J|A|C|S COMMUNICATIONS

Published on Web 02/01/2010

Diagonal-Free 3D/4D HN,HN-TROSY-NOESY-TROSY

Tammo Diercks,*,† Vincent Truffault,‡ Murray Coles,‡ and Oscar Millet†

NMR platform, CiC bioGUNE, Parque Tecnológico de Bizkaia, Ed. 800, 48160 Derio, Spain, and Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

Received December 14, 2009; E-mail: TDiercks@cicbiogune.es

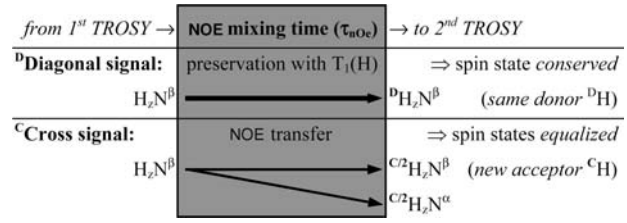
Protein structure elucidation by NMR spectroscopy relies on establishing spatial proximity between protons via cross-signals observed in a nuclear Overhauser effect spectroscopy (NOESY) spectrum. The first focus is on H^N because of the manageable number of amide $H^N-H'^N$ NOE cross-signals. Still, their unambiguous distinction requires editing via the associated ^{15}N frequencies with their superior dispersion, particularly in helical and partially unfolded proteins. Encoding *both* ^{15}N dimensions then yields the best dispersed NOESY spectrum possible for [U-^{15}N]-labeled proteins and is critical for spectral assignment by "NOE walking", fast fold determination, derivation of a first structural model, and structure refinement. Moreover, ^{15}N dimensions show a maximal transverse relaxation-optimized spectroscopy (TROSY) effect[1] for resolution and sensitivity enhancement, particularly in large, deuterated proteins, making the 3D/4D (H)N,HN-TROSY-NOESY-TROSY[2] experiment optimal in both spectral dispersion and resolution. However, NOESY spectra generally contain intense, uninformative *diagonal* signals that are the major source of spectral overlap, baseline distortion, truncation artifacts, and t_1 noise, possibly obscuring or biasing critical NOE cross-signals. Effective diagonal peak suppression is therefore paramount to maximize the coverage and precision of structural information obtainable from NOESY spectra. Satisfying all requirements for the first time, we here present *diagonal-free* 3D/4D (H)N,HN TROSY-NOESY-TROSY as a fully optimized experiment for recording the pivotal $H^N-H'^N$ NOE contacts. For this purpose, we connect two H,N-TROSY modules[1,3,4] by a modified spin-state-selective ($\mathbf{S^3}$) transfer module[5] (after NOE mixing) to allow for suppression of diagonal signals by *o*rthogonal *s*pin-state selection ($\mathbf{oS^3}$).[6,7]

The $\mathbf{oS^3}$ principle exploits the fact that uniform H_z polarization is subdivided into *two distinct* types of $\mathbf{S^3}$ H_z polarization when coupled to a spin $^1/_2$ partner (i.e. ^{15}N) that may be in either its α *or* β spin state. On the level of *separate* H_zN^α *or* H_zN^β polarizations, the paths leading to diagonal signals (denoted with $^{\mathbf{D}}$ superscripts) or NOE cross-signals (with $^{\mathbf{C}}$ superscripts) can then be distinguished, as shown in Scheme 1.

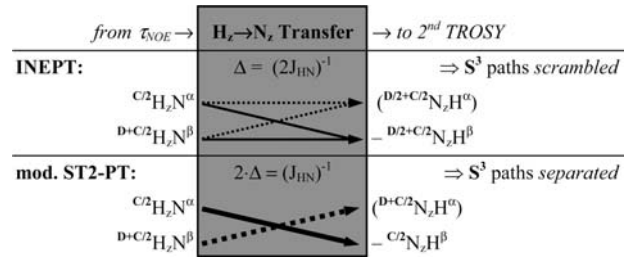
After selection of pure H_zN^β polarization by the first TROSY module, the diagonal path (thick arrow) *conserves* its coupled N^β spin state during τ_{NOE}, while stochastic NOE transfer to a different $^{\mathbf{C}}H$ acceptor proton polarization ($^{\mathbf{C}}H_z = {}^{\mathbf{C/2}}H_zN^\alpha + {}^{\mathbf{C/2}}H_zN^\beta$) entails an *equal distribution* (thin arrows) of α and β states for its coupled ^{15}N spin. Selecting for different (i.e. orthogonal) ^{15}N spin states before and after τ_{NOE} then lets pass the 50% NOE polarization with inverted N^α spin state but excludes all diagonal polarization with the conserved N^β spin state. Longitudinal T_1(H) relaxation does not affect the stability of the input N^β spin state and hence the diagonal suppression quality. Stochastic $N^{\beta/\alpha}$ spin flips due to T_1(N) relaxation contrarily lead to leakage of residual diagonal signal, but such "cross-talk"[6] between allowed and forbidden $\mathbf{S^3}$ paths is negligible in the most common case where $\tau_{NOE} \ll T_1$(N).

