A new broadband homonuclear mixing pulse for NMR with low applied power

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Broadband homonuclear mixing pulses with low radiofrequency power are essential for NMR spectroscopy of proteins and small molecules, especially for emerging applications in high field NMR. We have analytically designed a mixing pulse with high bandwidth-to-power ratio, using our recently developed multi-frame method. Here, we compare the new pulse, NF4 (mixing in the fourth nutating frame), to the best currently available sequence, focusing on the low-power regime. We use simulations and experiments to compare the two pulses' relaxation properties and bandwidth, and demonstrate that NF4 has approximately 1.35 times higher bandwidth, with similar effective relaxation. Therefore, NF4 is a good choice for broadband homonuclear mixing, particularly when the available radiofrequency power is limited. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4885853>]

I. INTRODUCTION

In NMR spectroscopy, radiofrequency (RF) pulses must be used at sufficiently low power levels and for sufficiently short times to avoid probe damage and sample heating.^{1–6} However, total correlation spectroscopy (TOCSY) mixing pulses require high RF power to cover the large ranges of chemical shift frequencies encountered in practice.⁷ This problem is exacerbated at high field, since bandwidth increases proportionally to field strength. High-field NMR spectrometers are becoming increasingly prevalent, and experiments that require very long mixing times⁸ or very high RF power levels⁹ are regularly proposed. Therefore, efficient broadband mixing sequences that can be implemented at low RF power levels are needed. Numerically optimized sequences of rectangular pulses, notably Decoupling In the Presence of Scalar Interactions (DIPSI)¹⁰ and Flip-flop Spectroscopy (FLOPSY),¹¹ remain ubiquitous in protein NMR, despite the availability of a range of newer shaped pulses.^{12–15} In particular, FLOPSY-16 is known for having high transfer efficiency over a broader range of chemical shift offset frequencies than other currently available sequences.^{5,7,9}

Before deploying a shaped pulse in place of FLOPSY or another sequence, it is important to understand the trade-offs involved. Of particular interest are the change in bandwidth, the robustness of the new pulse to RF inhomogeneity, and the signal losses from relaxation during the mixing period. A side-by-side comparison of a new shaped pulse with FLOPSY-16 in the same experimental situation provides the best test of any new shaped pulse's performance.

In this paper we directly compare a recently designed shaped TOCSY pulse to the widely used FLOPSY-16 se-

quence, using both simulations and identical experimental conditions. Our data suggest that for low-powered mixing, better performance is achieved with our proposed pulse.

The new pulse was designed using the multi-frame method,^{16,17} in which a series of rotating frame constructions facilitate the analytical optimization of pulse parameters. Each additional frame is created by suitable sine and cosine modulation in the RF field, so that the method is an iterative generalization of nutating-frame spectroscopy.¹⁸ Specifically, the pulse we tested was designed for high bandwidth and transfer efficiency, using four tilted, rotating frames of reference. We call it NF4—indicating that it operates by a spin-locking field applied in the fourth nutating frame. We show that NF4 allows a considerable increase in bandwidth over FLOPSY, and is subject to similar relaxation losses. Experimental tests of TOCSY transfer at low RF power demonstrate the larger bandwidth of the new pulse: at low power levels we observe FLOPSY failing to generate cross peaks near the edges of the spectrum, while NF4's spectrum at equal or lesser RF power levels includes all expected peaks.

The utility of NF4 is not limited to protein spectroscopy and could be extended to NMR spectroscopy of small molecules, natural products, and peptides that exhibit a wide range of chemical shifts (~220 ppm). There are several cases in the literature where fully ¹³C labeled or partially ¹³C labeled small molecules and natural products and peptides from bacteria are studied using NMR. Metabolomics is now routinely done on cancer and other human cell lines grown with ¹³C glucose,¹⁹ and NF4 for TOCSY mixing will be invaluable for mixing over a wide range of chemical shifts. The latest cryoprobe technology allows mixing at high RF power—up to 17 kHz.⁹ At this power level NF4 can faithfully mix up to 28 kHz, which is over 220 ppm on a 500 MHz instrument.

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II. MATERIALS AND METHODS

A. Pulse design using four nutating frames

Our new TOCSY pulse was created using the recently developed multi-frames method.^{16,17} Under this approach, we consider the chemical shift and the Cartesian components of the RF Hamiltonian,

$$H_{CS} + H_{RF} = \omega_0 I_z + u_0(t) I_x + v_0(t) I_y,$$

where ω_0 is the chemical shift offset frequency. We choose RF fields that are constant along the x-axis $u_0(t) = u_0(0)$ and modulated along the y-axis $v_0(t) = 2v_1(t) \cos(f_1 t) + 2u_1(t) \sin(f_1 t)$. We then transform the Hamiltonian into a tilted, rotating frame (i.e., a nutating frame). The tilt about the y-axis combines the I_z and I_x terms, and the rotating frame demodulates the sine and cosine terms, and also produces a counter-rotating (i.e., off-resonance) wave.

