Supporting Information

**Unambiguous Tracking of Protein Phosphorylation by Fast High-Resolution FOSY NMR**

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Selective Polarisation Transfer schemes

The FOSY experiments use a concatenation of highly selective polarisation transfer (SPT) steps to single out a specific coupled spin system, i.e. residue, by selecting several of its known nuclear frequencies. Various schemes for SPT have been reported that differ, e.g. in transfer mechanism, transfer time, achievable selectivity, susceptibility to relaxation and competing passive couplings (given by the local spin topology). Notably the latter demands vary significantly along the desired polarisation transfer pathway through the peptide spin system. To maximise efficiency and robustness, FOSY therefore implements and adjusts the respectively best suited SPT scheme for each required transfer step. The key for concatenation of the SPTs into the experiment polarisation flow is the newly introduced frequency selective and spin state selective polarisation transfer $S^4PT$, as described below.

**PI-SPT**

For a heteronuclear IS two spin-½ system with mutual scalar $J_{IS}$ coupling, the spin coherence produces an in-phase signal doublet with frequencies $\nu^I + J_{IS}/2$ and $\nu^I - J_{IS}/2$ as the coupled S spin may be aligned either parallel (S₀) or antiparallel (Sₙ) with the external magnetic field, respectively. An inversion pulse applied with high selectivity at the frequency $\nu^I - J_{IS}/2$ then creates $\gamma_S$ enhanced anti-phase $2I_S S_z = I_S S_z - I_S S_y$ magnetization. This scheme for magnetization transfer, which we abbreviate PI-SPT (Population Inversion for Selective Polarisation Transfer), is frequency selective for spin I and notably immune to any further (other than $J_{IS}$) passive scalar couplings of spin S. Furthermore, in proteins, the amide HN group forms a particular IS system with significant cross-correlated relaxation giving rise to a strong TROSY effect. For such systems it was shown that PI-SPT reaches the physical limit of efficiency for magnetization transfer, surpassing all broadband PT schemes, if the $H_{NM}$ TROSY polarisation component is selectively inverted by a CROP-shaped pulse. In FOSY experiments, we employ such optimal PI-SPT for the initial $H_x \rightarrow 2N_H$ transfer step.

For the following section, we note that term $2I_S S_z$ can be also presented as $2I_S S_z = I_S S_z - I_S S_y$, where $I_{ijb}$ present single spin states of spin I. Both $I_S S_z$ and $I_S S_y$ terms are directly used by the subsequent $S^4PT$ block without need for intermittent complete magnetization conversion of $2I_S S_z$ to spin S.

**HH-$S^4PT$ - Heteronuclear Hartmann-Hahn (HH) frequency Selective and Spin-State Selective Polarisation Transfer ($S^4PT$)**

In an isolated heteronuclear IS two-spin system with mutual $J_{IS}$ coupling, selective $I \rightarrow S$ Hartmann-Hahn polarization transfer (HH-SPT) can be achieved by simultaneous weak continuous wave (CW) irradiation at the exact $\nu^I$ and $\nu_S$ frequencies for a duration $\tau_{CW}=1/J_{IS}$ and with identical field strengths

$$B_{1,I} = B_{1,S} = \frac{I_S \gamma_S^{\nu_{I} - \nu_{S}}}{4}$$  

[S1]

where $n = 1, 2, \ldots$ and $n = 1$ provides the weakest radiofrequency field $B_1 = J_{IS} \cdot \sqrt{3}/4$, affording highest $\nu^I$ and $\nu_S$ frequency selectivity as well as maximal tolerance to relative $B_1$ miscalibration and mismatch. These latter benefits of frequency selective heteronuclear Hartmann-Hahn transfer are crucially important and in stark contrast to the broadband implementation of Hartmann-Hahn transfer, where the losses from $B_1$ mismatch (that scale with $B_1$ and are inevitable for separate probe coils for I and S) are exacerbated and effectively prevent a wider use in solution state NMR.

Further passive couplings $J_{SM}$ of the receiving spin S, however, may strongly compromise the efficiency of HH-SPT. If $J_{SM} = J_{IS}$, the detrimental effects can be efficiently suppressed by simply using a slightly stronger $B_1$ field, given by Eq. S1, for $n = 2$ or 3, in exchange for a small loss of frequency selectivity in the Hartmann-Hahn polarisation transfer. If $J_{SM} > J_{IS}$, however, the signal of spin S gets “broadened” beyond the extremely narrow HH-SPT bandwidth of approximately $B_1 = J_{IS}$ (see above) due to splitting by $J_{SM}$. Efficient HH-SPT then requires to separately irradiate both lines of the S signal doublet\(^\dagger\)–\(^\ddagger\) with the same weak $B_1$ field strength given by Eq. S1, i.e. CW irradiation at the three frequencies $\nu^I, \nu_S - J_{SM}/2$, and $\nu_S + J_{SM}/2$. Under these conditions, HH-SPT passes via separate parallel $I_x \rightarrow S_x M_y$ and $I_x \rightarrow S_y M_b$ pathways that are immune to $J_{SM}$ coupling evolution and can each be described by the HH-SPT formalism for an isolated two spin system.\(^\circ\) As the parallel heteronuclear Hartmann-Hahn polarisation transfer pathways are both ($\nu^I, \nu_S$) frequency selective and ($M_{opb}$) spin state selective, we propose the acronym HH-$S^4PT$. Importantly, the sign for $I_x \rightarrow S_x M_y$ and $I_x \rightarrow S_y M_b$ pathways in HH-$S^4PT$ can be controlled via the phase of the pertaining CW irradiation at $\nu_S - J_{SM}/2$ vs. $\nu_S + J_{SM}/2$. Thus, identical CW phases produce $S_x M_y + S_y M_b = S_z$ inphase transfer while opposite phases $S_x M_y - S_y M_b = 2S_y M_b$ achieve antiphase transfer. Furthermore, HH-$S^4PT$ can be generalized to larger than three-spin systems, where passive couplings “broaden” both spin I and S resonances, by adjusting the number of CW irradiation frequencies.
In FOSY experiments, we employ HH-SPT for direct $2N_iH_x \rightarrow 2CO_i^{−1}CA_i^{−1}$ antiphase-to-antiphase polarization transfer. Both the starting N and receiving CO spins show passive couplings ($^1J_{NN} = 90 \text{ Hz}$ and $^1J_{CO,CA} = 55 \text{ Hz}$) much larger than the active $^1J_{N,CO} = 15 \text{ Hz}$ coupling. Consequently, HH-SPT must enable the following four transfer pathways:

