Supporting Information Part B

Orthogonally spin-labeled rulers help to identify crosstalk signals and improve DEER signal fidelity

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1 NO-NO, NO-Gd and Gd-Gd rulers in a 1:1:2 ratio

sample	ruler	V_{stock} [µl]	$c_{stock(spin)}$ [µM]	$V_{d8\text{-}glycerol}$ [µl]	V_{final} [µl]	$c_{final(spin)}$ [µM]
NO-NO	NO-NO	20	100	20	40	50
	NO-Gd	-	-			-
	Gd-Gd	-	-			-
NO-Gd	NO-NO	-	-	20	40	-
	NO-Gd	20	100			50
	Gd-Gd	-	-			-
Gd-Gd	NO-NO	-	-	20	40	-
	NO-Gd	-	-			-
	Gd-Gd	20	200			100
NO-NO + NO-Gd	NO-NO	10	100	20	40	25
	NO-Gd	10	100			25
	Gd-Gd	-	-			-
NO-NO + 2xGd-Gd	NO-NO	10	100	20	40	25
	NO-Gd	-	-			-
	Gd-Gd	10	200			50
NO-Gd + 2xGd-Gd	NO-NO	-	-	20	40	-
	NO-Gd	10	100			25
	Gd-Gd	10	200			50
NO-NO + NO-Gd + 2xGd-Gd	NO-NO	6.67	100	20	40	16.67
	NO-Gd	6.67	100			16.67
	Gd-Gd	6.67	200			33.33

Table S1: Sample preparation of the NO-NO, NO-Gd and Gd-Gd rulers in a 1:1:2 ratio. Stock solutions of NONO, NOGd and GdGd rulers were prepared using spin counting information obtained from X-band continous wave (cw) EPR for nitroxide (NO) and Q-band field-swept echo (FSE) detected spectra for gadolinium (Gd) (data not shown). 50% v/v (20 µl) deuterated glycerol was added to all samples as cryoprotectant. The DEER data obtained on these samples are presented in Fig. 4 - 8 in the main text and Fig. SS1 - SS2.

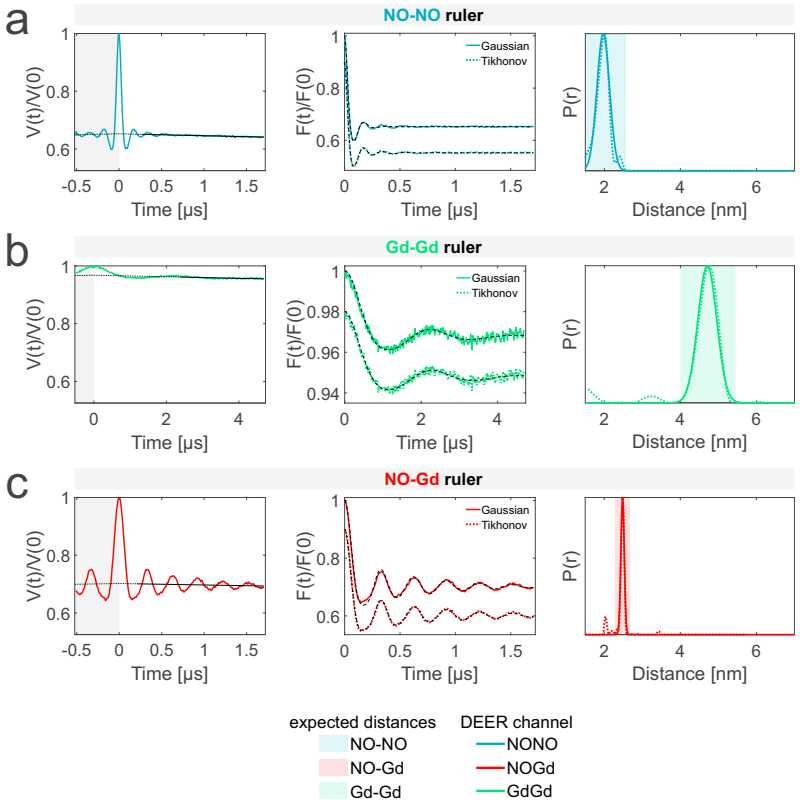

Figure S1: DEER data evaluation methods (related to Fig. 4 in the main text). Comparison of Gaussian fitting and Tikhonov regularization methods for DEER data evaluation. Left, primary data with background fit (gray areas are excluded from data evaluation); middle, form factors with fit; right, obtained distance distributions.