The nutating frame construction produces new *effective* chemical shift and RF Hamiltonians, as well as an off-resonance term arising from the counter-rotating wave. The effective RF pulse in the first rotating frame has Cartesian components $(u_1(t), v_1(t))$, which can be chosen freely. We can repeat this process arbitrarily many times by choosing u_k and v_k in the same way as u_0 and v_0 , before applying a y-phase spin-lock field in the final rotating frame. The effective chemical shifts in successive frames ω_k obey the recursion

$$\omega_k = \sqrt{\omega_{k-1}^2 + u_{k-1}^2} - f_k \quad (1)$$

so that the bandwidth of ω_k can be engineered from the original bandwidth and the pulse parameters. In particular, the effective bandwidth in frame k (determined by the extrema of ω_k) is at most half of the bandwidth in frame $k - 1$; the range of effective chemical shift offsets is decreasing exponentially with the number of sinusoidal modulations in the pulse.

Our design method neglects the off-resonance term generated by each rotating frame construction; however, in general corrections to lowest order in the resonance offset for counter-rotating waves (for example, the Bloch-Siegert shift²⁰) can be found using (e.g.) average Hamiltonian theory,^{21,22} the Magnus expansion,^{23,24} or the Floquet expansion,^{25,26} and included in the effective Hamiltonian in each constructed frame. Note that with corrections for off-resonance terms, (1) can be adjusted by adding the lowest order z- and x-component corrections to ω_{k-1} and u_{k-1} , respectively. There is no correction (to lowest order) in the y-component of the Hamiltonian.¹⁷ We find that the Bloch-Siegert corrections for the neglected off-resonance terms are small compared with the effective bandwidth in each nutating frame, and therefore insufficient to outweigh the exponential reduction in bandwidth bought about by the on-resonance terms, so that the recursive bandwidth-reduction scheme is not ruined by the neglected off-resonance terms. In particular, numerical simulation of magnetization trajectories under NF4 (see below), which use the full Hamiltonian including any off-resonance terms neglected in the theoretical analysis, reveals highly efficient spin-locking; the effective bandwidth under NF4 is near zero.

For the magnetization to be in phase at the end of the pulse, we require that the modulation frequencies $\{f_1, f_2, \dots, f_n\}$ occur in integer multiples. There exists a simple recursion to generate the corresponding modulation amplitudes $\{u_0, u_1, \dots, u_n\}$, optimized to cover the largest possible bandwidth.¹⁶

The resulting RF pulse has a closed form expression as a sum of sinusoids. For the present case of four rotating frames,

$$\begin{aligned} u_0(t) &= u_0, \\ v_0(t) &= 2u_1 \sin(f_1 t) + 4u_2 \cos(f_1 t) \sin(f_2 t) \\ &\quad + 8u_3 \cos(f_1 t) \cos(f_2 t) \sin(f_3 t) \\ &\quad + 16u_4 \cos(f_1 t) \cos(f_2 t) \cos(f_3 t) \cos(f_4 t). \end{aligned}$$

The RF amplitude $A(t)$ and phase $\phi(t)$ are then

$$\begin{aligned} A(t) &= \sqrt{u_0^2 + v_0^2(t)}, \\ \phi(t) &= \text{atan2}(v_0(t), u_0), \end{aligned}$$

where atan2 is the two-argument arctangent function. The root-mean-square RF amplitude is 3 kHz and the duration is $T = 3.477$ ms. The pulse can of course be repeated to achieve a desired mixing time. No super cycle is needed for these repetitions, and none was used in any of our simulations or experiments with NF4. The RF amplitude and duration can be inversely scaled to achieve any RF power, and the bandwidth changes proportionally to the amplitude. We chose to express the pulse parameters for root-mean-square RF amplitude of 3 kHz arbitrarily. The pulse parameters are given in Table I.

