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## Comment on mr-2021-60

Anonymous Referee #1

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Referee comment on "Analysis of conformational exchange processes using methyl-TROSY-based Hahn echo measurements of quadruple-quantum relaxation" by Christopher A. Waudby and John Christodoulou, Magn. Reson. Discuss.,  
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In the manuscript, Waudby and Christodoulou present a novel NMR strategy to probe chemical exchange processes on the order of microsecond to millisecond by analyzing the static magnetic field-dependence of the relaxation rates of a set of multiple-quantum (MQ) coherence of side-chain  $^1\text{H}$ - $^{13}\text{C}$  methyl groups. The proposed experimental strategy is based on the previously reported approaches analyzing double quantum (DQ) and zero quantum (ZQ) coherences of methyl  $^1\text{H}$ - $^{13}\text{C}$  correlations, as developed by Toyama *et al.* (Nat. Commun. 2017) and Gill *et al* (JBNMR 2011, JBNMR 2019). In this paper, the authors extend their methods by including the magnetic field-dependence of four-spin double quantum (DQ') and quadruple quantum (QQ) coherences to break the symmetry of the  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift contributions to the DQ and ZQ coherences, which enables to determine the values for the relative amplitudes of  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift differences between two exchanging conformers. The authors also show that the joint analyses of magnetic-field dependence of these MQ coherences and  $^{13}\text{C}$ - $^1\text{H}$  MQ/ $^1\text{H}$  SQ CPMG relaxation dispersion experiments enable the extraction of robust thermodynamic and kinetic parameters. The established methodology is successfully applied to 108-residue FLN5, the fifth immunoglobulin domain from the *Dictyostelium discoideum* filamin protein, and two distinct conformational exchange processes with the  $k_{\text{ex}}$  of  $\sim 870\text{ s}^{-1}$  and  $\sim 6,900\text{ s}^{-1}$  are identified.

The paper is very well and clearly written and most of the details, including the theoretical backgrounds and pulse programs/processing scripts, are provided. The approach presented here would be extremely useful for characterizing fast exchange processes, such as microsecond-order conformational dynamics and weak ligand-binding processes, and thus would greatly accelerate investigations of various biologically and chemically important systems. The idea of observing quadruple coherence is really fascinating and would attract great interest from the broad NMR community. Therefore, I strongly recommend publication in Magnetic Resonance.

I have a few suggestions for further improvement as follows.

(1) Throughout the paper, the authors assume that the conformational exchange processes affecting  $^{13}\text{C}$  and  $^1\text{H}$  chemical shifts are always completely correlated (*i.e.* they can be described with the same  $k_{\text{ex}}$  and  $p_{\text{B}}$ ). This should be clearly mentioned somewhere in the main text (though this is implied in lines 81-88 and line 100). I agree that this assumption is very reasonable in most cases, however, the  $^{13}\text{C}$  chemical shift is sensitive to the side-chain rotameric changes and  $^1\text{H}$  chemical shift is more sensitive to ring-current effects from the proximal aromatic rings, and these two processes can be attributed to two distinct conformational fluctuations with different thermodynamic and kinetic parameters. This was also suggested in the analyses of T4 lysozyme in the previous work by Toyama *et al.*

(2) Line 103 "While it is possible to determine the relative sign of  $\delta_{\text{C}}$  and  $\delta_{\text{H}}$  from the sign of  $\Delta\delta_{\text{MQ,ex}}$ , additional approaches are also required to determine their absolute signs."

Here, the authors pointed out that absolute signs of  $^{13}\text{C}$  and  $^1\text{H}$  chemical shift differences cannot be unambiguously determined by the previous approaches, however, the Hahn-echo analyses of ZQ, DQ', and QQ coherences presented in this paper also cannot provide the absolute signs in principle (as can be seen in two symmetric optimal values in Fig 5C and D). In that sense, this sentence may be a bit misleading.

(3) Line 126. It may be helpful to mention that the relaxation induced by the external deuterons is not considered here.

(4) Line 173. "We observe no fixed ordering of the various relaxation rates, and indeed in some cases four spin relaxation rates are slower than ZQ or DQ rates (e.g. for QQ relaxation in L701CD1, Fig. 2B, right hand panel)."

The QQ relaxation is the fastest in L701, I guess this should be written as L664CG2.

(5) Line 186. Here, the authors propose the F1-decoupled HSQC to obtain  $^{13}\text{C}$  CSA values. I wonder how effective this approach would be when considering the applications to more complex systems. such as high molecular proteins. In large proteins, the outer components of the quartet decay very rapidly and these outer lines are almost invisible, which might affect the accuracy of the 2D line-shape analyses as there is less information available. Furthermore, in large systems, the signal overlap can be much more severe. In such a case, the pseudo-4D type experiment as originally proposed by Toyama *et al* may be a preferable.

(6) Line 221 and Fig 4. Is the IP and AP scheme inverted in the pulse scheme in figure 4? Also, after the purge element, the phase of  $\phi_2$  should be on  $y$  to purge the fast-relaxing outer components as these outer components evolve with  $J$  and become orthogonal with respect to the slowly-relaxing central component.

(7) Line 280. Please label I743 and L701 on the structure.

(8) Line 285. Regarding the best-fit parameters displayed in Figs 6 C and D, how did the authors determine the absolute sign of the  $^{13}\text{C}$  and  $^1\text{H}$  chemical shift differences? The absolute sign of the  $^{13}\text{C}$  and  $^1\text{H}$  chemical shift differences cannot be determined from the analyses of Hahn-echo relaxation measurements and  $^{13}\text{C}$ - $^1\text{H}$  MQ/ $^1\text{H}$ -SQ CPMG dispersion experiments also do not provide the information of the sign of  $^{13}\text{C}$  and  $^1\text{H}$  chemical shift differences.

(9) Line 380. Please indicate the exact labeling pattern of “[ $^2\text{H}$ ,  $^{13}\text{CH}_3$  -ILV]-labeled” sample. I imagine the  $\text{CH}_3/\text{CD}_3$  labeling for Leu/Val would be important to reduce the effects of spin flips by the external protons.

(10) Regarding Fig S3, it would be worthwhile to mention that the slope is not exactly 1 and the offset has a non-zero value. It would be useful to comment on the potential sources of these deviations.