sample	experiment	NO-NO signal theor.	NO-NO signal exp.	NO-Gd signal theor.	NO-Gd signal exp.	Gd-Gd signal theor.	Gd-Gd signal exp.
NO-NO	NONO DEER	+	+				
	NOGd DEER						
	GdGd DEER						
NO-Gd	NONO DEER			$\mathbf{X}_1$	$\mathbf{X}_1$		
	NOGd DEER			+	+		
	GdGd DEER						
Gd-Gd	NONO DEER						
	NOGd DEER						
	GdGd DEER					+	+
NO-NO + NO-Gd	NONO DEER	+	+	$\mathbf{X}_1$			
	NOGd DEER			+	+		
	GdGd DEER						
NO-NO + 2xGd-Gd	NONO DEER	+	+			$\mathbf{X}_4$	
	NOGd DEER					$\mathbf{X}_2$	$\mathbf{X}_2$
	GdGd DEER					+	+
NO-Gd + 2xGd-Gd	NONO DEER			$\mathbf{X}_1$	$\mathbf{X}_1$	$\mathbf{X}_4$	
	NOGd DEER			+	+	$\mathbf{X}_3$	$\mathbf{X}_3$
	GdGd DEER					+	+
NO-NO + NO-Gd + 2xGd-Gd	NONO DEER	+	+	$\mathbf{X}_1$		$\mathbf{X}_4$	
	NOGd DEER			+	+	$\mathbf{X}_3$	$\mathbf{X}_3$
	GdGd DEER					+	+

Table S2: Theoretically possible versus experimentally detected DEER signal contributions: desired + / not-detectable/ crosstalk $\mathbf{X}_i$ / not-detected for NONO, NOGd and GdGd rulers mixed in a 1:1:2 molecular ratio for the orthogonal Q-band DEER setups given in Fig. 2. The crosstalk signals are classified as follows: $\mathbf{X}_1$ is an NO-Gd crosstalk signal in the NONO channel; $\mathbf{X}_2$ ($\mathbf{X}_3$) is a Gd-Gd crosstalk signal in the NOGd channel in absence (presence) of "real" NO-Gd signal; $\mathbf{X}_4$ is a Gd-Gd crosstalk signal in the NONO channel. In our setup, the number of experimentally observed crosstalk signals is by far smaller than the number of theoretically possible ones.

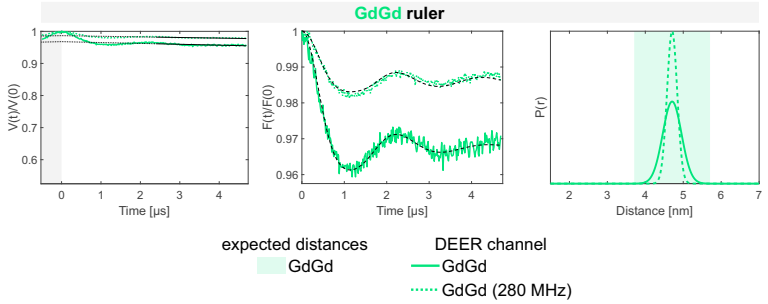

Figure S2: Comparison of GdGd DEER setups (related to Fig. 7 in the main text). Left, primary data with background fit (gray area is excluded from data evaluation); middle, form factors with Gaussian fit; right, obtained distance distributions. The data plotted as a green solid line were obtained using the standard GdGd DEER setup shown in Fig. 3(c) with the pump pulse placed on the maximum of the Gd spectrum and the observer 100 MHz lower in frequency. The second setup (green dotted line) is basically the same as the NOGd DEER setup shown in Fig. 3(b) with the observer on the spectral maximum and the pump placed 280 MHz higher in frequency but in this case with a pump pulse optimized in power to optimally excite the Gd. The data obtained with the standard GdGd DEER setup are characterized by a 3.5% modulation depth while the second setup just yields a 1.25% modulation depth due to the lower spectral density at the pump position.

