

SUPPORTING INFORMATION.

Improved Sensitivity for Long-Distance Measurements in Biomolecules: Five-Pulse Double Electron-Electron Resonance.

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S1. EXPERIMENTAL DETAILS

a. Pulse Dipolar Spectroscopy

The Ku-band (17.3 GHz) DEER/DQC spectrometer used in this work for DEER measurements is a modified version of our X/Ku band spectrometer ¹. It retains its original heterodyne quadrature mw pulse-forming channel and receiver, but additionally it has a DEER channel, which is a complete Ku-band homodyne transceiver with two mw pulse-forming channels capable of quadrature phase cycling with 2° accuracy and of generating arbitrary amplitude/phase modulated pulses with 300 MHz video bandwidth using a 1.2/2.4 Gsps arbitrary waveform generators (AWG), DBS2050A from Anlogic, Inc. The spectrometer is outfitted with a ~4 kW Ku band TWTA (176Ku, Applied systems Engineering, Inc.) and is capable of producing intense mw pulses as short as 1 ns. For DQC, π -pulses as short as 4 ns ($B_1 \sim 45$ G) can be generated in a dielectric resonator in samples with up to ~15 μ L active volume. The quadrature output of the receivers (or arbitrary waveform sampled at several key points including resonator) is multiplexed for recording into a 1 Gsps dual-channel signal averager, AP240 (Acqiris, Inc.). Sample temperature from 4 to 300 K is provided by CF935 liquid helium flow cryostat (Oxford Instrument, Inc.).

The spectrometer is capable of recording DEER and DQC signals in low concentration samples as can be judged based e.g. on our published data²⁻⁵. Below we show examples of DEER data obtained on model systems for testing its current limits (Figure S1). The standard level of sensitivity is sufficient to facilitate distance measurements in e.g. membrane proteins, wherein relaxation times are often short and spin concentrations are low. It also helps to make it practical to use pulse sequences that utilize a long time-scale, such as DEER-5, since the spin-echo is attenuated by phase relaxation by one to three orders of magnitude by the time of detection, yet the spin concentration has to be limited.

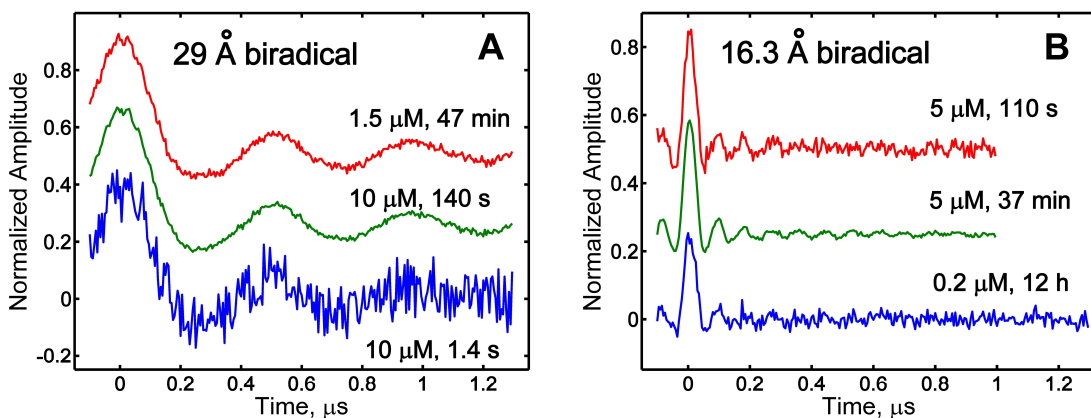


Figure S1. Raw Ku band (17.3 GHz) DEER signals recorded at 57 K on rigid biradicals in *o*-terphenyl-*d*₂₄. A pump pulse of 16 ns was used in all cases. Sample concentrations and the duration of the experiments are indicated on the figures. Normalized as in ref 4, dipolar signals in **A** and **B** are distributed vertically for clarity of presentation. The biradicals are the same as used in our past work⁶.

The DEER pulse setup uses the standard protocol with the detection pulse sequence applied at the lower field edge of the nitroxide EPR spectrum. The detection $\pi/2$ - π - π pulse sequence used 16 ns $\pi/2$ and 32 ns π -pulses. In 4-pulse DEER a 32-step phase cycle is used. Nuclear ESSEM was suppressed by four-point averaging, advancing interpulse periods in steps of a quarter period of nuclear modulation frequency. The pump pulses, separated by 70 MHz from the frequency of the detection pulse sequence, were applied in the center region of the spectrum. The variable position pump π -pulse has a width in the range of 28-30 ns, the additional pump pulse in DEER-5 was 12 ns or, alternatively, it was a shaped pulse; only Hermite⁷ and hyperbolic secant⁸ type pulses were tested.

b. Sample Preparation Details

The T4L double cysteine mutants, 8C/44C, 8C/128C, 65C/128C and 65C/135C, (with respective distances of 3.3, 3.8, 4.8, and 4.3nm) were produced following the procedure detailed by Georgieva et al.⁴ All final samples contained 30% or 40% (w/v) glycerol or glycerol- d_8 . The solutions were loaded into 1.8 mm I.D. capillary tubes and shock-frozen in liquid nitrogen prior to measurements. The protein concentration was estimated from UV absorbance at 280 nm⁴, and the spin concentration was estimated by comparison with the spin-echo amplitude of a standard reference sample containing 200 μ M TEMPOL in 50% (w/v) glycerol/H₂O³. Relaxation decay and amplitude loss caused by instantaneous diffusion⁹ were included as a correction factor (typically ~ 1.2 - 1.3). Very good agreement was found between the independently determined protein and spin concentrations. The modulation depth in 4-pulse DEER indicated close to 100% spin labeling efficiency, thereby allowing the referencing of protein concentration to spin concentration.