- $N_i^1H_x \rightarrow CO_i^{−1}CA_i^{−1}$
- $N_i^1H_x \rightarrow -CO_i^{−1}CA_i^{−1}$
- $-N_i^1H_x \rightarrow CO_i^{−1}CA_i^{−1}$
- $-N_i^1H_x \rightarrow -CO_i^{−1}CA_i^{−1}$

To sum up to the desired overall transfer:

$$2N_i^1H_x = N_i^1(H_x - H_y) \xrightarrow{\text{HH-SPT}} CO_i^{−1}(CA_i^{−1} - CA_i^{−1}) = 2CO_i^{−1}CA_i^{−1}$$

This is achieved by CW irradiation with identical $B_1$ (given by Eq. S1) at the four frequencies $\nu_N^i + \frac{^1J_{NN}}{2}, \nu_N^i - \frac{^1J_{NN}}{2}, \nu_{CO}^i - \frac{^1J_{COCA}}{2}, \nu_{CO}^i - \frac{^1J_{CO}}{2}$ and with inverted irradiation phases at $\nu_N^i - \frac{^1J_{NN}}{2}$ and $\nu_{CO}^i - \frac{^1J_{CO}}{2}$ (see Figures S2b and c). Since the nitrogen CWs are applied at two different frequencies, it is important to align their phases at the beginning. Similarly, the two carbon CWs are phase-aligned at the end.

**LSF-SPT**

Similar to the PI-SPT described above, for an IS spin system, the Longitudinal Single Field Selective Polarization Transfer module\(^5\-^7\) (LSF-SPT) uses a single radiofrequency and thus is frequency selective only for spin I. The LSF-SPT does not depend on frequency and passive couplings of spin S and it performs efficient transfer of spin I magnetization to antiphase coherence of spin I in respect to spin S ($I_z \rightarrow 2I_zS_z$). The LSF-SPT uses a continuous-wave irradiation of spin I at frequency $\nu_I$ with optimal duration $\tau_{\text{CW}} = \frac{\sqrt{2}}{2J_{IS}}$

and strengths $B_1 = \frac{J_{IS}}{2}$. As for HH-SPT, the use of CW irradiation in LSF-SPT allows SPT implementation to remove detrimental effects of passive scalar couplings of the starting I spin. Thus, for a three spin ISM system with passive couplings $J_{MS}=0$ and $J_{MI} > J_{IS}$, the LSF-SPT with the $\tau_{\text{CW}}$ and $B_1$ defined above is performed without decoupling of spin M by two CW’s at frequencies $\nu^I - J_{MI}/2$ and $\nu^I + J_{MI}/2$. Transfers $I_z \rightarrow 2I_zS_z$ and $2M_zI_z \rightarrow 2I_zS_z$ are achieved using the CW’s aligned at the start with the same and opposite phases, respectively. The LSF-SPT approach can be extended to more passive scalar couplings of spin I by combining the corresponding synphase and antiphase CW’s.

In contrast, small passive scalar couplings $J_{MI} < J_{IS}$ of spin I are handled by adjusting both $\tau_{\text{CW}}$ and $B_1$. For the desired $2C_i^{−1}CA_i^{−1} \rightarrow 2C_i^{−1}N_i^{−1}$ transfer by active $^2J_{CA,I-1N,I-1} = 12 \text{ Hz}$ in the presence of passive $^2J_{CA,I-1N,I} = 7 \text{ Hz}$ coupling, setting $\tau_{\text{CW}} = 43 \text{ ms}$ and $B_1 = 8 \text{ Hz}$ yields 72% transfer efficiency (disregarding relaxation) similar to broadband INEPT (compare with round parentheses in Fig. S1a). The competing smaller $^2J_{CA,I-1N,I}$ coupling results in some back-transfer to $2C_i^{−1}N_i^{−1}$ about three times less efficient than the desired transfer via $^1J_{CA,I-1N,I-1}$, Unlike broadband INEPT, LSF-SPT would enable even 100% transfer when selectively decoupling the $^{15}N$ frequency. Yet, this may be problematic if the $^{15}N^{+1}$ and $^{15}N^i$ frequencies are close and we, therefore, have not implemented $^{15}N^i$ decoupling in our experiments.
The 2D FOSY \(^1\)H-\(^{15}\)N \(\text{hnco(CA)NH}\) and \(^1\)H-\(^{13}\)C \(\text{hncoCA(N)H}\) experiments for deuterated proteins (Figure S1a) start with highly selective population inversion (PI-SPT, Figure S2a) to create \(\nu^i\)-frequency selected and \(\nu_H\) enhanced \(2H^1J^1N^1\) two-spin magnetization. Then, a hard 90° pulse on \(^{15}\)N produces anti-phase coherence \(2H^1J^1N^1\) for the subsequent selective Hartmann-Hahn polarisation transfer (HH-S\(^4\)PT step) \(2H^1J^1N^1 \rightarrow 2C_z^{A,i-1}A^{A,i-1}\). Then, chemical shifts of \(^{13}\)C\(^{A,i-1}\) or \(^{15}\)N\(^{A,i-1}\) are traditionally sampled during corresponding constant time periods followed by TROSY \(^1\)H\(^1\) detection. With two unknown frequencies (\(H^1\) and either \(N^1\) or \(CA^1\)) sampled and three known frequencies (\(H^1\), \(N^1\), \(CO^1\)) preselected, a pair of the 2D FOSY \(\text{hnco(CA)NH}\) and \(\text{hncoCA(N)H}\) spectra corresponds to a 3D FOSY \(\text{hncoCANH}\) (with both \(CA^1\) and \(N^1\) frequency sampling) that has the frequency dispersion of a pertaining 6D experiment sampling all six nuclei.