2 NO-NO, NO-Gd and Gd-Gd rulers in a 1:1:1 ratio

The data shown in this section are an independent repetion of the data presented in the main text at a different molar ratio. All samples were prepared based on rulers from the same batch but using newly prepared stock solutions creating an independent set of samples.

sample	ruler	V_{stock} [µl]	$c_{stock(spin)}$ [µM]	$V_{d8\text{-}glycerol}$ [µl]	V_{final} [µl]	$c_{final(spin)}$ [µM]
NO-NO	NO-NO	20	100	20	40	50
	NO-Gd	-	-			-
	Gd-Gd	-	-			-
NO-Gd	NO-NO	-	-	20	40	-
	NO-Gd	20	100			50
	Gd-Gd	-	-			-
Gd-Gd	NO-NO	-	-	20	40	-
	NO-Gd	-	-			-
	Gd-Gd	20	100			50
NO-NO + NO-Gd	NO-NO	10	100	20	40	25
	NO-Gd	10	100			25
	Gd-Gd	-	-			-
NO-NO + Gd-Gd	NO-NO	10	100	20	40	25
	NO-Gd	-	-			-
	Gd-Gd	10	100			25
NO-Gd + Gd-Gd	NO-NO	-	-	20	40	-
	NO-Gd	10	100			25
	Gd-Gd	10	100			25
NO-NO + NO-Gd + Gd-Gd	NO-NO	6.67	100	20	40	16.67
	NO-Gd	6.67	100			16.67
	Gd-Gd	6.67	100			16.67

Table S3: Sample preparation of the NO-NO, NO-Gd and Gd-Gd rulers in a 1:1:1 ratio. The preparation of the samples was performed as described in Table SS1. The data obtained on these samples are presented in Fig. SS3 - SS5.

