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Supporting Information to: Milliwatt Three- and Four-Pulse Double Electron Electron Resonance for Protein Structure Determination.

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Introduction

3-pulse versus 4-pulse DEER

Table S1: Maximum potential signal gain S_{gain} by performing a 3-pulse over a 4-pulse DEER experiment for different phase memory times T_{m} and τ_1-values (see Fig. 1) calculated using Eqn. (1). This model is a maximum estimation and strongly simplified. The realizable signal gains will be more moderate and strongly sample-dependent. ESEEM effects can lead based on their strength (amplitude) and type (frequency) to significant modulations of the observer echo depending on the sequence length and therefore strongly influence the achievable signal gain (see Fig. 7 for more details). Furthermore, the relaxation behavior of the 3-pulse observer echo and the refocused 4-pulse observer echo will be significantly different.[1–3]

T_{m} [µs]	S_{gain} ($\tau_1 = 0.2$ µs)	S_{gain} ($\tau_1 = 0.4$ µs)
1.10	1.4	2.1
1.00	1.5	2.2
0.90	1.6	2.4
0.80	1.6	2.7
0.70	1.8	3.1
0.60	1.9	3.8
0.50	2.2	5.0
0.40	2.7	7.4
0.30	3.8	14.4
0.20	7.4	54.6
0.10	54.6	2981.0

Resonators

Table S2: Properties of the resonators compared in this work. Λ_{ave} is the B_1 conversion factor (also known as resonator efficiency), Q_{L} is the loaded and Q_0 is the unloaded quality factor or Q-value of the resonator, β is the overcoupling parameter, and τ_{d} is the dead time calculated according to Eqn. (5) in the main text.

resonator	Λ_{ave} [mT/$\sqrt{\text{W}}$]	Q_0	β	Q_{L}	τ_d (calc.) [ns]
MS-3	0.4	1750	2.7	475	113
microhelix	3.2	220	4	44	8