S2. ANALYSIS OF DIPOLAR PULSE SEQUENCES

a) 5-Pulse DEER Signal and Dipolar Pathways

Here, we derive the expression for 4,5-pulse DEER, using the spin Hamiltonian, H , which for two spins a and b with Larmor frequencies Ω_a , and Ω_b we express in the frame of reference doubly-rotating with frequencies ω_1 and ω_2 of mw pulses, (cf. Slichter p.279 and assumptions¹⁰ therein) and in the limit of weak electron spin dipolar coupling, as appropriate for DEER. H in the absence of mw pulses takes the form:

$$H = \omega_a S_{za} + \omega_b S_{zb} + AS_{za}S_{zb}. \quad (\text{S1})$$

In eq S1 $A = \omega_d / (1 - 3\cos^2\theta)$, $\omega_d = \mu_0 \gamma_e^2 \hbar / 4\pi r^3$ is the electron spin dipolar coupling frequency as given in the main text; ω_a and ω_b are the respective Larmor frequencies offsets of a from the observer frequency, ω_1 and of b from the pump frequency, ω_2 . Spin a has its Larmor frequencies, Ω_a in the vicinity of ω_1 and does not interact with pump at ω_2 . Spin b , belonging to the rest of the spins, may or may not interact with the pump frequency ω_2 and is unaffected by ω_1 . We assume the following set of inequalities $\omega_d \ll \gamma_e B_{1a(b)} \ll |\omega_2 - \omega_1|$. The first inequality allows us to

neglect the dipolar coupling during the pulse, the second ensures that there is only a small overlap of pulse excitations at the two frequencies.

The pulse sequences for 5-pulse DEER depicted in Figure 1 (main text) can be expressed as:

$$P_{1a}(\pi/2) \xrightarrow{t_1} P_{2a}(\pi) \xrightarrow{t_2-t} P_{3b}(\pi) \xrightarrow{t} P_{4a}(\pi) \xrightarrow{\delta T} P_{5b}(\pi) \xrightarrow{t_2-t_1-\delta T} echo. \quad (S2)$$

Arrows denote free evolution for the duration of the time shown due to H , and $P_{k\mu}$ is the pulse propagator for k^{th} pulse applied at the frequency $\omega_{1,2}$, acting respectively upon spins at ω_{μ} ($\mu=a,b$). The refocused primary echo (RPE) formed by a spin a is produced by pulses 1, 2 and 4 at ω_1 via a $\mathbf{p} = (-1, +1, -1)$ coherence pathway. The first $\pi/2$ pulse produces $S_{a\pm}$ with standard amplitude factors (not shown) defined in the literature⁶, which then evolves according to eq. S2. The action of the rest (all π) pulses, $P_{k\mu}$ is well defined by the probability, $p_{k\mu}$ to flip a spin at ω_a or ω_b , respectively. The probability not to be flipped, $q_{k\mu}$, is $1-p_{k\mu}$. Free evolution of spin a is fully determined by the free evolution propagator $\exp[-i(H_z + \Omega_d)]$, with operators $H_z \equiv \omega_a S_{az}$ and $\Omega_d \equiv AS_{az}S_{bz}$. H_z and Ω_d commute and we can consider them separately. We can write for the free evolution of shift operators $S_{a\pm}$ due to H_z or Ω_d the following:

$$\begin{aligned} S_{a\pm} &\xrightarrow{H_{za}t} S_{a\pm} e^{\mp i\omega_a t} \\ S_{a\pm} &\xrightarrow{\Omega_d t} S_{a\pm} (\cos(At/2) \mp i2S_{bz} \sin(At/2)) \equiv S_{a\pm} D_{\pm t}, \end{aligned} \quad (S3)$$

where D_t has the following properties:

$$D_t \xrightarrow{P_{k\mu}} q_{k\mu} D_t + p_{k\mu} D_t^*, \quad D_t^* = D_{-t}, \quad D_{t_1+t_2} = D_{t_1} D_{t_2}. \quad (S4)$$

The amplitude $V(t)$ of the echo signal is given by the trace, $\text{Tr}(S_{a+}\rho(t))/\text{Tr}(S_{a+}S_{a-})$, where $\rho(t)$ is the density matrix measured at time t after the first pulse in the sequence. Therefore in the end we retain in ρ , only the terms in S_{a-} . We will follow the evolution of single-quantum in-phase coherence of spin 1, S_{1a-} created by the first $\pi/2$ pulse.

By repeatedly applying eqs S3-S4 to S_{a-} , the following sequence of transformations (omitting amplitude factors for spin a) produces the detectable density matrix element in DEER-5:

$$\begin{aligned}
& S_{a-} \xrightarrow{t_1} S_{a-} D_{t_1}^* e^{i\omega_a t_1} \xrightarrow{P_{2a}} S_{a+} D_{t_1}^* e^{i\omega_a t_1} \xrightarrow{t_2-t} S_{a+} D_{-t_1+t_2-t} e^{i\omega_a(t_1-t_2+t)} \xrightarrow{P_{3b}} \\
& S_{a+} (q_3 D_{-t_1+t_2-t} + p_3 D_{t_1-t_2+t}) e^{i\omega_a(t_1-t_2+t)} \xrightarrow{t} S_{a+} (q_3 D_{-t_1+t_2} + p_3 D_{t_1-t_2+2t}) e^{i\omega_a(t_1-t_2)} \\
& \xrightarrow{P_{4a}} \xrightarrow{\delta T} S_{a-} (q_3 D_{-t_1+t_2-\delta T} + p_3 D_{t_1-t_2+2t-\delta T}) e^{i\omega_a(t_1-t_2+\delta T)} \xrightarrow{P_{5b}} \\
& S_{a-} (q_3 (q_5 D_{-t_1+t_2-\delta T} + p_5 D_{t_1-t_2+\delta T}) + p_3 (q_5 D_{t_1-t_2+2t-\delta T} + p_5 D_{-t_1+t_2-2t+\delta T}) e^{i\omega_a(t_1-t_2+\delta T)} \xrightarrow{t_2-t_1-\delta T+t_e} \\
& S_{a-} (q_3 q_5 D_{-t_1+t_2-\delta T} + q_3 p_5 D_{t_1-t_2+\delta T} + p_3 q_5 D_{t_1-t_2+2t-\delta T} + p_3 p_5 D_{-t_1+t_2-2t+\delta T}) D_{-t_2+t_1+\delta T-t_e} e^{-i\omega_a t_e} = \\
& S_{a-} e^{-i\omega_a t_e} D_{t_e}^* (q_3 q_5 + q_3 p_5 D_{2t_1-2t_2+2\delta T} + p_3 q_5 D_{2t_1-2t_2+2t} + p_3 p_5 D_{-2t+2\delta T}).
\end{aligned} \tag{S5}$$