Diagonal suppression by $\mathbf{oS^3}$ is, however, impossible when $\mathbf{S^3}$ paths are remixed after τ_{NOE}. Yet, Scheme 2 shows that this is exactly what

Scheme 1



Scheme 2



happens when insensitive nuclei enhanced by polarization transfer (INEPT) is used for the required $H_z \rightarrow N_z$ polarization transfer after τ_{NOE} to feed the second TROSY module that eventually selects input N_zH^β polarization: INEPT mixes and converts *half* (thin arrows) of H_zN^α *and* H_zN^β into target $^{\mathbf{D/2+C/2}}N_zH^\beta$ polarization. Thus, 50% of the cross-polarization is read out along with 50% of the diagonal polarization that can no longer be excluded (terms in brackets and dashed pathways in Scheme 2 do not contribute to the final spectrum). A modified[5] ST2-PT module,[4] however, avoids mixing of $\mathbf{S^3}$ polarizations during $H_z \rightarrow N_z$ transfer. Again, half the H_z polarization is transferred to N_zH^β, but it now represents the *full* (thick arrow) H_zN^α component. Selective $H_zN^\alpha \rightarrow N_zH^\beta$ transfer (with spin-state inversion) then suppresses the diagonal signals that are absent in the input $^{\mathbf{C/2}}H_zN^\alpha$ polarization. Contrarily, setting for $\mathbf{S^3}$ $H_zN^\beta \rightarrow N_zH^\beta$ transfer (with spin states conserved) lets pass the *full* diagonal signal from the input $^{\mathbf{D+C/2}}H_zN^\beta$ polarization. Diagonal suppression by $\mathbf{oS^3}$ does *not* entail additional losses in NOE intensity (i.e. the same $^{\mathbf{C/2}}$ part is in all the N_zH^β terms), except from T_2 relaxation during the modified ST2-PT module, which is longer by $\Delta = (2J_{HN})^{-1}$.

Figure 1 shows the pulse sequence for (a) diagonal-free and (b) conventional 3D/4D HN,HN-TROSY-NOESY-TROSY, with the denoted modules gray-shaded if they do *not* preserve separate $\mathbf{S^3}$ pathways. This is irrelevant for the initial INEPT, but its use elsewhere in the sequence precludes $\mathbf{oS^3}$. To ensure $\mathbf{S^3}$ $H_z \rightarrow N_z$ polarization transfer after τ_{NOE}, a modified ST2-PT module must therefore replace the conventional INEPT. Only NOE transfer during τ_{NOE} then reshuffles the spin states, allowing its distinction by $\mathbf{oS^3}$ filtering from the spin-state-conserving diagonal path. For the rest, the TROSY modules before and after τ_{NOE} are identical. The modified[5] ST2-PT is a mirror image of the conventional ST2-PT[4] module (designed for $\mathbf{S^3}$ coherence

† CiC bioGUNE.
‡ Max-Planck-Institute for Developmental Biology.