In the 2D FOSY \(\text{hnccabcNH}\) experiment (Figure S1c), INEPT transfer from \(C^{A,i-1}\) to \(N^{A,i-1}\) is replaced by the LSF-S\(^4\)PT module \(\text{qcw6}\) (Figure S2d) for direct \(2C_z^{A,i-1}A^{A,i-1} \rightarrow 2C_z^{A,i-1}N_z^{A,i-1}\) transfer with concomitant residue type selective \(C^{B,i-1}\) decoupling\(^1\), as explained above. The additional frequency selection of \(C^{A,i-1}\) and \(C^{B,i-1}\) here produces an effective frequency dispersion equivalent to a 7D experiment. The employed field strength of the \(C^{B,i-1}\) decoupling of several hundred Hertz provides only relatively low resolution in the \(C^{B,i-1}\) dimension, but it is sufficient in most cases to identify the preceding residue type. To cancel out any perturbation of \(C^{A,i-1}\) coherence, we compensate the effects of \(C^{B,i-1}\) decoupling at the opposite side of the \(^{13}\)C\(^{A,i-1}\) resonance (Figure S2d). If the \(C^{B,i-1}\) frequency is unknown, the corresponding \(C^{A,i-1}\) frequency “splitting” by \(^1J_{CA,CB}\) coupling is handled in the same way as the other passive \(^1J_{DA,CD}\) coupling, i.e. by implementing the \(S\(^4\)PT\) module \(\text{qcw6}\) with quadruple frequency selective CW irradiation (Figure S2e), as explained above.

For non-deuterated proteins, broadband \(^1\)H decoupling in the \(\text{hnco(CA)NH}\) and \(\text{hncoCA(N)H}\) experiments (Figure S1a) is implemented by a pair of \(^1\)H inversion pulses (Figure S3a) that also ensures a return of both water and aliphatic proton magnetisations towards their thermal equilibrium. To similarly adapt the \(\text{hnccabcNH}\) experiment, we replace the module shown in Figure S1c by the block depicted in Figure S3b. The corresponding LSF-S\(^4\)PT sandwiches with and without \(^{13}\)C\(^{B,i-1}\) decoupling are depicted in Figure S3c and Figure S3d, respectively. Of note, as the LSF-S\(^4\)PT scheme employs irradiation only on \(^{13}\)C, it naturally leaves all (water and protein) \(^1\)H magnetization unaffected.