Taking the trace at spin-echo time, $t_e=0$, we find for the echo amplitude:

$$V(t) \propto \langle q_3 q_5 + q_3 p_5 \cos(A(t_2 - t_1 - \delta T)) + p_3 q_5 \cos(A(t_2 - t_1 - t)) + p_3 p_5 \cos(A(t - \delta T)) \rangle_{\text{angles}}. \tag{S6}$$

The angular brackets denote averaging of all (hidden) amplitude terms, p_k , q_k , and A over all molecular orientations and magnetic tensors. The amplitude factors not included into eq S6 after integrating with $\exp(-i\omega_a t_e)$ produce the echo shape. The expression in angular brackets excluding the first term represents the dipolar modulation. Note that each cosine term, $\cos(A t_d)$ after averaging has a maximum when its respective time variable, t_d passes through zero. This point corresponds to “refocusing” of dipolar coupling.

In eq S6 two terms are dependent on the variable t . In standard 4-pulse DEER ($p_5=0$, $q_5=1$, $t \rightarrow t_2 - t$) it is the third term which is dominant, but the fourth term which appears reversed in time could also be present if there is an overlap of excitations at ω_1 and ω_2 , (i.e. pulse 2 or 4 plays the role of 5 with respective $p_5 \ll p_3$). In 5-pulse DEER, $p_3 p_5 \sim 1$, $p_3 q_5 \ll 1$, therefore the fourth term becomes dominant and the third term can be minimized by adjusting pulses. The case of special interest, which is the focus of this work, occurs when $t_2 = 2t_1 \equiv \tau$, leading to:

$$V(t) \propto \langle q_3 q_5 + q_3 p_5 \cos(A(\tau - \delta T)) + p_3 q_5 \cos(A(\tau - t)) + p_3 p_5 \cos(A(t - \delta T)) \rangle_{\text{angles}}. \tag{S7}$$

In eq S7 t has the range $[0, 2\tau]$, and the 4th term uses this range, if $\delta T \ll \tau$, (Figure S2). But the 3rd term is symmetric vs. $t = \tau$, where dipolar coupling is refocused; so the cosine argument uses only half the range $[-\tau, \tau]$. Note that after ensemble averaging, because p_5 is a wider excitation than p_3 , the following holds: $\langle q_3 q_5 \rangle \sim q_5$, $\langle p_3 p_5 \rangle \sim p_3$, $\langle p_3 q_5 \rangle \ll p_3$, $\langle q_3 p_5 \rangle \sim p_5 - p_3$. Note that if the π

pulse 5 inverts spins over a wide spectral extent, the background in V_5 may become very small, i.e. this signal becomes deeply modulated.

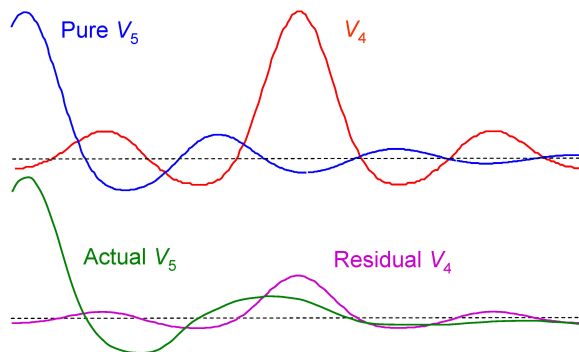


Figure S2. An illustration of signals given by eq S7. In the absence of any intermolecular effects, the pure 4-pulse DEER signal, V_4 recorded in the absence of pulse 5 (red) and the ideal 5-pulse DEER signal, V_5 for completely suppressed V_4 (blue) are shown. These signals will reside on a constant background and at low spin concentrations have nearly equal modulation depths, $\sim p_3$ if compared by shifting the signals, rather than by scaling. The two bottom curves show the attenuated residual contribution of V_4 (magenta) and V_5 containing this contribution (green).

The terms in D in eq S5 can be referred to as “dipolar pathways”. Each spectral selective π -pulse reverses time evolution for some spin pairs and leaves the rest unaffected, thereby doubling the number of pathways. It is not essential at what frequency the pulse is applied, since dipolar coupling is symmetric to spins. In DEER, as opposed to single-frequency techniques,^{6, 11} the pulses at the observer frequency have their positions fixed and only one coherence pathway is selected e.g. by phase cycling. Therefore dipolar pathways with time-dependence evolve only due to the variable positions of the pump pulses. However pulses at the observer frequency do contribute to this picture, since there usually is some small overlap of excitations at the observer and pump frequencies, leading to a number of weak spurious dipolar signals, commonly observed in DEER.

