Supplementary information

Selective Distance Measurements Using Triple Spin Labeling with Gd^{3+}, Mn^{2+}, and a Nitroxide

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1. Experimental Section

1.1 Protein preparation

Antennapedia homeodomain containing *p*-azido-L-phenylalanine (AzF) in position 22 was produced in *E. coli* using a pEVOL vector[1] with the aminoacyl-tRNA synthetase for AzF (AzF-RS).[2] The homeodomain gene, containing an amber stop codon in place of Phe22, was cloned into the T7 vector pETMCSI.[3] Both vectors were transformed into *E. coli* BL21(DE3). A 10 mL overnight culture was grown at 37 °C in the presence of 100 μg/mL ampicillin and 33 μg/mL chloramphenicol, and used to inoculate 1 L LB medium supplemented with 0.02% arabinose. At OD_{600} = 0.7 the cells from this culture were spun down gently and resuspended into 200 mL LB medium containing 0.02% arabinose and 1 mM AzF. Following incubation at 37 °C for another 30 mins, overexpression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was harvested after 3 h at 37 °C by centrifugation. The supernatant from the cell lysate was initially loaded onto a DEAE-Fractogel column (30 mL) equilibrated with buffer A (50 mM Tris-HCl, pH 7.5). After washing with three column volumes of buffer A, the homeodomain protein was eluted by a linear gradient of NaCl (0−1 M) in buffer A. Fractions containing homeodomain were pooled, dialyzed against three changes of 1 L buffer C (20 mM MES, pH 6.5) and further purified on a SP-650M column (30 mL). The column was washed with buffer C to remove unbound proteins, and the bound protein was eluted with a gradient of 0–1 M NaCl in buffer C. Fractions were analyzed by 10% SDS-PAGE, and protein fractions were pooled and dialyzed against click buffer (50 mM sodium phosphate, pH 8). The protein was concentrated to a final volume of about 0.15 mL using an Amicon ultrafiltration centrifugal tube (3 kDa molecular weight cutoff, MWCO).

1.2 Tagging of protein

The click reaction with the **C3-Gd** tag was conducted as described previously,[4] adding the solution of homeodomain in click buffer to the solution of **C3-Gd** tag, followed by addition of a premixed solution of $CuSO_4$ and BTTAA (copper(I)-binding ligand; BTTAA = 2-[4-({bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]acetic acid), NaCl, aminoguanidine, glycerol, and ascorbic acid to yield

a total reaction volume of 1 mL.

After the click reaction, the buffer was exchanged to buffer E (20 mM HEPES-KOH, pH 7.5) and the sample reduced by the addition of 50 equivalents of dithiothreitol (DTT). After incubation at room temperature for 1 h, the DTT was washed out by ultrafiltration. The reduced protein solution was added slowly into a solution of 5 equivalents of MTS-EDTA tag (Toronto Research Chemicals) in buffer E and kept at room temperature overnight. After the tagging reaction, the protein sample was exchanged to buffer E to remove the excess tag. Five equivalents of $MnCl_2$ solution were added, and then the protein sample was exchanged into EPR buffer (20 mM HEPES-KOH in D_2O, pH 7.5) in five steps of 3-fold dilution with EPR buffer and re-concentration using an Amicon ultrafiltration centrifugal tube (3 kDa MWCO). Finally, perdeuterated glycerol was added to a final concentration of 20% (v/v) to reach a final protein concentration of 0.1 mM.

