Point-by-point response

We would like to thank the editor all the reviewers for their comments. We believe that the overall quality of the revised manuscript has been further improved as a result of their constructive feedback. Below are our responses addressing the points raised.

Reviewer 1

Manuscript Summary:
Venditti and coworkers report a protocol for setting, acquiring and analyzing ^{15}N CPMG NMR data. This protocol is written clearly overall and will be a useful contribution to the user community who want to study conformational exchange in proteins. However, I have some major revisions listed below that must be addressed before the report can be considered suitable for publication.

Major Concerns:
My first main concern is with the fact that the article makes no connection with existing procedures for CPMG data acquisition and analysis. Conformational exchange in proteins has been studied using CPMG experiments for over two decades now, mainly by the Kay, Palmer and Torchia research groups. While a standardized protocol for such experiments would be a welcome addition to the literature, the protocol needs to be placed in the context of previous protocols which have been extensively cross-validated by multiple experimental approaches.

1. The TROSY CPMG experiment in not the obvious choice for measurement of ^{15}N CPMG dispersions. TROSY ^{15}N magnetization can convert to the AntiTROSY form because of spin-flips and solvent exchange, resulting in a deterioration of the CPMG profile. The "improved" experiment (Hansen et al JPhysChemB 2008) is better for smaller proteins where deuteration is not normally needed (< 15 kDa) and for IDPs (which have prevalent solvent exchange) because it uses in-phase Nx for measuring CPMG, while CW decoupling ^{1}H in the process. While deuteration improves TROSY CPMG dispersions, labs will be interested in detecting conformational exchange on small proteins without resorting to expensive deuteration. The way to do this would be to use the "improved" CPMG experiment above. The authors should thus measure dispersions with the "improved" sequence also and provide comparative protocols, if their protocols are to be considered as standard in the field.

The guest editor of this special issue of JoVE asked us to describe a protocol for acquisition and analysis of relaxation dispersion experiments based on our *2020 JMB* manuscript (on a 70 kDa enzyme) (https://doi.org/10.1016/j.jmb.2020.05.024). For this reason, we focused our discussion on the TROSY CPMG sequence and perdeuterated protein samples. However, we do agree with the reviewer that acknowledging CPMG methods optimized for lower molecular weight systems is important and would add to the present manuscript.

To address this point we have added the following text to the manuscript:

Page 2, lines 16-18
"*Several different pulse sequences and analysis protocols have been reported in the literature for ^{15}N CPMG experiments. Herein, the protocol implemented in our laboratory is described.*"

Page 2, line 28 – page 3, line 2
"*Overall, our protocol has been successfully applied to the characterization of conformational exchange in medium sized and large proteins.[4, 5, 9, 10] For smaller systems (< 20 kDa), the use of an HSQC-based pulse sequence[11, 12] is advisable.*"

2. It is important, while measuring CPMG profiles, to calibrate the CPMG 180 pulse carefully. I could not find this step anywhere in the protocol. We have found that ^{15}N 90 pulses at the CPMG power must be calibrated typically to an accuracy of 1%. Miscalibrating these pulses can result in quantitatively wrong profiles.

The reviewer is correct noting that calibration of the 180-degree pulse in the CPMG train is critical to ensure accurate dispersion profiles. We calibrate the pulse using a 100 mM ^{15}N urea standard. In addition, we double-check the calibration on our working sample by acquiring the first increment of a ^{1}H-^{15}N HSQC spectrum in which the ^{15}N 90-

degree pulse of the first INEPT block is switched to a 180-degree pulse. We have now added this information to the protocol (note to step 3.2):

"*NOTE: for optimal results it is important to calibrate the ^{15}N 90° pulse with great care. Calibration is usually accomplished using a 100 mM sample of ^{15}N–labeled urea in DMSO as described in the spectrometer manual. In addition, it is possible to double-check the calibration directly on the working NMR sample by acquiring the first increment of a ^{1}H-^{15}N HSQC spectrum in which the ^{15}N 90° pulse of the first INEPT block is switched to a 180° pulse. If the calibration is correct, a null should be obtained.*"

3. Recovering intensities from interleaved pseudo-3D experiments is one of the most important aspects of analyzing CPMG relaxation dispersion data. The authors' way of deriving intensities is to get intensities at the same spectral position in Sparky. Standard procedures in literature have been to fit the lineshape globally across all nuCPMG values keeping linewidth and frequency as global variables for all nuCPMG values of a single peak. Freely available software packages to do this include PINT (https://pint-nmr.github.io/PINT/), FUDA (https://www.ucl.ac.uk/hansen-lab/fuda/) and the nLinLS module in NMRPipe. The authors must quantitatively compare the CPMG dispersions obtained from their Sparky macro with those using the standard protocols in literature.