b) 5-Pulse DEER Sequence and its Extension to Multiple Pulses

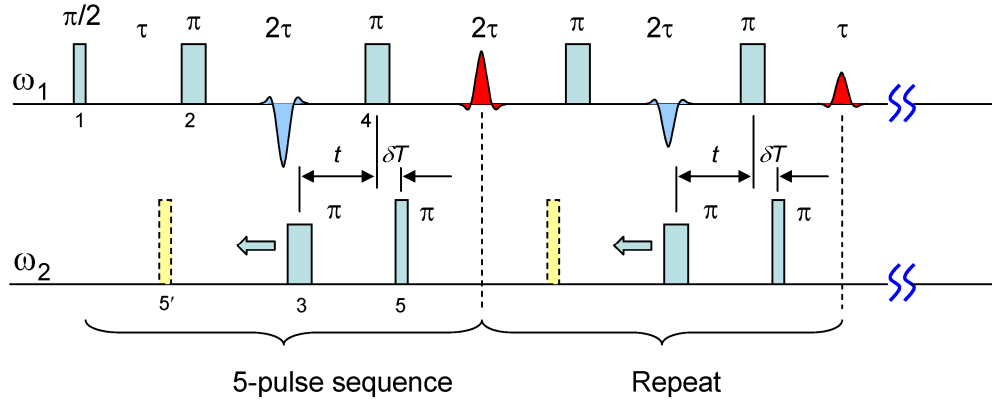


Figure S3. Five-pulse DEER (DEER-5) is shown with its possible extension to more pulses (denoted as “Repeat”). The upper pulse train is applied at the observer frequency, ω_1 and it has fixed pulse placement of the repeat pulse sequence¹². This sequence minimizes phase relaxation caused by nuclear spin diffusion due to protons. The lower pulse train is applied at the pump frequency, ω_2 to sample dipolar coupling by varying position of the pulse 3 scanning the range between observe pulses 2 and 4. The more intense fixed pulse 5 shifts the refocusing point of the dipolar signal to pulse 4, thereby utilizing the whole time interval from 2 to 4, as compared to only half in its absence. Due to the symmetry of the 5-pulse sequence, two essentially equivalent positions (5 or 5') of the additional π -pulse are possible. That is position 5 following pulse 4 or 5' before the pulse 2 may be used, as shown using dashed yellow boxes. Shifting pulse 5 (or 5') away from pulse 4 (or 2) as shown by a small amount, $\delta T \sim 50$ –150 ns, results in zero dead-time. The time interval δT is much smaller than τ and does not reduce the performance. The pulse sequence utilizes practically all the time available for dipolar modulation to progress. Both pulse positions were tested, but most results in this work were obtained using position 5' for technical reasons.

The 5-pulse sequence in Figure S3 refocuses the spin-echo twice, leading to a longer time period over which the echo can be detected. It can be extended by adding more pulses in blocks of 4, as shown, to the total of N blocks with $n=2N$ echo refocusing. However, this process is likely to be limited to an N of 2 or 3, since the number of dipolar pathways will grow rapidly and they cannot be separated by phase cycling (or by adjusting pulses). In addition, relaxation mechanisms unrelated to nuclear spin diffusion can become significant on a sufficiently long time-scale.

The principal signal is modulated as $\cos[NA(t-\delta T)] \equiv \cos[A(t'-N\delta T)]$, with $t' \equiv Nt$ being the respective time variable for dipolar evolution with this sequence. The time variable, t sweeps only a t_m/N extent of the full evolution period, nevertheless the sequence is nearly 100%

efficient, since t' spans t_m and $t_m \gg N\delta T$. Depending on the value of κ , 2 to 3 (cf. main text), t_m can be increased by factors of 1.4-1.6, 2-2.5, and 2.45-3.3 for $N=1,2$, and 3 respectively, unless other simple-exponential relaxation mechanisms become more significant. The extension to $N>1$ is well suited for spin pairs where one of two labels has a narrow ESR spectrum, which can be nearly completely excited, (e.g. trityl¹³). In the case of nitroxide pairs or other broad spectra, there will be contributions from unwanted pathways that can be minimized by referencing, as we demonstrate for the 5-pulse sequence. However, one would expect that these dipolar pathways (subharmonics of dipolar frequency) would merge into a non-oscillating background-like signal.

S3. INTERMOLECULAR EFFECTS

The signal in eqs S6-S7 is for an isolated pair of spins. A spin that contributes to the observed echo is surrounded by spins on other molecules, which produce intermolecular dipole-dipole coupling effects. We refer to the observed spins at ω_1 contributing to the echo as A-spins and to the rest as B-spins. If a B-spin is affected by the pump pulse(s), it contributes dipolar evolution according to the dipolar pathway specific for this particular pair. Different pairs may correspond to different types of dipolar signals. Therefore, for M pulses, we can separate the system into 2^M sub-ensembles with their partial concentrations $C_k = w_k C$, where w_k is the “weight” of the dipolar pathway in the signal expression such as exemplified by eqs S6-S7. One pathway corresponds to the spins that are not affected by pump pulses and thus is a constant background, which is the unaffected contribution to the echo. The rest 2^{M-1} are time-dependent dipolar signals. If there is just one type of dipolar signal ($M=1$) as in the simplest case of (the ideal) three pulse DEER (PELDOR), then in the case of a homogeneous spin distribution the signal is given by $\exp(-k_0 C_{eff} t)$, where $C_{eff} = pC$ ¹⁴. Here C is the (local) spin concentration in the sample and p is the probability for the spin to be flipped by the pump pulse, which in this case is equal to the weight of the sole dipolar pathway, if there is no overlap of pulse excitations between A and B spins. In spatial averaging over all spins by the Markoff method¹⁵ one takes the average $\langle 1 - \cos(At) \rangle_r$ over the distribution of B spins in the sample¹⁶. (Note, the notion “B spins” is not the equivalent to “all spins”). In the typical case of a homogeneous distribution, the averaging produces the term linear in $|t|$ in the exponent. The cases of more complex spin distributions were described in the literature¹⁷⁻¹⁸. In all cases the exponent is symmetrical with respect to the