DEER Setups

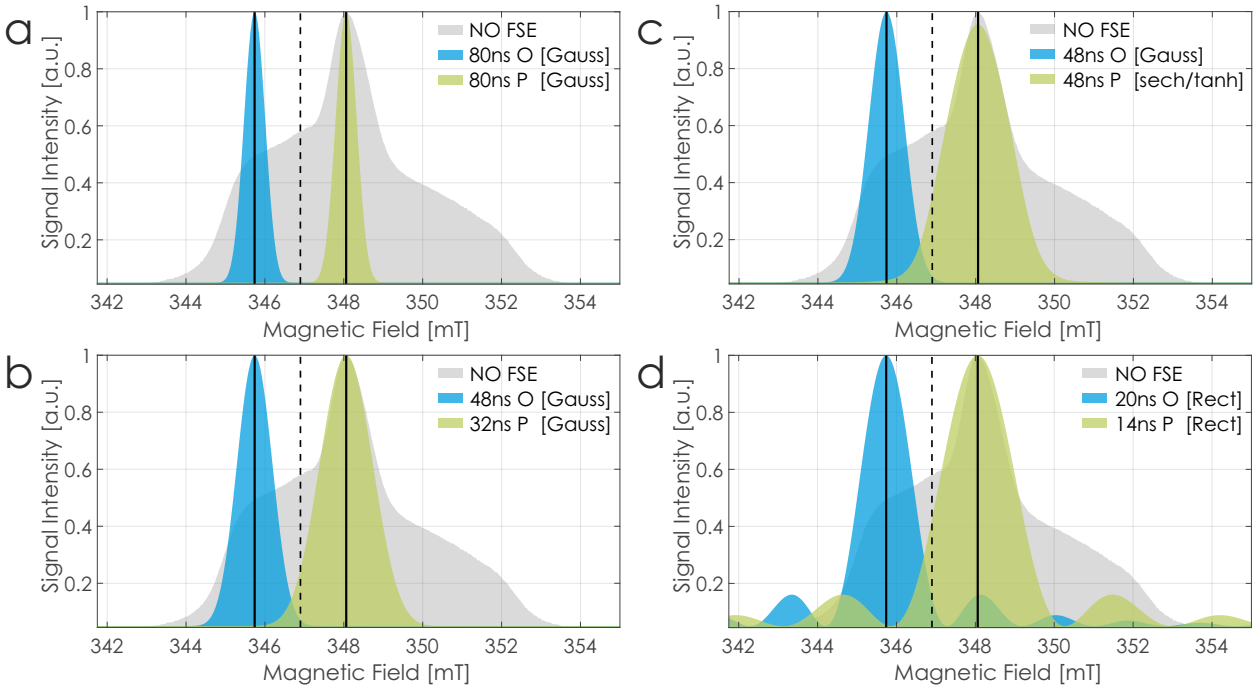

Figure S1: Illustrative representation of the utilized DEER setups. NO field-swept echo (FSE) spectra are shown as gray areas with superimposed pump (green) and observer (blue) π-pulse excitation profiles at their respective spectral positions. The excitation profiles were simulated using EasySpin version 5.2.8[4] with the provided "pulse" and "excite profile" functions. The excitation profiles are idealized π-pulses which are only indicative of the real excitation bandwidths since neither the spectral shape, nor the nonlinearity of the signal response or the resonator profile and the Q factor are taken into account. The vertical black lines indicate analogously to Figure 3 the placement of the experiment within the resonator dip, with the broken line being the center of the resonator.

B_1-field homogeneity of the microhelix

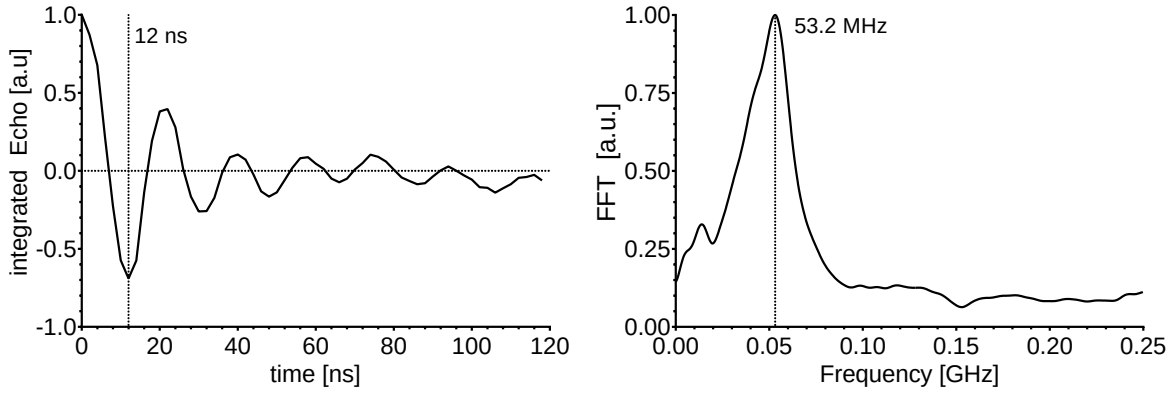

Figure S2: Transient nutation experiments were performed to assess the B_1 field homogeneity of the microhelix. (a) Nutation transient recorded at 555 mW of power for a nitroxide sample in a 0.4 mm OD amd 0.3 mm ID capillary at 50 K. The π-pulse time is 12 ns using a [6-400-12] ns rectangular pulse sequence after the nutation pulse to form the echo. (b) The fast Fourier transform (FFT) of the nutation experiment provides the magnetic induction inside the cavity which was determined to be 53.2 MHz.

DEER

Experimental Parameters

An overview of the utilized experimental parameters is given in Tab. S3. Please note that the product of number of phase cycles, h, m and n was kept constant between 3-pulse and 4-pulse experiments to ensure a comparability of the SNR.

The plotted 3-pulse and 4-pulse time traces (Fig. 3-5) appear to have different dipolar evolution times but this is caused by the different dead time delay values (d3) used in the sequences. In principle it should be possible to set d3 to zero in the 4-pulse DEER experiments (analogously to the 3-pulse experiments) effectively prolonging the time trace due to the low incident powers and connected dead times. This leads to a gain in the detection window of the 4-pulse experiment, as long as the there is no "2+1" signal present in the data[5].

The utilized dipolar evolution times are the same making the data comparable regarding their SNR, even though it has to be mentioned that the applied nuclear modulation averaging in 4-pulse DEER leads to a stepwise m×d31 prolongation of the the 4-pulse time trace that should lead to a small but hard to quantify decrease in SNR of the 4-pulse DEER time traces with respect to the 3-pulse DEER time traces.

The given microwave powers were measured independently at the microwave output of the bridge before being directed to the probehead. An illustrative representation of the utilized DEER setups for the different pulse lengths is given in Fig. S1.