For IDPs (like Tau) with their typically slow transverse relaxation, the \(^{15}\)N frequency selectivity can be further improved as shown in Figure S1b. Here, the initial PI-SPT module is replaced by a selective 90° pulse on \(^1\)H followed by HH-SPT employing simultaneously applied CW irradiation modules \(\text{scw1}\) (on \(H^1\)) and \(\text{scw2}\) (on \(N^1\)) for \(H^1 \rightarrow N^1\) magnetisation transfer. The directly appended HH-S\(^4\)PT step for \(N^1 \rightarrow 2C_z^{0,i-1}A^{A,i-1}\) is implemented by single \(\nu^i\) frequency selective (\(\text{scw3}\)) CW irradiation on \(N^1\) with concomitant selective \(^1\)H decoupling by \(\text{scw5}\). The use of \(\text{scw3}\) (Figure S1b) instead of double \(\nu^iH^1 + \frac{J_{HN}}{2}\) frequency selective irradiation \(\text{dcw3}\) (Figure S1a, S2b) results in a twice as high \(\nu^i\) frequency selectivity, while the \(\nu^iC^{-1}\) frequency selectivity of the same \(\text{dcw4}\) module (Figure S2c) is maintained. The block shown in Figure S1b can be used both for deuterated and non-deuterated proteins, and illustrate the versatility of the FOSY approach along with its possible fine tuning to a specific spin system. In this case, while the PI-SPT version of the experiment (Figure S1a) minimises relaxation losses and, thus, maximises sensitivity, its HH-SPT version (Figure S1b) optimises for frequency selectivity and effective spectral dispersion. As shown in Figure S7 for hTau40, both PI-SPT and HH-SPT versions of the experiment (Figure S1a and S1b) provided very similar results at 5°C and 15°C. The FOSY assignment walk for phosphorylated hTau40 was performed using the HH-SPT version (Figure S1b) as it offered enhanced \(^{15}\)N resolution while yielding marginally higher peak intensities at 15°C.
Figure S1. Inter-residue FOSY experiments for deuterated proteins in H2O-based buffer solution. Selective (Pi-SPT, HH-S4PT, LSF-S4PT) and broadband (INEPT, ST2-PT) transfer blocks are colored in blue and red, respectively, same as in Figure 1 of the article. a) The 2D FOSY 1H-15N hncoc(CA)NH and 1H-13CA hncoc(CA)NH experiments are acquired as the corresponding 2D planes of 3D FOSY-hncocCANH experiment depicted in the panel. Initial selective population inversion (Pi) of the TROSY component by a frequency selective pulse (applied at $\nu_H^{i} = \nu_{d_{2w3}}$) converts 2$H_x^i N_y^i$ antiphase magnetisation $^{1,2,4}$ that is converted into 2$H_x^i N_y^i$ coherence by a (unselective) 90° 15N pulse. The following HH-S4PT block with a double continuous-wave (dcw3, see Fig. S2) simultaneous transfers both 15N TROSY and anti-TROSY components without proton decoupling, i.e. the transfer (selective for 15N(i,j) and 13^{13}O(i,j)) 2$H_x^i N_y^i$ $\rightarrow$ 2$C_x^{ij-1} C_y^{ij-1}$ is achieved by a 66.7 ms 1H-SSPT block including: double selective at frequencies 15N(i,j)±46 Hz continuous waves (dcw3) with $B_{dcw3} = 14.5$ Hz beginning with aligned phases at; as well as double selective at frequencies 13^{13}O(i,j)±28 Hz continuous waves (dcw4) with the same $B_{dcw4} = 14.5$ Hz ending the phases aligned at. Constant-time States-TPPI evolution of 13^{13}CA(i,j) (including broadband 2$H$-decoupling) is implemented with $\delta_1$ delay and $\phi_1$. The subsequent INEPT step (in brackets) for simultaneous evolutions $2C_x^{ij-1} C_y^{ij-1}$ $\rightarrow$ 2$C_x^{ij-1} C_y^{ij-1}$ (with $2\zeta = (2-1/4)^{1/2} = 3.9$ ms) and $C_x^{ij-1} \rightarrow 2C_x^{ij-1} N_z^{j-1}$ (during $T_C < (2-1/4)^{1/2} = 26.6$ ms) is implemented with 1H$^2$ decoupling and optional States-TPPI type constant-time sampling of the 13^{13}CA(i,j) frequency (during $T_C \leq T_C$). The following INEPT step for $2C_x^{ij-1} N_y^{j-1} \rightarrow N_z^{j-1}$ transfer (during $T_{N_y} \leq (2-1/4)^{1/2} = 40$ ms) is concatenated with the final ST2-PT module and implemented with optional echo/anti-echo type constant-time sampling of the $N_z^{j-1}$ frequency (during $T_6 \leq T_N - 28$) with simultaneous alteration of three gradients $g_4^{*}E_{A4}, g_5^{*}E_{A5}, g_6^{*}E_{A6}$ ($E_{A4} = 1, 0.875; E_{A5} = 0.6677, 1; E_{A6} = 1, 0.6595$) and $\phi_6 = \gamma - y, \phi_7 = \gamma - y$. The constant-time delays $T_6$ and $T_{N_y}$ are 14.3 ms and 20 ms respectively; delays are $\zeta = 3/4 J_{CA}^{1/2}$ = 4.7 ms and $\delta = \Delta = 1/4 J_{NH}^{1/2} = 2.75$ ms. Pulses: Narrow and thick bars represent 90° and 180° high power pulses, respectively. Selective pulses on amide 1H: s1(90°) = 1 ms Sinc at the selected $\nu_H^i$; s2(90°) = 1.74 ms time reversed $EB_{BP}^2$, s3(180°) = 1.85 ms Re$B_{BP}$, and s4(90°) = 1.74 ms $EB_{BP}^2$ are applied at 8.5 ppm (centre of amide 1H). Selective pulses on 13^{13}C: s5(90°) = 600 ms Sinc and s6(180°) = 600 ms Sinc at the selected $\nu_C^{j0}$; s7(90°) = 500 ms QS and s8(90°) = 500 ms time reversed QS at 55 ppm (center of CA); s9(180°) = 200 ms Q3 at 39 ppm (centre of CA and CB); s10(180°) = 800 ms $I_{BP}^1$ at 173 ppm (centre of CO); s11(180°) = 600 ms $I_{BP}^1$ at 55 ppm (centre of CA). Gradients: All gradient pulses have smooth square (SMSQ) shape and 1ms duration except for $g_4^i, g_5^i, g_6^i$ (0.5 ms) and $g_7^i, g_8^i$ (0.3 ms). Relative gradient strengths: $g_1 = 57$, $g_2 = 47$, $g_3 = 37$, $g_4 = 80$, $g_5 = 30$, $g_6 = 30.13$, $g_7 = 15$, $g_8 = 60$, whereas 100% corresponds to 53.5 G/cm. Pulse phases (default = x): $\phi_1 = x, \phi_2 = 2(\gamma - y), 2(\gamma); \phi_3 = 4(x), 4(-x); \phi_4 = 8(x), 8(-x); \phi_5 = 16(x), 16(-x); \phi_6 = 2\gamma, \phi_7 = 2\gamma; \phi_{rec} = 4(x, -x, x, -x, x, x, x, x)$. b) Modification to double the $\nu_N$ selectivity. The indicated block replaces the pulse program segment in square brackets in panel (a). The initial Pi is replaced by a $\nu_N$ selective s1(90°) pulse followed by a HH-SPT block ($T_{scw3} = 11$ ms) employing simultaneous single frequency selective CW irradiation at $\nu_C^j$ (scw1) and $\nu_C^j$ (scw2) with identical strength $B_{scw3} = B_{scw2} = 39$ Hz. The subsequent dcw3 at $\nu_N^i$ is replaced by single frequency CW (scw5) with maintained $B_{scw3} = 14.5$ Hz and concomitant selective CW decoupling (scw5) at $\nu_N^i$ with $B_{scw5} = 700$ Hz. c) 2D FO-SSPT module (scw4 = 59 ms) employing quadruple frequency selective CW (qcw6; see Fig. S2d) at $\nu_N^i \pm 28$Hz with $B_1 = 6$ Hz and the frequency selective decoupling of 13^{13}CA with corresponding compensation irradiation.
Figure S2. Schematic representation of irradiation frequency schemes in the continuous-wave sandwiches used in the pulse sequence shown in Figure S1. Not shown are simple on-resonance single-frequency continuous-waves for scw1, scw2, scw3, and scw5. a) PI: Selective single pulse for population inversion of $^1$H TROSY component for $H_2^N \rightarrow 2H_2^N N_2^N$ polarization transfer. The pulse may have a Gauss or CROP shape, the latter is optimized for inversion in the presence of cross-correlated relaxation. b) dcw3: Double continuous-wave sandwich includes two continuous-waves with offsets $\nu_C^i \pm \frac{I_{CA}}{2}$ starting with opposite phases $\pm x$ (depicted as shapes pointing up/down). The $B_1$ strength for both continuous-waves is $\approx 15$ Hz. c) dcw4: Double continuous-wave sandwich includes two continuous-waves with offsets $\nu_{CA}^i \pm \frac{I_{CA}}{2}$ ending with opposite phases $\pm x$. The $B_1$ strength for both continuous-waves is the same $\approx 15$ Hz. d) qcw6: Quadro continuous-wave sandwich qcw6 for deuterated proteins with $^{13}C_{8,1}$ decoupling includes four continuous-waves: two with $\nu_{CA}^i \pm \frac{I_{CA}}{2}$ offsets ending with the opposite phases $\pm x$, and two decoupling continuous-wave at $\nu_{CB}^i \pm \frac{I_{CA}}{2}$ and compensation continuous-wave at $2\nu_{CA}^i - \nu_{CB}^i$ with opposite phase. The $B_1$ strength is 6 Hz for $^{13}C_{8,1}$ continuous-waves. For $^{13}C_{8,1}$ decoupling, $B_1 \gg \frac{I_{CA}}{2}$ (e.g. $B_1 = 300$ Hz) is selected to match/distinguish different types of amino acid. e) qcw6: Quadro continuous-wave sandwich for deuterated proteins without $^{13}C_{8,1}$ decoupling includes four $B_1 = 6$ Hz continuous-waves at $\nu_{CA}^i \pm \frac{I_{CA}}{2} \pm \frac{I_{CA}}{2}$ frequencies ending at phases $x, x, -x, -x$.