“dipolar refocusing point”. In 4-pulse DEER, one finds $\exp(-k_0 p_3 C |t - t_1|)$. In the general case of more than one distinct dipolar pathway, one has $\exp(-k_0 \sum_k (C_k |t_k|))$, where t_k is the time-variable for the given pathway, k . In 5-pulse DEER ($M=2$), the first term $q_3 q_5$ with no time dependence in eq S7 corresponds to-unaffected B-spins and contributes only to the background signal $\propto q_3 q_5 C$, i.e. the unperturbed part of the echo amplitude. One thus has a product of three exponentials originating from the three terms with time dependence in eq S6, of which only the last two depend on t .

$$V_{inter}(t) = \exp[-k_0 C \langle (q_3 p_5 | \tau - \delta T | + p_3 q_5 | t - \tau | + p_3 p_5 | t - \delta T |) \rangle_{\omega_2}] \quad (S8)$$

In this case angular brackets represent an integration over the spectrum, since there is no orientational correlation with randomly distributed surrounding spins, as opposed to eqs S6-S7 where each pair has its own orientation and conformation. The first term is responsible for some loss of the signal, limiting the concentrations that can be used, the second is relatively small, and the last gives about the same decay rate ($\sim k_0 p_3 C$) for both 4- and 5- pulse DEER.

In summary, several useful properties of 5-pulse DEER (DEER-5) following from eqs S6-S8 should be noted.

The DEER-5 signal (V_5) does not depend on the ratio of t_1 and t_2 , however the DEER-4 contribution (V_4) can be shifted in time within the second interval by varying the t_1 and t_2 . This may help in developing efficient numerical procedures for their separation as well as in adjusting this sequence for specific needs. For example, if $t_1 \ll t_2$ the two signals are time-reversed from each other, but with one of the dipolar signals, (V_5) being dominant. By adjusting pulses 3 and 5, the ratio of the two can be controlled, and then used in setting up the measurements and data processing, aimed at the principal case of $t_2 \approx 2t_1$. It is also possible to make V_5 and V_4 signals nearly coincide by using $\delta T \cong t_1$ and pulse 5 at 5' position.

The case of $t_1 \ll t_2$ allows the omission of pulse 1, thus creating yet another version of dead-time free PELDOR based on the primary echo and the V_5 type dipolar signal.

Importantly, when the harder pulse 5 provides broadband inversion, the constant offset (the first term in eq S6) in the DEER-5 signal diminishes and may become very small, since $q_5 \approx 0$ in the inverted region. However, taking the advantage of this requires maintaining a low spin concentration ($\lesssim 50 \mu\text{M}$) to avoid the loss of the signal due to the first term in the exponent of eq S8.

S4. DATA PROCESSING

With the example of the T4L 8/44 mutant, which give dipolar signals with time separation of 4- and 5-pulse signals, we show that the residual V_4 can be eliminated nearly completely. This is achieved first by using a stronger pump pulse 5, thereby suppressing V_4 by the factor of k . Then, the reference signal obtained by turning off pulse 5 is scaled down by k and subtracted out. Overall, this suppresses the content of V_4 by the factor of $K \sim 2\Delta k/k^2$, where Δk is the error in determining k . We find $\Delta k/k$ is ~ 0.1 or even less. A readily achieved k is 3-6, so K is about 15 to 30, which is sufficient for most purposes. The residual is then $\lesssim 5\%$ of the modulation depth, i.e. within the range of uncertainties in standard DEER such as residuals of baseline subtraction, small orientation effects, minor dipolar signals due to overlap of spectral excitation at the two frequencies, and baseline distortions due to pulse interaction in the TWTA. At any rate, the opportunity to measure longer distances much more efficiently greatly outweighs the possibility of introducing small distortions to the signal. Acquiring reference signal takes only a small fraction of the time needed to record a DEER-5 signal, since $k \sim 4$ usually can be achieved (or even ~ 6 with some care) and V_4 is highly symmetric, allowing one to fold it over the mid point, effectively shortening its recording by a factor of 2. Computations show that optimizing pulses at both frequencies and using the uniform region of B_1 in the resonator by controlling sample length may add another factor of 2-3, so that the subtraction step could be simplified or become unnecessary. In other words with a reasonable level of care, a sufficient K of more than 10 could be achieved instrumentally.

We find that k is not highly sensitive to the amplitude of pulse 5, and it suffices to set it within a 2 dB range, which is not a challenge. This enables referencing with another, “easy” sample, such as used in this example, which may be selected to have not a highly oscillating dipolar signal corresponding to a distance $\sim 30\text{-}35$ Å. (For example, a not very rigid biradical in deuterated *o*-terphenyl). This would be desirable for referencing the case of a long distance when only one or two periods of dipolar frequency may develop, and the signal may not oscillate at all on the longest time-scale available to DEER-5. The subtraction, illustrated in Figure S4 is performed on normalized data sets in a manner that makes it insensitive to “not-so-high” concentrations. It is thus desirable for the local spin concentration not to exceed 100-200 μM . (Higher concentrations would require pulse sequence based on softer pulses). The signal decay

due to intermolecular dipolar interactions has in the exponent $-k_0 C p t$ with $k_0 = 10^{-3} \mu\text{M}^{-1} \mu\text{s}^{-1}$ ¹⁹, therefore for average spin concentrations less than 100 μM and for typical values of $2\tau \lesssim 10 \mu\text{s}$ and $p \cong 0.2$, the exponent is ≤ 0.1 for V_4 and ≤ 0.2 for V_5 . Thus the exponential can be taken as additive linear slope.

