Sensitivity-enhanced double-TROSY experiment for simultaneous measurement of one-bond $^{15}\text{N}^{1\text{H}}$, $^{15}\text{N}^{13}\text{C}'$ and two-bond $^{1}\text{H}^{13}\text{C}'$ couplings

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Abstract

A recently published experiment for the measurement of $^{1}J_{\text{HN}}$, $^{1}J_{\text{NC}'}$, and $^{2}J_{\text{HC}'}$ coupling constants [J. Am. Chem. Soc. 125 (2003) 11504] was modified to yield a double-TROSY experiment which selects 1 of the 16 multiplet components from a $^{15}\text{N}$-HSQC spectrum recorded of a uniformly $^{15}\text{N}$/13C-labelled protein. Subspectra containing any 1 of the 16 multiplet components can be generated allowing accurate coupling constant measurements. The experiment is sensitivity enhanced, turning all magnetization components precessing during the evolution time into observable magnetization during the detection time. The experiment is discussed with regard to the previously published $^{a}/^{b}$-filtered HN($^{a}/^{b}$-NC$'$-$J$) experiment [J. Magn. Reson. 140 (1999), 32] which measures the same coupling constants.

Keywords: Couplings constants; TROSY; Spin-state selective editing; Sensitivity enhancement

1. Introduction

Residual dipolar couplings of weakly aligned molecules provide important long range restraints for protein structure determination by NMR [1]. Many different experimental schemes have been designed for the accurate measurement of coupling constants in uniformly $^{15}\text{N}$/13C-labelled proteins. Accurate measurements of, for example, $^{1}J_{\text{NC}'}$ coupling constants can be obtained with a quantitative long-range correlation experiment [2]. A particularly intuitive representation of this and other coupling constants is, however, presented by the multiplet fine-structure observed in a $^{15}\text{N}$-HSQC spectrum. The $^{1}J_{\text{NC}'}$ and $^{2}J_{\text{HC}'}$ coupling constants can be resolved particularly well, if the low-field component of the $^{1}J_{\text{HN}}$ doublet is selected in the $^{15}\text{N}$-dimension of the $^{15}\text{N}$-HSQC spectrum [3]. A recently published experimental scheme splits the $^{15}\text{N}$-HSQC spectrum differently into two subspectra with cross-peaks displaced by $^{1}J_{\text{NC}'}$ and $^{2}J_{\text{HC}'}$ coupling constants [4]. This experiment facilitates the measurement of $^{1}J_{\text{NC}'}$ in situations of overlapping doublet components in the $^{15}\text{N}$-dimension, but abandons the advantage of the narrower line width associated with TROSY peak components [4]. Here we present a generalized TROSY version of this experiment which separates the multiplet components of the $^{15}\text{N}$-HSQC cross-peaks into 16 different subspectra, allowing the facile measurement of the $^{1}J_{\text{HN}}$, $^{1}J_{\text{NC}'}$, and $^{2}J_{\text{HC}'}$ coupling constants from the narrowest multiplet components. The experiment belongs to the class of $^{a}/^{b}$-filtered HN($^{a}/^{b}$-NC$'$-$J$) experiments [5] in their TROSY version [6]. Since TROSY-type spin-state selection is applied twice, it may be referred to as a double-TROSY experiment. Similar to the original scheme [4], it is sensitivity enhanced. It is compared to the HN...
(α/β-NC′-J)-TROSY experiment which achieves the same type of subspectral editing [6].

2. Methods

Fig. 1 shows the pulse sequence of the sensitivity-enhanced double-TROSY experiment discussed here. The experiment returns the water back to the z-axis before data acquisition [7,8]. Two water-selective pulses at the start and at time point e ensure that the water magnetization is stored as z-magnetization also during the evolution time and the following A delays. The evolution of protein magnetization is readily described in terms of Cartesian product operators [9]. Our description assumes a three-spin system with the operators H, N, and C representing the spins of an amide proton, amide nitrogen, and carbonyl carbon, respectively. In a shorthand notation, we omit the standard normalization factors of anti-phase coherences.