Table S3: Overview of the parameters used to perform all DEER experiments on the NO-NO ruler presented in the main text. The designation of the variables follows the conventions typically used in Bruker Xepr PulseSPEL scripts for DEER experiments. All DEER experiments at T = **50 K** were performed using the following parameters: 16-step phase cycle, srt = 1000 µs (shot repetition time), d2 = (τ_{dip} =) 1400 ns (dipolar evolution time), d30 = 2 ns (pump pulse time increment), pg = observer π-pulse length (observer echo integration window) and transmitter (TM) level: 50%. AWG [%] corresponds to the utilized AWG pulse amplitude as defined in Xepr. **4-pulse DEER** experiments were performed using the following parameters: h = 12 (shots per point), d1 = (τ_1 =) 400 ns (for primary observer echo), d3 = 280 ns (dead time delay), m = 8 (nuclear modulation averaging) with a stepping of d31 = 16 ns. The shaped pump pulse is a 48 ns sech/tanh pulse which is swept in frequency from -40 to -90 MHz. **3-pulse DEER** experiments were performed using the following parameters: d3 = 0 ns, h = 96 and m = 1. The DEER experiments performed at T = **100 K** just differ in the chosen dipolar evolution time which is in this case d_{dip} = 800 ns.

resonator	T [K]	DEER exp.	P (π) [ns]	O (π/2) [ns]	O (π) [ns]	n	signal	noise	SNR	att. [dB]	AWG [%]	power [mW]
MS3	50	4p GDEER	80	80	80	1	0.17	0.002	90	3	n/a	44742
mh	50	4p GDEER	80	80	80	1	0.17	0.022	8	33	65	43
mh	50	4p GDEER	32	48	48	1	0.43	0.039	11	25	72	273
mh	50	4p GDEER	48 (shaped)	48	48	1	0.55	0.042	13	25	100	277
mh	50	3p GDEERS	32	48	48	36	0.37	0.004	92	25	77	275
mh	50	4p GDEER	32	48	48	36	0.37	0.007	53	25	77	275
mh	50	3p RDEERS	14	20	20	36				22	90	555
mh	50	4p RDEER	14	20	20	36				24	100	350
mh	100	3p RDEERS	14	20	20	36	0.38	0.022	17	21.5	88	622
mh	100	4p RDEER	14	20	20	36	0.38	0.045	8	21.5	88	622

3-pulse DEER

Raw Data

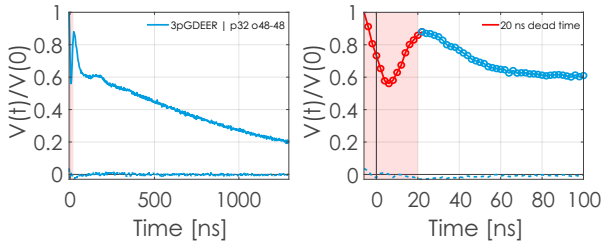

Figure S3: 3-pulse DEER raw data (related to Fig. 3(a)). (left) Raw data of the 3-pulse DEER time trace. (right) Magnification of the first part of the time trace. Data points are highlighted with circles (increment = 2 ns). The data in red area were excluded from data evaluation.

DEER-Stitch

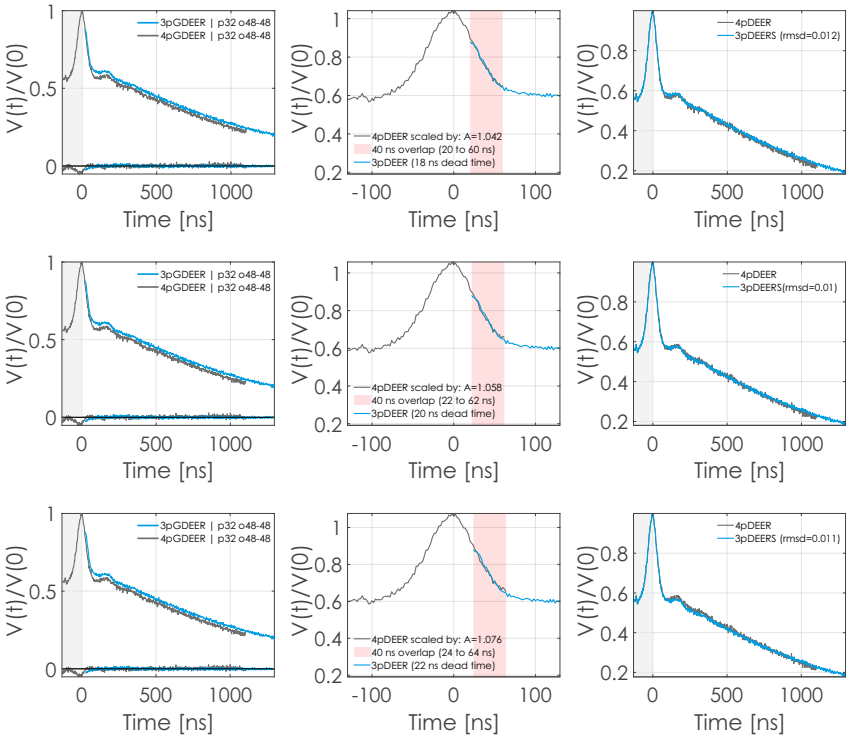

Figure S4: DEER-Stitch[6] using different stitching parameters (related to Fig. 3).