Using the recursion Eq. (1), we see that for a wide range of chemical shifts—approximately 10.5 kHz—the effective chemical shift ω_4 is less than 1/6 of the applied spin-locking field u_4 in the final frame. That is, there exists a multi-rotating frame of reference (the fourth nutating frame) in which the apparent Hamiltonian is $H^{(4)} = u_4 I_y + \omega_4 I_z$, with $|\omega_4| \leq u_4/6$. Under this Hamiltonian, the spins are effectively spin-locked to the y axis. Moreover, the highly contrived multi-rotating frame aligns with the original frame at stroboscopic times (up to a tilt about the y-axis) so we achieve effective spin-locking in the original frame of reference too.

For networks of coupled spins, each spin with chemical shift in the active bandwidth is effectively spin-locked. The effective J -coupling is fully maintained between spin pairs near the main diagonal and decreases along the off-diagonal of a two-dimensional spectrum. Inspection of Fig. 1 shows that the coupling is present in a wide region about the main diagonal.

TABLE I. The amplitudes u_k and frequencies f_k of the modulations in each frame in kHz. The pulse time $T = 3.477$ ms, so that all sinusoidal modulations average out during the pulse (i.e., Tf_k are integers). The five amplitudes and four frequencies, along with the pulse time T , completely determine the pulse shape for NF4.

	$k = 0$	1	2	3	4
u_k	1.9285	1.0880	0.7450	0.2755	0.0734
f_k	...	4.0260	1.7254	0.8627	0.2876
Tf_k	...	14	6	3	1

FIG. 1. Simulated transfer of magnetization for two isotropically coupled spins with $J_{IS} = 35$ kHz. The mixing time is $t_m = 1/(2J_{IS})$ and the root-mean-square RF amplitude is 3 kHz. (a) FLOPSY-16 transfer $I_z \rightarrow S_z$. (b) NF4 transfer $I_y \rightarrow S_y$. Contour levels show the proportion of in-phase magnetization originally on the I spin that has migrated to the S spin at time t_m . Both pulses have good transfer efficiency near the main diagonal. NF4 has a wider bandwidth along the main diagonal.

B. Simulations of TOCSY transfer

We have simulated the TOCSY transfer as a function of the offset frequencies of the two isotropically coupled spins with $J_{IS} = 35$ Hz. Fig. 1 compares the transfer $I_y \rightarrow S_y$ for NF4 with $I_z \rightarrow S_z$ under FLOPSY-16. The simulations were performed in MATLAB and relaxation effects and RF inhomogeneity were neglected during the mixing time.

Note that the unitary propagator (solution to the time-dependent Schrödinger equation) was simulated for one period of the pulse, t_p , and then the propagator for the desired mixing time t_m was calculated via $U(0, t_m) = U(0, t_p)^{t_m/t_p}$. In experiments, we can only repeat the *complete* pulse, i.e., t_m/t_p must be an integer; however, in simulations we can set t_m/t_p to a non-integer to ensure that $t_m = 1/(2J_{IS})$. In Fig. 1 we set NF4 to have $t_m/t_p = 4.12$, and we set FLOPSY-16 to have $t_m/t_p = 0.91$. Note that the duration of NF4 is only 22% as long as FLOPSY-16 per repetition, so that in experiments a desired mixing time can be chosen more precisely with NF4.

Fig. 2 shows traces of the transferred magnetization onto the S spin, for three different offsets of the I spin. We simulated both pulses for a whole (integer) number of repetitions, and adjusted the J -couplings so that the mixing time was $t_m = 1/(2J_{IS})$. This simulation includes RF inhomogeneity uniformly distributed between $\pm 10\%$.

Clearly, transfer is achieved for a larger range of offsets under NF4 along the main diagonal. The width along the anti-diagonal is similar for the two pulses—FLOPSY is about 8% wider on resonance, in terms of full-width at half-maximum measured in Fig. 2(a). However, when spin I is 2 kHz off-resonance, NF4 is 6% wider than FLOPSY (Fig. 2(b)). Moreover, NF4 continues to function properly for offsets outside the working bandwidth of FLOPSY (Fig. 2(c)).

C. Autorelaxation by invariant trajectories

FLOPSY-16 transfers longitudinal magnetization $I_z \rightarrow S_z$, while NF4 transfers transverse magnetization $I_y \rightarrow S_y$. Longitudinal relaxation (R_1) is generally slower than transverse relaxation (R_2), suggesting the possibility that NF4 could produce more rapid loss of signal than FLOPSY. How-

FIG. 2. Simulated transfer of magnetization from a spin which is (a) on resonance, (b) 2 kHz offset, and (c) 4 kHz offset. FLOPSY-16 transfers (dashed lines) are $I_z \rightarrow S_z$, while NF4 transfers (solid lines) are $I_y \rightarrow S_y$. Transfers in time $1/(2J_{IS})$ are plotted as a function of offset frequency of the S spin, averaged over 20 values of RF inhomogeneity uniformly distributed between $\pm 10\%$. The root-mean-square RF amplitude is 3 kHz, and $J_{IS} = 31.85$ for FLOPSY and $J_{IS} = 36$ for NF4, so that the mixing time is an integer multiple of the pulse time. At $\omega_I = 4$ kHz FLOPSY-16 is not effective, while NF4 still transfers magnetization to a broad range of offsets ω_S .

ever, simulation of the magnetization trajectories *during* the pulse reveals very similar autorelaxation performance.