1.3 Oligonucleotide preparation

Oligonucleotides were purchased from Integrated DNA Technologies (IDT) and dissolved in H_2O to make 10 mM stock solutions. The oligonucleotide d(CTCTAATGGCTT*TC) was ordered with a phosphorothioate (PT) group between the last two deoxythymidine nucleotides (indicated by the star), and the complementary strand was ordered without a PT modification. Separation and identification of the R_P and S_P diastereomers were carried out as described previously.[5]

1.4 Oligonucleotide labeling

A 5 mM DMSO solution of IAM-PROXYL (Sigma-Aldrich) was added to an equal volume of 1 mM solution of the PT oligonucleotide in H_2O. The solution was then diluted 3-fold by transfer into a mixture of 2:1 (v/v) TEAA (pH 6.5) and DMF. The ligation reaction was conducted at 50 °C for 8 h. Excess tag and unligated DNA were removed by HPLC purification, using a semi-preparative C18 HPLC column with a linear MeOH gradient (30–100%) in 40 mM ammonium carbonate (pH 8.0). Prior to concentrating by lyophilization, the buffer of the DNA fractions was exchanged for water by ultrafiltration. Double-stranded DNA was formed by combining equimolar amounts of single-stranded DNA in H_2O at room temperature. The annealed duplex was then concentrated and exchanged to EPR buffer (20 mM HEPES-KOH in D_2O, pH 7.5). The

buffer exchange was performed using an Omega membrane centrifugal tube (1 kDa molecular weight cutoff) and perdeuterated glycerol was added to a final concentration of 20% (v/v) to reach a final oligonucleotide concentration of 0.1 mM.

1.5 Protein-DNA complex

The complex between the double-tagged homeodomain and the single-tagged DNA was formed by combining equimolar amounts of DNA and protein in EPR buffer at room temperature. Perdeuterated glycerol was added to a final concentration of 20% (v/v) to reach a final concentration of the protein-DNA complex of 0.1 mM.

1.6 Modelling of the DEER distance distributions

The tags were modeled on the homeodomain by generating rotamer libraries of the tags as described previously, setting the dihedral angle χ_6 of the AzF-**C3** conjugate to 180°.[4]

1.7 Spectroscopic measurements

All pulse EPR measurements were performed on a homebuilt spectrometer at 95 GHz and 10 K.[6-7] The ED-EPR measurements for the nitroxide used $\pi/2$ and π pulse lengths of 30 ns and 60 ns, respectively, and a repetition rate of 30 ms. For the measurement of Gd^{3+} and Mn^{2+} we used $\pi/2$ and π pulses of 15 ns and 30 ns and a repetition time of 800 µs. Under these conditions the nitroxide signal is saturated. For all ED-EPR measurements τ was set to 500 ns. To differentiate between the signals from Gd^{3+} and Mn^{2+} ions, the field was positioned on the respective peaks and the MW power was controlled by a pin diode attenuator while measuring the Rabi nutation such that a 30 ns pulse rotated the magnetization by π.

All DEER distance measurements were performed using the standard 4-pulse DEER sequence[8] with a repetition time of 800 µs and evolution times of 3-5 µs. The parameters for the different measurements are summarized in Table S1. All DEER data were analyzed with DEERAnalysis[9] using Tikhonov regularization.

Table S1. Summary of experimental conditions for the various DEER measurements

Spin-pair	Pump position	Detection frequency	Detection π/2, π (ns)	Pump π (ns)
Gd-Mn	peak of Gd	ν(pump)-100 MHz (single-mode cavity)	15, 30	15
Gd-NO	peak of NO	ν(pump)-695 MHz (dual-mode cavity)	30, 60	92.5
NO-Mn	peak of NO	ν(pump)-120 MHz (single-mode cavity)	15, 30	20