The procedure described in this protocol is simple and gave us good results in several practical applications (see for example *2012 JBC*, *2015 Nat Commun*, *2018 Biophys J*, *2020 J Mol Biol*.). We do agree with the reviewer that there are more accurate methods to recover signal intensities from the CPMG experiment. We now acknowledge the existence of these methods in the protocol (note to step 4.6):

"*Note: more accurate protocols that use lineshape fitting for recovering intensities from interleaved pseudo-3D experiments have been described in the literature.[14] Freely available software for lineshape fitting is available at https://pint-nmr.github.io/PINT/ (PINT), https://www.ucl.ac.uk/hansen-lab/fuda/ (FUDA) and within NMRPipe (nLinLS module) and SPARKY (it module).*"

As this contribution is intended to describe the protocol used in our laboratory to measure relaxation dispersion profiles (validated in previous research articles), we believe it is beyond the scope of the present manuscript to compare different methods reported in the literature.

4. The standard procedure for curve fitting of CPMG data is by numerical integration of Bloch-McConnell equations. This procedure provides much more flexibility and accuracy than the Carver Richards analytical equations, without significantly slowing down the fitting. For example, the pulse width of the CPMG 180 pulse can be explicitly accounted for by using Bloch-McConnell equations. Similarly, TROSY/AntiTROSY interconversion via spin flips can also be accounted for through terms in the Bloch McConnell equations, and this is very important in TROSY based ^{15}N CPMG experiments. Again, software packages such as Chemex (https://github.com/gbouvignies/ChemEx) and CATIA (https://www.ucl.ac.uk/hansen-lab/catia/) are freely available to carry out this data fitting. The authors should provide a comparison between their fitting method and these standard methods.

The reviewer is correct noting that the Bloch-McConnell equations provide more flexibility in fitting the CPMG curves. Indeed, in our lab we routinely use the Bloch-McConnell equations to model NMR data (see for example *2017 Angew. Chem. Int. Ed.* and *2020 ChemCatChem*). However, as this protocol is shaped on our *2020 JMB* manuscript, we have decided to present the Carver Richards equations for consistency. We now acknowledge the existence of other software for fitting the data using the Bloch-McConnell equations (note to step 5.5):

"*…Additional software packages such as Chemex (https://github.com/gbouvignies/ChemEx) and CATIA (https://www.ucl.ac.uk/hansen-lab/catia/) are available to carry out data fitting using the Block-McConnell equations*"

My second main concern relates to the fact that there is no mention in the protocol about how to evaluate the reliability of the fit parameters. Parameters that have been estimated from the fit of CPMG data have to be closely looked at, because there can be severe correlations in parameters that preclude a reliable estimate in certain cases. For example, in the fast exchange limit, p_b and dw_{ab} are tightly correlated, so that only k_{ex} can be determined. While running the matlab scripts associated with this protocol, I noticed that there is no module for evaluating reliability and no error estimates are provided as far as I could see. The authors need to add two things to the fitting routine:

We agree that obtaining accurate parameters (especially p_b and dw_{ab}) from fitting of the CPMG data is a challenging task. We have recently started acquiring data at multiple temperatures and model the temperature dependence of p_b and k_{ex} in order to increase the accuracy on these fitted parameters (see for example *2020 J Mol Biol*). As we envisioned this protocol to serve as a starting point for researchers that are approaching the field, we opted for neglecting this issue. However, following the reviewer rightful concern we now describe tools that are incorporated in our fitting script to evaluate the reduced chi squared and errors on the fitted parameters. Please see below.