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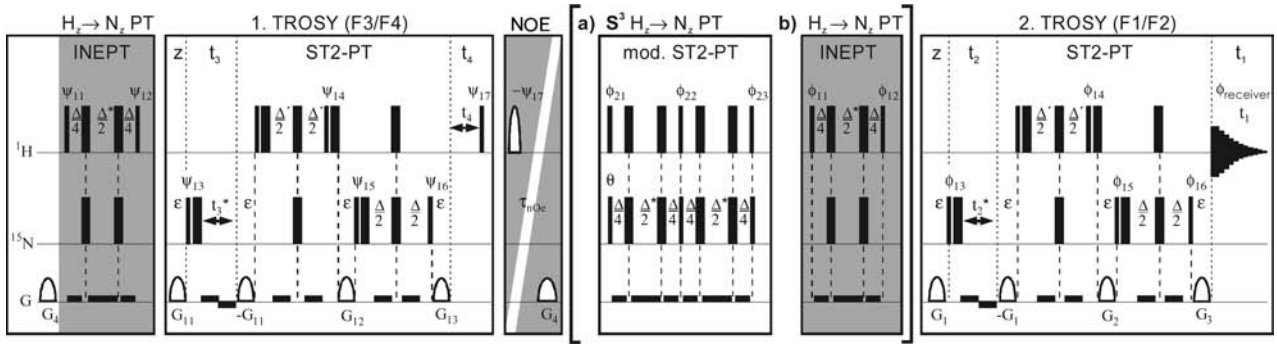

Figure 1. HN,HN-TROSY-NOESY-TROSY (a) with and (b) without diagonal suppression by **oS³**. The denoted modules are gray-shaded if they do *not* preserve separate **S³** pathways. During τ_{NOE}, NOE transfer equalizes the $N^{\beta/\alpha}$ spin states, but diagonal polarization preserves its N^{β} spin state (see Scheme 1). This distinction is obliterated by subsequent INEPT readout (b) (see Scheme 2) but can be preserved using the modified[5] ST2-PT module[4] (a). Diagonal signals can thus be excluded ($\theta = y$) or passed ($\theta = -y$). All versions ensure on-resonant water flip-back, include the $\gamma_N N_z$ polarization,[4] and allow for CLEAN TROSY[8] in F2, F3 (by adjusting delay Δ') and F1, F4 (by adjusting phases ϕ_{16}, Ψ_{16}). Phases: $\Psi_{11} = -y$; $\Psi_{12} = x$; $\Psi_{13} = y, -y$; $\Psi_{14} = y$; $\Psi_{15} = 8x, 8(-x)$; $\Psi_{16} = 8(-y), 8y$; $\Psi_{17} = 2x, 2(-y)$; $\phi_{11} = -y$; $\phi_{12} = \phi_{22} = 4x, 4(-x)$; $\phi_{21} = -x$; $\phi_{23} = 4(-y), 4y$; $\phi_{13} = 2y, 2(-y)$; $\phi_{14} = y$; $\phi_{15} = 4x, 4(-x)$; $\phi_{16} = 4(-y), 4y$; $\phi_{receiver} = (x, -x, -y, y), (-x, x, y, -y), (-x, x, y, -y), (x, -x, -y, y)$. Delays: Δ, $\Delta' \leq (2 \cdot J_{HN})^{-1}$. Delays marked with * are extended by $p90 \cdot 4/\pi$, where p90 is the duration of the preceding 90° pulse; $t_4 = p180(^{15}N) - p90(^{1}H) \cdot 4/\pi$ remains fixed in the 3D [H]N,NH-edited version. Gradient integrals: G_4, $G_{1,11}$ = arbitrary; $G_{2,12} = 0.437G_{1,11}$; $G_{3,13} = 0.291G_{1,11}$ (echo). Antiecho selection in F2(F3) requires setting $G_3(G_{13}) = 0.582G_1(G_{11})$ and inverting $\phi_{11} + \phi_{21} + \phi_{14} + \phi_{15}$ ($\Psi_{11} + \Psi_{12} + \Psi_{14} + \Psi_{15}$). Antiecho selection in F4 requires setting $\Psi_{17} = 2x, 2y$ and $G_{13} = 0.582G_{11}$ (echo in F3) or $0.291G_{11}$ (antiecho in F3). G_1 and G_{11} must *not* be identical to avoid artifacts from interference of gradient echoes. All 180° pulses are *b*roadband *i*nversion *p*ulses (BIPs)[9] to enhance sensitivity and tolerance toward B_{rf} heterogeneity, miscalibration, and offset. The water-selective 90° pulse ($-\Psi_{17}$) in τ_{NOE} is optional if radiation damping is sufficiently fast.