Following the initial INEPT period, the term H$_2$N$_x$ is present at the start of the evolution time $t_1$. This magnetization evolves under the $^{15}$N chemical shift and the coupling constants $^1J_{HN}$ and $^1J_{NC}$, generating eight terms at the end of the evolution time (time point a in Fig. 1). Couplings to $^{13}$C are refocused by the 180°($^{13}$C) pulse. All eight terms are listed in the left-most column of Table 1. In addition, Table 1 lists the transformations of each of these terms until the detection period (time point b in Fig. 1). The 90°($^{15}$N) pulse following the evolution time stores half of the $^{15}$N magnetization as longitudinal terms while the other half decays by transverse relaxation until time point b. The period 2$A$ between time points a and b serves only as a relaxation delay. The following 90°($^{15}$N) pulse turns transverse N magnetization into longitudinal magnetization and vice versa. As a result, both halves of $^{15}$N-magnetization have experienced the same amount of transverse and longitudinal relaxation at time point c (Fig. 1). During the second period $2A = 1/(2J_{NC})$, half of the terms change from in-phase to antiphase magnetization with respect to the C′ spin and vice versa. This provides the basis for C′-spin-state selection by cycling of the phases $\phi_3$–$\phi_5$.

Table 1 shows that all eight terms result in observable magnetizations. Each term represents different in-phase and antiphase multiplicity with respect to the $^1J_{HN}$, $^1J_{NC}$, and $^2J_{HC}$ coupling constants. Two coupling constants are involved in each dimension ($^1J_{HN}$ and $^1J_{NC}$ in the F$_1$ dimension, and $^1J_{HN}$ and $^2J_{HC}$ in the F$_2$ dimension). Consequently, there are four possible combinations of in-phase and antiphase multiplicity in each dimension, totalling 16 combinations in the 2D experiment. Sixteen different data sets, A$_1$–D$_4$, must therefore be recorded and combined in appropriate manner to generate all 16 possible subspectra where each contains

![Fig. 1. Pulse sequence of the sensitivity-enhanced [15N–1HN]-double-TROSY experiment for measurement of one-bond 15N–13C′ and two-bond 13C–1H and 15N–1H couplings. Narrow and wide bars represent 90° and 180° pulses, respectively, with phase $\pi$, unless indicated otherwise. The 180°(13C) pulse drawn with a broken line is required only for recording data sets A$_2$–D$_2$ and A$_4$–D$_4$. Open and filled bell shapes on the 1H channel represent water-selective 90° pulses with the shape of Gaussian and sine functions, respectively. The carrier frequencies in the 1H, 15N, 13C, and 13C′ channels are positioned at 4.77 (water resonance), 120, 178, and 58 ppm, respectively. The pulse width of the 180°(13C) pulses is adjusted to sqrt(3)/(2$\Delta$), where $\Delta$ is the frequency difference in Hz between the 13C and 13C′ carrier frequencies. The delays are $\tau = 2.7$ ms, $A = 16$ ms. Selection of a single multiplet component requires the recording of a minimum of four data sets A–D, e.g., A$_1$–D$_1$. The complete set of 16 subspectra containing the 16 different multiplet components individually requires the recording of all data sets A$_1$–D$_2$ and A$_4$–D$_4$. $\phi_1$ and $\phi_2$ are cycled as $\phi_1 = x$, $-x$; $\phi_2 = x$, $x$, $x$, $x$, $-x$. In addition, the phase cycles of the different data sets are: (A$_1$,2) $\phi_1 = x, \phi_4 = y, \phi_3 = x, \phi_5 = y, \phi_{rec} = x, -x$; (B$_1$,2) $\phi_1 = -x, \phi_4 = y, \phi_3 = x, \phi_5 = y, \phi_{rec} = x, -x$; (C$_1$,2) $\phi_1 = x, \phi_4 = x, \phi_3 = y, \phi_5 = y, \phi_{rec} = x, -x$; (D$_1$,2) $\phi_1 = -x, \phi_4 = x, \phi_3 = y, \phi_5 = y, \phi_{rec} = x, -x$. The linear combinations required for subspectral editing are listed in Table 2. Quadrature detection in the indirect frequency dimension is achieved by incrementing $\phi_1$ with each increment of the evolution time $t_1$ in the manner of States-TPPI. Pulsed field gradients with a sine-bell shape are applied with 500 ms duration for $G_{1,2,3,4,5,6}$ and 1 ms for $G_2$, using the strengths of $G_{1,2,3,4,5,6} = 5, 15, 5, 7, 5, 28$ G/cm.](image_url)
Table 1
Relevant product operator terms at selected time points of the pulse sequence of Fig. 1*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Term</th>
<th>(\sigma (a))</th>
<th>(\sigma (b))</th>
<th>(\sigma (c'))</th>
<th>(\sigma (d'))</th>
<th>(\sigma (e))</th>
<th>(\sigma (f))</th>
<th>(\sigma (g))</th>
<th>(\sigma (h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1</td>
<td>(-H_N \cos(\Theta_{2/1}) \cos(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(H_N, z)</td>
<td>(-H_N, z)</td>
<td>(-H_N, z)</td>
<td>(-H_N, z)</td>
<td>(-H_N, z)</td>
<td>(-H_N, z)</td>
<td>(-H_N, z)</td>
</tr>
<tr>
<td>A1</td>
<td>2</td>
<td>(H_N \sin(\Theta_{2/1}) \cos(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(H_N, x)</td>
<td>(-H_N, x)</td>
<td>(-H_N, x)</td>
<td>(-H_N, x)</td>
<td>(-H_N, x)</td>
<td>(-H_N, x)</td>
<td>(-H_N, x)</td>
</tr>
<tr>
<td>A1</td>
<td>3</td>
<td>(H_N \sin(\Theta_{2/1}) \sin(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
</tr>
<tr>
<td>A1</td>
<td>4</td>
<td>(H_N \cos(\Theta_{2/1}) \sin(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
<td>(-N)</td>
</tr>
<tr>
<td>A1</td>
<td>5</td>
<td>(N_C \cos(\Theta_{2/1}) \cos(\pi J_{HN1} t) \sin(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
</tr>
<tr>
<td>A1</td>
<td>6</td>
<td>(N_C \sin(\Theta_{2/1}) \cos(\pi J_{HN1} t) \sin(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
</tr>
<tr>
<td>C1</td>
<td>1</td>
<td>(-N_C \sin(\Theta_{2/1}) \sin(\pi J_{HN1} t) \sin(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
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<tr>
<td>C1</td>
<td>2</td>
<td>(-N_C \cos(\Theta_{2/1}) \sin(\pi J_{HN1} t) \sin(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
</tr>
<tr>
<td>C1</td>
<td>3</td>
<td>(-N_C \sin(\Theta_{2/1}) \sin(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
</tr>
<tr>
<td>C1</td>
<td>4</td>
<td>(-N_C \cos(\Theta_{2/1}) \cos(\pi J_{HN1} t) \cos(\pi J_{NC1} t))</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
<td>(-N, C)</td>
</tr>
</tbody>
</table>