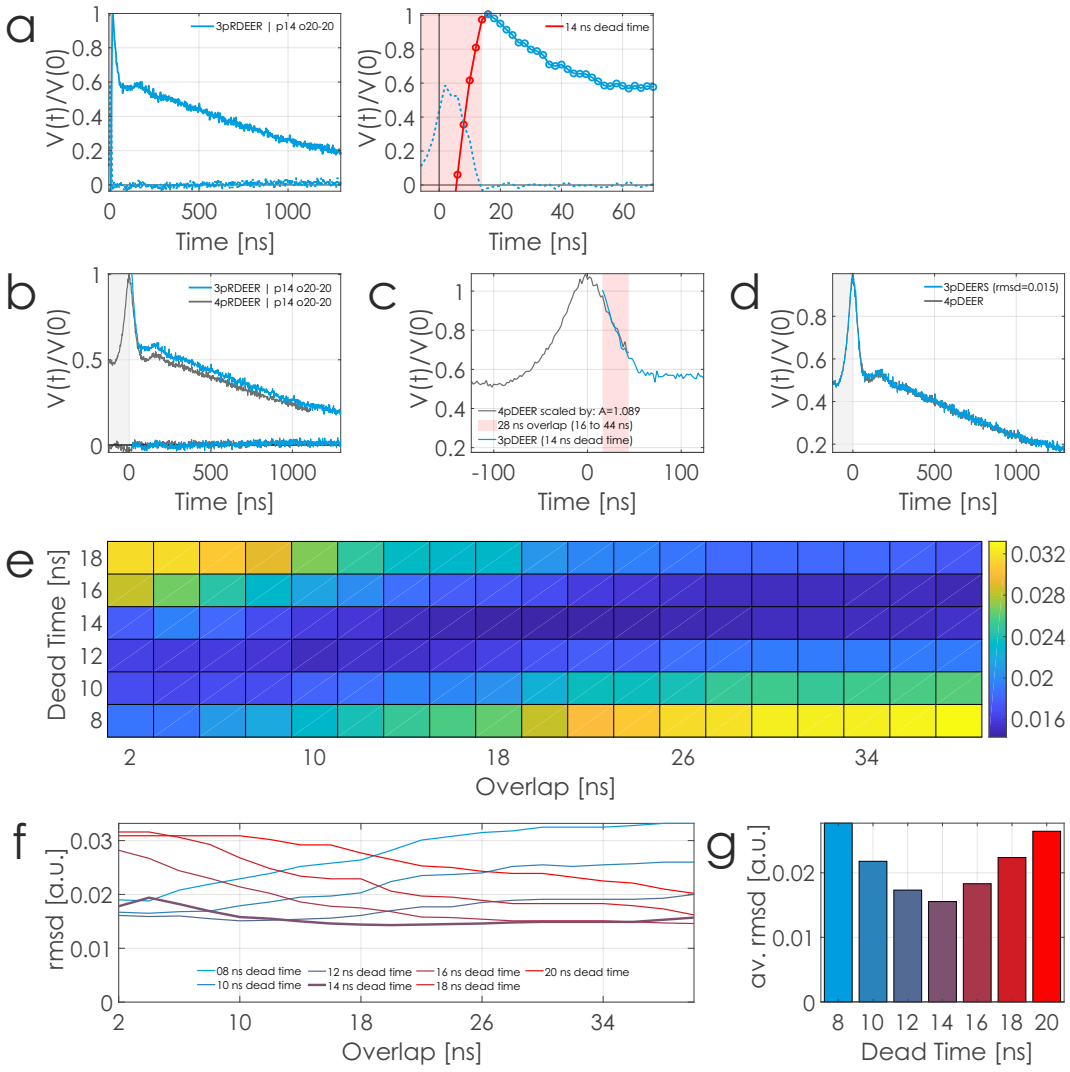

Figure S5: DEER-Stitch[6] of 3-pulse DEER data recorded using rectangular pulses (related to Fig. 3). (a) Raw data of the 3-pulse DEER time trace (left). Magnification of the first part of the time trace (right). Data points are highlighted with circles (increment = 2 ns). The data in red area were excluded from data evaluation. (b) 3-pulse and 4-pulse DEER experiments performed with the microhelix using 555 and 350 mW of power. (c) Stitching of the 4-pulse to the 3-pulse data using a 28 ns overlap and assuming 14 ns dead time and (d) comparison of the stitched 3-pulse and the 4-pulse time trace via rmsd. (e) 3D plot showing the rmsd between the stitched 3-pulse and the 4-pulse time trace in dependence of the chosen dead time and overlap between the time traces. (f) rmsd as a function of the overlap for a fixed dead time and (g) average rmsd in the given overlap range (2 to 40 ns) for different dead times. The rmsd analysis shows that the dead time is (14 ± 2) ns when using a 14 ns rectangular pump pulse alongside 20 ns rectangular observer pulses. An overlap of 28 ns between the time traces is sufficient to have a stable stitching result.

Temperature Dependence of the Phase Memory Time

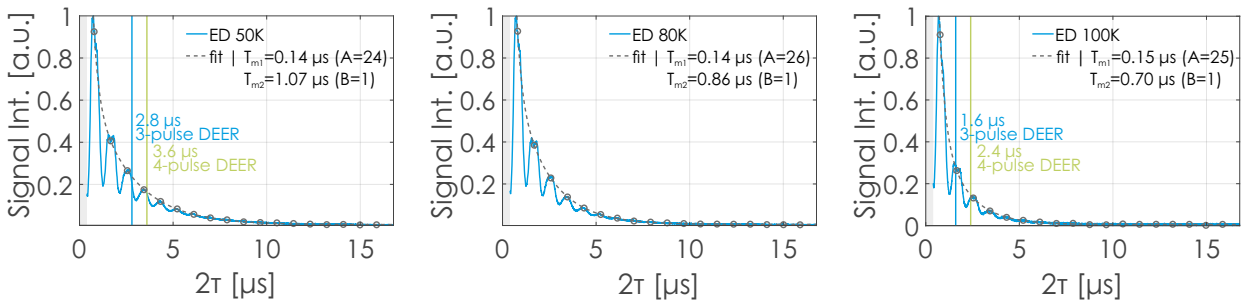

