# $^{1}$H, $^{13}$C, and $^{15}$N NMR Pulse Width Determination for Labeled Proteins in $H_2O$ Solution: Nutation Pulse Sequences Including WATERGATE and Analysis by MUSASHI

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We developed MUSASHI, a method to determine NMR 90 degree pulse width by nonlinear least square curve fitting. A nutation experiment is carried out and then the data set is sent to MUSASHI program. We report new nutation pulse sequences for labeled proteins in water including WATERGATE scheme aimed at obtaining data for MUSASHI. The new sequences and MUSASHI make it possible to obtain $^{1}$H, $^{13}$C, and $^{15}$N pulse width required to double- and triple-resonance experiments in a short time.

In the last decade, triple-resonance NMR experiments got much popular and are routinely used for solution structure analysis of proteins. Especially, in structural genomics, so large numbers of $^{13}$C and $^{15}$N-labeled proteins are mass-productively prepared and their double- and triple-resonance experiments are successively carried out with a very high throughput. Because these triple-resonance experiments are more complicated than a single pulse experiment and larger numbers of pulses are used, the accumulation of the inaccuracy of pulse widths finally causes large amount of signal loss, even if the degree of the inaccuracy of each pulse width would be small. Therefore, spectrometers should be calibrated by the signals from the sample, namely the solute protein itself rather than standard samples, i.e., 90° pulse widths should be determined prior to such measurements.[1] However, the signal intensities of proteins are generally smaller than those of small molecules in such standard samples (i.e., 1% $^{13}CH_3I$) and solvent suppression must be done with high efficiency. As we described previously,[2] our group developed a method named MUSASHI (MUltiple Spectra Analyzing System with Hyper Intelligence) to determine $^{1}$H 90° pulse width by means of nonlinear least square curve fitting. In MUSASHI, a nutation experiment varying pulse duration gradually is measured, the resulting data are sent to the program and used to determine the pulse width as to fit the model function below,

$$f(t) = A\sin(\omega t + B)\exp(-t/C) + D \qquad (1)$$

where $f(t)$ is intensity of the spectra, $t$ is pulse duration, $A$, $B$, $C$, $D$, and $\omega$ are parameters determined by MUSASHI. In this letter, we report a set of $^{1}$H, $^{13}$C, and $^{15}$N nutation pulse sequences with a water suppression scheme and the analyses by MUSASHI. The combination of nutation data with an excellent solvent suppression and very sensitive detection of 90° pulse width results in very high throughput to set experimental conditions.

In order to have spectra with very good water suppression, we applied W5-WATERGATE[3] water suppression technique. In WATERGATE, the W5 composite pulse-train gives almost null-excitation around the frequency offset (water resonance) and 180° flip angle in the other region. This implies that the signals around the water resonance are disappeared because they are dephased by two field gradient pulses, while the other resonances far from the water resonance like in aliphatic region are observed because they are re-phased by 180° flip given by W5 and two field gradient pulses. In the case of $^{1}$H pulse width determination in Figure 1, the duration of the first $^{1}$H pulse (p1) is varied in the nutation experiment, while the W5 pulses are fixed and should be derived from another experiment done prior to the nutation experiment. On the other hand, in the case of $^{13}$C and $^{15}$N (X-nucleus) pulse width determination, both p1 and W5 $^{1}$H pulses are fixed, while p2 (X-nucleus) are changed. The signal intensities of $^{1}$H attached to X-nucleus vary as the pulse duration of X-nucleus increases, whereas those of $^{1}$H not attached to X-nucleus are unchanged throughout the nutation experiment.

We successfully obtained the 90° pulse widths of $^{1}$H, $^{13}$C, and $^{15}$N with the pulse sequences depicted in Figure 1 and MUSASHI calculations from nutation data[4–7] shown in Figure 2. In the implementation we show here, the residual solvent signal is well suppressed and this results in good convergence. Although Bax proposed an indirect method to determine 90° pulse width[8] and this method has been widely used for $^{13}$C and $^{15}$N pulse width determination, it has a drawback that not only $^{1}$H attached to X-nucleus but also the other $^{1}$H modulate during $1/2\,J$ delay. In our sequence, 180° flip implemented by W5 refocuses all $^{1}$H magnetization except for those around water resonance.

