Advanced NMR Training Course: February 27th, 2004

National Program for Genomic Medicine High-Field NMR Core Facility,
The Genomic Research Center, Academia Sinica

Course Topic: TROSY and NMR of Large Proteins

by

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Molecular weight distribution of the NMR structures deposited in the PDB as 12/1997

Intrinsic Problems of Solution NMR of Large Molecules

- Many more signals spectral overlap
- Slow tumbling fast transverse relaxation rate (short $T_2$, fast decay of signal) poor signal to noise (s/n)

Linewidth $\Delta \nu_{1/2} = 1/(\pi T_2)$

Pictures from M. Sattler, http://www.embl-heidelberg.de/nmr/sattler/teaching/
Current Techniques Designed to Attack the Size Limit

NMR hardware, new NMR methods, advanced in molecular biology

• Isotope labeling ($^{15}$N, $^{13}$C), 3D, 4D triple resonance experiments: overlap
• NMR hardware: bigger magnets (overlap, s/n)
• Cryogenic probe: s/n (optimal 4-fold increase)

• TROSY, CRINPT: overlap, s/n
• Deuteration: overlap, s/n
• Selective isotope labeling: overlap

• Line-narrowing by low viscosity solvent: overlap, s/n
• Segmental Labeling: overlap
• Residual dipolar coupling: extra angle and long distance information
• Cross saturation: identify binding surface
### Impact of TROSY on Solution NMR

<table>
<thead>
<tr>
<th>Molecular size</th>
<th>NMR pulse sequence</th>
<th>NMR signal</th>
<th>Resonances in spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) WITHOUT TROSY</td>
<td><img src="image1" alt="Diagram" /></td>
<td><img src="image2" alt="Diagram" /></td>
<td>$\Delta v \sim \frac{1}{T_2}$</td>
</tr>
<tr>
<td>(b) WITH TROSY</td>
<td><img src="image3" alt="Diagram" /></td>
<td><img src="image4" alt="Diagram" /></td>
<td><img src="image5" alt="Diagram" /></td>
</tr>
</tbody>
</table>

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Application of TROSY NMR on Biological Systems

Figure from K. Pervushin in *EMBO Practical NMR Course 2003*
http://www.embl-heidelberg.de/nmr/sattler/embo/coursenotes.html

Nucleic acids and small proteins
(fine details of 3D structures, dynamics)

Oligomeric proteins
Large single chain proteins
β-barrel membrane proteins
Polytopic α-helical membrane proteins
(bb and sc assignment, 3D folds, dynamics)

Protein/protein and
protein/nucleic acid interactions
(chem. shift mapping, surface mapping,
drug discovery)

Large supramolecular complexes
(detection of individual resonances)

The estimated range of molecular weights of biological systems the most effectively studied by TROSY NMR
Transverse Relaxation-Optimized Spectroscopy (TROSY)

1. Main relaxation source for $^1$H and $^{15}$N: dipole-dipole (DD) coupling and, at high magnetic fields, chemical shift anisotropy (CSA).

2. Different relaxation rates (line width) for each of the four components of $^{15}$N-$^1$H correlation.

3. The narrowest peak (the blue peak) is due to the constructive canceling of transverse relaxation caused by chemical shift anisotropy (CSA) and by dipole-dipole coupling at high magnetic field.

4. TROSY selectively detect only the narrowest component (1 out of 4).
TROSY-HSQC and Conventional HSQC

**TROSY-HSQC**

1. No $^1$H decoupling during $^{15}$N evolution.
2. No $^{15}$N decoupling during $^1$H acquisition.
3. Use the TROSY-HSQC pulse sequence to selectively observe the most slowly relaxing component.

**Conventional HSQC**

In both $^1$H and $^{15}$N dimensions, both J-split components are mixed by hetero-nuclear decoupling. This collapses each $^{15}$N and $^1$H doublet into a single peak in each dimension.