2. T_1 measurements

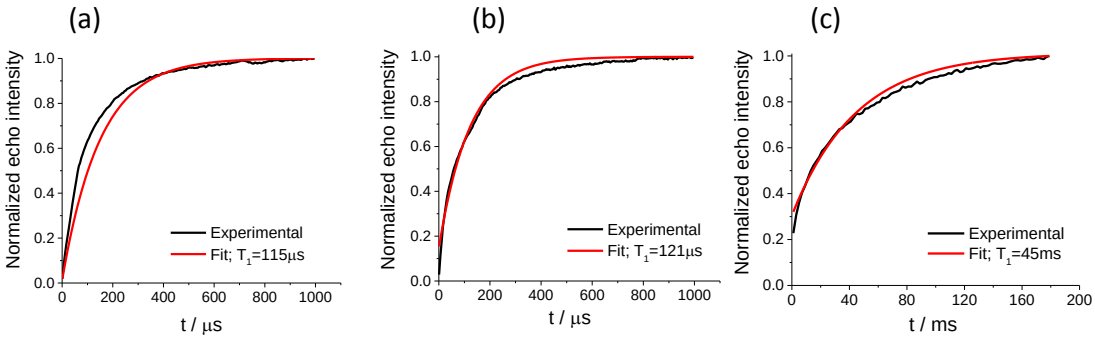

Figure S1. Saturation recovery measurements on the A*ntennapedia* homeodomain–DNA complex carried out at different magnetic fields to select the T_1 values of the different spin labels. (a) Measurements carried out on the maximum of the Gd^{3+} spectrum, selecting mainly the signal from Gd^{3+}. Experimental parameters: saturation pulse length 200 µs, echo detection pulses 15/30 ns, τ=0.8 µs, repetition time 2 ms. (b) Measurements carried out on the second Mn^{2+} peak, selecting contributions from Mn^{2+} and, to a lesser extent, Gd^{3+}. Otherwise, the experimental conditions were the same as in (a). (c) Measurement selecting mainly the nitroxide signal, setting the field to the maximum of the nitroxide spectrum. Experimental parameters: saturation pulse length 10 ms, echo detection pulses 30/60 ns, τ=0.8 µs, repetition time 250 ms. The red trace in each panel corresponds to an exponential fit of the data and the extracted T_1 values are noted on each panel. The fits are not perfect as the echo recovery deviates from a single exponential, but they clearly demonstrate the much longer T_1 time of the nitroxide spectrum compared with Gd^{3+} and Mn^{2+}.

3. ED-EPR spectra of *Antennapedia* homeodomain with and without DNA

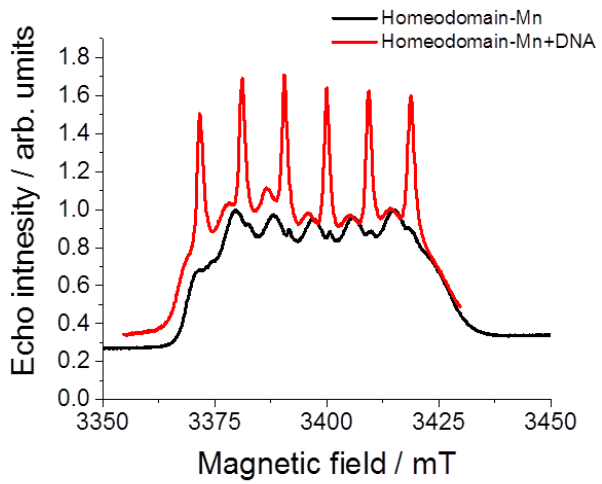

Figure S2. ED-EPR spectra of the Mn^{2+} central transition of the *Antennapedia* homeodomain tagged with MTS-EDTA-Mn^{2+} in the absence (black) and presence of DNA (red). The presence of the DNA led to the release of some of the Mn^{2+} ions, as indicated by the sharp peaks. Minor peaks in the Gd^{3+}-Mn^{2+} DEER distance distribution observed in the presence of DNA may arise from non-specific association of the released Mn^{2+} ions with the DNA. The free DNA sample did not show any signals from Mn^{2+} ions.