* Experiments A1 and C1 are recorded with different phase cycles (Fig. 1, Table 2). In particular, the receiver phase in C1 is shifted by 90° with respect to that in A1. The lower panel shows only those terms which differ from those of experiment A1 due to the different phase cycle.

b For a three-spin system consisting of \(^1H\), \(^{15}N\), and \(^{13}C\). The table reports all eight terms present at the end of the evolution time \(t\), and their evolution during the remaining part of the pulse sequence. Trigonometric factors are listed only once. Normalization factors of product operators were omitted for simplified presentation.

c Assuming \(\Delta = 1/(4^1 J_{NC})\).

d Assuming \(\tau = 1/(4^1 J_{HN})\).

e Terms changing sign when the phase \(\phi_1\) changes sign.

f Terms changing sign when the phase \(\phi_2\) changes sign.

g Terms changing sign when a 180°(C) pulse is applied.

3. Experimental

For experimental verification, spectra were recorded with a sample of \(^{15}N/^{13}C\)-labelled cytoplasmic domain of immunoglobulin-\(\beta\) [11]. The peptide was aggregated under the conditions used, resulting in a rotational correlation time \(\tau_c\) of about 7 ns at 25 °C. Fig. 2A displays one of the cross-peaks observed in a \(^{15}N\)-HSQC spectrum recorded without decoupling during \(t_1\) or \(t_2\), except that \(N-C^\alpha\) couplings were refocused during \(t_1\) with a \(C^\alpha\)-selective 180° pulse. The cross-peak displays only 8 of the 16 multiplet components in the fashion of an E.COSY experiment, because the C' spin is a passive spin during \(t_1\) and \(t_2\) and no pulses were applied at the C' frequency [12,13]. Figs. 2B-E show the same cross-peak in the subspectra a1–d1 (Table 2) recorded with the double-TROSY experiment of Fig. 1. The four multiplet components have different signs in the different subspectra. Summation of all four spectra results in a spectrum containing the multiplet component which is most high-field in the \(^1H\) dimension and most low-field in the \(^{15}N\)-dimension (subspectra \(z_1\) in Table 2). The four multiplet components are displaced by \(J_{HN}\) and \(J_{HC}\) in \(F_2\) and \(J_{NH}\) and \(J_{NC}\) in the \(F_1\) dimension. Separation of the multiplet components into different subspectra allows the accurate measurement of these couplings with minimal spectral overlap. The four panels \(z_1\) to \(z_1\) aligned along the diagonal of Fig. 3 display the corresponding subspectra. The subspectra \(z_1\) and \(z_1\)
contain the narrowest and most intense peaks of the multiplet. They are displaced with respect to each other by $J_{NC}$ and $J_{HC}$ (15.6 and 4.0 Hz, respectively, for the cross-peak displayed in Fig. 3).