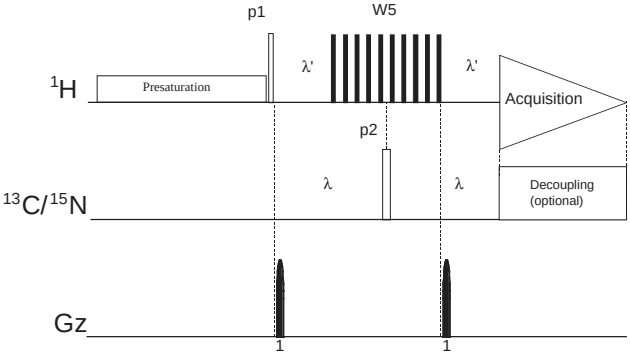


**Figure 1.** Pulse sequence diagram for nutation experiment with W5-WATERGATE aimed at pulse width determination of labeled proteins in water solution. Closed squares are pulses for W5 selective excitation[2] and pulse width are fixed and should be known prior to this experiment. For $^{1}$H, p1 was varied for the sake of nutation experiment and neither $^{13}$C nor $^{15}$N pulse is applied, while for $^{13}$C and $^{15}$N pulse width determination, the pulse width of p1 was fixed and p2 was varied. The $^{1}$H, $^{13}$C and $^{15}$N carrier were centered at 4.7 ppm (water), 35 ppm, and 120 ppm, respectively. The delay employed was $\lambda' = 5$ ms for $^{1}$H, $\lambda = 3.4$ ms for $^{13}$C, and $\lambda = 5.3$ ms for $^{15}$N. The duration and maximum strength of the half-sine-shaped gradient pulses along with $z$ axis was g1 = (1 ms and 120 mT/m). Presaturation was optionally used to help water suppression. Composite pulse decoupling can also optionally used for $^{13}$C and $^{15}$N.

