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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 #1

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This manuscript focuses on DEER distance measurements between Gd(III) and a nitroxide (NO) radical, often referred to as orthogonal spin labeling. One of the motivations for using such labeling schemes is the ability to carry out selective distance measurements , for example if a biomolecule is labeled with one NO and one Gd(III), then one can probe intra molecular distance via Gd-nitroxide and intermolecular distances (which can arise from oligomerization) by Gd(IIII)-Gd(III) or NO -NO distance measurements. This approach was introduced already in 2012 (DOI: 10.1039/C2CP40282C, Phys. Chem. Chem. Phys., 2012, 14, 10732-10746, which unfortunately is not referenced by the authors). Other reasons maybe increased sensitivity compared to Gd(III)-Gd(III) and elimination of the effect of the dipolar pseudosecular terms on the DEER

modulation frequencies in the case of short distances. In this work the authors used three model compounds with two NO, one NO and one Gd(III) and two Gd(III)-Gd(III) and use them to evaluate how selective are the Gd(III)-Gd(III), Gd(III)-NO and NO-NO distance measurements , while exploiting the different spectral and spin dynamics properties, which have been highlighted in earlier works. This work does not present any new original ideas but using well defined model compounds that can be mixed in a control manner they clearly show expected pitfalls and when they can be overcome and when not. These arise from the spectral overlap of Gd(III) and NO throughout the spectral width of the NO. The authors refer to the consequences of this overlap in various pulse set-ups for DEER as "cross talk" . The value of this manuscript is mainly "educational' as it nicely highlights all issues involved in such measurements on controlled samples. The authors borrowed from optics the nomenclature of color channels to accompany their explanations and in the figs use the associated colors, which again has educational value. I think that after appropriate revisions following the comments below this manuscript will be of value to practitioners of DEER and therefore I recommend publication.

1. In Fig. 3 the bandwidth of the pump and observe pulses are assumed to be the same but I think that this is incorrect, the bandwidth of the an echo detection sequence (two or three pulses) is not the same as just that of the pi pulse. This is even mentioned by the authors (page 13, line 220). Please calculate the correct bandwidth and change Fig. 3.

2. The manuscript is very qualitative and its level can be increased by calculating the predicted modulation depth for NO and Gd(III) at the relevant pump frequencies and compare to the observations. As they have the full lineshape of the Gd(III) and the NO this can be easily done. Similarly, they can account for the degree of overlap for the observe sequence for the different conditions. Such calculations can actually serve to guide the experimental optimized set up. In table S2 the authors mention "Theoretically possible" but as they did not do any theoretical calculations, this term is inappropriate.

3. The presentation of normalized distance distributions without the uncertainties evaluated by validations are misleading. For example in Fig. 1c the trace is very noisy and the modulation depth is small, yet the distance distribution is nice and intense just like the one below. This is just one example but is occurs in many of the Figs. This should be corrected, the P(r) values should be noted on the Y axis and uncertainties should be shown. In Fig. S1 they show that there is no real difference between Gaussian and Tikhonov regularization . So if they chose Tikhonov regularization this maybe easier to show.

4. Why was the Gd(III) pulse taken as 24 ns, when there is enough power to shorten it and improve SNR.

5. Please explain why you choose to add the Gd-Gd ruler in a twice as much concentration, is this to enhance the “cross talk” ?

6. The spectrometer artifact is worrisome – it is larger than the cross talk. What is the source of the artifact and why it appears only in the red channel?

7. P. 3 line 67 : You should use TM (phase memory time) and not T2. Also the differences in phase memory time of Gd(III) and NO is not very different. If you know of cases where it has been used to filter NO and Gd(III) please give a ref.

8. It is more appropriate to cite the original papers than a review. There are not so many examples of Gd(III) –nitroxide distance measurements so better give credit to the original papers and not a review.

9. In general the referencing is rather poor, focusing on self-citations. The omission of the work of Lovett is one example. Another one is the omission of distance measurements between three different spins (Gd(III), nitroxide and Mn(III) (Goldfarb group) and the reference mentioned at the beginning of this evaluation. P. 2 line 35 please give a reference to the DD software as well when mentioning Gaussian fits.

10. Isn’t the easiest way the identify the X2 and X3 crosstalk is just running a Gd(III)-

Gd(III) set up and see that it is the same distance as observed in the cross talk.

11. Please shorten the conclusions – no reason to have a two page conclusion that just repeat the results. Should be short and to the point.

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