Reply to Comments from Alberto Collauto

The authors propose a very interesting application of a high-bandwidth, high-power W-band setup to measure undistorted Gd(III)-Gd(III) DEER traces even for short distances, condition under which the mixing of the (…) states caused by the pseudo-secular terms of the dipolar Hamiltonian results, under normal measuring conditions, in dampening of the dipolar modulation. A very interesting conclusion is proposed suggesting the use of the already available Gd(III)-based tags with low zero-field splitting even for short distances, provided that both the pump pulse and the detection sequence completely avoid the excitation of the central transition.

The manuscript is well written, and definitely suitable for publication on Magnetic Resonance; the conclusions are substantial and nicely supported by the presented data and analyses. However, there are some points that I would like to be addressed by the authors.

We thank Dr Collauto for his kind words above, and careful reading of the manuscript. We reply to his helpful and interesting comments below.

1. **The style of the references is not homogeneous: in some cases the full DOI hyperlink is reported, whereas in other ones only the DOI number is displayed; some references make use of journal abbreviations, whereas in other ones the full journal title is mentioned. Besides, the absence of spacing and/or indentation makes it really hard to find a specific item. Moreover, references having the same first author are not always listed chronologically. I advise to follow thoroughly the author guidelines.**

This appears to have been a problem with ENDNOTE. We have now corrected any inconsistencies in the referencing.

2. **As far as I could see, no specific literature for Gd(III) labelling of DNAs has been cited although reference has been made in the text to this application (line 49).**

We have now added a reference.

3. **I found the nomenclature proposed in Table 1 rather unclear; for example, why is a 10 ns-long pump pulse set to the maximum of the central transition once identified as P1 and once identified as P3 (6.0 nm Gd ruler)? I would find easier for the reader to have the relevant experimental conditions reported for each experiment (pulse length and frequency offset) in the figure caption or as inset, and, to improve the readability of the manuscript, I would consider moving the sensitivity considerations reported in Table 1 to the supporting material.**

We have used such nomenclature P1 and P3 to emphasize that these two positions, although having similar pulse lengths, and positioned at the central transition, are at different frequencies. Note that the observer frequency is kept at 94 GHz and the pump frequency is varied. We chose this naming scheme only after considering many alternatives.

With regard to the sensitivity, this is mentioned in the title and high concentration sensitivity is emphasized in the abstract, and we are not aware of any experimental results that show a higher concentration sensitivity for these systems. So we feel it is an important part of the manuscript. These numbers allow other groups to directly compare sensitivity. The paper is not just about measuring short distances by having probe and pump away from the central

transition. It is the fact that one can still make the measurement with very high sensitivity that we feel makes the result useful and interesting.

4. **Is the (rather lengthy) discussion about the echo decay traces relevant for the purpose of this paper? After all, the measurements were performed on the maximum of the central transition, whereas the DEER detection sequence was always placed at spectral positions where the largest contribution to the echo comes from other transitions. A possible solution could be to move this section to the supporting material.**

We would claim that relaxation times and thus discussion of echo decay traces is highly relevant to sensitivity with regards to the practicality and design of potential experiments made at low sample concentrations. We completely agree it would be better to give the relaxation times at offset frequencies. At the time this was an oversight. Unfortunately, immediately after the experiments the spectrometer was rebuilt to incorporate a wideband AWG and then we had the lab lock-down, and it has not been possible to make these measurements since.

5. **A high sensitivity of the experimental setup is claimed. However, a rather large sample amount (around 75 µL of a 40 µM solution, hence 3 nmol) was used compared to the typical ones used for conventional W-band or Q-band spectroscopy (around 5 µL of a 40 µM solution, hence 0.2 nmol; 15 times smaller!), or even X-band spectroscopy (around 20 µL of a 40 µM solution, hence 0.8 nmol). An extension of the proposed approach to applications where the limiting factor is the sample amount, such as investigations inside cells or on systems that are challenging to express and/or label, is therefore in my opinion still not straightforward.**