1) A module for testing reliability: this is done by estimating the reduced chi2 surface independently as a function of p_b and k_{ex}. Only if the reduced chi2 increases significantly (typically 1 unit above the minimum redchi2) within meaningful exchange parameters can p_b, k_{ex} and dw_{ab} be reliably extracted from CPMG data.

We now describe how to obtain the reduced chi2 at specific values of k_{ex} and p_b in point 5.6 (see below)
Please note that in order to obtain reliable values of k_{ex} and p_b for this dataset, we had to fit data acquired at multiple temperatures and model the temperature-dependence of k_{ex} and p_b (*2020 J Mol Biol*).

5.6) Test the reliability of the fitted parameters estimating the reduced χ^2 as a function of p_b and k_{ex}.
Note: the reduced χ^2 is provided in the output file. p_b and k_{ex} can be restrained to specific values by using lower and upper bounds in the fitting procedures. In our scripts the lower and upper bounds for p_b are lb(2) and ub(2), respectively. The lower and upper bounds for k_{ex} are lb(3) and ub(3), respectively

2) A module for evaluating errors IF the parameters can be obtained reliably from the fit, as determined in 1: standard ways for error estimation in literature are bootstrapping, jack-knifing, Monte Carlo or the covariance matrix based methods.

In our script the error on the fitted parameters is computed by Monte Carlo simulation of synthetic datasets from the estimated experimental error. The procedure is not explained in the protocol (step 5.7):

5.7) Estimate the error on the fitted parameters. This can be done by setting the value of MC_fac in our script to 1 and repeating the fitting multiple times (typically >20 repeats). The error on each parameter is estimated as the standard deviation of the distribution.
Note: setting MC_fac to 1 generates a synthetic dataset in which a Gaussian distributed error (calculated based on the experimental error) is added to the experimental data

The authors must also include in their protocol, a section on how to determine robustness and errors of parameters obtained from CPMG data. All these points have been discussed in literature in detail and will significantly improve the protocol.

We agree with the reviewer that these are very important points. These issues are now covered in sections 5.6 and 5.7 (please see above).

Minor Concerns:
1) The ^{15}N CPMG experiment can be taxing on the probe. The authors could add a section identifying which parameters should be set carefully. For example, d30 values typically do not exceed 50 ms and nuCPMG values for ^{15}N experiments do not exceed 1-2 kHz (depending on spectrometer and probe).

We describe this issue in step 3.5:

*3.5) Use the command "vclist" to create a list of integer numbers corresponding to the parameter n in Figures 1a and 1b. Each entry in the list would correspond to a different CPMG field (ν_{cpmg}) according to $\nu_{cpmg} = 4 \times n / d30$. **Make sure** that the first number in the list is 0 (this corresponds to the reference experiment for which the CPMG block is skipped and d30 = 0 s) and that **you do not use numbers larger than 1000 x d30 / 4. Numbers larger than this threshold result in ν_{cpmg} > 1 kHz and might damage the probe**.*

2) Temperature must be calibrated very carefully in CPMG experiments since data is usually acquired on two or more spectrometers. Since k_{ex} and p_b are very sensitive to temperature, care must be taken to limit to ensure that the difference in temperature between the two spectrometers is not more than 0.3 C or so. This can be indicated in the protocol.

We highlight this important issue at page 11, lines 6-9:

"*Another issue to consider carefully is the acquisition temperature. Indeed, as the RD data are usually measured at multiple static fields, it is crucial that the acquisition temperature is consistent among all spectrometers used. Therefore, it is highly recommended to check the temperature calibration before setting up the experiment.*"

3) The importance of other nuclei can briefly be mentioned in the end of the Discussion. Just like we would hardly make conclusions on the structure of a protein using ^{15}N chemical shifts alone, it is not best practice to make conclusions on other species detected with CPMG experiments based on ^{15}N experiments alone.