During a RF pulse, magnetization will follow a trajectory that is (in general) not always aligned with the longitudinal axis nor always contained in the transverse plane. Therefore, it is subject to a mixture of R_1 and R_2 relaxation effects. The method of invariant trajectories predicts relaxation based on simulated or calculated periodic magnetization trajectories on the Bloch sphere.^{7,27–29} The transverse weight σ_T is the time-averaged component of magnetization in the x - y plane,

$$\sigma_T = \frac{1}{T} \int_0^T n_x^2(t) + n_y^2(t) dt,$$

where $\vec{n}(t)$ is the periodic magnetization vector under the RF pulse. The transverse weight determines the effective relaxation rate R_e via

$$R_e = \sigma_T R_2 + (1 - \sigma_T) R_1.$$

We have simulated the transverse weight under NF4 and FLOPSY-16 as a function of offset frequency (Fig. 3). Simulations assumed an isolated spin without relaxation, for one period of the applied RF pulse. We also averaged over RF inhomogeneities uniformly distributed between $\pm 10\%$. Under

FIG. 3. Comparison of the transverse weight of (a) FLOPSY-16 and (b) NF4 as a function of offset frequency. Simulations used an average RF amplitude of 3 kHz applied to an isolated spin. The time-averaged projection of the magnetization trajectory onto the x - y plane during one period is plotted, averaged over 20 values of RF inhomogeneity between $\pm 10\%$. The initial magnetization is $\rho(0) = I_z$ for FLOPSY and $\rho(0) = I_y$ for the four-frame pulse. During the pulses the spins spend a similar proportion ($\sim 2/3$) of their time near the transverse plane, and are therefore subject to similar weightings of R_2 versus R_1 autorelaxation.

either pulse, and for a wide range of offset frequencies, the magnetization vector projects onto each of the three axes for approximately equal amounts of time. This leads to a transverse weight of about 2/3 for both FLOPSY and NF4. We conclude that signal loss due to autorelaxation is similar for FLOPSY and NF4.

D. NMR experiments

The first set of comparative NMR experiments were carried out on a synthetic hexa-peptide with the amino acid sequence PIFHAG, with the first five residues ^{13}C - and ^{15}N -labeled,¹⁶ using a Bruker 500 MHz spectrometer equipped with a cryogenically cooled TXO probe. The TOCSY experiments were performed with proton and nitrogen decoupling during the indirect and direct dimension carbon evolution. The shaped amplitude and phase profiles of the NF4 mixing pulse were finely discretized due to spectrometer software requirements (the discretization sampling time was 7/500). The pulse shape was repeated 9 times, without any supercycling scheme, to achieve a mixing time of 9T. The FLOPSY-16 sequence was repeated 2 times. This ensured equal mixing times for the two sequences. The list of mixing times at each RF field strength is given in Table II. The experiments were done at 298 K. The spectra were processed and plotted with NMRPipe,³⁰ and are presented in Fig. 4.

At the highest RF amplitude tested (3 kHz) all the expected intra-residue cross peaks were generated by both mixing sequences. However, we observe that NF4 generates all cross peaks for RF levels of at least 2.2 kHz, while FLOPSY requires the full 3 kHz. We conclude that the working bandwidth of NF4 is approximately 35% larger than for FLOPSY-16 (although this single metric does not really capture the differing shape of the two sequences' transfer efficiencies along the main diagonal and the anti-diagonal).