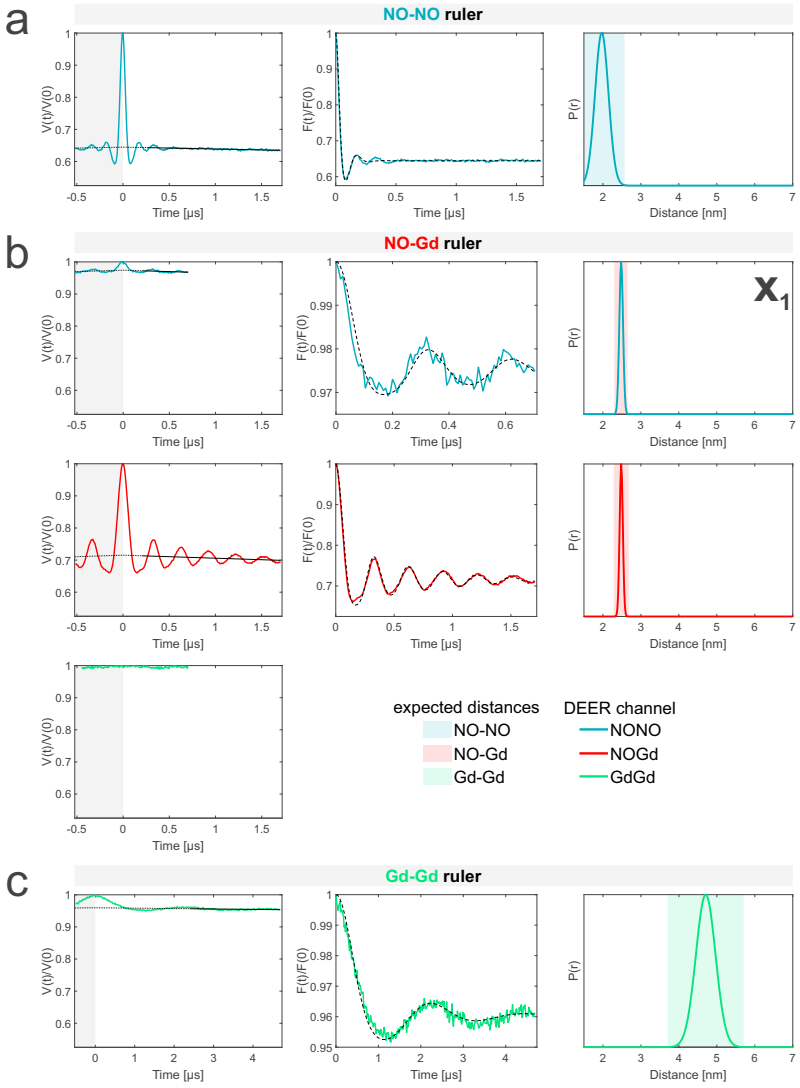

Figure S3: Characterization of the isolated rulers (related to Fig. 4 in the main text). The DEER setups are introduced in Fig. 3. Left, primary data with background fit (gray areas are excluded from data evaluation); middle, form factors with Gaussian fit; right, obtained distance distributions. The time traces, form factors and distance distributions recorded with the NONO DEER channel are colored in blue, those recorded with the GdGd channel are green, and those recorded with the NOGd channel are red. Regions in which distances can be theoretically expected based on the rulers present in the specific sample are represented as shaded blue, green and red areas in the distance distributions. "$\mathbf{X}_i$" refers to a specific crosstalk signal.