Figure S6: Temperature dependence of the phase memory time T_{m} of the NO-NO ruler sample. Shown are 2-pulse echo decay (ED) transients acquired at different temperatures. The transients were shifted by 400 ns (gray shaded areas) to account for the blind spot created by the initial sequence length of 2τ. The maxima of the deuterium ESEEM-modulated transients are indicated by gray circles (period: $\tau_{2\mathrm{H}} = 444$ ns corresponding to 2.25 MHz) and were fitted using a biexponential fit function. Given are the two fitted decay constants $T_{\mathrm{m1,2}}$ (already divided by "2" to account for the 2τ-axis) with the corresponding amplitudes (A, B) of the exponential for each temperature. The fitted fast decay constant T_{m1} can be attributed to various diffusion processes within the sample, while T_{m2} is the actual phase memory time of the sample. The vertical lines are indicating the sequence lengths of a 3-pulse (blue) and a 4-pulse DEER experiment (green) with a 1.4 or 0.8 µs dipolar evolution time, respectively. The corresponding DEER data are shown in Fig. 4 and 5 in the main text.

T4 lysozyme 68R1/140R1

Continous wave EPR

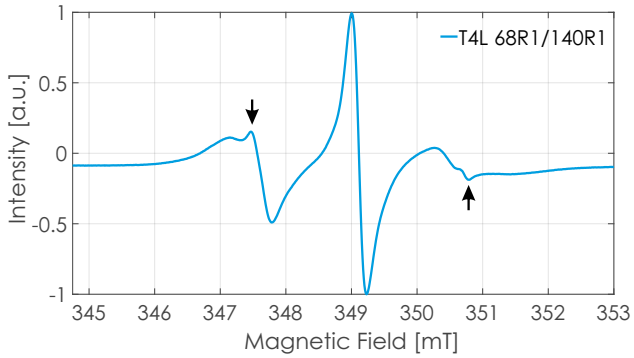

Figure S7: Continuous wave EPR spectrum of the T4 lysozyme 68R1/140R1 sample. The labeling efficiency is 106% with the surplus attributable to a small fraction of free label present in the sample (indicated by black arrows).