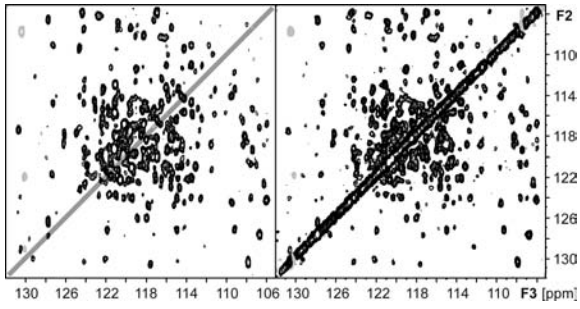

Figure 2. F2(^{15}N)/F3(^{15}N) projections of 3D [H]N,HN-TROSY-NOESY-TROSY spectra of maltose binding protein (MBP, 40 kDa) recorded (left) with diagonal suppression by **oS³** and (right) conventionally using the pulse sequences shown in Figure 1a,b, respectively. The suppressed diagonal (left) is traced in gray; its absence reveals several new NOE cross-signals with near-degenerate ^{15}N shifts. The spectra were recorded using [U-^{15}N/^{13}C, 70% ^{2}H]-labeled MBP (0.8 mM, 298 K) on a 800 MHz Bruker AVANCE III spectrometer with a TCI cryoprobe; 24 × 40 × 512 complex points were acquired during 30 h with 16 scans, $\tau_{NOE} = 150$ ms, $\tau_{interscan} = 1.5$ s.

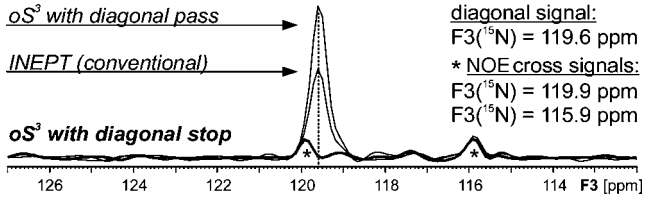

Figure 3. F3(^{15}N) rows extracted at F2(^{15}N)/F1(^{1}H) = 119.6/7.02 ppm from the 3D [H]N,HN-TROSY-NOESY-TROSY spectra of MBP recorded with the pulse sequences shown in Figure 1. The superposition confirms that the conventional INEPT version (Figure 1b) already suppresses diagonal signals by half in comparison with the **oS³** version (Figure 1a) set to pass the diagonal signals ($\theta = -y$), while diagonal suppression ($\theta = y$) entails no discernible loss of NOE intensity and is virtually complete. This allows unbiased identification of an NOE signal at F3(^{15}N) = 119.9 ppm that is otherwise completely obscured by the intense diagonal signal.

transfer in the opposite N→H direction), with an initial 90°(ϕ_{21}) ^{1}H pulse to convert H_z polarization to H_y coherence and a final 90° ^{15}N pulse to store the N_yH^{β} TROSY coherence as N_zH^{β} polarization for the subsequent *z* filter. Inverting either of these framing pulses inverts all HN magnetization. Inverting any of the other four 90° pulses swaps **S³** pathways, thus switching between diagonal stop or pass, while their pairwise inversion again inverts all HN magnetization.

Figure 2 shows that diagonal suppression in the new 3D **oS³** [H]N,HN-TROSY-NOESY-TROSY spectrum is virtually complete. This allows easy identification and unbiased quantification of various new $H^N-H'^N$ NOE contacts with similar ^{15}N shifts. A comparison of signal intensities (Figure 3) confirms that the conventional INEPT version eliminates 50% of the diagonal magnetization relative to the **oS³** version set for diagonal pass, while full diagonal suppression by **oS³** comes without noticeable losses in the NOE cross-signal intensities.

Diagonal-free **oS³** HN,HN TROSY-NOESY-TROSY thus offers optimal spectral dispersion and exploitation of the TROSY effect in two ^{15}N dimensions combined with specific elimination of the unwanted diagonal signals without compromising the NOE intensities. We therefore believe the experiment may become the standard for measuring crucial $H^N-H'^N$ NOE contacts, particularly in large deuterated, helical, membrane, or partly unfolded proteins or in nucleic acids. A key application could be studies of symmetric homodimers, where specific suppression of diagonal signals A−A may even reveal directly underlying, indicative NOE ipso contacts A−A′.

Acknowledgment. T.D. acknowledges a bizkaia:Xede grant.

Supporting Information Available: Concise product operator analysis of **S³** transfer pathways for the presented experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA910523Q