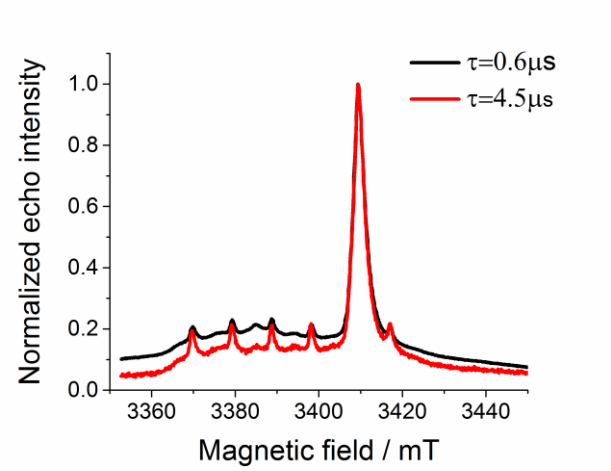

Figure S3. ED-EPR spectra of the *Antennapedia* homeodomain-DNA complex carried out under the same conditions as the spectrum shown in Figure 2c, recorded with the two τ delays indicated in the figure. The relative contribution of the Mn^{2+} impurity, which is characterized by the narrow lines, increased for longer τ delays due to its longer phase memory time.

4. Primary DEER data, nitroxide–Gd^{3+} DEER and validation results

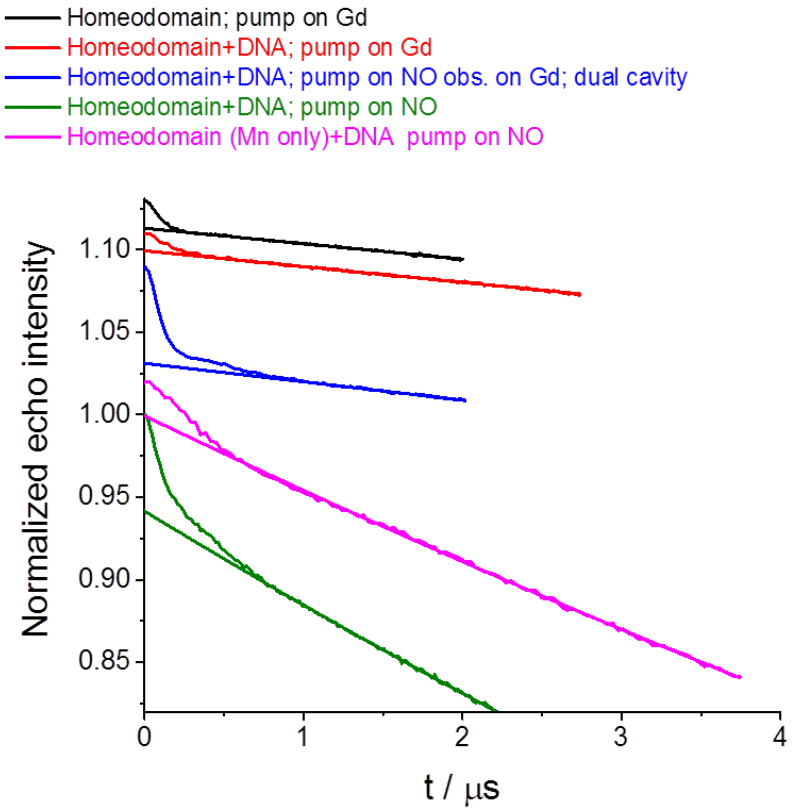

Figure S4. Raw data of the DEER measurements presented in Figure 3 in the main text along with the corresponding background decay functions used in their analysis. The color legend is given in the figure. The traces were shifted up with respect to each other to allow better comparison.