We highlight the importance of acquiring data on other nuclei at page 11, lines 18-24:

"*Finally, it should be noted that, although our manuscript focuses solely on the application of ^{15}N RD data for the investigation of protein conformational dynamics, several other experiments were described in the literature to measure RD curves on different nuclei and other biological and non-biological molecular systems.[18, 19, 21-23] In particular the use of different nuclei is extremely important, as it allows a more dense sampling of the protein structure and provides information on side-chain dynamics that are largely disregarded by the ^{15}N-based experiment presented in this protocol.[5, 21, 23]*"

4) Figure 1D: An ideal dispersion curve actually has plateaus at both low and high frequency sides. Such a curve will reliably yield p_b, k_{ex} and dw_{ab} values from the fit. The dispersion curve in the top left of Figure 1D will only yield kex reliably.

What we meant is that R_{ex} is large (and easily detectable) when $k_{ex} \sim dw$. We agree with the reviewer that using the word "ideal" is misleading. We have now corrected Figure 1D to say "*RD curve with large R_{ex}*"

5) Figure 1A: I believe the operator at the beginning and end of the CPMG experiment is 2HzSx (anti-phase transverse nitrogen coherence)? Also, the Palmer P-element is missing in the CPMG block. If this is intended merely as a schematic, that is fine.

We just use this figure as schematic to introduce the CPMG pulse train.

Reviewer 2

Manuscript Summary:
This paper describes a protocol for recording and processing CPMG relaxation dispersion data. The authors supply the pulse sequence and parameters necessary to record the data.

Minor Concerns:
The authors specifically address the TROSY version, but for studies of smaller proteins this may not be the most sensitive approach. It would be good to include a non-TROSY experiment as well. The authors might wish to discuss artifacts in the experiment and limitations with the Carver Richards expression which does not take into account differences between ^{15}N T_1 and T_2 relaxation rates, pulse offset effects etc, which can become more critical as fields increase.

The guest editor of this special issue of JoVE asked us to describe a protocol for acquisition and analysis of relaxation dispersion experiments based on our *2020 JMB* manuscript (https://doi.org/10.1016/j.jmb.2020.05.024). For this reason, we focused our discussion on the TROSY CPMG sequence and the Carver Richards equations. However, we do agree with the reviewer that acknowledging the CPMG methods optimized for lower molecular weight systems and the limitations associated with the Carver Richards equations is important and would add to the present manuscript.

To address this point we have added the following text to the manuscript:

Page 2, lines 16-18
"*Several different pulse sequences and analysis protocols have been reported in the literature for ^{15}N CPMG experiments. Herein, the protocol implemented in our laboratory is described.*"

Page 2, line 28 – page 3, line 2
"*Overall, our protocol has been successfully applied to the characterization of conformational exchange in medium sized and large proteins.[4, 5, 9, 10] For smaller systems (< 20 kDa), the use of an HSQC-based[11, 12] pulse sequence is advisable.*"

Page 11, lines 10-17
"*For what concerns the analysis of the RD curves, it is important to stress out that the procedures and fitting scripts presented here make use of the Carver-Richards equations. While this is the most common procedure applied in the literature for quantitative modeling of the RD data, the Carver Richards equations incorporate a number of approximations and are limited to the two-site exchange case.[17] If a particular dataset requires the more rigorous Bloch-McConnell matrices for data modelling, the fitting procedure should be modified accordingly.[18-20] In the protocol above, a few freely available software packages are listed that perform data modeling using the Bloch-McConnell theory.*"

Reviewer 3

Manuscript Summary:
The authors elaborate on the measurement of the relaxation dispersion for the ^{15}N spins (the TROSY component) of protein amide groups, while also providing its pulse program and software for the processing. The last author has already published many papers on research using the method, and I therefore believe that the described content is guaranteed.
I read the draft a few times and wondered if the following minor points might be incorrect. I have an impression that the other parts form an excellent work.

Major Concerns:
None

Minor Concerns:
The pulse program, trosy_15N_cpmg.vv, contains the following comment.
;the cpmg field is B=1/(4*d3)=4vc/T.
Please check if this can be a mistake of B = 4*cpmgLoop/T = 2vc/T.

The rationale is as below.
The following comment in the pulse program means that vc equals to 2*cpmgLoop.
;There will be a total of 4*vc pulses in T corresponding to 8*d3*vc.
If I were correct, "vc" should be twice the number that is written in the vclist. However, I am afraid that this expression is rather confusing because the shared spelling part "vc" may make some users think by mistake that vc is the same as the number that they have input in vclist.
In the pulse program used by the authors, the constant time T (= d30) for relaxation is divided into two equal CPMG blocks. In the so-called relaxation compensation method, the "n" in the text corresponds to "cpmgLoop" in the pulse program, which represents the repeat number during each T/2 period, instead of total repeat number during the whole T.