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Interference of Relaxation between DD and CSA Relaxation

• DD relaxation is field-independent. However, $\text{CSA} \propto B_0^2$, therefore at high magnetic fields, CSA relaxation can be comparable to DD relaxation, and the interference effect on relaxation can be observed.
TROSY, 40 kDa, 750 MHz

Decoupled HSQC (during t1 & t2)

Non-decoupling HSQC

TROSY-HSQC

Linewidth:
60% reduction in $^1$H,
40% reduction in $^{15}$N

If perdeuterated:
Expected reduction
40-fold for $^1$H &
10-fold for $^{15}$N

The Sensitivity and Resolution Gain by TROSY and Deuteration

\( u^{-2}H,^{15}N\text{-Gyrase-45 (45 kDa), 750 MHz} \)

TROSY Effect is Field Dependent and Motion Dependent

- Optimal field strength: 1 GHz for amide NH; 600 MHz for CH in aromatic moieties (500-800 MHz applicable).

- Relaxation rate for the narrowest component of the doublet:
  \[ R_{1212} = (p - \delta_S)^2(4J(0) + 3J(\omega_S)) + p^2(J(\omega_1 - \omega_S) + 3J(\omega_1) + 6J(\omega_1 + \omega_S)) + 3\delta_1^2J(\omega_1), \]
TROSY Application

- The implication of TROSY technique is revolutionary and wide spreading for large molecules (>20 KDa). Virtually all $^1$H,$^{15}$N-HSQC-based double resonance and triple resonance experiments gain sensitivity and spectral resolution via TROSY.


Pushing the Size Limit by TROSY, Deuteration, and Selective Isotope Labeling

723 a.a., 81.4 kDa

TROSY-HSQC of U-[\(^2\text{H},^{15}\text{N}\)]-MSG at 800 MHz

MSG: Malate synthase G

>95% \(^1\text{H}\), \(^{15}\text{N}\), \(^{13}\text{C}_\alpha\), \(^{13}\text{CO}\), \(^{13}\text{C}_\beta\) assigned !!!

3D NOESY-[$^1$H, $^{15}$N, $^1$H]-ZQ-TROSY

110 kDa, [70% $^2$H, U-$^{15}$N]-DHNA

Diagonal peaks in NOESY are suppressed!

Pervushin et al.  
*PNAS USA, V96, P9607, (1999)*
SEA (Solvent Exposed Amides)-TROSY


71 kDa, [U-2H, $^{15}$N]- P450

This technique has become very popular for drug screening since binding mainly occurs on the surface of a protein.
Deuteration

- Reduce relaxation ($\gamma_D / \gamma_H = 1/6.5$). (a maximal 16 fold reduction).
- Reduce number of signals.
- Suppress spin-spin diffusion.
Impact of Deuteration on Relaxation and Sensitivity

Figure from D. Nietlispach in “EMBO NMR course, 2003”.

Figure from M. Sattler, http://www.embl-heidelberg.de/nmr/sattler/teaching/
Some Notes on TROSY

• Intrinsic 50% loss in sensitivity (the sensitivity improved version*) due to rejection of some coherence pathway. However, for large proteins (>20 kDa) at high magnetic fields, the detection of the most slowly relaxing peak compensates for the loss of sensitivity. Instead sensitivity and resolution are both gained.

• TROSY effect is field strength dependent. Optimal field strength: 1 GHz for amide NH; 600 MHz for aromatic moieties (500-800 MHz).

• Minimal of phase cycling need to be complete for the selection of the narrowest component. (1 out of 4).

• The larger a protein (< 200 kDa), the more pronounced line-narrowing effect by TROSY.

*Pervushin et al., J. Biomol. NMR, 1998, 12:345-348
TROSY via INEPT Transfer Becomes Ineffective for Very Large Molecule (>200 kDa)

• Due to very fast transverse (T2) relaxation for large proteins (>200 kDa). TROSY via INEPT through-bond scalar coupling transfer becomes inefficient.

• For M.W. >200 kDa, use cross correlation between dipole-dipole coupling and CSA relaxation to transfer in-phase $^1$H coherence to $^{15}$N coherence in $^{15}$N-$^1$H moieties (CRIP).

• Cross relaxation enhanced polarization transfer (CRINEPT=CRIP+INENP) become more effective for very large molecules.