Figure S3. Modifications of the FOSY experiment (Figure S1) for non-deuterated proteins in H$_2$O-based buffer. The part in round brackets in Figure S1 is replaced by a block: a) For FOSY-hnocANH (Figure S1a,b), continuous broadband $^1$H$^N$-decoupling is replaced by a pair of unselective $^1$H inversion pulses to refocus $J_{CA}$ evolution and return water and protein proton magnetization to thermal equilibrium. b) For FOSY-hncocabNH (Figure S1c) experiment with fixed $^{13}C_{8,1}$ and selective $^{13}C_{8,1}$ decoupling, continuous $^1$H$^N$-decoupling is omitted while the quadruple $\nu_{CA}^i$ frequency selective CW irradiation module (qcw6) is replaced by hcw7 CW sandwich analogue. HCW7: hexo continuous-wave sandwich employing selective $^{13}C_{8,1}$ decoupling used in (b). hcw7 consists of four 6 Hz CWs at $\nu_{CA}^i \pm \frac{I_{CA}}{2} \pm \frac{I_{CA}}{2}$ frequencies ending with phases $x, -x, x, -x$ depicted as shapes pointing up (x) and down (-x), and two CWs $B_1 \gg \frac{I_{CA}}{2}$ (e.g. $B_1 = 300$ Hz), one for selective $^{13}C_{8,1}$ decoupling at $\nu_{CB}^i \pm \frac{I_{CA}}{2}$ frequency and a compensation CW with the opposite phase at $2\nu_{CA}^i - \nu_{CB}^i$. d) ocw7: octo continuous-
wave sandwich without $^{13}$CBI decoupling optionally replacing hcw7 in (b) including eight 6 Hz CWs at $\nu^{\text{IC}}_{\text{CA}} \pm \nu^{\text{JS}}_{\text{CA}} \pm \nu^{\text{JC}}_{\text{CA}} \pm \nu^{\text{IC}}_{\text{CB}}$ frequencies ending with phases x, x, -x, x, x, -x, -x depicted as shapes pointing up (x) and down (-x).