Four times as many data sets are required to generate all 16 subspectra shown in Fig. 3 (Table 2), half of which must be recorded with an additional $180^\circ/C_0$ pulse (Fig. 1). These spectra do not contain new information and, except for the spectra $a_2$ and $b_2$, are less suited for coupling constant measurements because of their broader linewidths.

### 4. Discussion

The double-TROSY experiment of Fig. 1 was derived from the sensitivity-enhanced E.COSY $^{15}N-^{1}H$ HSQC experiment [4]. Since only $180^\circ$ pulses are applied to $C'$, the magnetization transfer efficiency of the experiment corresponds to that of a two-spin system. Therefore, the experiment can be performed in a way that all eight different magnetization components present at the end of the evolution time $t_1$ are transferred into observable magnetization at the start of the acquisition time [14].

Four times as many data sets are required to generate all 16 subspectra shown in Fig. 3 (Table 2), half of which must be recorded with an additional $180^\circ(C')$ pulse (Fig. 1). These spectra do not contain new information and, except for the spectra $a_2$ and $b_2$, are less suited for coupling constant measurements because of their broader linewidths.

### Table 2

Linear combinations yielding subspectra of the double-TROSY experiment

<table>
<thead>
<tr>
<th>Phase cycle</th>
<th>Linear combinations and subspectra$^{ab}$</th>
<th>Step-I</th>
<th>Step-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: $\phi=x$, $\psi=y$, $\phi=x$, $\phi=x$</td>
<td>$a_1 = A_1 + B_1$</td>
<td>$b_1 = (A_1 - B_1)^\omega$</td>
<td>$\alpha_1 = a_1 + b_1 + c_1 + d_1$</td>
</tr>
<tr>
<td>B: $\phi=-x$, $\psi=y$, $\phi=x$, $\phi=x$</td>
<td>$c_1 = C_1 + D_1$</td>
<td>$d_1 = (C_1 - D_1)^\omega$</td>
<td>$\gamma_1 = a_1 + b_1 - c_1 - d_1$</td>
</tr>
<tr>
<td>C: $\phi=x$, $\psi=x$, $\phi=y$, $\phi=y$</td>
<td>$a_2 = A_2 + B_2$</td>
<td>$b_2 = (A_2 - B_2)^\omega$</td>
<td>$\alpha_2 = a_2 + b_2 + c_2 + d_2$</td>
</tr>
<tr>
<td>D: $\phi=-x$, $\psi=x$, $\phi=y$, $\phi=y$</td>
<td>$c_2 = C_2 + D_2$</td>
<td>$d_2 = (C_2 - D_2)^\omega$</td>
<td>$\gamma_2 = a_2 + b_2 - c_2 - d_2$</td>
</tr>
<tr>
<td>A: $\phi=x$, $\psi=y$, $\phi=-x$, $\phi=x$</td>
<td>$a_3 = A_3 + B_3$</td>
<td>$b_3 = (A_3 - B_3)^\omega$</td>
<td>$\alpha_3 = a_3 + b_3 + c_3 + d_3$</td>
</tr>
<tr>
<td>B: $\phi=-x$, $\psi=y$, $\phi=-x$, $\phi=x$</td>
<td>$c_3 = C_3 + D_3$</td>
<td>$d_3 = (C_3 - D_3)^\omega$</td>
<td>$\gamma_3 = a_3 + b_3 - c_3 + d_3$</td>
</tr>
<tr>
<td>C: $\phi=x$, $\psi=x$, $\phi=-y$, $\phi=y$</td>
<td>$a_4 = A_4 + B_4$</td>
<td>$b_4 = (A_4 - B_4)^\omega$</td>
<td>$\alpha_4 = a_4 + b_4 + c_4 + d_4$</td>
</tr>
<tr>
<td>D: $\phi=-x$, $\psi=x$, $\phi=-y$, $\phi=y$</td>
<td>$c_4 = C_4 + D_4$</td>
<td>$d_4 = (C_4 - D_4)^\omega$</td>
<td>$\gamma_4 = a_4 + b_4 - c_4 + d_4$</td>
</tr>
</tbody>
</table>

$^a$ Filled and open circles represent positive and negative peaks, respectively. Small dots indicate unobservable multiplet components.