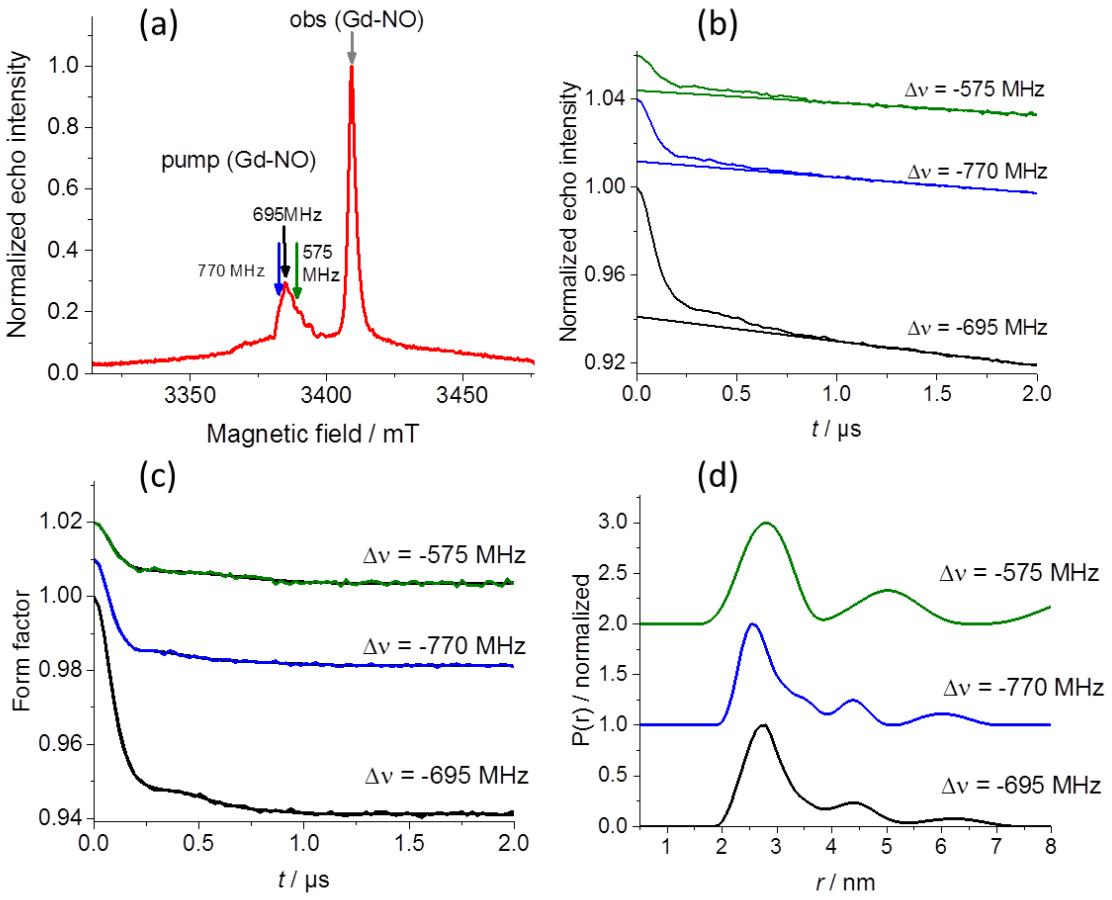

Figure S5. DEER measurements of the nitroxide–Gd^{3+} distance on the *Antennapedia* homeodomain-DNA complex with all three spin labels, with pumping at different frequencies of the nitroxide spectrum to check for orientation selection. Measurements were performed with a dual mode cavity. (a) ED-EPR spectrum measured at 25 K using $\pi/2$ and π pulse lengths of 15 ns and 30 ns and a repetition time of 30 ms. The positions of the pump and observe pulses are indicated together with their frequencies. (b) Primary DEER data with the background decay function. (c) DEER traces after removal of the background decay along with the fit obtained with Tikhonov regularization using the program DeerAnalysis[9] (black thin trace). (d) Corresponding distance distributions.