Experimental Parameters

Table S4: Overview of the SNR and microwave power parameters used to perform all DEER experiments on the T4 lysozyme sample presented in the main text. The designation of the variables follows the conventions typically used in Bruker Xepr PulseSPEL scripts for DEER experiments. All DEER experiments were performed at T = **50 K** using the following parameters: pg = observer π-pulse length (observer echo integration window) and transmitter (TM) level: 50%. AWG [%] corresponds to the utilized AWG pulse amplitude as defined in Xepr. More experimental parameters, in particular the averaging times, are given in Tab. S5

resonator	T [K]	DEER exp.	P (π) [ns]	O (π/2) [ns]	O (π) [ns]	n	signal	noise	SNR	att. [dB]	AWG [%]	power [mW]
mh	50	3p RDEERS	14	20	20	32	0.51	0.004	128	23.0	100	440
mh	50	4p RDEER	14	20	20	32	0.52	0.008	64	23.0	100	440
mh	50	3p RDEERS	14	20	20	16	0.48	0.012	40	23.5	100	392
mh	50	4p RDEER	14	20	20	8	0.50	0.013	39	23.5	100	392
mh	50	3p RDEERS	14	20	20	16	0.45	0.008	56	23.5	100	392
mh	50	4p RDEER	14	20	20	8	0.48	0.020	24	23.5	100	392
mh	50	3p RDEERS	14	20	20	64	0.45	0.020	23	24.0	100	350
mh	50	4p RDEER	14	20	20	32				24.0	100	350

Table S5: Overview of the concentrations, as well as averaging parameters used to perform all DEER experiments on the T4 lysozyme sample presented in the main text. The designation of the variables follows the conventions typically used in Bruker Xepr PulseSPEL scripts for DEER experiments. The abbreviations given in the table stand for: protein concentration (c_{prot}), deuterated glycerol (dGly), refocusing time for the primary observer echo ($d1 = \tau_1$), dipolar evolution time ($d2 = \tau_{dip}$), number of points (NOP), number of scans (n), shots per point (h), nuclear modulation averaging parameter (m, in steps), phase cycling (PC, in steps), shot repetition time (srt), and total averaging time (τ_{av}) where $\tau_{av} = [NOP \times n \times h \times m \times PC \times srt]$. It has to be noted that the averaging time for 3-pulse and 4-puulse DEER experiments was kept constant in order to make these experiments comparable. Also the third and fourth experiment have the same number of averages per point, the difference in total averaging time is a result of the different NOP used in these experiments. All experiments were performed at 50 K using $d30 = 2$ ns (pump pulse time increment). **4-pulse DEER** experiments were performed using the following parameters: $h = 12$ (shots per point), $m = 8$ (nuclear modulation averaging) with a stepping of $d31 = 16$ ns and $d3 = 280$ ns (dead time delay), apart fromt the 500 ns time trace where $d3 = 80$ ns was used. **3-pulse DEER** experiments were performed using the following parameters: $d3 = 0$ ns and $m = 1$. More experimental parameters, in particular the used microwave powers, are given in Tab. S4

DEER exp.	c_{prot} [µM]	dGly [%]	d1 [ns]	d2 [ns]	NOP	n	h	m	PC	srt [µs]	τ_{av} [h]
3pRDEERS	500	50		2000	920	32	96	1	16	1000	12.56
4pRDEER	500	50	400	2000	920	32	12	8	16	1000	12.56
3pRDEERS	250	33		1200	610	16	768	1	1	1000	2.08
4pRDEER	250	33	200	1200	620	8	12	8	16	1000	2.12
3pRDEERS	250	33		1400	620	16	768	1	1	1000	2.12
4pRDEER	250	33	200	1400	620	8	12	8	16	1000	2.12
3pRDEERS	100	50		1400	620	64	768	1	1	1000	8.47
4pRDEER	100	50	200	500	270	32	12	8	16	1000	3.69

