 6, it’s a so-called ‘reverse INEPT’)

Where is the cross-peak going to be?
What multiplet fine structure will it have?
Do we expect diagonal peaks and what does this mean for the sensitivity of the experiment?
Will $^{15}$N equilibrium magnetization become observable?

**The HSQC spectrum allows the measurement of heteronuclei (i.e. non-$^1$H spins) at the sensitivity of a $^1$H NMR experiment.** $^{15}$N-HSQC and $^{13}$C-HSQC spectra probably are the most frequently recorded 2D spectra.
$^{15}$N-HSQC spectrum of a uniformly $^{15}$N-labelled protein. Each cross-peak corresponds to one NH group. Most NH groups in proteins are amides.
3D NMR spectroscopy
The pulse sequences of NOESY and $^{15}$N-HSQC can be concatenated:

The FID is recorded during the detection time $t_3$. $t_1$ and $t_2$ are evolution times which are independently incremented to yield a 3D data set in the time domain. Fourier transformation ($t_3 \rightarrow F_3$, $t_2 \rightarrow F_2$, $t_1 \rightarrow F_1$) yields the 3D spectrum in the frequency domain (i.e. with three chemical shift axes). $F_1$ and $F_3$ display $^1$H chemical shifts, $F_2$ displays $^{15}$N chemical shifts.

Cross-peaks in a 3D NOESY-$^{15}$N-HSQC spectrum are much better resolved than in a 2D NOESY. The 3D spectrum is most useful for 100% $^{15}$N-enriched proteins.

- Would one expect more signals in a 3D NOESY-$^{15}$N-HSQC than in a 2D NOESY?
- What is the purpose of the $180^\circ$($^{15}$N) pulse in the middle of the $t_1$ time?
4D NMR spectroscopy

The pulse sequences of $^{13}$C-HSQC, NOESY and $^{15}$N-HSQC can be concatenated to yield a 4D NOESY spectrum:

F₁, F₂, F₃ and F₄ display the $^{13}$C, $^1$H(C), $^{15}$N and $^1$H(N) chemical shifts, respectively.
The 4D spectrum offers MUCH better resolution. A shows a \(^1\text{H}^-\text{H}\) plane from the 4D spectrum. B shows a region from the 2D NOESY spectrum. Many cross-peaks overlap in the 2D spectrum. There are only a few peaks in the 4D plane shown in A. The other peaks are in different planes.

Note that the 4D plane shows more than 7 ppm for each of the \(^1\text{H}\) axes, whereas the plot of the 2D spectrum shows just a small region (1 ppm for each axis). The peak marked with a box in A is at the position of the cross in B.