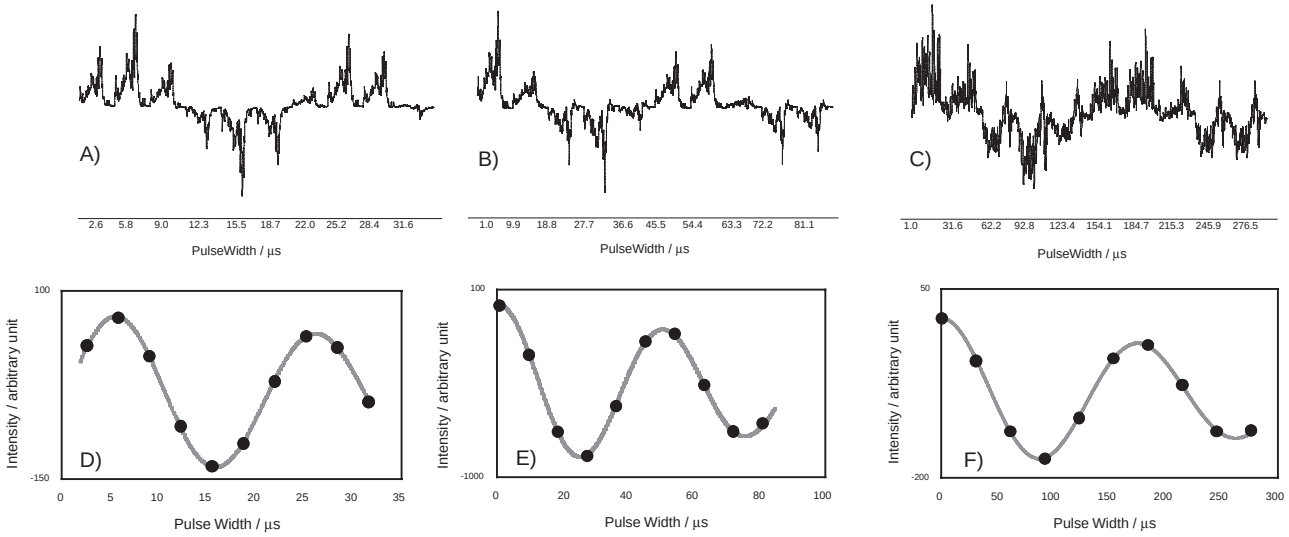


**Figure 2.** Nutation data of $^{13}C/^{15}N$ labeled chlorella ubiquitin measured by the pulse sequences depicted in Figure 1 and their curve fitting results by MUSASHI. The nutation data of A) $^{1}H$, B) $^{13}C$, and C) $^{15}N$ are shown. In all three cases, data were sampled at ten different pulse widths. The integral of signals in the region of $-1 \sim 3$ ppm of A) and B) were considered in MUSASHI calculation,[1] whereas the integral in $6 \sim 9$ ppm of C) was considered. The integrals of the nutation experiments (closed circle) and the fitting-results by MUSASHI (curve) corresponding to D) $^{1}H$, E) $^{13}C$, and F) $^{15}N$ are shown. The resulting parameters for $^{1}H$ were $A = 113.65$ (arbitrary unit), $B = -0.176$ rad, $C = 84.52\,\mu s$, $D = -41.65$ (arbitrary unit) and $\omega = 0.3047$ rad/μs. Those for $^{13}C$ were $A = 487.6$ (arbitrary unit), $B = 1.440$ rad, $C = 140.89\,\mu s$, $D = -478.7$ (arbitrary unit) and $\omega = 0.1253$ rad/μs, while those for $^{15}N$ were $A = 102.61$ (arbitrary unit), $B = 1.478$ rad, $C = 444.1\,\mu s$, $D = -91.9$ (arbitrary unit) and $\omega = 0.03596$ rad/μs. The 90° pulse width obtained were 5.29 μs($^{1}H$), 13.56 μs($^{13}C$) and 46.24 μs ($^{15}N$).

The indirect nutation curves are cosinusoidal in both Bax's and our methods, in other words, the intensity is the highest at the zero pulse duration and goes to null at the 90° pulse width. Because MUSASHI uses a model function as shown in eq 1 and it has a phase parameter $B$ therein, MUSASHI does not care whether the curve has the phase of the sine or cosine curve. As same as discussed previously,[2] because MUSASHI finds the pulse width not only from the null point of the nutation experiment but also from the total data points of the nutation experiment, it has a higher sensitivity and gives the result in a fewer data points (therefore shorter total experimental time) than the conventional method. In the example we showed here (Figure 2), the pulse widths of $^{13}C$ and $^{15}N$ are obtained from ten points in each nutation experiment, the total experimental time required to determine the pulse length is shorter than half the time of the conventional method. This makes it possible to calibrate RF settings of triple-resonance experiments in ten minutes.

In the case of the $^{15}N$ pulse width determination, we can take an advantage of "integral option" of MUSASHI.[2] In MUSASHI calculation, we can use not only the signal intensity of a peak but also the integral of the certain region of the spectra. Generally speaking, $^{1}H$ chemical shifts of most amide protons and most aromatic protons in proteins are spread around 6–9 ppm. In the $^{15}N$ nutation experiment shown in Figure 1, as $^{15}N$ pulse width gets longer, the signal intensities of protons attached to $^{15}N$ varied, while protons attached to (aromatic) carbons stay at the same intensities. It is not so easy to find the point where amide proton intensities disappear in Figure 2c, on the other hand, it is very easy for MUSASHI to find the pulse width because unchanged intensities result in the intensity offset in MUSASHI calculation (the term $D$ in eq 1) and this does not affect the results of the curve fitting.

**References and Notes**

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3 M. Liu, X. Mao, C. Ye, H. Huang, J. K. Nicholson, and J. C. Lindon, *J. Magn. Reson.*, **132**, 125 (1998).
4 0.9 mM $^{13}C/^{15}N$ uniformly (~98%) labeled chlorella ubiquitin solution was purchased (Chlorella Industry Co. Ltd., Tokyo, Japan). All the NMR experiments are done on a JNM-ECA600 spectrometer (JEOL LTD., Akishima, Japan) operating at 14.1 T. The temperature of the solution was set to 25 °C. The data of which the direct observing $^{1}H$ axis is Fourier transformed are used for further nonlinear curve fitting calculation.
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6 The $^{1}H$ pulse width determination shown here may sound nonsense because this requires knowing the pulse width for W5 prior to the measurement. The accurate 90° pulse width of the protein sample is obtained by this pulse sequence, as far as the pulse widths used for W5 are in the range of 50–200% of the real pulse widths. Considering an advantage of the excellent water suppression and rapid 90° pulse width determination of protein samples against disadvantage of the acceptable sensitivity loss (in addition, MUSASHI is more sensitive than the conventional method[2]), we conclude that it is meaningful to use this method.
7 For all the experiments shown here, we used a spectrometer with multiple sequencer system.[3,4] Depending on a spectrometer to use and the range of pulse duration to be varied, the implementation of the pulse sequences shown in Figure 1 for $^{13}C$ and $^{15}N$ may be complicated because the order of the fifth $^{1}H$ pulse in the W5 pulse train and $^{13}C$ or $^{15}N$ pulse may have to be inverted as $^{13}C$ or $^{15}N$ pulse width gets longer.
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