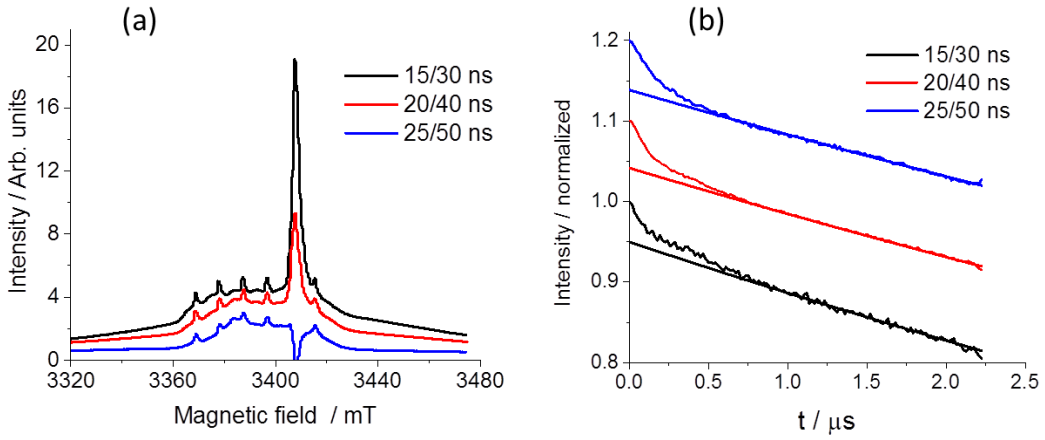

Figure S6. (a) ED-EPR spectra recorded with the pulse lengths indicated on the figure. (b) Primary DEER traces along with the background function used, recorded with different observe pulse lengths.