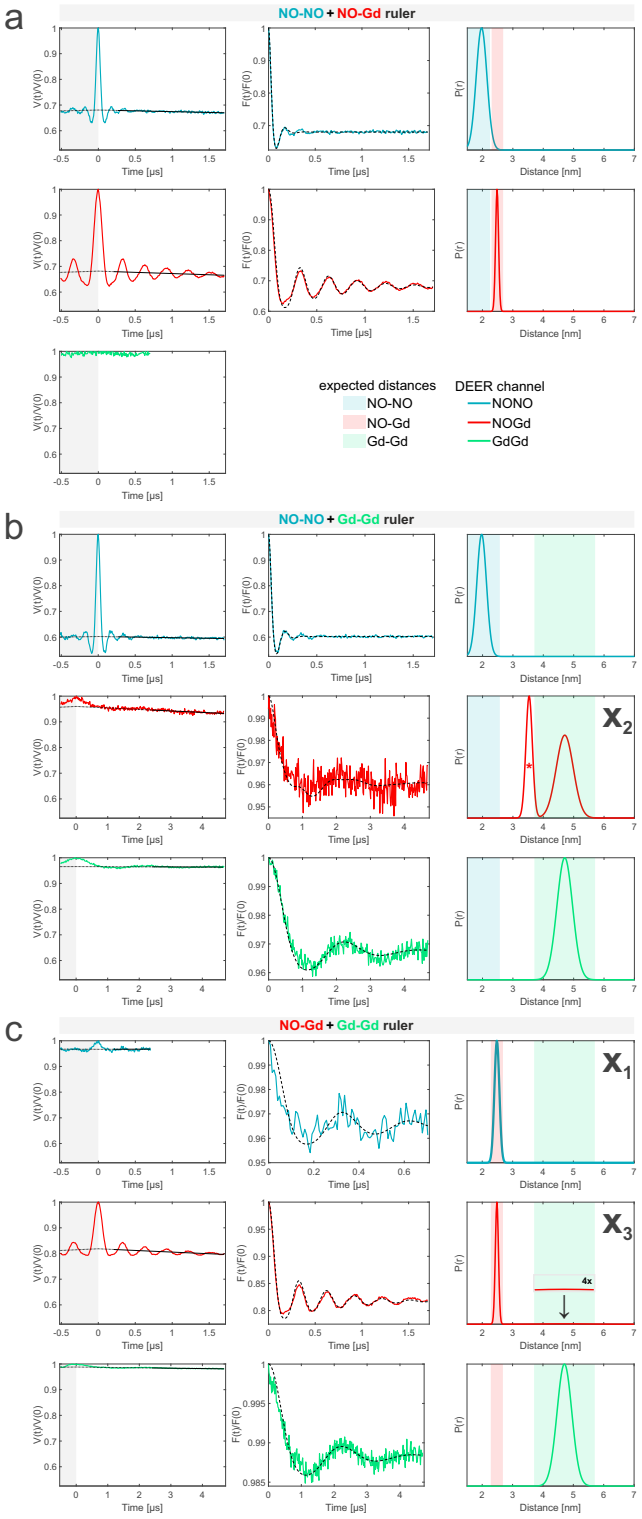

Figure S4: Pairwise mixtures of rulers in a 1:1 ratio (related to Fig. 5 in the main text). The DEER setups are introduced in Fig. 3. Left, primary data with background fit (gray areas are excluded from data evaluation); middle, form factors with Gaussian fit; right, obtained distance distributions. Color coding as in Fig. S3. "$\mathbf{X}_i$" refers to specific crosstalk signals. (a) Sample containing the NO-NO and the NO-Gd rulers mixed in a 1:1 molar ratio. (b) Sample containing the NO-NO and the Gd-Gd rulers mixed in a 1:1 ratio. The NOGd DEER channel contains a GdGd crosstalk signal $\mathbf{X}_2$. The distance indicated with an asterisk originates from a spectrometer-specific artifact signal. (c) Mixture of the NO-Gd ruler with the Gd-Gd ruler in a 1:1 ratio. NO-Gd crosstalk signal in the NONO channel ($\mathbf{X}_1$) and Gd-Gd crosstalk signal in the NOGd channel in presence of an NO-Gd signal ($\mathbf{X}_3$).

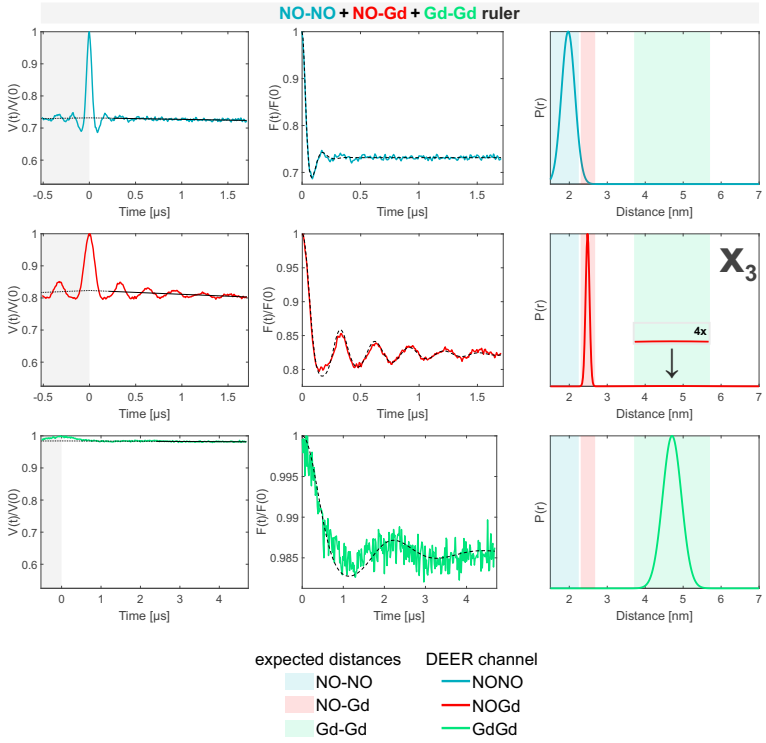

Figure S5: Mixture of NO-NO, NO-Gd and Gd-Gd rulers in a 1:1:1 ratio (related to Fig. 6 in the main text). The DEER setups are introduced in Fig. 3. Left, primary data with background fit (gray areas are excluded from data evaluation); middle, form factors with Gaussian fit; right, obtained distance distributions. Color coding as in Fig. S3. "$\mathbf{X}_i$" refers to a specific crosstalk signal.