$^b$ The subscript '90' denotes a 90° phase correction of the data set in both dimensions. This can be achieved in the time domain by exchanging the real and imaginary part of each complex data point and taking the complex conjugate.

$^c$ The data sets $A_2-D_2$ and $A_4-D_4$ are recorded with the $180^\circ(C')$ pulse indicated by a broken line in Fig. 1.
pling constant measurements is important for the accuracy of the measurements, because the observed splittings are systematically reduced when the coupling partner is subject to relaxation and the linewidth is comparable to the magnitude of the coupling constant [17,18].

Fig. 2. Selected region of $^{15}$N–$^1$H correlation spectra recorded with an about 0.4 mM solution of uniformly $^{15}$N/$^{13}$C-labelled cytoplasmic domain of immunoglobulin-β at pH 5.7 and 25 °C. The data were recorded on a Bruker AV 800 NMR spectrometer using $t_{\text{max}} = 215$ ms and $t_{\text{mix}} = 128$ ms and a sweep width of 2381 Hz in the indirect dimension. (A) $^{15}$N–$^1$H HSQC spectrum recorded without $180^\circ(1H)$ pulse during $t_1$ and without $^{15}$N-decoupling during $t_2$; only the evolution caused by the $^1J(^{15}$N,$^{13}$C) scalar coupling was refocused by a $180^\circ(13C)$ pulse in the middle of the $^{15}$N evolution time $t_1$. Each $^{15}$N–$^1$H cross-peak displays eight of the 16 possible multiplet components shifted by $^1J(1H,15N)$, $^1J(15N,^{13}$C), and $^2J(1H,^{13}$C) with respect to each other. Asterisks identify multiplet components from an overlapping cross-peak. (B–E) Subspectra $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ obtained from the $^{15}$N–$^1$H double-TROSY experiment recorded with the pulse sequence of Fig. 1, following linear combination as shown in Table 2. Four data sets (A1–D1) were recorded with a total experimental time of 6 h, using 4 scans per FID. Positive and negative contours are represented by solid and dashed lines, respectively.

Fig. 3. Selected region from 16 different subspectra recorded with the $^{15}$N–$^1$H double-TROSY experiment of Fig. 1. The region encompasses the 16 different individual multiplet components of the cross-peak shown in Fig. 2A. For example, the four multiplet components present in Figs. 2B–E are found in the spectra aligned along the diagonal. The subspectra are identified with the Greek characters and Roman numerals used in Table 2. The experimental conditions were the same as those used to record the subspectra shown in Fig. 2. Asterisks identify multiplet components of an overlapped cross-peak.

pling constant measurements is important for the accuracy of the measurements, because the observed splittings are systematically reduced when the coupling partner is subject to relaxation and the linewidth is comparable to the magnitude of the coupling constant [17,18].
The phase cycle of Fig. 1 was chosen so that equilibrium $^{15}$N magnetization adds to the intensities of the conventional TROSY components and subtracts from the intensities of the anti-TROSY components on a spectrometer following the Bruker phase conventions. Since all terms present at the end of the $t_1$ evolution period are converted into terms observable during $t_2$, the sensitivity of the double-TROSY experiment corresponds to that of the generalized sensitivity-enhanced TROSY experiment [19–22], if relaxation during the delay $4\Delta$ can be neglected.