There was a typo in the comment at the top of the pulse program file (we apologize for that). Now the typo is fixed.
The delay d3 is one-half the spacing between centers of the 180-degree pulses. As there are 8*vc pulses in T (we have 2 CPMG blocks and 4*vc refocusing pulses per each block), T=16*d3*vc and CPMGfield=1/(4*d3)=4vc/T. We thank the reviewer for noticing this typo.

Line 56:
In "(ii) set up and acquisition and the NMR experiments",
The word "and" appears twice.
Should this be "... in the NMR experiments"?

We have fixed this typo

Line 80:
"protocols are available" should be "proteins are ...".

Line 85:
"located the in the folder" Please remove the first "the".

We have fixed this typo

Line 147:
"in you working directory" should be ".. your ..".

We have fixed this typo

Line 228:
"on the us-ms timescale" should be ".. (Greek letter micro)s-ms ..".

We have fixed this typo

In figure 2:
At the top of the last panel, "kkb" should be "kba".

We have fixed this typo

Reviewer 4

Manuscript Summary:
This protocol is quite interesting and useful. I think it could help those who want to conduct transverse relaxation dispersion measurements and interpretation.

Major Concerns:
The approach Singh et al. describe privileges the TROSY-based experiment. However very efficient sequences are implemented in the routine pulse program catalog of the Bruker spectrometers (the one used also by the authors). Those sequences are both HSQC-based and TROSY-based. So the authors should specify which is the difference between the sequence they provide and the one found in the Bruker pulse sequence library (trhncorexf3gp). Importantly, the proposed sequence is accompanied by specific "include" files whose compatibility with Bruker corresponding scripts should be stated to know if problems can arise upon execution.

We thank the reviewer for the comment. We now acknowledge the existence of a similar sequence in the Bruker portfolio. We also describe the major differences between the Bruker sequence trhncorexf3gp and our pulse program:

Page 10, last line – page 11, line 5
"*In this respect, it is also important to mention that a pulse sequence similar to the one provided with the present protocol is present in the standard pulse sequence portfolio of the spectrometer (file name: trhncorexf3gp). The main difference between the two files is that the standard sequence is based on a TROSY-HNCO experiment, while our experiment is based on a TROSY-HSQC experiment and does not require* ^{13}*C labeling of the sample.*"

As for the include file, we have implemented our sequence on more than 10 spectrometers located on both the Iowa State and NIH campuses. As to this date, we have not observed any compatibility issue.

The description of the data processing procedure is useful and based on well-known software (either freely available or not). As for any protocol, I would suggest emphasizing the importance of reading the original papers because a recipe is useful only if one masters the very meaning of an experiment and the data thereof.

We completely agree with the reviewer. We have emphasized the importance of studying the relevant literature in the discussion section (page 10, lines 17-20):

"*Below some important aspects regarding the acquisition and analysis of RD experiments are discussed. However, for a more in depth description of the experiment and data analysis, careful studying of the original literature is highly recommended.*[3, 8, 11, 15, 16]"

Minor Concerns:
Finally I would suggest an editing of Figure 2. The authors choose and hand-drawn style that is somehow peculiar. It does seem to attempt an imitation of the illustrations of the book "Nuts & Bolts Approach" by Fukushima and Roeder or the famous drawings of Ray Freeman. The results here are not at that level, unfortunately.

We have prepared revised Figure 2 accordingly.

Editor's comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise the following lines to avoid previously published work: 23-26, 62-63.

Done

3. Please provide an institutional email address for each author.

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Vincenzo Venditti [venditti@iastate.edu]

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Bruker, Topspin, etc.

Done

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Done

7. Line 80: Please revise "protocols" as "proteins".

Done

8. Line 82: Please specify the amount of NMR sample required.

Done

9. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done
We highlighted steps 2.1-3.13

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Done

11. Please sort the Table of Materials in alphabetical order.

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