DEER

500 µM protein concentration

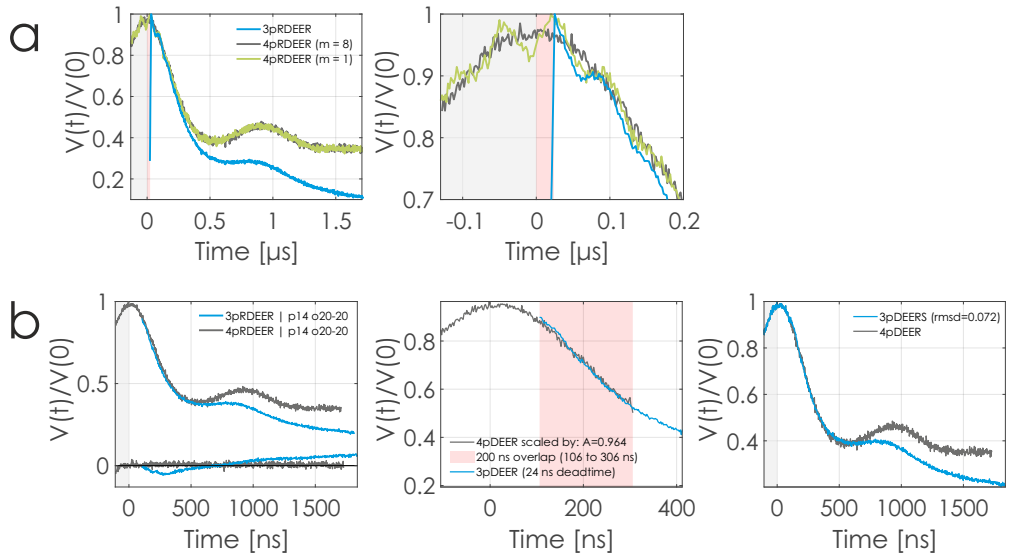

Figure S8: Comparison of 3-pulse and 4-pulse DEER experiments using rectangular pulses obtained on the T4 lysozyme sample at 500 µM protein concentration with 50% v/v deuterated glycerol (related to Fig. 6). (a) Shown are three time traces, a 4-pulse DEER time trace recorded using nuclear modulation averaging of 8-steps (m = 8) with a 16 ns increment (d31) plotted in gray, a 4-pulse DEER time trace recorded without nuclear modulation averaging (m = 1) in green and the corresponding 3-pulse DEER time trace recorded using the same parameters without nuclear modulation averaging in blue. The green time trace clearly shows strong ^{1}H-ESSEM effects that modulate the DEER signal around the zero time. This is unfortunate and a sample-dependent effect. Nuclear modulation averaging allows to fully suppress these signals by stepwise varying the length of the time trace (gray). Also the 3-pulse data (blue) are suffering from these ESEEM signals but it was not possible to experimentally remove them via nuclear modulation averaging or phase cycling. (b) Shows the stitching of the DEER data, as described before. Due to the strong ESEEM effects 104 ns of the otherwise 24 ns deadtime 3-pulse DEER time trace had to be removed. Therefore and also due to the much lower dipolar frequency compared to the NO-NO ruler sample, a larger area (200 ns) was needed to obtain a good stitching result. This still requires just a 300 ns 4-pulse DEER time trace to stitch the 2000 ns 3-pulse DEER time trace. An evaluation of the stitched 3-pulse and the 4-pulse data is presented in Fig. 6. All experimental parameters are given in Tab. S4 and S5.

250 μM protein concentration

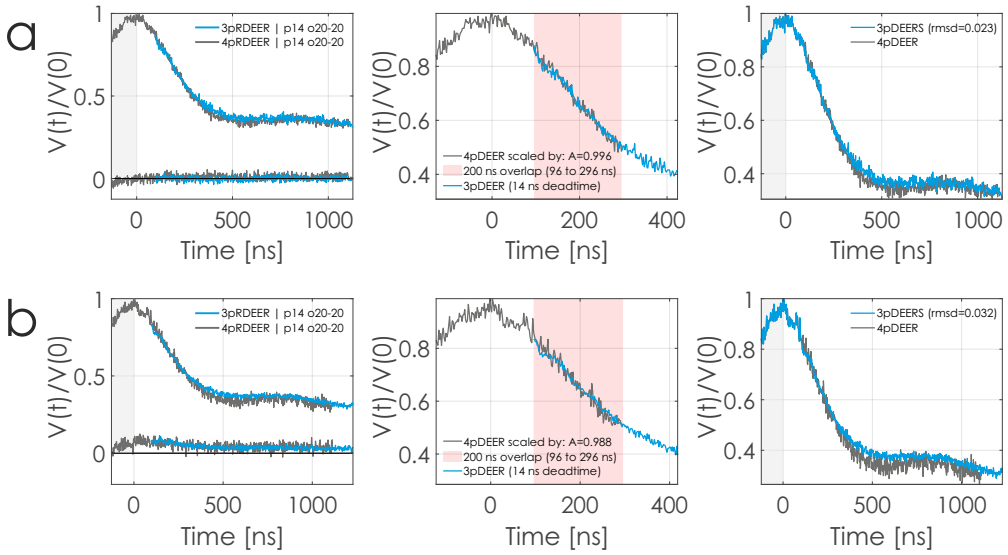

Figure S9: Stitching of 3-pulse and 4-pulse DEER data obtained using rectangular pulses on the T4 lysozyme sample at 250 μM protein concentration with 33% v/v deuterated glycerol (related to Fig. 7). (a) Stitching of the 1.2 μs and (b) 1.4 μs time traces. All time traces have deadtimes of 14 ns. 104 ns were removed in order to exclude the ^{1}H-ESSEM from the 3-pulse data. 200 ns of overlap between the time traces was used, requiring a 300 ns 4-pulse DEER time trace in total to stitch the 3-pulse data. All experimental parameters are given in Tab. S4 and S5.