The difference in practical performance is clearly illustrated with the mixing patterns of isoleucine and alanine peaks, which are labeled in Fig. 4(h). With FLOPSY, the mixing between the C^α and C^β nuclei of alanine (separated by approximately 30 ppm) is observed at 2.2 kHz and above. However, with NF4 this cross peak can clearly be seen with a 1.8 kHz RF field in Fig. 4(b). Similarly, the mixing between isoleucine C^δ and C^α carbons fails below a field strength of 3 kHz for FLOPSY. However, with NF4 the isoleucine pat-

FIG. 4. 2D-TOCSY spectra recorded using FLOPSY-16 (left column) and NF4 (right column). The sample is a synthetic hexa-peptide PIFHAG, with the first five residues ^{13}C - and ^{15}N -labeled. Spectra were recorded in a 500 MHz spectrometer equipped with a cryoprobe. The mixing times are given in Table II. The root-mean-square average RF amplitude is (a) and (b) 1.8 kHz, (c) and (d) 2.2 kHz, (e) and (f) 2.6 kHz, and (g) and (h) 3.0 kHz. The carrier frequency, number of scans, sample, and display settings are the same for all spectra, and the mixing times are approximately equal at each RF field strength. NF4 generates all cross peaks for RF amplitude levels of at least 2.2 kHz, whereas FLOPSY-16 requires 3 kHz amplitude to generate all cross peaks. The isoleucine and alanine patterns are labeled in (h).

TABLE II. The mixing times used in the experiments. These were kept approximately equal at each power level. NF4 has approximately 2/9 of the duration of FLOPSY-16, therefore NF4 was repeated 9 times and FLOPSY-16 was repeated 2 times. The mixing time is inversely proportional to the RF amplitude.

RF amplitude (kHz)	FLOPSY mixing time (ms)	NF4 mixing time (ms)
3.0	31.41	31.29
2.6	36.26	36.11
2.2	42.81	42.67
1.8	52.35	52.16

tern is weakly generated at 1.8 kHz and clearly observed at 2.2 kHz. This is because of the increased bandwidth of NF4 along the main diagonal, shown in Figs. 1 and 2. Although the C^δ and C^α nuclei are 50 ppm apart they are connected via sequential bonds with smaller separations, and each of the directly coupled spin pairs in isoleucine maintains a strong coupling under NF4 even at low RF power.

The second set of experimental tests was carried out on a 2 mM ^{15}N - ^{13}C labeled ubiquitin sample in Tris buffer at pH 7.5, under the same experimental conditions as were

FIG. 5. 2D-TOCSY spectra recorded using FLOPSY-16 (left column) and NF4 (right column). The sample is uniformly ^{13}C - and ^{15}N -labeled ubiquitin. Spectra were recorded in a 500 MHz spectrometer equipped with a cryoprobe. The mixing times are given in Table II. The root-mean-square average RF amplitude is (a) and (b) 2.2 kHz, and (c) and (d) 3.0 kHz. The carrier frequency, number of scans, sample, and display settings are the same for all spectra, and the mixing times are approximately equal at each RF power level. We observe that at both RF power levels, cross peaks appear over a broader range of chemical shift offsets with NF4 than with FLOPSY-16.

used for the hexa-peptide sample. The results are presented in Fig. 5. Again, the spectra obtained with NF4 show cross peaks over a larger bandwidth at each RF power level. The difference is particularly striking at a root-mean-square RF amplitude of 2.2 kHz (Figs. 5(a) and 5(b)), in which FLOPSY-16 has clearly not produced mixing over the required bandwidth, while NF4 has generated many more cross peaks around the edges of the spectrum. When the RF level is 3 kHz (Figs. 5(c) and 5(d)) both pulses perform well; however, there is still evidence of higher bandwidth for NF4. For example, at the edges of the spectra there are connectivities to the resonance at 9 ppm, and to the resonance at 72.5 ppm, that are observed in Fig. 5(d) but not in Fig. 5(c).

III. CONCLUSION

We anticipate that NF4 will be especially useful for experiments on high field spectrometers, in which transferring polarization over relevant bandwidths remains a challenge due to RF power limitations. Other experiments that will benefit from increased bandwidth-to-power ratio include CACA-TOCSY,⁸ which requires a mixing duration of the order of hundreds of ms to create weak inter-residue cross peaks between weakly coupled neighboring C^α in the presence of strongly coupled C^β . The long mixing duration means that the main worries in this experiment are sample heating and probe arcing. Generally, experiments that are sensitive to sample heating from RF pulses,⁶ and high bandwidth applications in protein and small molecule NMR, can also be improved by using NF4.

We conducted a direct comparison of the FLOPSY-16 sequence with NF4, first using simulations, and then under

identical experimental conditions. We found that the mixing bandwidth of NF4 is about 35% greater, while the magnetization trajectories under the two pulses have similar transverse weight (and therefore similarly weighted mixtures of R_1 and R_2 relaxation). Furthermore, NF4 is robust to reasonable RF inhomogeneity. Because of these favorable properties, we propose that NF4 is a better choice for use in broadband TOCSY applications than other available pulses.

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