We can only agree that it would be nice to have both extremely high concentration sensitivity and very little sample. However, this comment does not appear to take into account the significant loss of concentration sensitivity for small volume cavities, especially at lower frequencies. If you are not sample limited then (with some caveats) maximising sample volume, at a given frequency, will always give a larger signal. We have now attached data in the SI where we show measurements taken at Q-band at high power (150 W), using Bruker's large volume Q-band cavity (with comparable sample volume to that used here – 50-60 µL). For 2.1 nm, with both pump and probe on one side of the central transition, the concentration sensitivity is reduced by around a factor of 72, compared, to the W-band measurement corresponding to P3O in the paper. One might expect the concentration sensitivity of the small volume Q-band resonator (quoted) to be down a further factor of 4. The concentration sensitivity of the X-band resonators quoted are likely to be very significantly worse. Of course, for systems that are difficult to express, having 50 uL sample volumes is not necessarily trivial – but as discussed in the paper there are potentially relatively straightforward ways to further improve sensitivity (and hence potentially reduce volume and improve absolute sensitivity) and still reach sub-µM sensitivity. So we believe this to be a very promising and flexible approach. That is not to say there aren't other promising approaches, like the W-band resonator approach taken by our collaborators at the Weizmann Institute. But we believe it will be very challenging to significantly improve the sensitivity at X-band and Q-band to make them competitive, both in terms of absolute and concentration sensitivity for these types of samples. We will add a line in the discussion.

6. **Throughout the main paper and the SI plots belonging to the same figure have different sizes and are not always aligned (see for example Figures 3 a/b, 5, S3, S4, S5).**

We will endeavour to correct this with the copy editors in the final version.

7. **Table 1: the shot repetition time should be given in time units; what is reported is the shot repetition rate.**

Many thanks – we have corrected this typo and changed to shot repetition rate.

8. **Table 1: why was the shot repetition rate decreased from 3 kHz to 1 kHz for some of the measurements on the 2.1 nm Gd ruler (see Table 1)? Are measurements available to justify this choice?**

The simple answer is that the 1 kHz measurements could have been made with a repetition rate of 3 kHz (or an even higher rate as Stefan Stoll suggests) and we would have had the same signal to noise in less time. At the time we were being very conservative in our choice. That is one of the reasons we give sensitivity measures to allow different results to be compared. Results were not repeated, because immediately afterwards the experiments, the spectrometer front-end and detection system was rebuilt to incorporate a wideband AWG, which was a major change. We then had the COVID lab shut-down (and are still affected by it).

9. **Table 1: what was the used value of $\tau 1$ for the DEER experiments?**

The value is 300 ns and has been added to the figure caption.

10. **Were the DEER measurements performed with or without a phase cycling of the $\pi/2$ pulse? If without, which precautions were taken so as to have no constant offset of the DEER traces?**

We can measure with phase cycling, but these specific measurements were actually taken without phase cycling (for technical reasons). Instead, offsets were removed by separate automatic measurements of the baseline, on either side of the echo. This baseline was then subtracted (at the cost of a slight reduction in S/N). We would add that offsets are known to be very low, and signals were relatively high in these experiments and so the correction was rather small.

11. **In Figure S3a the intermolecular contribution for the experimental condition P3O3 has been modelled as an increasing function, a clearly unphysical assumption (as also stated by the authors). The analysis of these experimental data has to be repeated by taking an exponential decaying function. Furthermore, the primary data are displayed only for t >= 0; is this the way in which the data were recorded? If so, why? If not, it would be advisable to plot the whole data, in such a way that the maxima of the recorded traces are visible.**

We chose to show the increasing intermolecular contribution (to be consistent with other results where contributions were chosen to give the best Pake pattern). However, we agree that this just casts an unnecessary question mark on that result. We have now fitted using a decaying contribution. This leads to a slightly less optimum Pake pattern, but effectively exactly the same distance and distance distribution. The underlying problem is of course that the oscillations have not died out by the end of the trace, and so it is difficult to fit the background with absolute confidence. It is not clear why this trace has a slightly different background. However, we would point out that the intermolecular contributions in all these

traces are much smaller than have been observed before in experiments on these samples and thus potential errors in estimating distributions are correspondingly much smaller.

We chose to only show $t > 0$, as the traces are very long and thus the period where $t < 0$ is correspondingly very short relative to the total trace, and correspond to only a few points, with little extra information content. Nevertheless, we have now added these points.