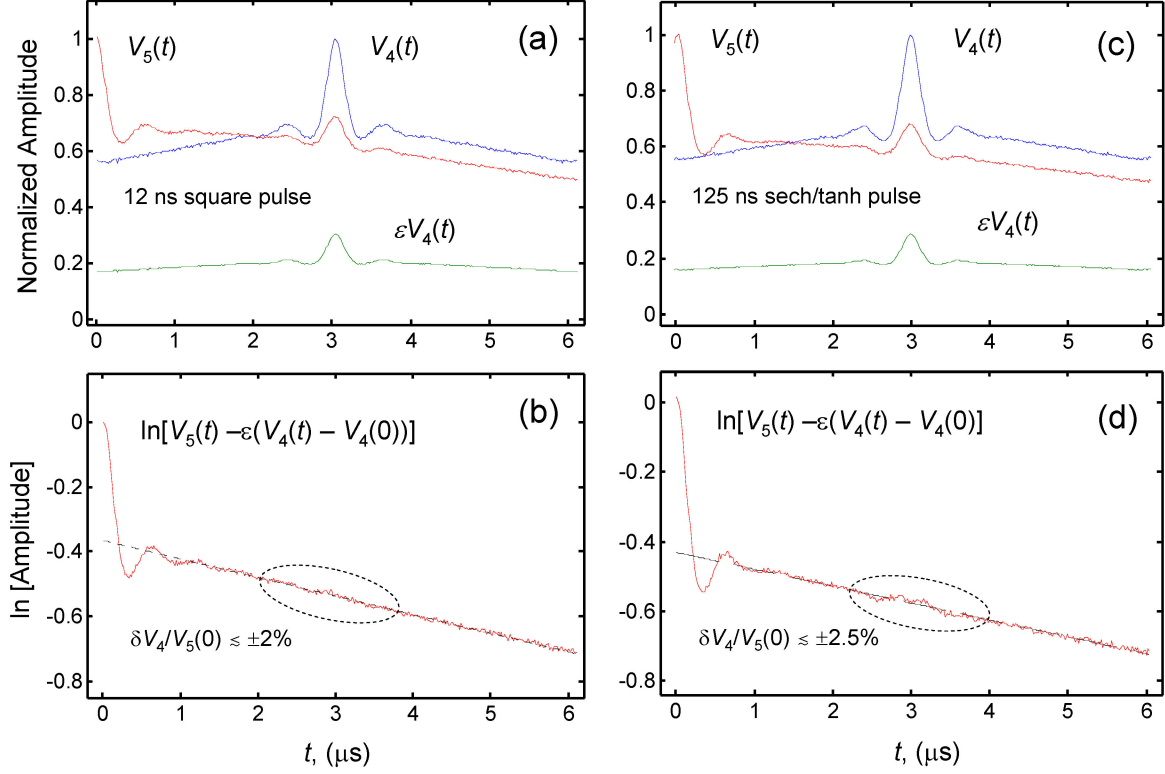


Figure S4. We show the removal of unwanted dipolar signal by using subtraction of the reference 4-pulse signal. The subtraction is performed on the data normalized to unit amplitude at their maxima, thereby relating the dipolar modulations of the two signals. (a) Normalized signal data for the case of a 12 ns pulse 5 are plotted for V_5 and the reference, V_4 . V_4 is then scaled down by the factor $\varepsilon = k^{-1}$ and subtracted out from V_5 . Panel (b) shows the result. The residual (ca. $\pm 2\%$ of modulation amplitude) is enclosed in the dashed oval. Such a sample with good separation of both signals can be used to reference another sample where signals merge into each other and cannot be distinguished visually. Panels (c, d) are similar to (a, b) but a hyperbolic secant pulse was used for pulse 5. A small improvement ($\sim 10\%$) relative to the square pulse (cf. panel c vs. panel a) could be noticed in the instrumental suppression of V_4 , but the subtraction is less perfect due to a less defined refocusing point for the dipolar signal in this case (cf. panel d vs. panel b). The data for V_4 and V_5 were recorded for 1 and 4 h, respectively at 17.3 GHz and 60 K on $\sim 80 \mu\text{M}$ T4L 8/44 double mutant prepared in H_2O buffer containing 40% (v/w) glycerol.

The subtraction can be performed iteratively in two steps. The first step, depicted in Figure S4, uses a linear approximation for the intermolecular signal. The value of k determined

in this way can be applied to the case of more substantial concentration effects where the non-linearity can be accommodated by performing one iteration that includes modification of V_5 by $\exp(\varepsilon a|t-\tau|)$, with a determined from the raw V_4 , and of V_4 by $\exp(bt+\varepsilon a|t-\tau|)$, with b determined from V_5 processed first in the linear approximation, thus essentially removing any error caused by intermolecular effects.

It would be highly desirable to suppress V_4 just by instrumental efforts, by making pulse 5 have more precise inversion everywhere where $q_3 \neq 0$. However, it is a challenge to automatically produce such pulses routinely at the required level of accuracy. So far, pure shaped pulses that we have applied (modified Hermite and hyperbolic secant types) did not provide major improvement compared to a 12 ns rectangular pulse. They were not modified to compensate for subsequent distortions, so with further effort we expect their efficiency could be substantially increased. The goal is to achieve $k \gtrsim 15$, thereby largely eliminating the need for referencing. On the other hand, developing a robust automated procedure, which can use the reference efficiently and accurately with conventional pulses, is a viable alternative that allows the method to be easily exercised with standard hardware. Note, that this is not an ill-posed problem, but one that handles the case of separating well parameterized data, which can be manipulated experimentally by adjusting pulses and pulse sequence, as noted in Subsection S3.

S5. OTHER EXAMPLES

Here we show long-distance measurements on a protein in low-concentration and in completely protonated environment, (Figure S5). In addition, the pulse was mistuned to provide a substandard level of unwanted signal suppression. The echo amplitude decayed by a factor of ~ 100 at $t_m = 8 \mu\text{s}$, so this example undoubtedly tests the limits of this technique. The tests used the 65/135 mutant of T4L, well characterized by DEER⁴, and DQC²⁰, although the latter used a disadvantageous setup.