Proline – Selective experiments
The triple resonance experiments used in this work are based on the BEST-TROSY intra-HNCA and HNCOCA experiments\textsuperscript{5} with modifications for proline selection as described by Solyom et al.\textsuperscript{10} Thus, a selective 8.25 ms long REBUP\textsuperscript{11} shape $^{15}$N inversion pulse covering the distinct proline chemical shift range 138±2.77 ppm (250 Hz) is applied during the constant-time $^{13}$C\textsuperscript{4} chemical shift evolution period ($T_e = 2/\nu_{\text{CA}}$ = 56 ms). This pulse is omitted in alternating scans such that the $\nu_{\text{CANPRO}}$ coupling either evolves or not, while the receiver phase is inverted for pairwise subtraction of scans. In the resulting spectrum, signals are only observed if the corresponding $^{13}$C\textsuperscript{4} couples with a $^{15}$N proline spin inverted by the REBUP pulse. Thus, modified iHNCA and HNcoCA difference experiments contain signals only for residues X preceding (XP) or succeeding (PX) a proline, respectively.

Experimental NMR technical details
All NMR experiments were recorded on an 800 MHz Bruker AVANCE III HD spectrometer equipped with a 3 mm TCI $^1$H/$^{13}$C/$^{15}$N cryoprobe. Spectra acquisition, processing, and analysis were performed using TopSpin 3.5 (Bruker BioSpin). 2D FOSY-hncoCA(N)H experiments were recorded with 150 × 1024 complex data points; 2D FOSY-hnco(CA)NH and 2D FOSY-hnccacbNH experiments were recorded with 65 × 1024 complex data points. The spectral widths for $^{13}$CA, $^{13}$N, and $^{1}$HN were 30 ppm, 26 ppm, and 14 ppm, respectively. The interscan relaxation delay was 0.5 sec, and the number of scans ranged from 2 to 32, depending on the signal intensity. The proline-selective experiments were recorded with 128 × 2048 complex data points and a $^{15}$N spectral width of 26 ppm for the PX spectrum vs. 64 × 1024 complex data points and a $^{15}$N spectral width of 41 ppm for the XP spectrum. The standard 3D BEST-TROSY HNCO experiment, obtained from the IBS library\textsuperscript{12}, was recorded with 100 × 100 × 2048 complex data points and spectral widths of 10 ppm for $^{13}$CO, 26 ppm for $^{15}$N, and 16 ppm for $^{1}$HN. A short recycle delay of 0.5 s was used in all experiments.

Protein expression and purification

*E. coli* BL21 (ADE3) Star\textsuperscript{TM} (Novagen) cells were transformed with a modified pET28b plasmid harboring full length hTau40 protein fused to an amino-terminal His-SUMO Tag (purchased in *E. coli* codon optimized-form from GenScript). $[^{15}$N,$^{13}$C] or $[^{1}$H,$^{15}$N,$^{13}$C] isotope enriched protein was produced using 2xM9 minimal medium\textsuperscript{13} supplemented with $[^{15}$N,$^{13}$C]H\textsubscript{4}Cl (1 g/l) and D-($^{1}$H,$^{13}$C) or D-($^{1}$H,$^{13}$C)-glucose (2 mg/l) as the sole nitrogen and carbon sources, respectively, in either H\textsubscript{2}O or D\textsubscript{2}O based growth medium. All isotopically labelled material was purchased from Merck. The transformed cells were grown at 37°C in medium supplemented with 50 µg/ml kanamycin until reaching an OD\textsubscript{600} = 0.8. Expression was induced by adding 1 mM isopropyl-thiogalactoside (IPTG) (Thermo Scientific) for 16 h at 22°C. Cells were harvested by centrifugation (5000 × g, 30min) and subsequently resuspended in lysis buffer (20 mM NaP\textsubscript{4}, 500 mM NaCl, pH 7.8). Cells were lysed by four passes through an Emulsiflex C3 (Avestin) homogenizer and the cleared lysate was purified with a 5ml HisTrap HP column (GE Healthcare). hTau40 eluted within a 150 mM imidazole step. Fractions containing hTau40 were pooled and dialyzed overnight against human SenP1 cleavage buffer (20 mM TrisHCl, 150 mM NaCl, 1 mM DTT, pH 7.8). After dialysis, SenP1 protease (Addgene #16356)\textsuperscript{14} was added and enzymatic cleavage was performed for 4 hours at room temperature. His-SUMO-Tag and hTau40 were separated by a second HisTrap HP column step and fractions containing cleaved hTau40 in the flow-through were collected, concentrated, and subsequently purified by gel filtration using a HiLoad 10/60 200pg (GE Healthcare) pre-equilibrated with NMR buffer (25 mM sodium phosphate buffer pH 6.9, 50 mM NaCl, 1 mM EDTA). The pure hTau40 fractions were concentrated to about 500 µM, flash-frozen in liquid nitrogen, and stored at -80°C till usage.

Phosphorylation of Tau protein
GSK3β kinase was purchased from SignalChem (ab60683). In vitro phosphorylation reactions were carried out as described before.\textsuperscript{15} Briefly, 150 µM $[^{1}$H,$^{15}$N,$^{13}$C] hTau40 was mixed with 0.04mg/ml of GSK3β in phosphorylation buffer (20 mM HEPES, 2 mM ATP (Thermo Scientific), 25 mM MgSO\textsubscript{4}, 2 mM EDTA, pH 7.4). The reaction was performed at 25°C for 12 hours, followed by gel filtration (Superdex200 Increase 10/300 GL, GE Healthcare) with NMR buffer. Under the chosen conditions, GSK3β kinase phosphorylated hTau40 to near completion (>95%), as assessed by NMR spectroscopy. The final NMR sample of phosphorylated hTau40 was then concentrated to 150 µM and measured at 15°C.
NMR samples
We used three NMR samples of identical 150 µM concentration: (i) phosphorylated [U−2H,15N,13C] hTau40, (ii) U−2H,15N,13C hTau40, and (iii) [U−15N,13C] hTau40, all dissolved in the same NMR buffer (25 mM sodium phosphate buffer pH 6.9, 50 mM NaCl, 1 mM EDTA, 1H2O/2H2O 9:1).