In principle, our double-TROSY experiment generates subspectra identical to those obtained by the $\alpha/\beta$-filtered HN($\alpha/\beta$-NC'-J) TROSY experiment [6]. The main difference between the two experiments is the position of the $t_1$-evolution period in the pulse sequence. In our experiment and the experiment by Ding and Gronenborn [4], the $t_1$-evolution period is placed right after the initial INEPT delay, whereas it is located after the $\alpha/\beta$-filter element in the experiment by Permi and Annila [6]. Consequently, the latter version does not require the relaxation compensation period $2\Delta$ and is thus shorter and contains fewer pulses. The $\alpha/\beta$-filtered HN($\alpha/\beta$-NC'-J) TROSY scheme by Permi and Annila [6] thus appears to be the better experiment. Combined with the generalized TROSY scheme [19,20] and an optional 180°($C'$) pulse before acquisition, this scheme would also afford 16 different subspectra as in Fig. 3.

Differences between the double-TROSY experiment of Fig. 1 and the $\alpha/\beta$-filtered HN($\alpha/\beta$-NC'-J) TROSY scheme by Permi and Annila [6] pertain to the presence of cross-talk between the different subspectra, which arises when the coupling constants deviate from the value 1/(2$J$) chosen for the delays $2\tau$ and $2\Delta$. Since the double-TROSY experiment of Fig. 1 combines the $\Lambda$ delays with the TROSY scheme in a single mixing period, the experiment offers alternative opportunities for suppression of cross-talk which are different from the established strategies applicable to the experiment by Permi et al. [23,24]. For example, we found that rapid exchange of amide protons with water at high pH affected primarily the product operators containing H$_2$ at time point b (Fig. 1), resulting in cross-talk from multiplet components which are separated by $J_{HN}$ from the selected components. This cross-talk could be reduced substantially by shortening the delay between time points d and e, which reduced the size of those terms which were less affected by amide proton exchange.

Cross-talk generated by different residual dipolar couplings, when measurements are performed with weakly aligned proteins, can be viewed as a result of $J$-mismatch, where the effective one-bond coupling constants do not match the delays optimized for single values of $J_{HN}$ and $J_{NC}$. In the absence of relaxation effects, $J$-mismatch is predicted to generate cross-talk of similar amplitude as the $\alpha/\beta$-filtered HN($\alpha/\beta$-NC'-J) TROSY experiment by Permi and Annila [6]. For example, subspectrum $\alpha_1$ (Table 2) is expected to display about 3% peak intensity from subspectrum $\beta_1$ and about 6% of peak intensity from the subspectra $\alpha_3$ and $\beta_3$, if $J$-mismatch is produced by one-bond $^1$H-$^{15}$N and $^{15}$N-$^{13}$C couplings of 65 and 12 Hz in an experiment with delays optimized for $J_{HN} = 92.6$ Hz and $J_{NC} = 15.6$ Hz, respectively (disregarding relaxation and the contribution from $^{15}$N-steady-state magnetization). Any other cross-talk is at least one order of magnitude smaller. Despite the complex mixing period, no observable magnetization is created by the additional magnetization transfer pathways opened by $J$-mismatch.

Quite generally, cross-talk from doublet components separated by $J_{HN}$ is attenuated by the enhanced relaxation rates of the anti-TROSY components and the contribution from $^{15}$N-steady-state magnetization. If a complete set of subspectra has been recorded, linear combination of the relevant subspectra can of course be used to compensate for cross-talk in individual cross-peaks [25], including cross-talk arising from different relaxation rates of the eight terms during the TROSY mixing period $4\tau$ [23]. Availability of additional subspectra would further assist in the coupling constant determination by providing several independent measurements and access to the $J_{HN}$ coupling constant from the narrowest multiplet components of the cross-peak. Since dipole–dipole cross-correlation effects between the $^1$H$_{\text{N}}$-$^{15}$N and $^1$H$_{\text{N}}$-$^1$H spin pairs result in asymmetric H$^N$ multiplets in the $F_2$ dimension [26], the $J_{HN}$ coupling constant is more accurately measured in the $F_1$ dimension [27]. In contrast, measurements of $J_{HC}$ coupling constants in the $F_2$ dimension of the double-TROSY experiment would not be affected by this particular dipole–dipole cross-correlation effect, since these coupling constants can be measured from multiplet components corresponding to the same $^{15}$N-spin state. Dipole–dipole cross-correlation effects between $^{13}$C$_t$-$^1$H$^N$ and $^1$H$_{\text{N}}$-$^1$H$^N$ spin pairs, however, could introduce a small $^{13}$C$_t$-spin-state dependent asymmetry to the cross-peaks in the $F_2$ dimension, affecting the measurement of $J_{HC}$. Clearly, these caveats do not apply to uniformly $^{15}$N/$^{13}$C/$^2$H-labelled proteins.

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