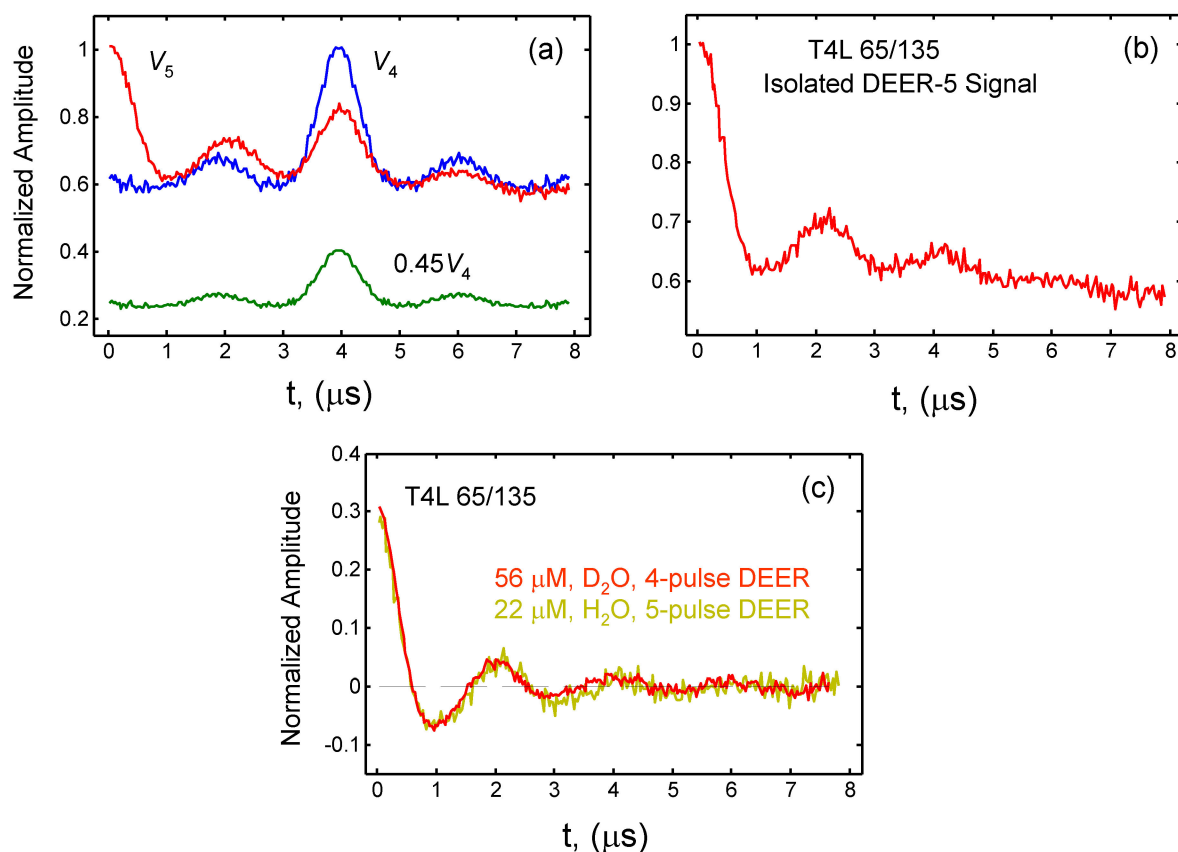


Figure S5. The moderately long distance signal ($46 \text{ \AA}$) was recorded using $8 \mu\text{s}$ time-scale on $22 \mu\text{M}$ T4L 65/135 mutant prepared in H_2O buffer. Panel (a) shows the subtraction in the case of not exactly tuned pulse 5, leaving substantial 4-pulse signal. The isolated 5-pulse DEER signal (b) after subtracting the homogeneous background is compared with the data⁴ on a $56 \mu\text{M}$ sample prepared in D_2O in (c). The data averaging took 10.8 h for the D_2O sample and for the H_2O sample it was 11 h including 2.2 h for recording the reference data.

Another example is of long-distance measurements in the absence of matrix deuteration and was conducted on a very long rigid biradical. It shows that referencing with another “easy” sample

using the same instrumental setup is viable. Given the greater complexity of the 5-pulse sequence, a more elaborate and robust algorithm based on parameterization should be devised and such work is in progress. Before this goal is fully addressed, the recommendation is to maintain the concentration as low as practical.