12. Figures 5d and 7b: what do the black arrows highlight?

The arrows indicate 94 GHz which is the centre frequency of the EIK. This was added to be a guide to the eye, to show how the pump and probe pulses were positioned relative to the centre frequency. We have added a note in the captions.

13. Table S1: which distribution of E values was taken to fit the experimental data shown in Figure 2? Were the simulations perform assuming a monomodal distribution of D values around +D or a bimodal distribution of D values around ±D? (I am not able to deduce this information from lines 197-199 of the main text).

Whilst some previous studies on Gd-spin labels samples have needed a bimodal distribution centred on +D and –D to simulate the observed spectra, in this study, we found we could get an excellent fit by using a monomodal distribution around D. We will add a note to make it clear what we have done.

14. Table S2: is the time corresponding to a decay of the echo signal to 10% of its initial value given as τ or 2τ? In which units is this value reported?

This parameter is a function of 2τ, and the units are μs. We have clarified this.

15. Captions of the Tables S2/S3: what is x? Was the dead time 2τ 0 taken into account for the fit of the echo decay curves? (This is relevant as the traces were fitted with a non-exponential function).

The x parameter corresponds to time and it has now been changed to t to make this clear. The dead time was taken into account in the fits. However, we didn't observe a major difference to the fits, both with and without the dead time.

16. Table S3: a bi-exponential behaviour of the inversion recovery curves has been reported. Were other kind of experiments attempted aimed at minimizing the role of spectral diffusion? Besides, a T1 value resulting from a mono-exponential fit of the experimental traces has been reported but no comparison between the biexponential and mono-exponential fits is shown in Figure S2.

At 40 uM concentration we do expect much (intermolecular) spectral diffusion, although we cannot completely rule it out. We do show a comparison in Figure S2, but we will make this clearer.

17. Figure S2: because of the poor resolution I can hardly see the experimental data points.

That is partly because the fit is so good (R^2=0.9999), but we have now changed the way the experimental data is presented to hopefully make this clearer.

18. Figure S2a: what was the minimum used value of τ? This can't be deduced from the figure, where the first point of the decay trace has been set at 2τ = 0.

The value of the interpulse τ was 300 ns and a note has been added to the figure caption.

19. Figure S2b: the inversion recovery curves have not been collected till a plateau corresponding to the full recovery of the echo signal has been observed. This may result in severe uncertainties in the estimation of the longitudinal relaxation rate by fitting of the experimental data (Table S3).

We agree that a slight error is possible, but the results are entirely consistent with Ref [Phys Chem Chem Phys, 18, 19037-19049] and were only used to estimate viable repetition rates.

20. Caption of Table S2 and lines 312-313 of the main text: why the fit of the echo decay curves has been described as a "sum of two stretched exponential functions" although for one of the components the exponent has been fixed to 1?

Many thanks. We agree the term "stretched" is confusing in this context and has been changed.

21. Figure S3: given the amount of free space on the page, I would consider useful to quickly recap, maybe in the form of a table, the relevant settings corresponding to the different traces.

Tables have been added showing the relevant settings corresponding to the different traces.

22. Figures S4, S5, S6: in my opinion, a reminder to the legend of Figure 2 for what concerns the color code used in the simulation of the EDFS-EPR spectra would be useful.

We have now added the legends, that were shown in Figure 2.

23. In my opinion, it would be useful to add the frequency response of the EIKA, which dominates the bandwidth of the system, to the plots in the supporting materials showing the excitation profiles of the pump and detection pulses. This would make immediately clear to the reader where the pulses have been positioned within the bandwidth of the transmission chain.

We have carefully considered this suggestion, but on balance we feel that this would make the resulting figures too cluttered. We will however point out in the caption that 94 GHz represents the centre frequency, and has been designated by a black arrow, where appropriate.

24. Figures 3, 5, 7, S4, S5, S6: how was the excitation profile of the detection sequence calculated?

An effective B_1 was calculated, derived from the length of the π/2 pulse that gave the largest signal, (allowing for the fact we are dealing with a high spin system). The predicted excitation profile for a refocussed echo was then calculated using simple spin mechanics, (Phys Chem Chem Phys., 2007, 1895-1910). The resulting excitation profile is narrower than the excitation profile of a simple π pulse or a simple Hahn spin echo, as expected.