100 µM protein concentration

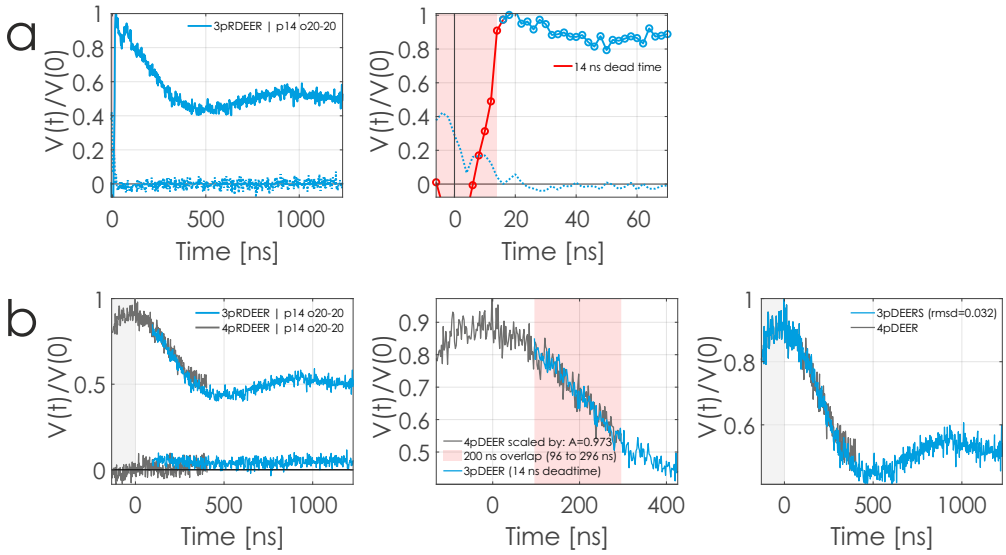

Figure S10: 3-pulse and 4-pulse DEER data obtained using rectangular pulses on the T4 lysozyme sample at 100 µM protein concentration with 50% v/v deuterated glycerol (related to Fig. 8). (a) Raw data of the 3-pulse DEER time trace (left). Magnification of the first part of the time trace (right). Data points are highlighted with circles (increment = 2 ns). The data in red area were excluded from data evaluation. (b) Stitching of the 3-pulse DEER data with the parameters found in Fig. S14 (b) for the same time trace length (14 ns dead time, 200 ns overlap).

References

(1) Soetbeer, J.; Hülsmann, M.; Godt, A.; Polyhach, Y.; Jeschke, G. Dynamical decoupling of nitroxides in o-terphenyl: a study of temperature, deuteration and concentration effects. *Phys. Chem. Chem. Phys.* **2018**, *20*, 1615–1628.

(2) Soetbeer, J.; Millen, M.; Zouboulis, K.; Hülsmann, M.; Godt, A.; Polyhach, Y.; Jeschke, G. Dynamical decoupling in water–glycerol glasses: a comparison of nitroxides, trityl radicals and gadolinium complexes. *Phys. Chem. Chem. Phys.* **2021**, *23*, 5352–5369.

(3) Bahrenberg, T.; Jahn, S. M.; Feintuch, A.; Stoll, S.; Goldfarb, D. The decay of the refocused Hahn echo in double electron–electron resonance (DEER) experiments. *Magn. Reson.* **2021**, *2*, 161–173.

(4) Stoll, S.; Schweiger, A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.* **2006**, *178*, 42–55.

(5) Teucher, M.; Bordignon, E. Improved signal fidelity in 4-pulse DEER with Gaussian pulses. *J. Magn. Reson.* **2018**, *296*, 103–111.

(6) Lovett, J.; Lovett, B. W.; Harmer, J. DEER-Stitch: Combining three-and four-pulse DEER measurements for high sensitivity, deadtime free data. *J. Magn. Reson.* **2012**, *223*, 98–106.