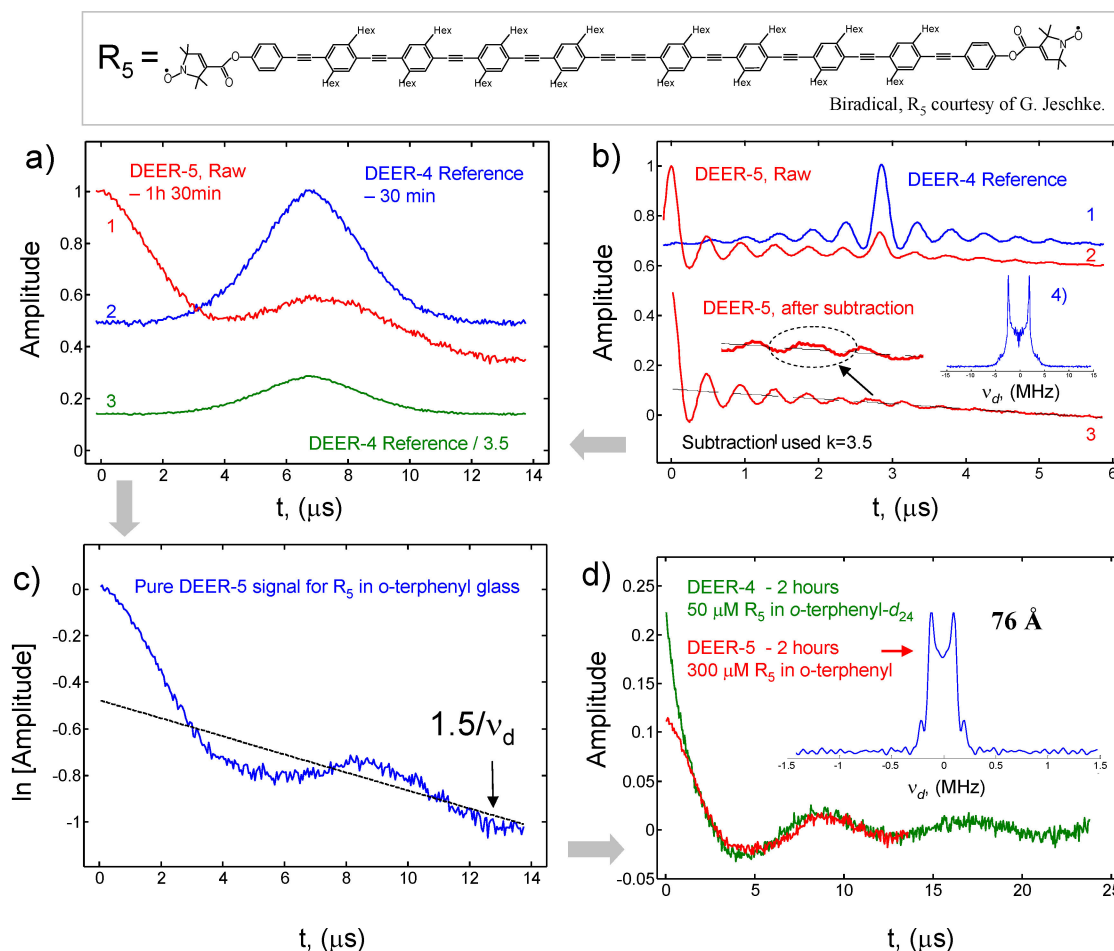


Figure S6. The long distance ~ 76 Å was measured on the rigid biradical R_5 ²¹⁻²² at 55 K in *o*-terphenyl glass, as shown in panels (a), (c). The data in (a) were referenced using a 29 Å rigid biradical R_2 ⁶ dissolved in *o*-terphenyl- d_{24} glass as shown in (b). The high SNR data in (b) permits accurate determination of the scaling parameter, k . Note that 15 μ s is about the upper limit for *o*-terphenyl glass, however the 14 μ s used is sufficient for accurate distance analysis providing 1.5 periods of dipolar frequency. Using a shorter t_m of 12 μ s enables one to record higher SNR data than shown just in 10 min. (d) Shows the comparison of the DEER-4 and DEER-5 data obtained in deuterated and non-deuterated matrices, respectively. In *o*-terphenyl- d_{24} a quick 4-pulse DEER experiment was conducted. The signal can be recorded overnight on the vastly extended time-scale of 36-40 μ s, still yielding good SNR. Even in this case of complete system deuteration some advantage of 5-pulse DEER for such long t_m 's could be noticed.

S6. METHOD COMPARISON

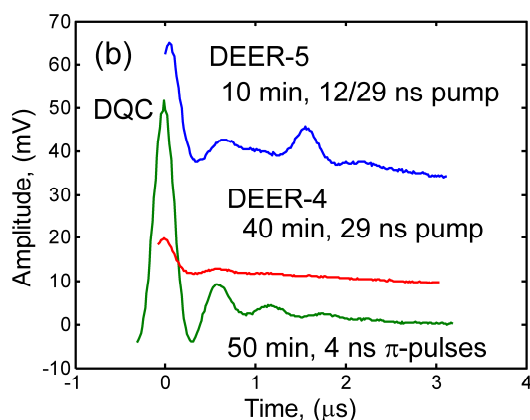


Figure S7. Shown in the Figure are raw signals from three PDS methods recorded on the same 40 μM T4L 8/44 sample. SNR (for the modulated part of the signal) after 10 min of data collection was, respectively, 44, 158, and 172 for DEER-4, DEER-5, and DQC. DEER-4 has decayed by a factor of 25 at 3 μs . After 4 μs DEER-5 dominates and can be readily recorded up to 8 μs without deuteration

In Figure S7 we compare DEER-5 with the two significant PDS methods. Raw data from 4-pulse DEER (middle), 6-pulse DQC (bottom), and 5-pulse DEER (top), were taken for the same 10 μL sample of 40 μM T4L MTSL-labeled 8/44 cysteine mutant and for the same receiver settings. The sample was prepared in H_2O buffer containing $\sim 30\%$ (w/v) glycerol. Data were collected at 60 K in Ku-band (17.3 GHz). Both DEER sequences used a 29 ns main pump pulse 3, and the width of the additional pump pulse 5 in the 5-pulse sequence was 12 ns. DQC used 2 ns $\pi/2$ and 4 ns π pulses, corresponding to a B_1 of ~ 45 G. The DQC signal is about a factor of 9 greater than the modulated part in 4-pulse DEER. The ratio of SNR's in DQC and 4-pulse DEER is a factor of ~ 4.0 due to a wider signal bandwidth used to record the DQC signal. Unprocessed raw DEER-5 contains the unwanted signal pathway, appearing as a central hump. At $t_m = 3$ μs , used in recording the signals, nuclear-spin diffusion is already significant, attenuating the echo in 4-pulse DEER by the factor of ~ 25 . It dominates phase relaxation at larger t_m . SNR in 5-pulse DEER is already close to DQC, which itself features some degree of suppression of nuclear spin diffusion. Beyond ~ 5 μs , both, standard 4-pulse DEER and DQC cannot detect a useful signal, but 5-pulse DEER is still very efficient.

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