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# *Interactive comment on* "Orthogonally spin-labeled rulers help to identify crosstalk signals and improve DEER signal fidelity" *by* Markus Teucher et al.

**Anonymous Referee #2**

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Teucher et al. describe a systematic investigation of distance distribution artifacts that can occur in orthogonally spin-labelled biomacromolecules when specific spins cannot be exclusively addressed but the pulses also excite other spins unintentionally. There are no new concepts or experiment designs in this manuscript but the declared aim is to provide a strategy for identifying and possibly removing 'false positive' distance contributions. While the results do not bring many surprises, this could have been a worked example of how one can thoroughly identify and quantify these artifacts in the distance distribution. However, with the current lack of quantification and error estimation in the analysis this is almost entirely anecdotal with limited value to practitioners. Once the artifacts are quantified and the most important avenues for their suppression



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explored and experimental uncertainties are given this may become publishable. In the current state publication would be premature.

Model system

1 You find a much broader distance distribution of the NO-NO ruler than found for the homologous ester-linked structures. As this is unlikely to be rooted in real backbone flexibility (cf. Jeschke JACS 2010 cited in here) and acid amides will not be more flexible than esters either I would suspect a distribution in small exchange couplings. How does the fast motion cw EPR compare with ester-linked rulers? It seems odd to generate a new structure for this study and not fully investigate its spectroscopic properties.

DEER setup

2 You describe the experiments insufficiently to allow independent reproduction of the results. How were the power levels calibrated? You write reducing the AWG output from 100% to 22% corresponds to 12 dB attenuation. Is this frequency independent, is this with the TWT in saturation at 100%? With some of your results not showing the expected microwave power response it is important to understand how the settings were optimized and controlled. Were all pulse power levels optimized via nutation experiments at the respective frequencies to account for the limited resonator bandwidth? This is not clear from the current description. What are the expected differences in nutation between Gd and NO in the NONO and GdNO channels? The contribution from the central transition is not all out dominant at these pump frequencies.

Distance analysis

3 Increase the size of your figure panels. Six-panel wide figures with uniformly scaled distance distributions and DEER signals make it very hard to see the detail of the data. Many of the figures are only 3 panels wide with lots of white space around the data panels.

4 Gaussian fitting does indeed allow a much more stable parametrized analysis. The comparison with Tikhonov Regularization must be extended to the pure rulers (Fig S3). None of the Gaussian fits is particularly good so that the model free analysis has to be shown.

5 Nevertheless, the Gaussian fits allow straightforward quantification of contributions of different distances to the modulation depth. This should be don't throughout and replace the qualitative discussion (see below). The GdGd ruler should contribute to the NONO channel. The signal may be too weak to detect but this should at least be mentioned here. Looking at the spectral overlap a contribution of the GdNO ruler to the NONO DEER does not seem "surprising" at all.

Modulation depths

6 NONO DEER gives 35% and NOGd 30%. You must provide error estimates. Is the difference significant? These are both synthetic rulers with 100% nitroxide labelling. One might assume the modulation depths should be identical unless you can give reasons for the opposite. This needs to be quantitatively addressed.

7 Once the modulations depths are quantified and Gaussian contributions to the distances have been fitted it is straightforward to quantify the contributions of the different spin pairs to the DEER signal in question. This is currently only qualitative (e.g., line 140 "with a slightly smaller modulation depth"). The quantified depths can then be compared with the predictions from the respective modulation depths of the pure rulers. I expect to see a table with the different experiments and samples listing the expected and experimentally found modulation depths and contributions of individual rulers expected and found. Finally, you can add the pure ruler DEER signals in the calculated ratios and show that the contributions are similar to experiment and that the analysis does or does not recover the artifact.

Channels and cross-talk

8 I fail to see the benefit this new nomenclature brings over previous descriptions. There may be some point in the choice of these terms but this should be explained comprehensively as currently it only unnecessarily adds to the confusion. Especially assigning the same distance contributions different cross-talk names whether found with a corresponding spin pair present seems arbitrarily expanding the complexity. What is the added value?

9 According to figures S4 and S5 you only see the GdGd contribution to GdNO experiment in the equimolar samples and no other crosstalk at all. This means doubling the content of GdGd ruler was done to see the other artifacts at all and is biased from the outset. You should be transparent and explicit about this from the outset when describing the setup and results.

10 You derive conclusions from data you refuse to show. This violates basic research transparency and either the data needs to be added or the statements removed (line 193, 233-234)

11 You attribute the GdNO contribution in the NONO experiment to both contributions of Gd to the echo and to the pumped spins. This is based on a 12 dB pump power reduction not altering the modulation depth. How large is the Gd echo at 50K and the chosen refocused echo position? Is it not more likely that the pumping of Gd far off the maximum seems to be invariant to the power levels used in agreement with the data of further experiments (see below)?