Deuteration is not strictly required for IDPs with their favourably slow T2 relaxation and we, therefore, present the experiments (Figures S1-S3) and results (Table S2) for both deuterated and non-deuterated proteins. However, deuteration is favourable and clearly advantageous for FOSY experiments as well as for their conventional broad-band 5D-7D analogues. Thus, elimination of the homonuclear 3J(HN,HA) coupling (ca. 7 Hz) nearly doubles the signal resolution in the amide protein dimension and pertaining S/N. Furthermore, deuteration removes the notable contribution of aliphatic protons to the T2 relaxation of amide protons and, especially, of 13CA spins, thus improving the S/N by 10-30% even for the highly flexible IDPs.

Table S1. Peptide sequence stretches of hTau40 conforming with the general motif (P/G)-Xn-p(S/T)-Xn-(P/G), with X ≠ (P/G), as traced out by the presented FOSY assignment approach. This list was filtered for stretches containing the phosphorylation sites reported in literature16 and/or known kinase consensus recognition motifs. Progressing one sequence position at a time, the X amino acid can be classified as preceding a proline (XP), following a proline (PX), following a glycine (GX) or none of the above, ruling out peptide sequences that cannot be mapped based on the derived sequence motif. The table shows a tick mark (✓) for those residues in each stretch that conform with the amino acid type indicated by the delineated FOSY assignment protocol. Thus, starting signals ‘a’ and ‘d’ (Figure S4) could be unambiguously mapped to the phosphorylated residues pS404 and pS409 after only three and two FOSY steps tracing out the unique GXTp(S/T)p and PXXp(S/T)p motifs, respectively (shown bold).

| hTau40 phosphorylation sites by GSK3β16 | Local sequence stretches | FOSY step | Start signal = ‘a’ | Start signal = ‘d’ |
|----------------------------------------|--------------------------|------------|-------------------|-------------------|
|                                       |                          | (S/T)P      | -1 -2 -3          | (S/T) X X PX |
| T175                                  | PAKTP                    | ✓           | ✓ ✓              | 0 -1 -2 -3       |
| T181                                  | PKTP                     | ✓           | ✓                | 0 -1 -2 -3       |
| S199                                  | GDRSGYSSP                | ✓           | ✓ ✓ ✓            | 0 -1 -2 -3       |
| T205                                  | PGTP                     | ✓           |                   | ✓ ✓              |
| S212                                  | PGSRSTTP                 | ✓           | ✓ ✓              | 0 -1 -2 -3       |
| S214                                  | PSLP                     | ✓           |                   | ✓ ✓              |
| T217                                  | PTP                      | ✓           |                   | ✓ ✓              |
| T231                                  | PKKVAVVRTP               | ✓           | ✓ ✓              | 0 -1 -2 -3       |
| S235                                  | PKSP                     | ✓           |                   | ✓               |
| S262                                  | GSTENLKHQP               | ✓           |                   |                 |
| S356                                  | GSDLNITHYP               | ✓           |                   |                 |
| S396                                  | GAIEIVYKSP               | ✓           | ✓ ✓              | 0 -1 -2 -3       |
| S400                                  | PVVSG                    | ✓           | ✓ ✓              | 0 -1 -2 -3       |
| S404                                  | GDTSP                    | ✓           | ✓ ✓ ✓            | 0 -1 -2 -3       |
| S409                                  | PRHLŠN                   | ✓           | ✓ ✓ ✓ ✓          | 0 -1 -2 -3       |
Table S2. Chemical shift assignments obtained by the delineated FOSY approach for sequence stretch 398VVSDTS404 for phosphorylated deuterated [U-2H, 15N, 13C] p-2H-tau40, and 398VVSDTS403 for unphosphorylated deuterated [U-2H, 15N, 13C] 2H-tau40 and unphosphorylated protonated [U-15N, 13C] 1H-tau40 samples. Exact chemical shifts were obtained from the 3D BEST-TROSY HNCO, 2D FOSY-hnco(CA)NH, and 2D FOSY-hncoCA(N)H spectra of deuterated (Figure S1) and non-deuterated (Figure S3) hTau40. The sequential assignment walk started from the newly appearing pS404 peak for the p-2H-tau40 sample, whereas for the unphosphorylated 2H-tau40 and 1H-tau40 the initial T403 peak in HNCO spectrum was identified using the values taken from published assignment.17