• Riek et al. “Polarization transfer by cross-correlated relaxation in solution NMR with very large molecules”, PNAS USA V96, 4918-4923, 1999.
CRIPT or CRINEPT Offers Higher Sensitivity for Very Larger Molecules (>200 kDa)

**CRIPT**: Cross relaxation-induced polarization transfer (between DD coupling and CSA relaxation).

**CRINEPT**: Cross relaxation-enhanced polarization transfer (CRIPT+INEPT).

**INEPT**: Insensitive nuclei enhanced by polarization transfer.

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**Graph a**

- **Axis**: $A_{rel}$ vs. $\tau_c [\text{ns}]$
- Curves for CRINEPT, CRIPT, and INEPT.

**Graph b**

- **Axis**: $A_{rel}$ vs. $^{1}\text{H}$ frequency $[\text{MHz}]$
- Curve for CRIPT.

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**Legend**

- Inset: CRIPT, CRINEPT, INEPT.
CRINEPT-TROSY for Very Larger Molecules (>200 kDa)

In (b): No splitting in the $^{15}\text{N}$ dimension since $^{1}J_{NH}$ is decoupled during the $^{15}\text{N}$ evolution.
A 470 kDa Complex by Solution NMR

900 kDa Complex by Solution NMR

72 kDa

472 kDa

900 kDa

TROSY

CRIPT-TROSY
Only one out of several domains is isotope labeled. Spectra are therefore simplified.

Xu et al, *PNAS USA*, 1999, V96, P388
• Measure $\theta$ from residual dipolar coupling to provide additional structural constraint.

• Usually residual dipolar coupling is averaged to zero due to the isotropic motion of a protein in solution.

• Align a paramagnetic protein by high magnetic field strength. (Tolman et al. (Prestegard’s lab), *PNAS USA*, 1995, V. 92, P9279).

Residual Dipolar Coupling introduces an extra splitting in the $^{15}$N dimension in a non-$^1$H decoupled HSQC spectra.

- Residual dipolar coupling introduces an extra splitting in the $^{15}$N dimension in a non-$^1$H decoupled HSQC spectra.

Angle: Structural Refinement; relative orientation

Long Distance Measurement:
Inter-nuclear distance up to 12 A was measured by residual dipolar coupling.

Tolman et al. *PNAS USA*, (1995), V. 92, P9279

NMR of Encapsulated Proteins Dissolved in Low-Viscosity Fluids

\[ \tau_c = \eta v / kT \]

- To arrange for a protein to tumble at a faster rate \((\text{smaller } \tau_c)\) by reducing the viscosity of the bulk solvent.
  - \(\eta_{\text{propane}} = 0.1 \times \eta_{\text{H2O}}\)

- Use the reverse micelle technique to solublize proteins.

- Pioneered by J. Wand’s lab.

Wand et al., *PNAS USA*, 1998, V 95, P15299
High Pressure Mixing Apparatus for Preparing Protein Sample Under Pressure

Designed for preparing protein samples in liquid propane

Native-Like Structure for Chymotrypsin-Bound $^{15}$N-OMTKY3 in Reverse Micelles Dissolved in Propane

Red: in H$_2$O
Blue: in reverse micelles dissolved in propane

600 MHz $^1$H, $^{15}$N-HSQC spectra of chymotrypsin-bound $^{15}$N-OMTKY3

Cross Saturation Method for Determining the Interfaces of Large Protein–Protein Complexes

The residues of protein I at the interfaces acquires change in $^1$H, $^{15}$N chemical shifts due to saturation transfer from protein II.

Cross Correlation and TROSY Effect

- **Cross correlation**: Two different types of interactions interacts with each other. DD-DD, DD-CSA, CSA-CSA cross correlation. (compared to “Cross relaxation: NOEs”)

- **TROSY** takes advantage of the interference relaxation between chemical shift anisotropy (CSA) and dipole-dipole coupling (DD-coupling) for large molecules at high magnetic fields.