12 When reducing the pump power in the GdNO experiment this does not seem to alter the GdGd contribution significantly but the GdNO contribution. You state its distance peak intensity increases but contradict this in the next paragraph by stating its modulation depth reduces. You must quantify the contributions (see above) to make quantitative statements. The statement of “optimized pump power” seems peculiar as the modulation depth reduces with contradicting this more optimum setup. It seems the dependence of the modulation depth on the pump power on Gd away from the

maximum is not understood and largely invariant to pump power if not contradicting the predicted trends. The discussion has to reflect this. The power dependence of the spectra in Fig 8 indicate that none of your spins is experiencing the nominal flip angles at 100%.

Spectrometer-specific artifact

13 You should be able to see this artifact in its pure form using a sample of free Gd and NO spin label. How do you know it is an artifact? How do you know it is spectrometer-specific? How many other instruments with the same nominal configuration have you tried?

GdNO DEER

14 The main potential advantage of NO detected Gd pumped DEER is that 50 K can be used for fast repetition on the nitroxide and diminishing contributions of Gd to the refocused echo as transverse dephasing should be fast. This should definitely be compared experimentally with the other GdNO DEER setup used in here but is not even mentioned. The experiment in Fig 8 done at 50 K will be insightful in first instance. The sentence "...but experimentally impracticable for samples containing NO and Gd spins due to the prohibitively long shot repetition time of the experiment and the small modulation depths expected." in the conclusion should be adapted in the light of this.

Conclusion

15 The conclusion should not repeat the findings at length but conclude the relevant achievement with respect to the state of the art and the resulting implications and several points of discussion should be moved to the relevant section: -GdGd crosstalk in NONO DEER is likely to be diminished by a negligible Gd refocused echo at 50K and this is why the NO detected GdNO DEER and the Gd transverse dephasing at 50 K need to be given for comparison. -The suggestion to produce new samples lacking certain spins to prove crosstalks is directly opposed to this manuscript's aim. If

you make these samples anyways why bother with identifying crosstalks? The GdNO DEER pumping Gd will likely be more cost-effective. -GdGd crosstalk in the GdNO channel can be identified by a minor change in modulation depth upon pump pulse power reduction but if the modulation depth collapses to ∼15% how do I exclude the presence of GdGd crosstalk?

Minor

-"The term orthogonal refers to spin labels that are spectroscopically distinguishable from each other and can be addressed and/or detected independently, e.g. via distinct resonance frequencies, relaxation behavior or transition moments." It would be very helpful to readers if at least one example per concept (frequency, relaxation and nutation filtering) could be given rather than none at all.

-In section 1.3 you quantify the spectral widths and relative nutation frequencies but not relaxation differences. You can help the reader by giving longitudinal and transverse magnetization decay constants for both spins at 10 and 50 K to follow this rationale.

-Caption figure 4: “Regions in which distances can be theoretically expected”. Outline the theory and how this determines where distances can be expected in practice.

- “Accordingly, we suggest that the dominant signal contribution at 2 nm arising from the NO-NO ruler masks the NO-Gd crosstalk signal.” This can easily be checked by synthesizing data from the two pure rulers in the corresponding ratio and analyzing it.

-Figure S1 You seem to observe some orientation correlation in the GdNO ruler, does the small short-distance spike in the Tikhonov distance distribution correspond to double the frequency of the main peak?

The manuscript has a plethora of general statements that need modification or at least significant context:

-You give 8 nm as upper limit for DEER which is half the current maximum claimed in literature.





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-Your discussion of background correction relies on a homogeneous distribution of spins. This should at least be mentioned.

-You should clarify the definition of the form factor, when comparing the initial definition by Milov et al. and the more recent use by Jeschke this means different things.

-The multi-spin problem leads to ghost peaks as you rightly state, but it also leads to loss of intensity and resolution at longer distances.

-Your definition of spectroscopically orthogonal seems ambiguous. As it is impossible to independently address the nitroxide it would fall outside the definition of being orthogonal to the Gd.

-Spectral overlap between metal ion and nitroxide is common for Gd, Mn, Fe but not for Cu.

-"Nitroxides (NO) and GdIII-based spin labels (Gd) are the most commonly used orthogonal spins for DEER experiments on biomolecules." Please provide evidence for this statement. The selective citation practice does not back this up.

-“For the Gd-Gd crosstalk signals in the NOGd DEER channel, which are the most relevant unwanted signals in the analysis of complex protein mixtures. . .” There should be evidence provided for this assertion.

-“Q band currently offers the highest sensitivity to perform the three-channel DEER experiments with samples containing both NO and Gd spin labels on a commercial spectrometer.” There is justification or references needed for this statement.

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