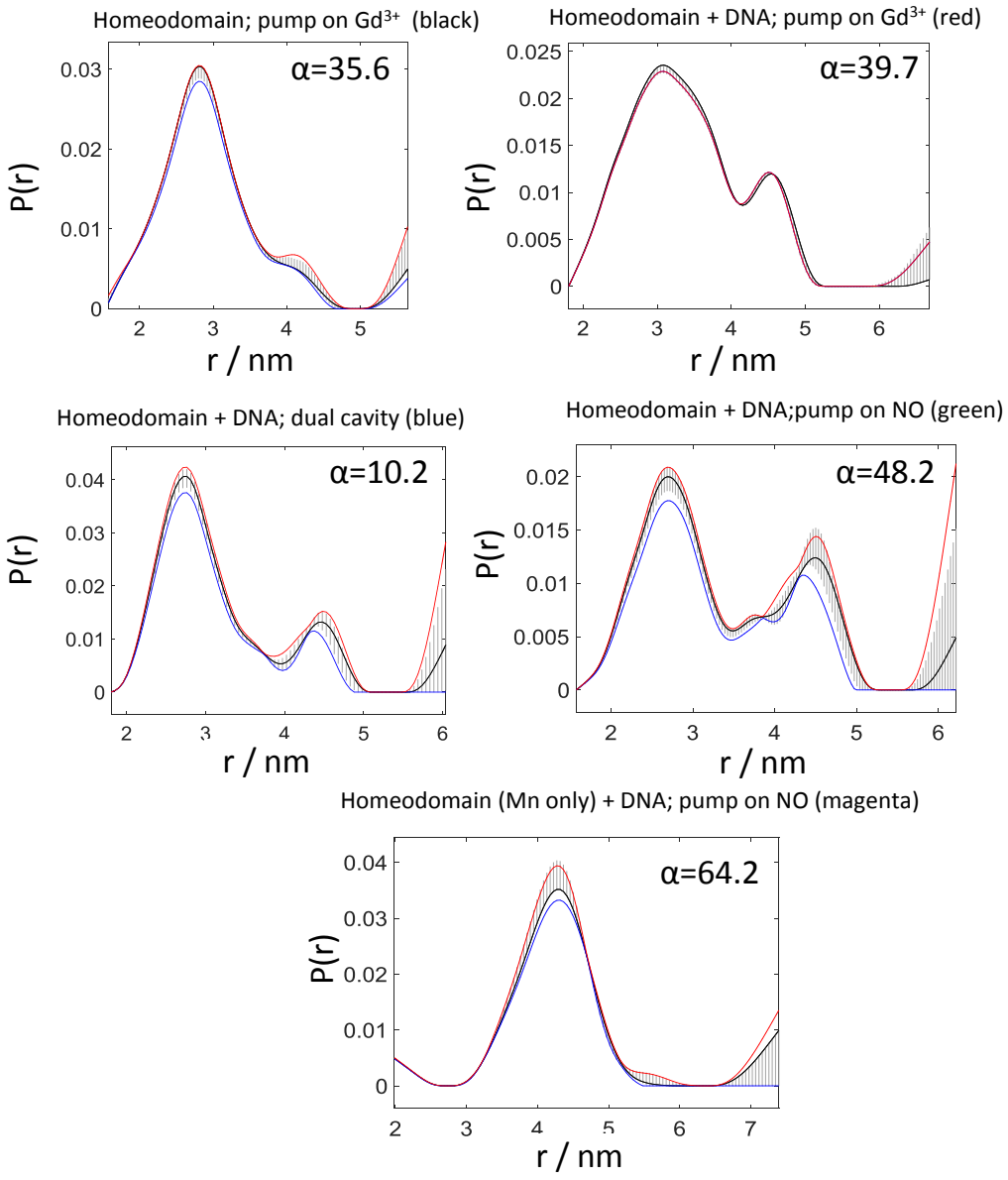

Figure S7. Validation of distance distributions performed using the validation package in DEERAnalysis.[9] The title of each panel refers to the color code used in Figure 3 in the main text and in Figure S4. The Tikhonov regularization parameter used is indicated in each panel.

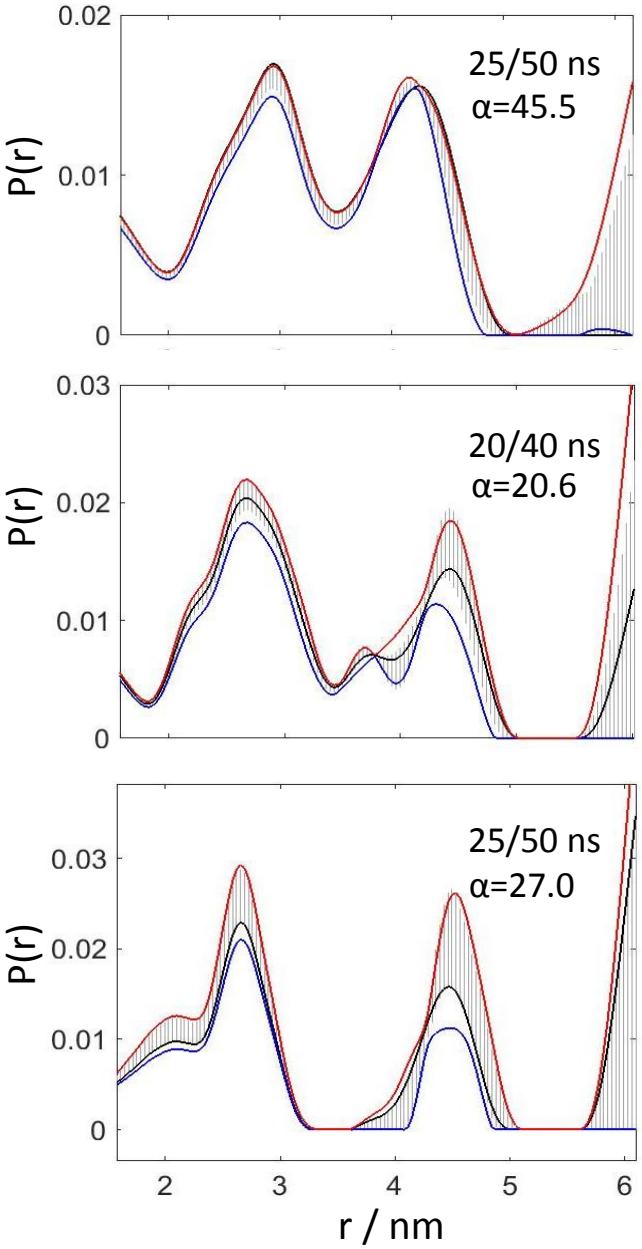

Figure S8. Validation of distance distributions performed using the validation package in DEERAnalysis[9] for the data shown in Figure 4 in the main text. The Tikhonov regularization parameter used is indicated in each panel.

References

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