How does the “line-narrowing” (reduction in line width) occur?
Chemical Shift Anisotropy

1. \( \sigma \) is directly related to the electron density at a distance \( r \) from the nucleus by Lamb’s equation.
2. There are three principle components of the tensor: \( \sigma_{11}, \sigma_{22}, \sigma_{33} \) (or \( \sigma_{xx}, \sigma_{yy}, \sigma_{zz} \)).
3. Isotropic shift tensor: \( \sigma_{iso} = \frac{1}{3} (\sigma_{11} + \sigma_{22} + \sigma_{33}) \).
4. The shift tensor \( \sigma \) is related to the Larmor frequency \( \omega_0 \): \( \omega_0 = B_0 (1 - \sigma) \gamma / 2\pi \)

Figures taken from p34 and p40 of “Biomolecular NMR Spectroscopy” by Jeremy N.S. Evans
Transverse Relaxation and TROSY

Consider two scalar coupled spins 1/2, I and S

\[ p = \frac{1}{2\sqrt{2}} \gamma_I \gamma_S h / \Gamma_{IS} \]

DD coupling between spin I and spin S

\[ \delta_I = \frac{1}{3\sqrt{2}} \gamma_I B_0 \Delta \sigma_I \]
\[ \delta_S = \frac{1}{3\sqrt{2}} \gamma_S B_0 \Delta \sigma_S \]

Chemical shift anisotropy of I and S

\( \gamma_I, \gamma_S \): gyromagnetic ratios of I and S; \( h \): Planck constant / 2\( \pi \); 
\( r_{IS} \): inter-nuclear distance between I and S; \( B_0 \): magnetic field strength 
\( \Delta \sigma_I, \Delta \sigma_S \): the differences between the axial and the perpendicular principle components of the axially symmetric chemical shift tensors of I and S.

\[ \gamma^{1H} \sim 10 \times \gamma^{15N}, \quad \gamma^{1H} \sim 4 \times \gamma^{13C}, \quad \gamma^{1H} \sim 6.5 \times \gamma^{2H} \]
Transverse Relaxation and Interference Effect by Cross Correlation between DD and CSA

\[ R_{1212} = (p - \delta_s)^2 (4J(0) + 3J(\omega_S)) + p^2 (J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)) + 3\delta_I^2 J(\omega_I), \]

\[ R_{3434} = (p + \delta_s)^2 (4J(0) + 3J(\omega_S)) + p^2 (J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)) + 3\delta_I^2 J(\omega_I), \]

• \( R_{1212} \) and \( R_{3434} \) are the transverse relaxation rates of the individual components of the “S” doublet (i.e. \( ^{15}\text{N} \), and let \( ^1\text{H}^\text{N} \) be the “I” spin) in a single quantum spectrum.

• \( J(w) \) represent the spectral density functions at the frequencies indicated.

• In the slow-tumbling limit, only terms in the \( J(0) \) need to be considered.

• Recall that \( J(w) \) is the power (energy) available to bring about relaxation as a function of molecular tumbling.

• If CSA and DD coupling are comparable, i.e. \( p \approx \delta_s \), The relaxation rate of \( R_{1212} \) becomes small (slow relaxation), and the resonance at \( \omega_s^{12} \) relaxes slowly even for large molecules.
Relaxation of Transverse Magnetization:

Fast Exchange limit

\[
\frac{(\Omega_1 - \Omega_2)^2}{k} = \Delta\Omega^2 \tau_c
\]

Figure modified from that by C. Griesinger with permission.
Dipolar Relaxation: \( \frac{(3\cos^2\theta - 1)}{2} \)

Dipolar Coupling between I and S

Figure modified from that by C. Griesinger with permission.
Dipolar Relaxation II

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Figure modified from that by C. Griesinger with permission.
CSA Relaxation of S

Figure modified from that by C. Griesinger with permission.
CSA Relaxation of S and Dipolar Coupling between I and S: Interference by Cross Correlation

Dipolar Coupling between I and S and CSA of S
Interference of Relaxation Cross Correlation between Dipolar and CSA Relaxation

\[ 2D_{\parallel} \]

\[ I_{\alpha} \]

\[ I_{\beta} \]

\[ \sigma_{zz} \]

\[ -D_{\parallel} \]

\[ \sigma_{xx} \]

\[ \theta = 0 \]

\[ \theta = 90 \]

Solution average

\[ \frac{I_{\alpha} + J(IS) + I_{\beta}}{3} \]

\[ \left( \sigma_{xx} + \sigma_{yy} + \sigma_{zz} \right) / 3 \]

TROSY selects the narrowest component!

Figure modified from that by C. Griesinger with permission.