|       | p-2H-tau40 | 2H-tau40 | 1H-tau40 |
|-------|------------|----------|----------|
| Ser-404 | 1H^N       | 8.43     | 8.00     | 8.00     |
|        | 15NH       | 120.6    |          |          |
| Thr-403 | 13CO       | 174.2    | 176.6    | 176.6    |
|        | 13CA       | 61.1     | 53.8     | 54.2     |
|        | 1H^N       | 7.92     | 8.00     | 8.00     |
|        | 15NH       | 114.1    | 114.7    | 114.8    |
| Asp-402 | 13CO       | 176.3    | 176.6    | 176.6    |
|        | 13CA       | 53.9     | 53.8     | 54.2     |
|        | 1H^N       | 8.08     | 8.06     | 8.06     |
|        | 15NH       | 120.8    | 120.8    | 121.0    |
| Gly-401 | 13CO       | 173.7    | 173.6    | 173.7    |
|        | 13CA       | 44.7     | 44.7     | 45.1     |
|        | 1H^N       | 8.27     | 8.27     | 8.28     |
|        | 15NH       | 111.6    | 111.6    | 111.8    |
| Ser-400 | 13CO       | 174.8    | 174.8    | 174.8    |
|        | 13CA       | 57.8     | 57.7     | 58.2     |
|        | 1H^N       | 8.27     | 8.25     | 8.26     |
|        | 15NH       | 120.4    | 120.3    | 120.6    |
| Val-399 | 13CO       | 176.0    | 176.0    | 176.0    |
|        | 13CA       | 61.4     | 61.4     | 61.8     |
|        | 1H^N       | 8.11     | 8.12     | 8.12     |
|        | 15NH       | 125.3    | 125.3    | 125.6    |
| Val-398 | 13CO       | 176.2    | 176.2    | 176.2    |
|        | 13CA       | 61.8     | 61.8     | 62.3     |
|        | 1H^N       | 8.00     | 8.00     | 8.01     |
|        | 15NH       | 121.4    | 121.4    | 121.6    |
Figure S4. 2D $^1$H,$^{15}$N (top) and $^1$H,$^{13}$CO (bottom) projections of the 3D BEST-TROSY HNCO spectrum of unphosphorylated hTau40 (cyan) and phosphorylated p-hTau40 (black). All identified new and shifted signals are annotated by letters ‘a’ to ‘g’. Peaks ‘a’ to ‘c’ and ‘d’ to ‘g’ were mapped to the pS404 and pS409 phosphorylation sites and their preceding peptide stretches, respectively.
Figure S5. (top) Superposition of 2D $^1$H,$^{15}$N projections from the proline selective PX- (red), XP-(yellow), and regular BEST-TROSY HNCO (black) spectra of p-hTau40. The newly (after phosphorylation) appearing signals ‘a’ and ‘d’ are the starting points for local FOSY assignment of the pertaining two phosphorylation sites. (bottom) Superposition of all nine 2D FOSY-hnco(CA)NH experiments acquired to successively correlate signals ‘a’ with ‘b’ to ‘c’ and ‘d’ with ‘e’ to ‘g’. Each individual spectrum is coloured differently, occasionally revealing multiple peaks in a single spectrum due to overlap of the selected frequencies.
Figure S6. FOSY assignment walk for the second phosphorylation site in p-hTau40. The walk in the 2D $^1$H,$^{15}$N plane starts from the newly appearing signal 0 (corresponding to peak ‘d’ in the HNCO spectrum in Figure S4) and proceeds via the indicated three FOSY steps (-1 to -3) until reaching the signal from a PX- or GX- type residue. This traces out a PXXp(S/T) motif that can be mapped to either PQLAT$^{427}$, PVDLS$^{316}$, or PRHL$^{409}$ stretches in the hTau40 primary sequence. FOSY-hncocacbNH is then used to narrow down on the pertaining amino acid type, indicating a Leu (not Ala) for signal -1, His for signal -2, and Arg for signal -3. Thus, PRHL$^{409}$ is unambiguously confirmed as correct assignment. This stretch is also the only one of the three alternatives that was shortlisted as a known GSK3β-mediated phosphorylation site (Table S1).
Figure S7. Signal slices (along the detected HN dimension) from 7.5 minute-long 2D FOSY-hnco(CA)NH spectra of hTau40 (U-2H, 13C, 15N) recorded using (green) relaxation optimized PI-SPT (Figure S1a) and (blue) 15N frequency selectivity optimized HH-SPT (Figure S1b) versions of the experiment at two temperatures a) 15°C and b) 5°C. The comparison shows all signal traces for the complete FOSY walk along the residue segment T403-V399 (Table S2) and illustrate the quality and more than sufficient signal-to-noise ratio (S/N > 10) in all spectra, the very similar sensitivity of both experiment versions, and an only marginal effect of amide proton exchange with water (from virtually identical S/N at 15°C vs. 5°C).
Figure S8. Frequency histogram of the number of peptide stretches conforming with the general motifs (P/G)-X^n-(P/G) **(dark red line)** and (P/G/S/T)-Z^n-(P/G/S/T) **(filled orange)** in hTau40 versus the length n in X^n and Z^n stretch, where X and Z is any amino acid except P/G and P/G/S/T, respectively. These residue types, P/G/S/T are easily defined: P - from the Pro-selective experiments, and G/S/T - during the assignment walk in 2D FOSY-hnco(CA)NH / FOSY-hncoCA(N)H spectra using the sign of the signal (for Gly) and the distinct ^13^C\(^\alpha\), ^15^N chemical shifts of Gly, Ser, Thr. In the case of (P/G)-X^n-(P/G) motif, there are a few stretches where n >10 that would require more than 10 FOSY steps for assignment. However, their size also makes their motif unique and thus assignable. If we consider the presence of S/T that not only are the phosphorylatable residues but also have distinct chemical shifts compared to all other amino acids, then n < 5 for the vast majority of cases, and thus assignable by FOSY in a couple of hours.
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